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Bacteriohopanoid inventory of *Geobacter sulfurreducens* and *Geobacter metallireducens*

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ABSTRACT

Geobacter spp. are ubiquitous prokaryotes in diverse sedimentary environments, coupling the oxidation of various carbon compounds with the reduction of metals, such as ferric iron. One specific attribute of such species, namely *Geobacter metallireducens* and *Geobacter sulfurreducens*, is their ability to synthesize hopanoids. Hopanoids are among the most specific and abundant molecular fossils in the sedimentary record and are commonly used to trace bacterial activity in recent to sub-recent sediments. The production of polyfunctionalized bacteriohopanepolyols (BHPs) by *G. sulfurreducens* and *G. metallireducens* is known, but the intact BHPs were not identified. In this study, the complete hopanoid inventory of both species was investigated. Among the hopanoids, bacteriohopanetetrol cyclitol ether was the major compound in both, while guanidine-substituted bacteriohopanetetrol cyclitol ether and bacteriohopanetetrol glucosamine were also found in high abundance in *G. sulfurreducens*, but only in trace amounts in *G. metallireducens*. Interestingly, the BHP inventories are similar to those of some aerobic or facultative anaerobic methylotrophic bacteria, but have been identified in anaerobic cultures of *Geobacter* for the first time. The ubiquity of *Geobacter* spp. in anoxic sediments provokes us to speculate that such species may be among the predominant sources of composite BHPs in many sedimentary environments.

1. Introduction

Prokaryotes have shaped sedimentary environments since life evolved on the early Earth. As prokaryotes rarely leave behind morphological fossils, most information about early life derives from molecular fossils, commonly referred to as lipid biomarkers (e.g. Summons et al., 1999; Brocks et al., 2003; Brocks and Schaeffer, 2008). Lipid biomarkers are compounds that are specific to certain groups of organisms based on their production in modern representatives of these groups.

Among the most specific biomarkers of bacteria are hopanoids. Only an estimated 10% of the Bacteria have the ability to produce them (Pearson et al., 2007, 2009). Although many phyla of the bacterial domain are potentially capable of producing hopanoids, only few effectively synthesize them (Ourisson et al., 1979; Rohmer et al., 1984; Pearson et al., 2007, 2009; Talbot et al., 2008). Their function is still not well understood, but they are believed to enhance membrane stability and integrity (Ourisson et al., 1979, 1987; Jahnke et al., 1992; Berry et al., 1993; Kannenberg and Poralla, 1999; Welander et al., 2009). The characteristic pentacyclic carbon skeleton is diagnostic for all hopanoids and resists thermal degradation to a great extent. Due to this stability, hopanoids are among the oldest molecular fossils found in the rock record (Brocks et al., 1999, 2003) and are commonly prevalent in mature sediments and crude oils (Ourisson et al., 1979; Ourisson and Albrecht, 1992). They are also useful tracers in recent environments, where the distribution of diverse bacteriohopanepolyols (BHPs) has been used to reconstruct bacterial community structure (e.g. Talbot and Farrimond, 2007; Cooke et al., 2008; van Winden et al., 2012).

It was originally assumed that hopanoids were produced only by aerobic bacteria and a few facultative anaerobes (Rohmer et al., 1984; Neunlist et al., 1985). Only much later were they detected in microbial mats and deposits that formed in anoxic environments at methane seeps (e.g. Thiel et al., 2003), drawing attention to potential hopanoid production by anaerobic bacteria. Finally, they were also found in enrichment cultures of anaerobic ammonium-oxidizing (anammox) planctomycetes (Sinninghe Damsté et al., 2004) and sulfate reducing bacteria (Blumenberg et al., 2006). The production of hopanoids by *Geobacter metallireducens*, a strict anaerobe, and *Geobacter sulfurreducens*, usually an anaerobic organism, but able to tolerate or even use low amounts of O_2 (Lin et al., 2004), was reported in two independent studies (Fischer et al., 2005; Härtner et al., 2005). Members of the Geobacteraceae have been detected in various anoxic environments from freshwater, marine and estuarine settings to subsurface aquifers and organic- and metal-polluted environments (Cummings et al., 2003 and references therein). Their ubiquity provoked the

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suggestion that these bacteria might be among the most dominant hopanoid producers in many anoxic environments (cf. Fischer et al., 2005). Fischer et al. (2005), as well as Härtner et al. (2005), confirmed the presence of polyfunctionalized BHPs in *G. sulfurreducens* by measuring the HIO_4 cleaved products, but intact BHPs were not examined.

In this study, we have determined the inventory of hopanoids, including BHPs, in *G. sulfurreducens* and *G. metallireducens*, using gas chromatography–mass spectrometry (GC–MS) and high performance liquid chromatography–mass spectrometry (HPLC–MS), and discuss the implications of the findings for the interpretation of hopanoid signatures in sediments and sedimentary rocks.

2. Materials and methods

2.1. Bacterial strains and growth conditions

G. metallireducens (Lovley and Phillips, 1988; Lovley et al., 1993) and *G. sulfurreducens* (Caccavo et al., 1994) belong to the Deltaproteobacteria. They can both grow by way of complete oxidation of organic matter coupled to dissimilatory Fe(III) reduction. *G. metallireducens* was originally isolated from freshwater sediments of the Potomac River and was obtained for this study from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSMZ No. 7210, type strain). *G. sulfurreducens*, originally isolated from surface sediment of a ditch, was taken from the laboratory culture collection of the Tuebingen geomicrobiology group.

