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Targeted genomic detection of biosynthetic pathways: anaerobic production of hopanoid biomarkers by a common sedimentary microbe

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ABSTRACT

The lipid biomarker principle requires that preservable molecules (molecular fossils) carry specific taxonomic, metabolic, or environmental information. Historically, an empirical approach was used to link specific taxa with the compounds they produce. The lipids extracted from numerous, but randomly cultured species provided the basis for the interpretation of biomarkers in both modern environments and in the geological record. Now, with the rapid sequencing of hundreds of microbial genomes, a more focused genomic approach can be taken to test phylogenetic patterns and hypotheses about the origins of biomarkers. Candidate organisms can be selected for study on the basis of genes that encode proteins fundamental to the synthesis of biomarker compounds. Hopanoids, a class of pentacyclic triterpenoid lipid biomarkers, provide an illustrative example. For many years, interpretations of biomarker data were made with the assumption that hopanoids are produced only by aerobic organisms. However, the recent discovery of ^{13}C-depleted hopanoids in environments undergoing anaerobic methane oxidation and in enrichment cultures of anammox planctomycetes indicates that some hopanoids are produced anaerobically. To further examine the potential distribution of hopanoid biosynthesis by anaerobes, we searched publicly available genomic databases for the presence of squalene-hopene cyclase genes in known obligate or facultative anaerobes. Here we present evidence that *Geobacter sulfurreducens, Geobacter metallireducens,* and *Magnetospirillum magnetotacticum*, all bacteria common in anoxic environments, have the appropriate genes for hopanoid biosynthesis. We further show that these data accurately predict that *G. sulfurreducens* does produce a variety of complex hopanoids under strictly anaerobic conditions in pure culture.

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INTRODUCTION

Biomarker lipids are natural products that carry taxonomic or metabolic information. The degree of species-specificity and the ability to assign environmental interpretations to these compounds depend on the extent to which their sources are understood. Traditionally our understanding of biomarker distributions – including both taxonomically unique and more generic compounds – has been discovered empirically, without prior knowledge of the putative genetic capacity to make the biomarker in question (e.g. Volkman *et al.*, 1980; Rohmer *et al.*, 1984; Volkman *et al.*, 1992). Broad surveys of numerous taxa (Rohmer *et al.*, 1984) or specific studies targeted at likely candidate organisms (Volkman *et al.*, 1980; Volkman *et al.*, 1994*)* are used to determine the origins of biomarker lipids. Without the resources to examine all of the natural products made by organisms living today (never mind all the species that have ever existed), these studies of model organisms have provided an incomplete foundation for interpreting environmental data. The degree to which this random survey approach leads to a robust data set is limited by the number of taxa that are screened and by the effort involved in cultivating many species. Therefore we recently have begun to use publicly available genomic data to provide additional insight into the biosynthesis and phylogenetic distribution of lipids (Pearson *et al.*, 2003). This genomics approach also comes with limitations. The primary shortcomings are the small number of complete genomes in current databases and bias in the diversity of organisms chosen for sequencing. The advantages, however, are the rapidly increasing number of

available genomes and the ability to select organisms in a direct and efficient, rather than random, manner for cultivation and lipid analysis. Here we focus on an old and persistent question of organic geochemistry: are there species that live exclusively or predominantly as anaerobes, which usually inhabit sedimentary environments and which also produce hopanoids?

Hopanoids are pentacyclic isoprenoid lipids derived from the acyclic triterpene squalene. The carbon skeletons of hopanoids, hopanes, are resistant to degradation and are preserved in abundance in sedimentary rocks and petroleums from the late Archean to the present (Brocks *et al.*, 2003a). Hopanoid biosynthesis is widely distributed in bacteria (Rohmer *et al.*, 1984), and the 2-methyl and 3-methyl isomers in particular have been used as biomarkers for cyanobacteria (Summons *et al.*, 1999) and aerobic methylotrophs (Zundel & Rohmer, 1985; Summons & Jahnke, 1992), respectively. Although many of the hopanoid-producing bacteria contain measurable quantities of the C_{30}-hopanoids diploptene and diplopterol, the major hopanoid products in bacterial membranes are C_{35}-bacteriohopanepolyols (Rohmer *et al.*, 1984). The concentration of bacteriohopanepolyols in bacterial cells is comparable to sterol concentrations in eukaryotes, reflecting the presumed role of these compounds in membrane rigidity and permeability (Ourisson *et al.*, 1987).

