

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

W. W. FISCHER,¹ R. E. SUMMONS² AND A. PEARSON¹

¹Department of Earth and Planetary Sciences, Harvard University, Cambridge, Massachusetts, 02138, USA

²Department of Earth, Atmospheric, and Planetary Sciences, Massachusetts Institute of Technology, Cambridge, Massachusetts, 02139, USA

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 ¹³C-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.

Received 6 December 2004; accepted 1 February 2005

Corresponding author: W. W. Fischer. Tel.: 617/495-7602; fax: 617 495 8839; e-mail: wfischer@fas.harvard.edu

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 petroleum 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 C₃₀-hopanoids diploptene and diplopterol, the major hopanoid products in bacterial membranes are C₃₅-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 archaeobacteria). 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., *Rhodospseudomonas* 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 ($\delta^{13}\text{C}$ 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 CH₄. 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 CO₂ 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 *Rhodospirillum rubrum* 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 inocu-

lated 1 : 10 into hopanoid-free *Geobacter*-fumarate medium (ATCC medium 1957) prepared anaerobically under a mixture of 20% CO₂, 80% N₂ and containing 10 μ L of the redox indicator resazurin (2 mg mL⁻¹ stock) and 50 μ L 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 \times 10 mL for bulk culturing; resazurin remained colourless, indicating O₂ 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 $\geq 97\%$ identical to the 16S rDNA gene from the complete published genome of *G. sulfurreducens* strain PCA (Méthé *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 LiAlH₄ (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(trimethylsilyl)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 2 β -methyl and 3 β -methyl counterparts

Species	Accession no.	Score (bits)	E-value	Sequence* [599 . . . 605]
<i>G. sulfurreducens</i> PCA	NC_002939	615	e^{-175}	TGFPKFF (c1)†
		478	e^{-136}	TGFPRVF (c2)
<i>G. metallireducens</i> GS-15	NZ_AAAS02000041	628	e^{-179}	TGFPKYF (c1)
		469	e^{-131}	TGFPRVF (c2)
<i>M. magnetotacticum</i> MS-1	NZ_AAAP01003383	433	e^{-120}	TGFPRVF
<i>Bacillus cereus</i> ATCC 10987	NC_003909	282	$4e^{-75}$	TGLPGGF

*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.

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

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 C₃₂ 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), C₃₅ hopanoids that are hydroxylated, or otherwise functionalized, in the terminal four carbon atoms of the side chain. Compound II was accompanied by another C₃₂ 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 C₃₁ 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 C₃₀-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