Both were cultivated in an anoxic medium. For *G. metallireducens*, DSMZ medium 579 was used, containing 55.9 mM Fe(III) citrate, 28.0 mM NH_4Cl, 4.4 mM $NaH_2PO_4{\cdot}H_2O$, 1.3 mM KCl, 30.5 mM NaOAc and 10 ml trace element solution of DSMZ medium 141. The medium for *G. sulfurreducens* contained 5.6 mM NH_4Cl, 3.7 mM KH_2PO_4, 0.7 mM $CaCl_2{\cdot}2H_2O$, 2.0 mM $MgSO_4{\cdot}7H_2O$, 10 mM NaOAc and 10 ml trace element solution of DSMZ medium 141. After autoclaving and cooling under N_2/CO_2 (90/10), 22 mM HCO_3^- buffer, autoclaved separately under a N_2/CO_2 atmosphere, was added to each medium. The following solutions were then added from anoxic sterile stocks to each medium: 10 ml l^{-1} vitamin solution of DSMZ medium 141, and 1 ml l^{-1} selenite tungstate solution (Tschech and Pfennig, 1984). The *G. metallireducens* medium was additionally amended with 0.75 μM $Na_2WO_4{\cdot}2H_2O$; 40 mM Na_2-fumarate was added as electron acceptor to the medium for *G. sulfurreducens* from a sterile stock solution. The pH of the final medium was adjusted to 6.8–7.0. Cultures were inoculated from fresh pre-cultures. Cultures were grown in 500 ml medium in 1 l Schott bottles. Incubation was at 28 °C in the dark.

Fe(III) reduction in *G. metallireducens* was monitored using the spectrophotometric ferrozine assay (see below). Cultures were harvested during late exponential growth phase after 2 days incubation, when ca. 90% of Fe(III) was reduced to Fe(II). For *G. sulfurreducens*, optical density (OD) at 540 nm (Analytik Jena, Spekol 1300) was taken as a measure of growth stage. Cells were harvested in late exponential growth phase after 2 days incubation (OD_{540} ca. 0.5) via centrifugation at 10,000*g* under anoxic conditions (Herolab, HiCen 21), frozen and lyophilized (Christ, Alpha 1–4). The material from three independent cultures (500 ml each) was combined to provide the amount of biomass required for lipid analysis. Because cultures were not analyzed separately, the data represent an average of the combined triplicates.

2.2. Quantification of iron (ferrozine assay)

Fe(II) and Fe(total) were quantified using the spectrophotometric ferrozine assay modified from Stookey (1970) as described by Hegler et al. (2008). Quantification for all Fe-containing cultures was done before inoculation and during Fe(III) reduction by the cultures.

2.3. Extraction and derivatization of lipids

All glassware was baked at 400 °C for 4 h before use. The freeze dried material was extracted (4×) with dichloromethane (DCM)/MeOH (3:1 v/v) by sonication (15 min) at room temperature. Three internal standards (2-methyloctadecanoic acid, 1-nonadecanol and 5α-cholestane) were added to all samples before extraction. The extracts were combined and washed with DCM-extracted water. The organic phases were collected, combined and dried with Na_2SO_4. The solvent was removed under reduced pressure. Aliquots of the total lipid extracts (TLEs) were acetylated by reaction with Ac_2O and pyridine (1:1 v/v) for 1 h at 60 °C and subsequent incubation at room temperature overnight. Further aliquots of the TLEs were saponified (3 h, 80 °C) with 6% KOH in MeOH. After saponification, neutral lipids were extracted (4×) from the saponification extract with *n*-hexane. Neutral lipids were separated into three fractions according to polarity via column chromatography (Supelco LC–NH_2 glass cartridges; 500 mg sorbed): hydrocarbons (F1) were eluted with hexane, ketones (F2) and esters with hexane/DCM (3:1 v/v) and alcohols (F3) with DCM/Me_2CO (9:1 v/v). The alcohol fractions were acetylated with Ac_2O and pyridine (1:1 v/v) for 1 h at 60 °C and subsequent incubation at room temperature overnight.