Twenty years ago, a seminal survey of the distribution of hopanoids in more than 90 cultured strains of prokaryotes revealed that their biosynthesis is not universal (Rohmer *et al.*, 1984). Roughly half of the bacterial strains surveyed contained hopanoids, and there did not appear to be any clear phylogenetic or metabolic affinities associated with their production. Hopanoids were found in some, but not all, cyanobacteria, Gram-positive (*Bacillus* spp., *Streptomyces* spp.) and Gram-negative bacteria (numerous genera of proteobacteria). The only prokaryotic groups that categorically did not contain hopanoids were the purple and green sulphur bacteria and the archaea (then called archaebacteria). Despite the lack of a requirement for molecular oxygen during biosynthesis, hopanoids were not found in any species classified as an obligate anaerobe. This observation was among the most distinguishing characteristics of this study and of subsequent reports (Neunlist *et al.*, 1985; Ourisson *et al.*, 1987). Although hopanoids were not found in any strict anaerobes, they were produced by some facultative aerobes including all investigated members of the purple non-sulphur bacteria (*Rhodomicrobium* spp., *Rhodopseudomonas* spp., and *Rhodospirillum* spp.) and the fermentative α-proteobacterium *Zymomonas mobilis* (Rohmer *et al.*, 1984; Neunlist *et al.*, 1985; Ourisson *et al.*, 1987). Despite the observation that hopanoids occurred in the purple non-sulphur bacteria, the absence of hopanoids in anaerobes became a commonly held assumption in biomarker studies. The relative scarcity of non-sulfidic, photic zone anoxia – conditions amenable to anaerobic production of hopanoids by Rhodospirillaceae and relatives – may have contributed to the relative neglect of this known anaerobic source. Instead, the primary interpretation applied to geological samples is that hopanoids are principally created by aerobic bacteria in oxic environments. This assumption has since been applied to argue for methane hydrate destabilization and release into the water column during the late Quaternary (Hinrichs, 2001; Hinrichs *et al.*, 2003). The detection of hopanoids in 2.7–2.5 billion year old sedimentary successions in Western Australia also has been used to argue for the advent of oxygenic photosynthesis in the late Archean (Brocks *et al.*, 2003a, 2003b).

However, several recent biomarker studies have yielded additional data that is at odds with the classic interpretation of hopanoid sources, suggesting that hopanoids are created by anaerobes in at least some sedimentary environments (Elvert *et al.*, 2000; Pancost *et al.*, 2000; Thiel *et al.*, 2001; Thiel *et al.*, 2003). In each of these studies, hopanoids were found in samples taken from environments mediating the anaerobic oxidation of methane (AOM). Stable carbon isotopic analyses ($\delta^{13}C$ values) of individual compounds revealed strong ^{13}C depletion in acyclic isoprenoid lipids such as crocetane, archaeol, and *sn*-2 hydroxyarchaeol, derived from archaea presumed to be the primary consumers of CH_4. In addition, ^{13}C-depleted hopanoids also were reported in these samples. The hopanoid distribution in AOM environments does not include 3-methylhopanoids (a biomarker for aerobic methylotrophs (Zundel & Rohmer, 1985; Summons & Jahnke, 1992)). Furthermore, synthesis of hopanoids by aerobic bacteria in these instances would require that the hopanoids were produced remotely and subsequently transported into the AOM system; this explanation also seems unsatisfactory. It would be difficult to generate the level of ^{13}C depletion in these hopanoids anywhere other than within the AOM system. However, it remains unknown what phylogenetic group(s) of bacteria could be potential sources of the hopanoids.