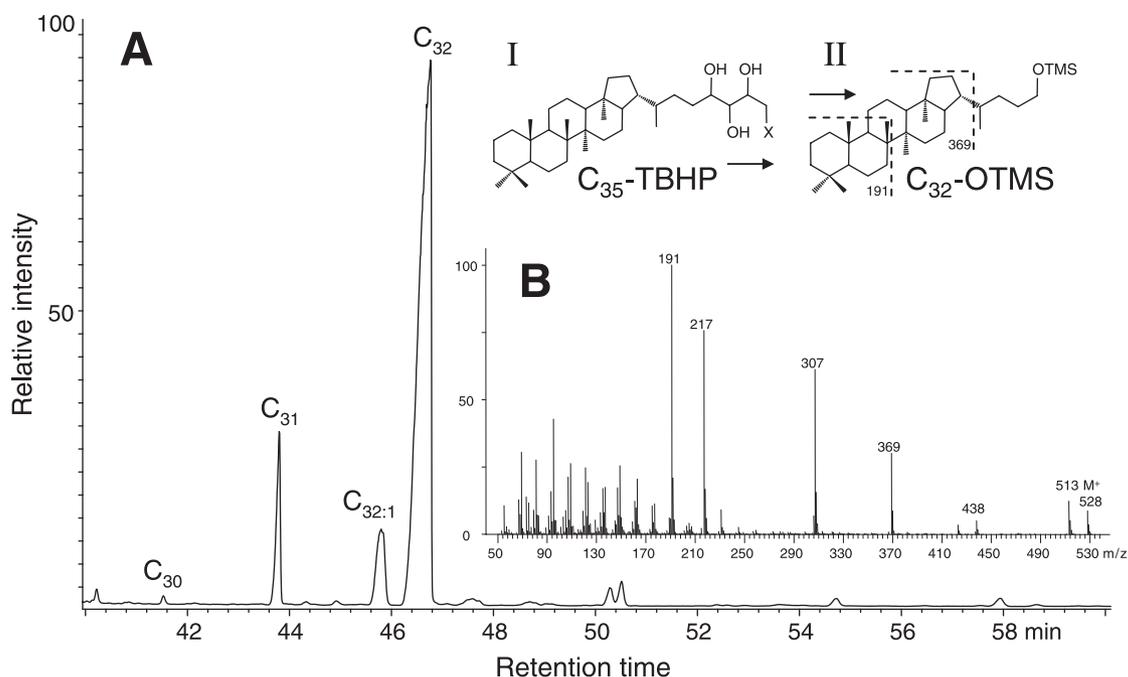


Fig. 1 (A) GC-MS total ion chromatogram revealing the variety of hopanoids produced by *Geobacter sulfurreducens*. (B) Mass spectrum from the largest peak at 46.5 min denotes the TMS derivative of 17 β , 21 β -bishomohopan-32-ol (C_{32} -OTMS). During the oxidation–reduction sequence, the vicinal functional 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, NH₂, 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
17 β (H), 21 β (H)-bishomohopan-32-ol	C_{32}	191(100), 217(75), 307(62), 369(29), 528(10; m ⁺)
17 β (H), 21 β (H)-bishomohopen-32-ol*	$C_{32:1}$	191(80), 215(72), 369(64), 526(5; m ⁺)
17 β (H), 21 β (H)-homohopan-31-ol	C_{31}	191(100), 205(80), 293(40), 369(20), 514(10; m ⁺)
17 β (H), 21 β (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 squalenohopene 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 (Méthé *et al.*, 2003). Once thought to be a strict anaerobe, *G. sulfurreducens* can tolerate and grow under low concentrations of O₂ (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 O₂ is not required for the synthesis of bacteriohopanepolyols, and thus the presence of O₂ 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. ¹³C-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 by-products 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, Rhodospirillaceae, 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 C₃₅ 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*.

ACKNOWLEDGEMENTS

We thank Alex Bradley for help with the GC-MS; and Meredith Fisher for guidance with PCR, cloning, and sequencing. This work was supported by NSF grant EAR-0311937 to A.P., and by NASA Exobiology grant NAG5-1236 to R.E.S. W.W.F. thanks the Agouron Institute for support. Sequence data for *Geobacter sulfurreducens*, *Geobacter metallireducens*, and *Magnetospirillum magnetotacticum* available in GenBank were produced by The Institute for Genomic Research (<http://www.tigr.org>) and by the Joint Genome Institute (<http://www.jgi.doe.gov>). Sequencing of all of these strains was accomplished with funding from the US Department of Energy.