2.4. GC–MS

Lipids in acetylated TLEs and fractions F1 to F3 were identified and quantified via GC–MS with an Agilent 7890 A GC system coupled to an Agilent 5975 C inert MSD mass spectrometer at the University of Vienna. A split/splitless injector (310 °C) and a 30 m HP-5 MS UI fused silica column (0.25 mm i.d., 0.25 μm film thickness) were used with He as carrier gas. The GC temperature programme was: 60 °C (1 min) to 150 °C at 10 °C/min, then 150 °C to 325 °C (held 35 min) at 4 °C/min. This programme was used for fractions F1 to F3. Acetylated TLEs were additionally run with a 15 m DB-5HT fused silica column (0.25 mm i.d., 0.10 μm film thickness) with He as carrier gas. The GC temperature programme was: 80 °C (3 min) to 200 °C at 15 °C/min, then 200 °C to 250 °C at 10 °C/min, then 250 °C to 360 °C (held 20 min) at 15 °C/min. All acetylated TLEs were run in parallel in full scan (*m*/*z* 50–800) and selected ion monitoring mode (*m*/*z* 191, 205) for analysis of trace amounts of pentacyclic triterpenoids, including bacteriohopane-32,33,34,35-tetrol (BHT). The acetylated TLEs were analyzed for identification and quantification of the components; 5α-cholestane was used as internal standard for quantification of the TLEs using the total ion current (TIC). The response factors of the various compounds were assumed to be identical, although it has been shown recently that response factors of various GC-amenable hopanoids may vary significantly (see Sessions et al., 2013). Fractions F1 and F3 were analyzed to detect possible artifacts that may have been introduced during acetylation of the TLE. Identification was based on comparison of retention times and mass spectra with published data and reference compounds.

2.5. HPLC–APCI–MS

BHPs in the acetylated TLE in MeOH/propan-2-ol (60:40 v/v) were analyzed at Newcastle University as described by van Winden et al. (2012). A Thermo Finnigan Surveyor HPLC system, equipped with a Phenomenex Gemini C_{18} 5 μm HPLC column (150 mm, 3.0 mm i.d.) and a security guard column of the same material was used. The programme was: 0.5 ml/min at 30 °C with

90% A and 10% B (starting at 0 min); 59% A, 1% B and 40% C (at 25 min); isocratic (to 40 min), returning to starting conditions over 5 min and stabilizing for 15 min, with A = MeOH, B = water, C = propan-2-ol. A Thermo Finnigan LCQ ion trap MS instrument equipped with an atmospheric pressure chemical ionization (APCI) source operated in positive ion mode was used. HPLC–MS was performed in data-dependent mode with two scan events: SCAN 1 over the whole spectrum (m/z 500–1300) and with SCAN 2, a data-dependent MS^2 spectrum recorded for the most abundant ion from SCAN 1.

The relative abundances of BHPs were corrected for differences in response factors where N-containing BHPs showed a response 1.5× that of non-N-containing BHPs (cf. van Winden et al., 2012 and references therein). No internal standard was added for BHP analysis. BHP quantification was done indirectly by correlating GC–MS and HPLC–APCI–MS data, using GC-amenable free BHT as the scale, which was quantified via GC–MS with the internal standard (5α-cholestane). The content quantified using GC was assumed to equal the content from HPLC, although it recently has been shown that BHP content measured using HPLC–MS is higher by at least a factor of 2 (Sessions et al., 2013). The content of the other BHPs was then calculated from relative abundance in the HPLC runs. Without standards for each hopanoid, the large potential error in the quantification calls for caution when comparing these values with values in the literature.

2.6. Quantification of lipids

For quantification of lipid compounds in *G. sulfurreducens* (producing only cells), amount of lipid per wt. of sample was back-calculated to amount per g carbon. The ratio of carbon wt. per dry wt. was assumed to equal 0.5 (Bratbak and Dundas, 1984) and the amount obtained per wt. of sample was divided by 2. For quantification of lipid compounds in *G. metallireducens* (producing cells and minerals), amount is given only per wt. of sample.

2.7. BLAST analysis

Protein BLAST searches of *Rhodopseudomonas palustris* strain TIE-1 HpnP sequence (responsible for C-2 methylation of hopanoids) and *Methylococcus capsulatus* strain Bath HpnR sequence (responsible for C-3 methylation of hopanoids) were run against the Integrated Microbial Genomes database (IMG of DOE's Joint