Further evidence for production of hopanoids by another group of anaerobes comes from enrichment cultures containing a high proportion of anammox planctomycetes (Sinninghe Damsté *et al.*, 2004). These bacteria have a unique metabolism consuming both nitrite and ammonia to yield dinitrogen (Strous *et al.*, 1999) and are strictly anaerobic. Although no pure cultures have been isolated, the enrichment cultures contain sufficient concentrations of hopanoids that production by the planctomycetes, rather than by satellite species, is likely. A relatively large depletion in ^{13}C is observed for the hopanoids in these cultures. This also is consistent with a source from the planctomycetes: the unique planctomycete lipids called ladderanes have a ^{13}C fractionation relative to CO_2 of 32–47‰, similar to the isotopic values of the hopanoids (Schouten *et al.*, 2004). Anammox bacteria may contribute hopanoids to sediments in anaerobic environments.

In summary, the production of hopanoids in anaerobic systems is becoming apparent, and therefore, the challenge is how to find additional species that might be responsible. The availability of hundreds of complete and partial microbial genomic sequences provides a more directed approach than

random screening of anaerobic cultures. It is possible to discover a candidate organism using a database search, scanning for genes encoding proteins necessary for the biosynthesis of hopanoids. This approach was applied previously to identify a sterol biosynthetic pathway in a planctomycete (Pearson *et al.*, 2003). Because contamination or coculturing can be a significant issue for natural products studies, here we also expand on the method of Pearson *et al.* (2003) to include microscopic observation and cloning and sequencing. This establishes the purity of the culture, thus definitively linking a species with its lipid product.

The approach is widely applicable: (1) find a candidate organism by searching genomic databases for genes encoding proteins exclusive to the synthesis of a particular biomarker; (2) grow and demonstrate a pure culture of the candidate strain; and (3) extract and analyze lipids from the culture to confirm production of the compound of interest. Among the requirements of this approach are that at least one enzyme exclusive to the biosynthetic pathway must be known, the protein amino acid sequence of this enzyme must exhibit significant homology between species, and it is only possible to search among organisms that have been sequenced and made available to the public. Here we apply this method to yield further insight into the anaerobic taxa responsible for production of hopanoids.

METHODS

The protein amino acid sequence for squalene-hopene cyclase of *Synechocystis* sp. PCC 6803 (GI:16330570, Accession NP_441298) was compared using the BLAST search program against all complete and partial prokaryotic genomes available through the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) using translated protein nucleotide BLAST (tBLASTn). All putative squalene-hopene cyclases (similarity scores 1000–370; expect values < $1e^{-100}$) were compiled and those belonging to known aerobes were eliminated. Facultative aerobes such as *Rhodopseudomonas* spp. also were discarded, as hopanoid production in these species is already known. The only species remaining after elimination of obligate and facultative aerobes were *Geobacter sulfurreducens* PCA (genomic sequence available at http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=ggs); *Geobacter metallireducens* GS-15, currently available only as a whole-genome shotgun sequence (http://genome.jgi-psf.org/draft_microbes/geome/geome.home.html); and *Magnetospirillum magnetotacticum* (http://genome.jgi-psf.org/draft_microbes/magma/magma.home.html). This search was conducted in early 2004. We selected *G. sulfurreducens*, a δ-proteobacterium and common anaerobe, as a candidate strain to examine the biosynthesis of hopanoids by this ecologically important anaerobic group.

