Anaerobic 3-Methylhopanoid Production By An Acidophilic Phototrophic Purple Bacterium

Marisa Mayer (✉ mhmayer@stanford.edu)

Stanford University School of Earth Energy and Environmental Sciences <https://orcid.org/0000-0002-0493-0163>

Mary N. Parenteau

NASA Ames Research Center

Megan L. Kempfer

University of Oklahoma: The University of Oklahoma

Michael T. Madigan

Southern Illinois University Carbondale

Linda L. Jahnke

NASA Ames Research Center

Paula V. Welander

Stanford University School of Earth Energy and Environmental Sciences

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Abstract

Bacterial lipids are well preserved in ancient rocks and certain ones have been used as indicators of specific bacterial metabolisms or environmental conditions existing at the time of rock deposition. Here we show that an anaerobic bacterium produces 3-methylbacteriohopanepolyols (3-MeBHPs), pentacyclic lipids previously detected only in aerobic bacteria and widely used as biomarkers for methane-oxidizing bacteria. Both *Rhodopila globiformis*, a phototrophic purple nonsulfur bacterium isolated from an acidic warm spring in Yellowstone, and a newly isolated *Rhodopila* species from a geochemically similar spring in Lassen Volcanic National Park (USA), synthesized 3-MeBHPs and a suite of related BHPs and contained the genes encoding the necessary biosynthetic enzymes. Our results show that 3-MeBHPs can be produced under anoxic conditions and challenges the use of 3-MeBHPs as biomarkers of oxic conditions in ancient rocks and as prima facie evidence that methanotrophic bacteria were active when the rocks were deposited.

Introduction

Bacteriohopanepolyols (BHPs), or hopanoids, are triterpenoid lipids that help preserve membrane integrity and permeability in certain bacteria (Ricci et al. 2017). BHPs are also quite recalcitrant biomolecules; their hopane derivatives can be preserved in sedimentary rocks for billions of years, and because of this, have been exploited as biomarkers of past environmental conditions or particular microbial activities (Brocks et al. 2005). While BHPs are produced by metabolically-diverse bacteria, BHPs methylated in the A-ring are more restricted in their distribution and linked to particular bacterial taxa or aerobic metabolisms. For example, BHPs methylated at the C-2 position (2-MeBHPs) have traditionally been linked to cyanobacteria (Summons et al. 1999) whereas BHPs methylated at the C-3 position (3-MeBHPs) have been associated with strictly aerobic methanotrophic and acetic acid bacteria (Zundel and Rohmer 1985).

In 2007, 2-MeBHP production was reported in photosynthetically-grown cultures of the purple nonsulfur (PNS) bacterium *Rhodopseudomonas (Rps.) palustris* (Rashby et al. 2007), a widely distributed species that inhabits freshwater lakes and fertile soils (Harwood and Gibson 1988). PNS bacteria are a phylogenetically diverse group of anoxygenic phototrophs that preceded cyanobacteria on Earth by at least 500 million years, may have been responsible for the earliest of the banded iron formations (Widdel et al. 1993), and whose photosynthetic metabolism is strictly anaerobic (Hohmann-Marriott and Blankenship 2011). These *Alpha*- and *Betaproteobacteria* inhabit various aquatic environments, including lakes, wastewaters, hot springs, and marine and hypersaline waters and typically can conserve energy from both photosynthesis (anoxic/light) or respiration (oxic/dark) (Madigan and Jung 2009). Subsequent metagenomic analyses showed that *hpnP*, the gene encoding the enzyme that methylates BHP at the C-2 position, was present in microbes from samples of a variety of microbial ecosystems, suggesting that 2-MeBHP production is widespread in nature (Ricci et al. 2015). Moreover, lipid analyses of microbial mat samples from hypersaline environments detected 2-MeBHPs in various bacteria, including species of PNS bacteria (Jahnke et al. 2014). Collectively, these discoveries demonstrated the danger in unambiguously

linking the presence of 2-methylhopanes (the breakdown product of 2-MeBHP) in ancient sediments to cyanobacteria or to oxic conditions in general.