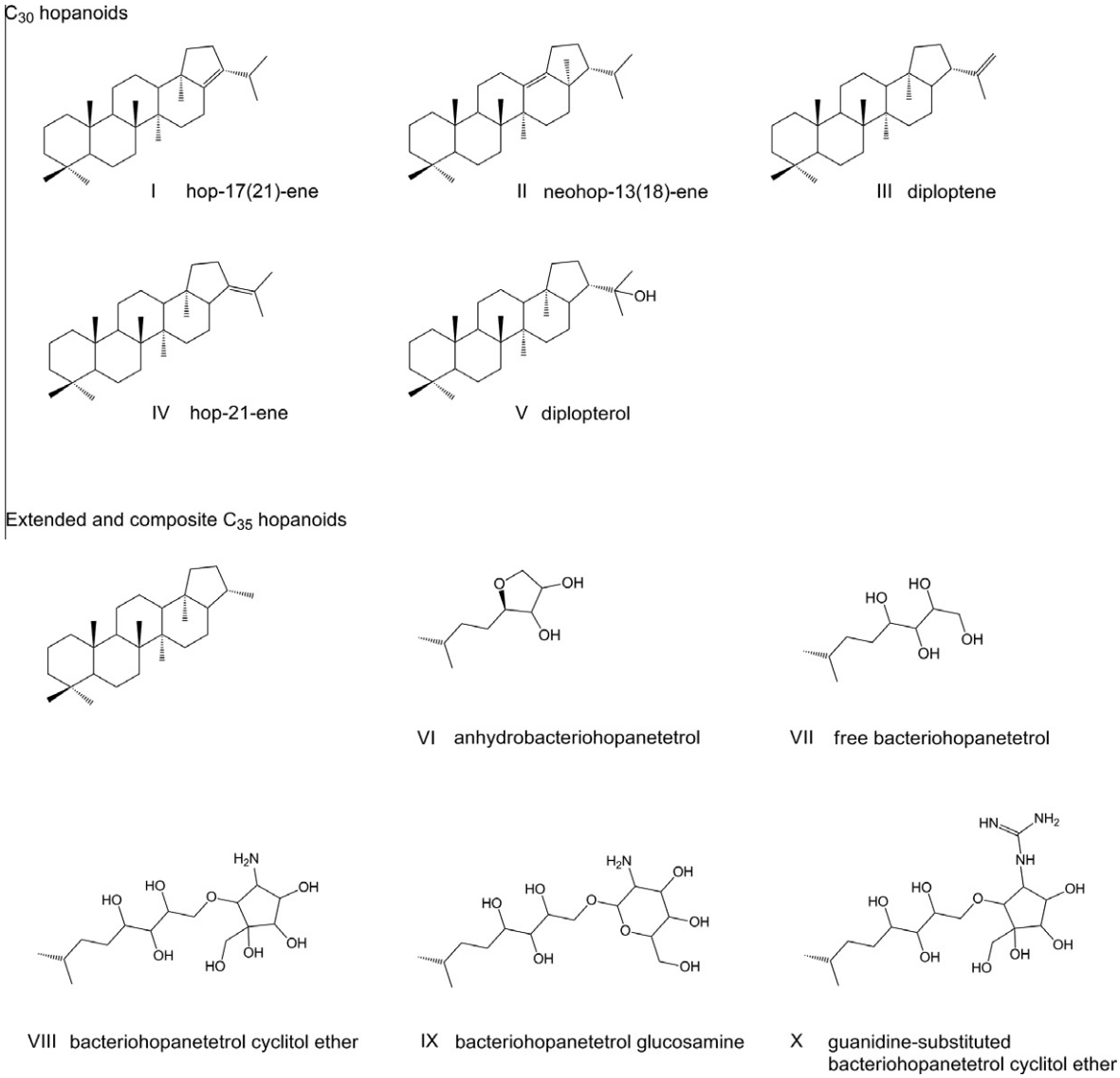

Fig. 1. Structures of hopanoids in *G. metallireducens* and *G. sulfurreducens*.

Genome Institute) of fully sequenced microbial genomes to search for homologues in *G. sulfurreducens* and *G. metallireducens*.

3. Lipid inventory of *G. sulfurreducens* and *G. metallireducens*

The hopanoids extracted from *G. sulfurreducens* and *G. metallireducens* can be subdivided into three groups: those without a side chain and 30 carbons (C_{30}), BHPs with a polyfunctionalized side chain and 35 carbons, and composite BHPs, where C-35 is linked to a complex moiety. The first group was only found in trace amount and comprised hop-22(29)-ene (diploptene) **III** (structures in Fig. 1) and hopan-22-ol (diplopterol) **V** (Table 1). Diploptene **III** was slightly more abundant in *G. sulfurreducens* than in *G. metallireducens* (Fig. 2; Table 1). Only a minor content of free bacteriohopanetetrol (BHT) **VII** was found in both cultures relative to the composite BHPs (see below). Absolute quantification of the C_{30} hopanoids was not possible due to additional production of C_{30} hopanoid hydrocarbons during derivatization (acetylation) or GC analysis. Diploptene **III** was abundant in the underivatized hydrocarbon fraction F1, but was found to be 4× more abundant in the TLE than in F1. Moreover, in the acetylated alcohol fraction (F3), hopenes (diploptene **III** and hop-21-ene **IV**) were present and even more abundant in F3 than in F1, revealing that they were artifacts probably produced by a combination of the derivatization (acetylation) of diplopterol (Welander et al., 2012; Eickhoff et al., 2013) and the injection with a split/splitless injector, which might also have been responsible for producing trace amounts of hop-17(21)-ene **I**, neohop-13(18)-ene **II** and hope-21-ene **IV** as a result of diplopterol dehydration (cf. Sessions et al., 2013).

G. sulfurreducens contained 12 mg hopanoids per g dry weight, corresponding to 6 mg per g carbon (Table 1). *G. metallireducens* synthesized 13 mg hopanoids per g dry wt. (Table 1), although the dry wt. comprised cells and minerals. This led to the assumption that the amount of hopanoids produced by *G. metallireducens* per g carbon was most likely higher (by an unknown factor) than that found for *G. sulfurreducens*. This is in agreement with a previous study in which *G. metallireducens* was found to produce 10× the amount of hopanoids produced by *G. sulfurreducens* (Härtner et al., 2005). The great majority of all hopanoids in both extracts were BHPs, predominantly composite BHPs (98% of all hopanoids; Table 1, Fig. 3). Free BHT **VII**, common in many hopanoid synthesizing bacteria, was present only in trace amount in both *Geobacter* species. While C_{30} hopanoids and free BHT were scarce in our cultures, three composite BHPs were found to be highly dominant