REFERENCES

- Anderson RT, Rooney-Varga JN, Gaw CV, Lovley DR (1998) Anaerobic benzene oxidation in the Fe (III) reduction zone of petroleum-contaminated aquifers. *Environmental Science and Technology* **32**, 1222–1229.
- Bazylnski DA (1999) Synthesis of the bacterial magnetosome: the making of a magnetic personality. *International Microbiology* **2**, 71–80.
- Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37**, 911–917.
- Brocks JJ, Buick R, Logan GA, Summons RE (2003a) Composition and syngeneity of molecular fossils from the 2.78–2.45 billion-year-old Mount Bruce Supergroup, Pilbara Craton, Western Australia. *Geochemica et Cosmochemica Acta* **67**, 4289–4319.
- Brocks JJ, Buick R, Summons RE, Logan GA (2003b) A reconstruction of Archean biological diversity based on molecular fossils from the 2.78–2.45 billion-year-old Mount Bruce Supergroup, Hamersley Basin, Western Australia. *Geochemica et Cosmochemica Acta* **67**, 4321–4335.
- Caccavo F, Jr, Blakemore RP, Lovley DR (1992) A hydrogen-oxidizing Fe (III)–reducing micro-organism from the Great Bay estuary, New Hampshire. *Applied and Environmental Microbiology* **58**, 3211–3216.
- Coates JD, Ellis DJ, Gaw CV, Lovley DR (1999) *Geothrix fermentans* general nov., sp. nov., a novel Fe (III)–reducing bacterium from a hydrocarbon-contaminated aquifer. *International Journal of Systematic Bacteriology* **49**, 1615–1622.
- Coates JD, Lonergan DJ, Lovley DR (1995) *Desulfuromonas palmitatis* sp. nov., a long-chain fatty acid-oxidizing Fe (III) reducer from marine sediments. *Archives of Microbiology* **64**, 406–413.
- Cummings DE, Caccavo F, Jr, Fendorf S, Rosenzweig RF (1999) Arsenic mobilization by the dissimilatory Fe (III)–reducing bacterium *Shewanella alga* BrY. *Environmental Science and Technology* **33**, 723–729.
- Cummings DE, Snoeyenbos-West OL, Newby DT, Niggemeyer AM, Lovley DR, Achenbach LA, Rosenzweig RF (2003) Diversity of Geobacteraceae species inhabiting metal-polluted freshwater lake sediments ascertained by 16S rDNA analyses. *Microbial Ecology* **46**, 257–269.
- Elvert M, Suess E, Greinert J, Whiticar MJ (2000) Archaea mediating anaerobic methane oxidation in deep-sea sediments at cold seeps of the eastern Aleutian subduction zone. *Organic Geochemistry* **31**, 1175–1187.
- Gibbs-Eggar Z, Jude B, Dominik J, Loizeau J-L, Oldfield F (1999) Possible evidence for dissimilatory bacterial magnetite dominating the magnetic properties of recent lake sediments. *Earth and Planetary Science Letters* **168**, 1–6.
- Härtner T, Straub KL, Kannenberg E (2005) Occurrence of hopanoid lipids in anaerobic *Geobacter* species. *FEMS Microbiology Letters* **243**, 59–64.
- Hinrichs KU (2001) A molecular recorder of methane hydrate destabilization. *Geochemistry Geophysics Geosystems* **2**, 2000GC000118. <http://www.agu.org/journals/gc> [Last access 25 February 2005]
- Hinrichs KU, Hmelo LR, Sylva SP (2003) Molecular fossil record of elevated methane levels in late Pleistocene coastal waters. *Science* **299**, 1214–1217.
- Holmes DE, Finneran KT, O'Neil RA, Lovley DR (2002) Enrichment of members of the family *Geobacteraceae* associated with stimulation of dissimilatory metal reduction in uranium-contaminated aquifer sediments. *Applied and Environmental Microbiology* **68**, 2300–2306.
- Hoshino T, Sato T (2002) Squalene-hopene cyclase: catalytic mechanism and substrate recognition. *Chemical Communications* **4**, 291–301.