G. sulfurreducens was obtained from the American Type Culture Collection (ATCC strain 51573). Cells were inoculated 1 : 10 into hopanoid-free *Geobacter*-fumarate medium (ATCC medium 1957) prepared anaerobically under a mixture of 20% CO_2, 80% N_2 and containing 10 μL of the redox indicator resazurin (2 mg mL^{-1} stock) and 50 μL of 5% w/v cysteine. Anaerobic conditions were maintained using the Hungate technique, and cells were propagated 1 : 10 (1 : 100 relative to the original inoculum) into 20 × 10 mL for bulk culturing; resazurin remained colourless, indicating O_2 levels below typical detection limits of 0.1 mg L^{-1}. Cells were harvested after 10 days by centrifugation. Visual inspection of formalin-fixed cells by fluorescent microscopy was performed using DAPI stain (4′,6-diamidino-2-phenylindole); only one morphology was visible. To further assess any contamination of the culture, genomic DNA was extracted from an aliquot of cultured cells, was amplified by polymerase chain reaction (PCR) using the universal bacterial primers 27F (5′-3′ AGA GTT TGA TCM TGG CTC AG) and 1492R (5′-3′ TAC GGY TAC CTT GTT ACG ACT T), and cloned using commercial products (Invitrogen TOPO® cloning kit). Twenty random clones were selected for sequencing. Of the 20 clones, 17 yielded usable sequence reads, all of which were ≥ 97% identical to the 16S rDNA gene from the complete published genome of *G. sulfurreducens* strain PCA (Methé *et al.*, 2003). No effort was made to reduce *Taq* errors or sequencing errors; this probably accounts for the difference between the observed 97% similarity and the commonly accepted species cutoff of 98% similarity.

Whole cells were extracted by the method of Bligh & Dyer (1959). The polyfunctionalized side chains of the bacteriohopanepolyols were cleaved to permit detection and analysis by gas chromatography-mass spectrometry (GC-MS). The total lipid extract was treated with periodic acid and the resulting aldehydes were reduced to primary alcohols by $LiAlH_4$ (similar to method of Rohmer *et al.*, 1984). Inherent to this oxidation–reduction method is a loss of information about the specific polyfunctionalized side chains as periodic acid cleaves sugars at vicinal-diol functions. However the method enables reliable positive detection of the hopanoid carbon skeleton. The product alcohols were derivatized to – OTMS ethers by heating with bis(trimethylsily)trifluoroacetamide plus 1% trimethylchlorosilane (BSTFA/TMCS) and pyridine at 60 °C for 1 h and analyzed by GC-MS. GC-MS analyses were performed on an Agilent 6890 GC coupled to a 5873 mass selective detector (MSD), equipped with a 60 m CP-Sil5 column (100% dimethylpolysiloxane, equivalent to DB-1). To further confirm the compounds present in *G. sulfurreducens*, the hopanoids in an additional aliquot of total lipid extract (TLE) were converted to acetate derivatives by heating with acetic anhydride and pyridine at 60 °C for 1 h. The acetates were analyzed by GC-MS along with similarly treated hopanoid concentrates from the cyanobacterium *Phormidium luridum* and the methanotroph *Methylococcus capsulatus*. These standard extracts contain a variety of tetra-, penta-, and hexafunctionalized hopanoids and their 2β-methyl and 3β-methyl counterparts

Table 1 BLAST results vs. squalene-hopene cyclase of *Synechocystis* sp. PCC 6803

Species	Accession no.	Score (bits)	E-value	Sequence* [599 . . . 605]
G. sulfurreducens PCA	NC_002939	615	e^{-175}	TG**F**PKFF (c1)†
		478	e^{-136}	TG**F**PRVF (c2)
G. metallireducens GS-15	NZ_AAAS02000041	628	e^{-179}	TG**F**PKYF (c1)
		469	e^{-131}	TG**F**PRVF (c2)
M. magnetotacticum MS-1	NZ_AAAP01003383	433	e^{-120}	TG**F**PRVF
Bacillus cereus ATCC 10987	NC_003909	282	$4e^{-75}$	TG**L**PGGF

*Standard numbering for SHC refers to *Alicyclobacillus acidocaldarius*; residue F601 is required for hopanoid production (Hoshino & Sato, 2002).