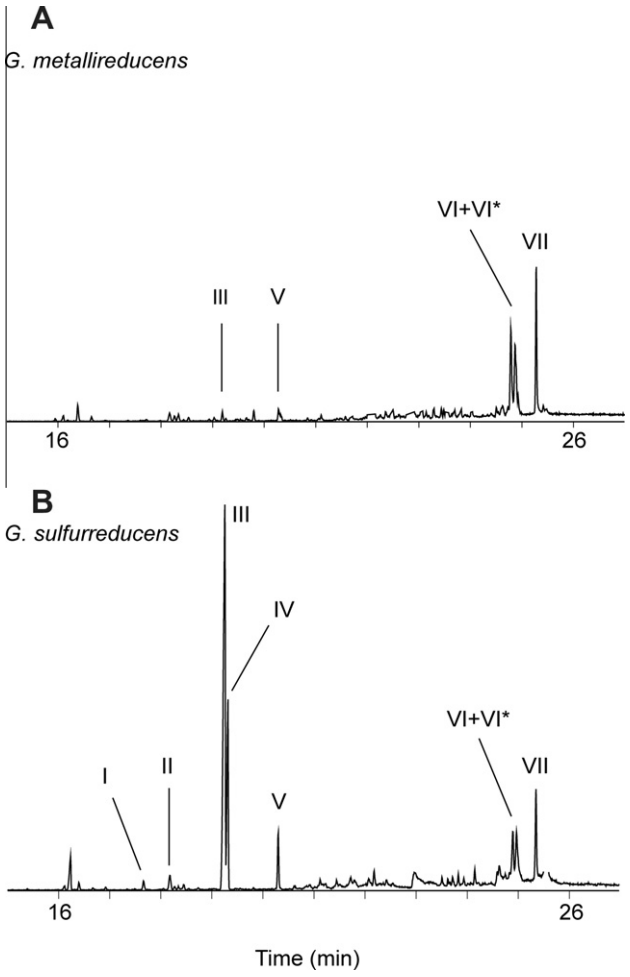

Fig. 2. GC ion chromatograms (m/z 191) of acetylated total lipid extracts of cultures of *G. metallireducens* grown with Fe(III)-citrate (A), and of *G. sulfurreducens* grown with fumarate (B) as electron acceptor. Cultures were harvested in late exponential growth phase. Numbered compounds: I, hop-17(21)-ene; II, neohop-13(18)-ene; III, diploptene; IV, hop-21-ene; V, diplopterol; VI, 32,35-anhydrobacteriohopanetetrol; VII, bacteriohopanetetrol; * stereo isomer. For structures see Fig. 1.

Table 1
Hopanoid inventory of *Geobacter metallireducens* and *Geobacter sulfurreducens* harvested at late stage exponential growth phase after 2 days. Relative abundance is given as % of total hopanoids (nd, not determined).

Compound[a,b]	*G. metallireducens*	*G. sulfurreducens*
C_{30} hopanoids		
III Diploptene	<1	2
V Diplopterol	<1	<1
C_{35+} hopanoids		
VII Free BHT[c]	<1	<1
VIII BHT–CE[c,d]	98	54
X Guanidine-substituted BHT-CE[c,d]	1	21
IX BHT-glucosamine[c]	<1	22
Absolute amount hopanoids		
mg/g Dry sample wt.	13	12
mg/g Carbon	nd	6

[a] C_{30} hopanoids and bacteriohopanetetrol were analyzed using GC–MS and C_{35+} hopanoids using HPLC–APCI–MS.
[b] For structures see Fig. 1.
[c] BHT, bacteriohopanetetrol.
[d] CE, cyclitol ether.

(Table 1). Interestingly, their relative abundance varied significantly between the two species. The major hopanoid synthesized by *G. sulfurreducens* was BHT-cyclitol ether **VIII** (54%; Table 1, Fig. 3); guanidine-substituted BHT-cyclitol ether **X** (21%) and BHT-glucosamine **IX** (22%) were produced in equal and still high abundance. In contrast, *G. metallireducens* produced almost exclusively BHT-cyclitol ether **VIII** (98%; Table 1, Fig. 3), which clearly distinguished the two strains from each other; 32,35-anhydrobacteriohopanetetrol (anhydroBHT) **VI** was detected as two stereoisomers **VI** and **VI*** during GC analysis only (Fig. 2). AnhydroBHT is known as a degradation product of free BHT and other composite BHPs (e.g. Bednarczyk et al., 2005; Talbot et al., 2005; Schaeffer et al., 2008, 2010). As it was not detected during HPLC analysis, it might have been generated as an artifact during GC analysis. Because of its low abundance vs. the composite BHPs, it does not have an impact on the quantification of the BHPs, the putative precursors of anhydroBHT. Fischer et al. (2005) predicted the synthesis of penta- and hexafunctionalized BHPs by *G. sulfurreducens*, because they found C_{30} and C_{31} hopanols after applying HIO_4 acid cleavage. In our cultures, we could not detect any penta- or hexafunctionalized BHPs. This, however, does not rule out their presence in trace amount in the analyzed cells or their production under different growth conditions.