- Jahnke LL, Embaye T, Hope J, Turk KA, Van Zuilen M, Des Marais DJ, Farmer JD, Summons RE (2004) Lipid biomarker and carbon isotopic signatures for stromatolite-forming microbial mat communities and *Phormidium* cultures from Yellowstone National Park. *Geobiology* **2**, 31–47.
- Kaberlein T, Lewis K, Epstein SS (2002) Isolating 'uncultivable' micro-organisms in pure culture in a simulated natural environment. *Science* **296**, 1127–1129.
- Kuypers MMM, Sliemers AO, Lavik G, Schmid M, Jorgensen BB, Kuene JB, Damste JSS, Strous M (2003) Anaerobic ammonium oxidation by anammox bacteria in the Black Sea. *Nature* **422**, 608–611.
- Lin WC, Coppi MV, Lovley DR (2004) *Geobacter sulfurreducens* can grow with oxygen as a terminal electron acceptor. *Applied and Environmental Microbiology* **70**, 2525–2528.
- Lovley DR, Phillips EJP (1986) Organic matter mineralization with reduction of ferric iron in anaerobic sediments. *Applied and Environmental Microbiology* **51**, 683–689.
- Methé BA, Nelson KE, Eisen JA, Paulsen IT, Nelson W, Heidelberg JF, Wu D, Wu M, Ward N, Beanan MJ, Dodson RJ, Madupu R, Brinkac LM, Daugherty SC, DeBoy RT, Durkin AS, Gwinn M, Kolonay JF, Sullivan SA, Haft DH, Selengut J, Davidsen TM, Zafar N, White O, Tran B, Romero C, Forberger HA, Weidman J, Khouri H, Feldblyum TV, Utterback TR, Van Aken SE, Lovley DR, Fraser CM (2003) Genome of *Geobacter sulfurreducens*: metal reduction in subsurface environments. *Science* **302**, 1967–1969.
- Neunlist S, Holst O, Rohmer M (1985) Prokaryotic triterpenoids. The hopanoids of the purple non-sulfur bacterium *Rhodomicoccus vannielii*: an aminotriol and its aminoacyl derivatives, N-tryptophanyl and N-ornithyl aminotriol. *European Journal of Biochemistry* **147**, 561–568.
- Ourisson G, Rohmer M, Poralla K (1987) Prokaryotic hopanoids and other polyterpenoid sterol surrogates. *Annual Reviews of Microbiology* **41**, 301–333.
- Pancost RD, Sinninghe Damsté JS, De Lint S, Van Der Maarel MJEC, Gottschal JC, Medinaut Shipboard Scientific Party (2000) Biomarker evidence for widespread anaerobic methane oxidation in Mediterranean sediments by a consortium of methanogenic archaea and bacteria. *Applied and Environmental Microbiology* **66**, 1126–1132.
- Pearson A, Brocks JJ, Budin M (2003) Phylogenetic and biochemical evidence for sterol synthesis in the bacterium, *Gemmata obscuriglobus*. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 15,352–15,315,357.
- Rajamani R, Gao J (2003) Balancing kinetic and thermodynamic control: The mechanism of carbocation cyclization by squalene cyclase. *Journal of the American Chemical Society* **125**, 12,768–12,712,781.
- Rappé MS, Connon SA, Vergin KL, Giovannoni SJ (2002) Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. *Nature* **418**, 630–633.
- Rohmer M, Bissleret P, Neunlist S (1992) The hopanoids, prokaryotic triterpenoids and precursors of ubiquitous molecular fossils. In: *Biology Markers in Sediments and Petroleum* (eds Moldowan JM, Albrecht P, Philp RP). Prentice Hall, New York, pp. 1–17.
- Rohmer M, Bouvier-Nave P, Ourisson G (1984) Distribution of hopanoid triterpenes in Prokaryotes. *Journal of General Microbiology* **130**, 1137–1150.
- Schouten S, Strous M, Kuypers MMM, Rijpstra WIC, Baas M, Schubert CJ, Jetten MSM, Damste JSS (2004) Stable carbon