†, *Geobacter sulfurreducens* and *Geobacter metallireducens*, each have two copies of SHC.

(Summons & Jahnke, 1992; Summons *et al.*, 1996; Summons *et al.*, 1999; Jahnke *et al.*, 2004; R. E. Summons, unpublished). Final identification of the hopanoids was determined by comparison of both the retention times and the respective mass spectra to published spectra of trimethylsilyl (TMS) ethers and to the standard *Phormidium* mixture as acetates.

RESULTS

BLAST similarity searches of the protein sequence for squalene-hopene cyclase (SHC) of *Synechocystis* sp. PC 6803 vs. microbial genomes submitted to the NCBI revealed that nearly 10% of all currently sequenced microbial genomes contained one or more putative SHC homologue (approximately 35 of more than 350 genomes; data not shown). Significant similarity was defined by an Expect Value smaller than $1e^{-100}$ and by detailed confirmation of critical functional motifs necessary for propagation of the cyclization reaction (Hoshino & Sato, 2002). This very specific cutoff ($1e^{-100}$ corresponded to amino acid sequence identity of 34%, positives 52%) separated the true SHCs from the paralogous genes for oxidosqualene cyclases. All sequences having Expect Values of intermediate or ambiguous significance (Expect Values between approximately $1e^{-35}$ and $1e^{-75}$), could be eliminated from candidacy as putative SHCs according to the absence of critical amino acids (Hoshino & Sato, 2002). In particular, this included numerous sequences from *Bacillus* spp. which lacked the critical phenylalanine, F601 (Table 1). This is consistent with prior reports that *Bacillus* spp. do not synthesize hopanoids (Rohmer *et al.*, 1984; Ourisson *et al.*, 1987); and it suggests that the cutoff between true SHCs and non-functional but similar homologues was placed correctly.

Of the sequences found, only three were from anaerobes having phylogenetic classification outside of the hopanoid-producing purple non-sulphur bacteria (e.g. *Rhodospirillum*, *Rhodomicrobium* [Neunlist *et al.*, 1985]). These three anaerobic or microaerophilic species were *Geobacter sulfurreducens*, *Geobacter metallireducens*, and *Magnetospirillum magnetotacticum*. Table 1 shows the BLAST search results for these species. It is also significant to note that no SHC homologues were found in any of the archaeal genomes currently available. Of the three species listed in Tables 1, *G. sulfurreducens* was selected for culturing and lipid analysis.

G. sulfurreducens synthesizes a variety of hopanoids, which are present in non-trace quantities (approximately 1 mg g^{-1} wet weight of culture; Fig. 1). The major product is a C_{32} derivative (II), which on the basis of comparison of its mass spectrum and retention time with the hopanols from *Phormidium luridum* we identified as 17β(H), 21β(H)-bishomohopan-32-ol. This compound is derived from (I) tetrafunctionalized bacteriohopanepolyols (TBHP), C_{35} hopanoids that are hydroxylated, or otherwise functionalized, in the terminal four carbon atoms of the side chain. Compound II was accompanied by another C_{32} homologue with a double bond ($C_{32:1}$). The mass spectral fragmentation pattern indicated that the unsaturation in the $C_{32:1}$ hopanoid occurs in the side chain, because the loss of this fragment yields a prominent ion at 369 m z^{-1}, corresponding to a saturated ring system (Fig. 1; Table 2). 17β(H), 21β(H)-bishomohopan-32-ol and its side chain unsaturated analog comprised 90% of the total hopanoids. The C_{31} hopanoid (9%), identified on the basis of retention time and mass spectrum, is 17β(H), 21β(H)-homohopan-31-ol (Table 2), which derives from pentafunctionalized precursors such as bacteriohopanepentol or related cyclitol ethers (Rohmer *et al.*, 1984). Finally, we identified traces of a C_{30}-derivative which is probably hopan-30-ol, derived from hexafunctionalized precursors. It is noteworthy that the distribution of cleaved hopanoids from *G. sulfurreducens* is very similar to that of *P. luridum*, with the exception that we did not detect any 2-methylhopanoids in *G. sulfurreducens*.