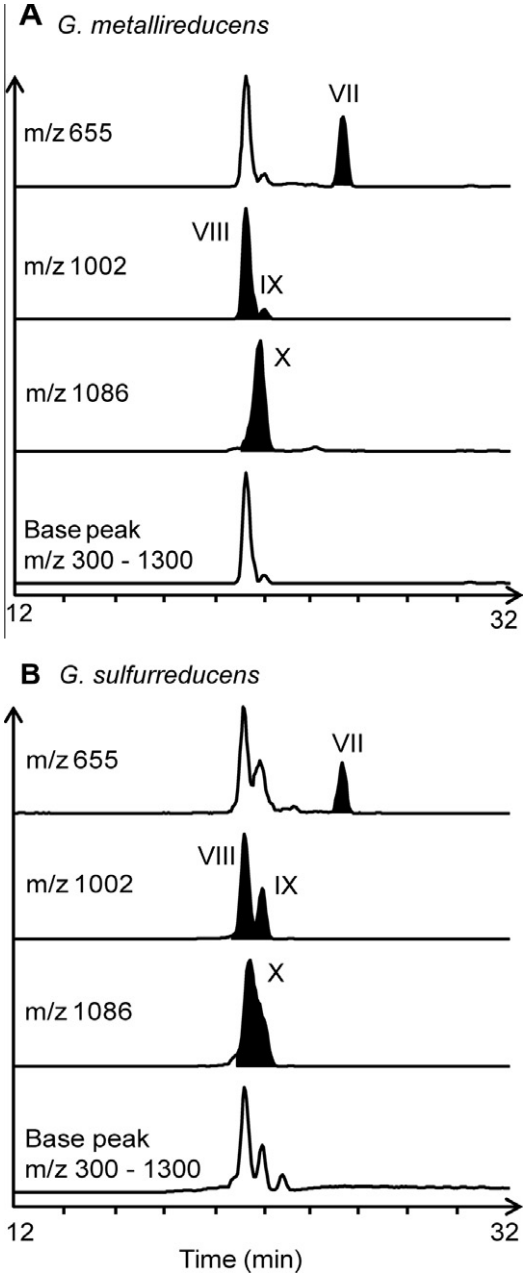

Fig. 3. HPLC–APCI–MS partial mass chromatograms showing bacteriohopanepolyols in acetylated total lipid extracts of cultures of *G. metallireducens* grown with Fe(III)-citrate (A), and cultures of *G. sulfurreducens* grown with fumarate (B) as electron acceptor. Numbered compounds: VII, free bacteriohopanetetrol (BHT); VIII, BHT-cyclitol ether; IX, BHT-glucosamine; X, guanidine-substituted BHT-cyclitol ether. For structures see Fig. 1.

Moreover, neither species produced C-2 or C-3 methylated hopanoids. These compounds are of interest because of their rarity among hopanoid synthesizing bacteria and the resulting potential as diagnostic biomarkers. C-2 methylated hopanoids are produced by various cyanobacteria (Rohmer et al., 1984; Summons et al., 1999; Talbot et al., 2008) and some Alphaproteobacteria (e.g. Bisseret et al., 1985; Vilchèze et al., 1994; Bravo et al., 2001; Rashby et al., 2007), while the HpnP enzyme necessary for their production is contained in several additional phyla (Welander et al., 2010). C-3 methylated hopanoid production has been found only in aerobic methanotrophs and acetic acid bacteria (Zundel and Rohmer, 1985a,b; Talbot et al., 2001), while the HpnR enzyme necessary for their production is contained in a diverse set of phyla (Welander and Summons, 2012). Based on amino acid sequences, both *Geobacter* strains contain proteins with relatively low homology to the C-2 methylase HpnP (*G. sulfurreducens*: 35.80% and 29.50% identity, *G. metallireducens*: 35.60% and 28.40% identity) and the C-3 methylase HpnR (*G. sulfurreducens*: 34.20%, 26.40% and 25.80% identity, *G. metallireducens*: 27.70%, 26.60% and 24.40% identity). The enzymes HpnP and HpnR are characterized as radical S-adenosylmethionine (SAM) domain proteins, as are the proteins identified in *Geobacter* with homologies to HpnP and HpnR. SAM domain proteins are a set of proteins that can catalyze diverse biochemical reactions that may or may not include C-2 or C-3 methylation of hopanoids. However, maximum likelihood trees constructed by Welander et al. (2010; HpnP) and Welander and Summons (2012; HpnR) show that *Geobacter* strains most likely do not contain genes for the enzymes necessary for C-2 or C-3 hopanoid methylation.