- isotopic fractionations associated with inorganic carbon fixation by anaerobic ammonium-oxidizing bacteria. *Applied and Environmental Microbiology* **70**, 3785–3788.
- Sinninghe Damsté JS, Rijpstra WIC, Schouten S, Fuerst JA, Jetten MSM, Strous M (2004) The occurrence of hopanoids in planctomycetes: implications for the sedimentary biomarker record. *Organic Geochemistry* **35**, 561–566.
- Spring S, Bazylinski DA (2000) Magnetotactic bacteria. In: *The Prokaryotes*. Published on the web at <http://www.springer-ny.com/>. Springer-Verlag, New York.
- Stein LY, La Duc MT, Grundl TJ, Nealson KH (2001) Bacterial and archaeal populations associated with freshwater ferromanganous micronodules and sediments. *Environmental Microbiology* **3**, 10–18.
- Strous M, Fuerst JA, Kramer EHM, Logeman S, Muyzer G, van de Pas-Schoonen KT, Webb R, Kuenen JG, Jetten MSM (1999) Missing lithotroph identified as new planctomycete. *Nature* **400**, 446–449.
- Summons RE, Jahnke LL (1992) Hopanes and hopanes methylated in ring-A: correlation of the hopanoids from extant methylotrophic bacteria with their fossil analogs. In: *Biology Markers in Sediments and Petroleum* (eds Moldovan JM, Albrecht P, Philp RP). Prentice Hall, New York, pp. 182–200.
- Summons RE, Jahnke LL, Hope JM, Logan GA (1999) 2-methylhopanoids as biomarkers for cyanobacterial oxygenic photosynthesis. *Nature* **400**, 554–557.
- Summons RE, Jahnke LL, Simoneit BRT (1996) Lipid biomarkers for bacterial ecosystems: studies of cultured organisms, hydrothermal environments and ancient sediments. *Evolution of Hydrothermal Ecosystems on Earth (and Mars?)*, CIBA Foundation Symposium 202, Wiley, Chichester, pp. 174–194.
- Talbot HM, Summons R, Jahnke L, Farrimond P (2004) Characteristic fragmentation of bacteriohopanepolyols during atmospheric pressure chemical ionization liquid chromatography/ion trap mass spectrometry. *Rapid Communications in Mass Spectrometry* **17**, 2788–2796.
- Talbot HM, Watson DF, Pearson EJ, Farrimond P (2003) Diverse biohopanoid compositions of nonmarine sediments. *Organic Geochemistry* **34**, 1353–1371.
- Thiel V, Blumenberg M, Pape T, Seifert R, Michaelis W (2003) Unexpected occurrence of hopanoids at gas seeps in the Black Sea. *Organic Geochemistry* **34**, 81–87.
- Thiel V, Peckmann J, Richnow HH, Luth U, Reitner J, Michaelis W (2001) Molecular signals for anaerobic methane oxidation in Black Sea seep carbonates and a microbial mat. *Marine Chemistry* **73**, 97–112.
- Vali H, Weiss B, Yi YL, Sears SK, Kim SS, Kirschvink JL, Zhang L (2004) Formation of tabular single-domain magnetite induced by *Geobacter metallireducens* GS-15. *Proceedings of the National Academy of Sciences (USA)* **101**, 16,121–16,126.
- Volkman JK, Barrett SM, Dunstan GA, Jeffrey SW (1992) C₃₀–32 alkyl diols and unsaturated alcohols in microalgae of the class Eustigmatophyceae. *Organic Geochemistry* **18**, 131–138.
- Volkman JK, Eglinton G, Corner EDS, Sargent, JR (1980) Novel unsaturated straight-chain C₃₇–39 methyl and ethyl ketones in marine sediments and a coccolithophore *Emiliania huxleyi*. In: *Advances in Organic Geochemistry, 1979* (eds Douglas AG, Maxwell JR.). Pergamon Press, pp. 219–227.
- Volkman JK, Barret SM, Dunstan GA (1994) C₂₅ and C₃₀ highly branched isoprenoid alkenes in laboratory cultures of two marine diatoms. *Organic Geochemistry* **21**, 407–413.
- Wendt KU, Schulz GE, Corey EJ, Liu DR (2000) Enzyme mechanisms for polycyclic triterpene formation. *Angewandte Chemie International Edition* **39**, 2812–2833.
- Zundel M, Rohmer M (1985) Hopanoids of the methylotrophic bacteria *Methylococcus capsulatus* and *Methylomonas* sp. as possible precursors for the C₂₉ and C₃₀ hopanoid chemical fossils. *FEMS Microbiology Letters* **28**, 61–64.