These findings indicate that the biosynthesis of hopanoids in *G. sulfurreducens* occurs under anoxic conditions, and that we were able to correctly predict the formation of hopanoids based on prior genomic sequence data. Additionally, the only 16S rDNA genes we were able to amplify from our culture are all from *G. sulfurreducens* with ≥ 97% similarity, demonstrating that contamination of the culture by unrelated bacteria is unlikely.

DISCUSSION

The detection of biosynthetic pathways for polycyclic triterpenoid lipids is particularly amenable to the genomic

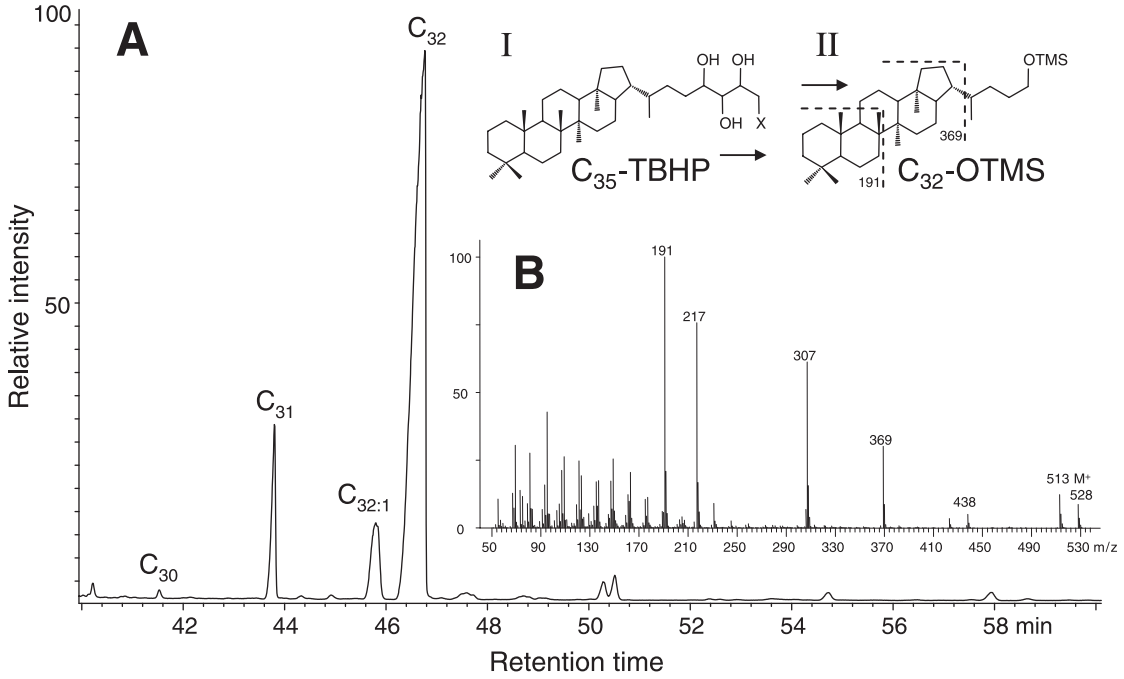

Fig. 1 (A) GC-MS total ion chromatogram revealing the variety of hopanoids produced by *Geobacter sulfurreducens*. (B) Mass spectrum from the largest peak at 46.5 min denotes the TMS derivative of 17β, 21β-bishomohopanol (C_{32}-OTMS). During the oxidation–reduction sequence, the vicinal functional groups of bacteriohopanepolyols are cleaved, leaving only the first hydroxy group. It is therefore inferred that the abundant C_{32}-OTMS product (II) is derived from tetrafunctionalized bacteriohopanepolyols (C_{35}-TBHP) (I); X = OH, NH_2, or sugar and amino acid derivatives. The presence of C_{30}, C_{31}, $C_{32:1}$, and the minor peaks eluting after 48 min point to a rich array of additional complex bacteriohopanepolyols in *G. sulfurreducens*.