4. Implications of the lipid patterns of *G. sulfurreducens* and *G. metallireducens* for the biomarker concept

Even though facultative anaerobic bacteria have been known for quite some time to produce hopanoids in laboratory cultures (Rohmer et al., 1984), hopanoids were not known to be produced by anaerobic bacteria in the environment. The first significant occurrence of hopanoids in anaerobes that are widespread in various environments was reported for cultured anammox planctomycetes (Sinninghe Damsté et al., 2004). Soon after, hopanoid production in anaerobic cultures of *G. sulfurreducens* and *G. metallireducens* was recognized (Fischer et al., 2005; Härtner et al., 2005). The ubiquity, high abundance and metabolic diversity of the Geobacteraceae led to the speculation that they might be one of the most prominent sources of hopanoids in anoxic environments. In various immature anoxic sediments tetrafunctionalized BHPs, including composite BHPs, are highly abundant (e.g. Innes et al., 1997; Farrimond et al., 2000; Talbot and Farrimond, 2007; Zhu et al., 2011). BHT-cyclitol ether is very common among the composite BHPs found in the environment, whereas BHT-glucosamine has only been found in a few environmental samples (Talbot and Farrimond, 2007; Coolen et al., 2008; Talbot et al., 2008; Kim et al., 2011). Based on our new lipid data, *Geobacter* could indeed be a major source of hopanoids in various anoxic environments, taking into account that the major portion of hopanoids produced by the two species investigated were tetrafunctionalized composite BHPs (Table 1). The less widespread occurrence of BHT-glucosamine and guanidine-substituted BHT-cyclitol ether, which has only been found in a few soil samples (Cooke, 2010), could be a result of fast degradation of these composite BHPs to free BHT, but this requires further investigation.

Both *Geobacter* species produced BHT-cyclitol ether as the major composite hopanoid, whereas BHT-glucosamine and guanidine-substituted BHT-cyclitol ether occurred in significant amount only in *G. sulfurreducens*. *G. sulfurreducens* is known to tolerate low levels of O_2, whereas *G. metallireducens* is strictly anaerobic. The new lipid data can now be used to better characterize sedimentary microenvironments, assuming that similar BHPs are produced under changing growth conditions. Since both *Geobacter* cultures were grown under strictly anoxic conditions and both were harvested in the exponential phase, it cannot be excluded that the inventory of composite BHPs may change under varying growth conditions, especially under microaerophilic conditions for *G. sulfurreducens*. However, as the BHP patterns of *G. sulfurreducens* and *G. metallireducens* were found to be rather similar, it seems more likely that compound abundance would change as a function of environmental conditions, with the combination of compounds remaining unchanged.

BHT-cyclitol ether, the most abundant BHP in both cultures, was first isolated as the major BHP from the facultative methanotroph *Methylobacterium organophilum* (Renoux and Rohmer, 1985). It is also produced by pink-pigmented facultative methylotrophs (PPFMs; Knani et al., 1994), *Zymomonas mobilis* (Renoux and Rohmer, 1985), the purple non-sulfur bacterium *Rhodoblastus acidophilus* (formerly *Rhodopseudomonas acidophila*) (Neunlist et al., 1988), strains related to *Burkholderia* (Cvejic et al., 2000), acetic acid bacteria including *Frateuria aurantia* (Joyeux et al., 2004) and by cyanobacteria (Talbot et al., 2008; Sáenz et al., 2012), often as major BHP. BHT-glucosamine is produced by mostly the same groups of bacteria as BHT-cyclitol ether, such as *Zymomonas* (Flesch and Rohmer, 1989), methylotrophic bacteria (Renoux and Rohmer, 1985; Knani et al., 1994), *Alicyclobacillus* (Langworthy et al., 1976; Rohmer et al., 1993) and some cyanobacteria (Talbot et al., 2008), but has not been found to be produced by purple non-sulfur bacteria or *Burkholderia*-like strains. While BHT-cyclitol ether, BHT-glucosamine and free BHT are produced by various groups of bacteria, guanidine-substituted BHT-cyclitol ether has been found here for the first time in a pure culture of bacteria other than methylotrophs (cf. Renoux and Rohmer, 1985; Knani et al., 1994). Otherwise, it has only been detected in a cyanobacterial enrichment culture (Talbot et al., 2008). Based on this apparently limited distribution, guanidine-substituted BHT-cyclitol ether is possibly more specific for *Geobacter* spp. than the other BHPs.

Interestingly, the hopanoid inventory of *G. sulfurreducens* is very similar to that of PPFMs (Knani et al., 1994) and *M. organophilum* (Renoux and Rohmer, 1985), with the exception of high amounts of 2-methyldiplopterol and free BHT in the latter two. PPFMs are closely related to the genus *Methylobacterium* (Green et al., 1988; Knani et al., 1994). They differ mainly in the ability of the latter to oxidize methane (Green and Bousfield, 1981) and are collectively referred to as PPFMs below. Methylotrophs are a remarkable group of bacteria because of their ability to use reduced carbon compounds with no carbon–carbon bonds (i.e. C_1 compounds) such as MeOH or $MeNH_2$ as sole carbon source. The genome of *G. sulfurreducens* predicts its capability to use C_1 compounds for energy generation (Methé et al., 2003). Both Geobacteraceae and PPFMs are distributed in various environments, can use a variety of carbon sources and have great metabolic versatility (Knani et al., 1994 and references therein; Methé et al., 2003; Aklujkar et al., 2009). Although they are not closely related, similar adaptations and metabolic pathways may possibly be a factor for the similarity in hopanoid patterns.

The most prominent difference between most bacterial strains mentioned above and *Geobacter* is that the former are mostly aerobic or facultative anaerobic bacteria, while both *Geobacter* strains were grown anaerobically. Although production of composite BHPs was known in a few anaerobic bacterial strains, the most common and abundant BHPs, such as free BHT, aminotriol, adenosylhopane and BHT-cyclitol ether (Neunlist et al., 1985, 1988; Flesch and Rohmer, 1989; Hermans et al., 1991; Rashby et al., 2007; Welander et al., 2012; this study), as well as several of the less common compounds, such as 2-Me-BHT, BHT-glucosamine, guanidine-substituted BHT-cyclitol ether, aminotetrol and aminopentol (Flesch and Rohmer, 1989; Hermans et al., 1991; Llopiz et al., 1992; Rashby et al., 2007; Blumenberg et al., 2012; this study) have now been detected in anaerobic cultures.