Table 2 Major ions of hopanol products analyzed as trimethylsilyl ethers

Compound	Abbreviation	Major ions
17β(H), 21β(H)-bishomohopan-32-ol	C_{32}	191(100), 217(75), 307(62), 369(29), 528(10; m^+)
17β(H), 21β(H)-bishomohopen-32-ol*	$C_{32:1}$	191(80), 215(72), 369(64), 526(5; m^+)
17β(H), 21β(H)-homohopan-31-ol	C_{31}	191(100), 205(80), 293(40), 369(20), 514(10; m^+)
17β(H), 21β(H)-hopan-30-ol	C_{30}	189(100), 191(80), 207(60), 279(40), 369(5), 500(5; m^+)

*Double bond in unidentified side chain location.

survey approach. This is not correspondingly true for other biosynthetic or metabolic pathways for which the necessary genes are either (1) unknown, or (2) poorly conserved in their sequence similarity. The production of pentacyclic and tetracyclic triterpenoids from the linear isoprenoid precursor, squalene, requires a very strict conformational homology of the cyclase enzymes squalenehopene cyclase and oxidosqualene cyclase, respectively (Wendt *et al.*, 2000; Hoshino & Sato, 2002; Rajamani & Gao, 2003). This leads to high similarity scores for sequence comparisons using statistical approaches such as BLAST. Because of this specificity, it is unlikely that we have overlooked additional anaerobic hopanoid producers among the nearly 400 microbial genomes that have been sequenced currently.

It is of particular interest that we find hopanoids in the Geobacteraceae, because these species have flexible metabolisms that are well-suited to anoxic environments. We assume that in addition to *Geobacter sulfurreducens*, *Geobacter metallireducens* also produces hopanoids. We did not grow *G. metallireducens* for this study, because the intensely orange-colored Fe(III) citrate medium for this species prevents rigorous monitoring of anaerobic growth conditions using the resazurin indicator.

The Geobacteraceae are found abundantly in anoxic environments (Cummings *et al.*, 2003). Members of this bacterial group have been detected in freshwater (Lovley & Phillips, 1986; Stein *et al.*, 2001), marine (Coates *et al.*, 1995) and estuarine sediments (Caccavo *et al.*, 1992), subsurface aquifers (Coates *et al.*, 1999), and environments with organic (Anderson *et al.*, 1998; Coates *et al.*, 1999) and metal (Cummings *et al.*, 1999; Holmes *et al.*, 2002) pollutants. It is possible that Geobacteraceae are responsible for much of

the *in situ* production of hopanoids in anaerobic sediments, given their ubiquity (Cummings *et al.*, 2003), abundance (e.g. Gibbs-Eggar *et al.*, 1999), and metabolic diversity.

G. sulfurreducens has the ability to anaerobically oxidize acetate completely to carbon dioxide using a wide variety of terminal electron acceptors, including metal ions, sulphur, and fumarate (Methé *et al.*, 2003). Once thought to be a strict anaerobe, *G. sulfurreducens* can tolerate and grow under low concentrations of O_2 (Lin *et al.*, 2004), which allows it to be better suited to fluctuating conditions within sediments. *G. sulfurreducens* has been detected in strictly anaerobic environments as well as in systems experiencing variable redox conditions; however, its predominant environmental growth niche appears to be anaerobic sediments. This suggests that most of the hopanoids produced by Geobacteraceae under *in situ* conditions would be produced in anaerobic zones and that sedimentary hopanoids synthesized by anaerobes could source from Geobacteraceae, planctomycetes, and the as yet unexplored *Magnetospirillum magnetotacticum*. This contrasts with hopanoid biosynthesis by the Rhodospirillaceae and relatives (Rohmer *et al.*, 1984; Neunlist *et al.*, 1985; Rohmer *et al.*, 1992); the anaerobic synthesis of hopanoids by these purple non-sulphur bacteria primarily would occur under the relatively unusual (on contemporary Earth) conditions of photic zone anoxia.