Bacteria with the pattern of composite BHPs most similar to that of *Geobacter* are aerobic PPFMs and some cyanobacteria. PPFMs are often found in association with aquatic or terrestrial plants and cyanobacteria thrive in the oxic photic zone of lacustrine or marine water columns, while *Geobacter* is found in aquatic or terrestrial anoxic sediments. Because the groups of bacteria with a similar BHP pattern do inhabit environments other than *Geobacter*, this pattern is apparently specific when found in anoxic environments. The recognition of the observed uncommon combination of composite BHPs in anoxic sediments may be taken as the first evidence for the presence of *Geobacter*. However, because hopanoids produced by PPFMs on plant surfaces or by cyanobacteria in the water column may eventually also end up in sediments, the source organisms cannot be determined with certainty (see also Talbot and Farrimond, 2007; Talbot et al., 2008). In the light of such ambiguity, other accompanying hopanoids might provide further evidence, allowing discrimination between inputs from the different bacterial groups. In such a scenario, high abundances of diplopterol, 2-methyldiplopterol and free BHT would indicate an input from PPFMs (Knani et al., 1994), even if the coexistence of *Geobacter* species could not be excluded. *Geobacter* species prefer freshwater environments, while *Desulfurumonas* species of the same order as the Geobacteraceae family are commonly responsible for metal reduction in marine environments (Roden and Lovley, 1993; Coates et al., 1995). Although marine nitrogen-fixing cyanobacteria produce a high amount of BHT-cyclitol ether (Talbot et al., 2008), as does *Geobacter*, they are of no concern for the biomarker concept in lacustrine environments favoured by *Geobacter*. Lacustrine cyanobacteria may produce some BHT-cyclitol ether, but a high abundance of free BHT is much more common in lacustrine cyanobacteria (Talbot et al., 2008), as is a high abundance of C-2 methylated BHPs, especially 2-Me-BHT (Summons et al., 1999; Talbot et al., 2008). Pentafunctionalized BHPs are also synthesized by some cyanobacteria. The presence of these specific hopanoids would identify an input from cyanobacteria, but again not excluding a possible coexistence of *Geobacter*.

The sediment depth at which hopanoids are found also needs to be considered. The composite BHPs produced by *Geobacter*, PPFMs, and cyanobacteria are easily degradable and the complex moiety at C-35 of the functionalized side chain will be lost quickly. The presence of composite BHPs in deeper sediments indicates either a terrigenous source (e.g. Handley et al., 2010; Sáenz et al., 2011) along with rapid transport through the water column and effective preservation in environments typified by high sedimentation rate, or sedimentary bacteria like *Geobacter* as source organisms. BHT-cyclitol ether has occasionally been reported, but the source organisms were not identified with certainty at a time when the production of these composite BHPs by anaerobic bacteria was unknown. On that basis, it had been suggested that these hopanoids in anoxic sediments derived from aerobic bacteria that flourished in the water column or the sediment surface (e.g. Innes et al., 1997; Farrimond et al., 2000). Only in a more recent study has an increase in the content of BHT-cyclitol ether with increasing depth in peat sediments been suggested to reflect an anaerobic source organism (van Winden et al., 2012). In anoxic settings, the ubiquitous and metabolically versatile *Geobacter* is a more likely source organism for BHT-cyclitol ether than aerobic bacteria thriving in the water column, although a terrigenous source and riverine transport in the form of particulate organic matter prior to sedimention cannot be excluded.

Because composite BHPs are prone to degradation and do not withstand diagenetic conditions, they are unlikely to occur in older, especially thermally mature, sediments. Based on the scenario that many geohopanoids derive from composite BHPs, it is possible that *Geobacter* is a major source of BHPs (especially free BHT) in anoxic environments. The composite BHTs found in *Geobacter* are precursors of BHT, C_{32} hopanols and C_{32} hopanoic acids – the most prevalent components in early diagenetic sediments – and hopanoid hydrocarbons (geohopanoids) in more mature sediments and sedimentary rocks. As a consequence, *Geobacter*-specific hopanoid markers are probably difficult to trace in older sediments. However, the characteristic inventory of composite BHPs in

Geobacter allows for the recognition of this important group of bacteria in young sediments using the biomarker concept.

5. Conclusions

Anaerobic cultures of *G. sulfurreducens* and *G. metallireducens* produce composite bacteriohopanepolyols (BHPs). The recognition of a pattern of composite BHPs specific for *Geobacter* is significant, calling for reinterpretation of findings of these intact composite BHPs in anoxic sediments. Such findings have been interpreted to reflect input from aerobic bacteria thriving in the water column or the uppermost sedimentary column, or from a terrigenous source. Based on our results from strictly anaerobic cultures of *Geobacter*, it seems more likely that these bacteria are the source of composite BHPs in anoxic sediments.

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