It has been known for some time that O_2 is not required for the synthesis of bacteriohopanepolyols, and thus the presence of O_2 in the environment is not a necessary interpretation when analyzing geologically important hopanoids. Nevertheless, biomarker studies proceeded with the assumption that these lipids were the products of aerobic bacteria, largely based on the early surveys of diverse bacterial taxa (e.g. Rohmer *et al.*, 1984). There are now numerous pieces of evidence suggesting that there could be widespread production of hopanoids in anaerobic systems. ^{13}C-depleted hopanoids from AOM sedimentary horizons (Elvert *et al.*, 2000; Pancost *et al.*, 2000; Thiel *et al.*, 2001; Thiel *et al.*, 2003) argue for *in situ* biosynthesis by organisms linked metabolically to the oxidation of methane or consumption of the immediate byproducts of this process. The presence of hopanoids in anaerobic planctomycetes argues for these species as sources of hopanoids in environments in which the anammox process is a significant component of the nitrogen cycle (e.g. the Black Sea, Kuypers *et al.*, 2003). Because of the widespread environmental distribution and diverse metabolism of the Geobacteraceae, it is possible that this group is indeed a primary source of these lipids in many anaerobic environments. Hopanoid biomarker data, both in more recent deposits (e.g. Hinrichs, 2001; Hinrichs *et al.*, 2003) and in the geological record (e.g. Brocks *et al.*, 2003a, 2003b), can no longer solely be interpreted to reflect the input of aerobic organisms or aerobic processes.

The biosynthesis of hopanoids in anaerobic environments may occur frequently in nature. In addition to biosynthesis by the Geobacteraceae, Rhodospirilliaceae, and planctomycetes, there may be other taxa also responsible for producing these lipids in anoxic water columns and sediments. Among these strains may be the sedimentary microbe, *M. magnetotacticum*. We did not grow this species as part of the present study, but the genomic search data indicate that *M. magnetotacticum* contains a protein homologous to squalene-hopene cyclase from other known hopanoid producers. This species also commonly grows anaerobically, in or near aerobic-anaerobic transition zones (Spring & Bazylinski, 2000); along with *G. metallireducens*, it also may be a major environmental source of biogenic magnetite (Bazylinski, 1999; Vali *et al.*, 2004).

High-throughput sequencing methods are increasing greatly the number of sequenced microbial genomes. There also have been significant advances in the ability to cultivate difficult species (e.g. Kaberlein *et al.*, 2002; Rappé *et al.*, 2002). This greater availability of raw data and of culturing approaches makes it probable that many new strains of hopanoid producers will be discovered in the near future; it is likely that more anaerobes will be found among them.

Although the production of bacteriohopanepolyols appears to be restricted to bacteria and is not present in archaea, the bacterial diversity of known hopanoid producers is taxonomically and metabolically widespread. Further metabolic or taxonomic information may be provided by studies of particular subsets of structures within the hopanoid lipid class. This may include an increased understanding of the phylogenetic distribution of methylhopanoids (Zundel & Rohmer, 1985; Summons & Jahnke, 1992; Summons *et al.*, 1999), or extend to the classification of the particular functional groups attached to the C_{35} position of the side chain (Talbot *et al.*, 2003; Talbot *et al.*, 2004).

Note added in proof. An additional paper has just appeared (Härtner *et al.*, 2005) which also demonstrates the presence of hopanoids in *G. sulfurreducens*. The salient conclusions of both papers are similar and reinforce the idea that Geobacteraceae produce hopanoids under anaerobic growth conditions. Härtner *et al.* (2005) do not address this question from a genomic perspective, and thus do not comment on *M. magnetotacticum*.

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