In contrast to 2-MeBHPs, evidence to date has shown that BHPs methylated at the C-3 position (3-MeBHPs) (Summons et al. 1999; Rashby et al. 2007) are synthesized only by bacteria that perform O₂-dependent metabolisms, with the most prominent producers being aerobic methane-oxidizing bacteria (methanotrophs) and acetic acid bacteria (Zundel and Rohmer 1985). The *hpnR* gene that encodes the C-3 BHP methylase has also been detected in a diverse array of aerobic bacteria further confirming the link to O₂-dependent metabolisms (Welander and Summons 2012). In our studies of hot spring microbial mats, we detected 3-MeBHPs in mat samples collected from a sulfidic and acidic (pH 3–4) spring in Lassen Volcanic National Park (California, USA) that was fed by a continuous discharge of warm volcanic water containing CO₂, H₂, and H₂S (Fig. 1a, b). The mat lacked cyanobacteria but contained a purple-red layer underneath a green algal layer. Knowing that 3-MeBHPs have not been reported from algae, we pursued the Lassen purple bacterium as the possible source of these lipids and, using standard enrichment and isolation techniques, obtained a pure culture of this phototroph (Fig. 1c). Here we show that pure cultures of this organism and its phylogenetic close relative produce a suite of 3-MeBHPs when grown under strictly anoxic conditions—the first report of the production of these hopanoid lipids in an anaerobic bacterium—and that their genomes encode the requisite enzymes for producing these lipids and methylating them in the C-3 position.

Materials And Methods

Organisms, isolation, and growth conditions

Lipid analyses and genomic studies were performed on axenic cultures of four purple nonsulfur bacteria: *Rhodopila* strain LVNP, *Rhodopila globiformis*, *Rhodopseudomonas palustris*, and *Rhodoblastus acidophilus* (the latter three species from the collection of MTM). *Rhodopila* strain LVNP was isolated from a microbial mat that formed in an acidic (pH 3.9), sulfidic spring in Lassen Volcanic National Park (near 40°27'09.5"N 121°32'13.3"W, Northern California, U.S.A.). The mat had an upper algal layer and a lower purple-red layer (Fig. 1). A sample of the purple layer was incubated in liquid medium (Pfennig 1974) under photosynthetic conditions at 25°C, and an axenic culture was eventually obtained from successive transfers of isolated colonies grown on plates of the same medium incubated in a Mitsubishi AnaeroPack 2.5L Rectangular Jar (Thermo Scientific Cat No. R685025). Anoxic conditions in liquid culture media were achieved by vigorously sparging under filtered argon (0.2 µm filter) for 5 min and then reducing media with sulfide (added from a sterile sodium sulfide solution neutralized to pH 7.5; final sulfide concentration in the medium was 0.14 mM) prior to inoculation. Liquid cultures were grown phototrophically in sealed tissue culture flasks incubated in the AnaeroPak Jar illuminated with incandescent light.

Lipid and genomic analyses

For lipid analyses, dense cell suspensions from liquid cultures were collected by centrifugation at 4,500 x g for 10 min at 4°C. Lipids were extracted by the modified Bligh-Dyer protocol as previously described (Jahnke et al. 1992) using both a monophasic ratio of water:methanol:chloroform (4:10:5) and physical agitation. Large cyclic lipids were treated with a redox procedure that yields both methylated and unmethylated BHPs and then analyzed as their acetate derivatives (Parenteau et al. 2014) by gas chromatography-mass spectrometry (GC-MS) (Jahnke et al. 1992). Hopanoids were identified from their precise mass measurements and fragmentation patterns and by comparison of relative retention time and mass spectra to previously published analytical data (Parenteau et al. 2014). Liquid chromatography-mass spectrometry (LC-MS) was performed as previously described (Talbot et al. 2003, 2007) and used to identify extended 3-MeBHPs and their derivatives.

Genomic DNA from *Rhodopila* LVNP was isolated using the Genomic Tip 500/G Kit (Qiagen Cat No. 10262) and sequenced by PacBio whole genome sequencing in collaboration with the 2016 Microbial Diversity Course (Marine Biological Laboratory, Woods Hole, U.S.A.). The genome was then assembled and annotated by the Joint Genome Institute annotation pipeline (Huntemann et al. 2015). Annotations of all potential hopanoid biosynthesis genes were confirmed by manual inspection.

Phylogenetic analyses of nearly complete (~ 1400 nucleotides) 16S rRNA genes amplified from genomic DNA were aligned with CLUSTAL X (Larkin 2007) and analyzed using BLASTN (Altschul et al. 1997) to generate a neighbor-joining tree using PHYLIP version 3.69 (Felsenstein 1989) with other details as previously described (Kempfer and Madigan 2012). HpnR sequences were identified in the Joint Genome Institute IMG microbial database by protein-protein BLAST (Altschul et al. 1990; Chen et al. 2019), aligned using MUSCLE (Edgar 2004), and maximum likelihood trees built using the PhyML model of evolution (Guindon et al. 2010; Lefort et al. 2017). The tree was then exported and annotated in iTOL (Letunic and Bork 2019).

Results

Characterization of a new *Rhodopila* isolate

Although *Rhodopila* (originally *Rhodopseudomonas*) *globiformis* has been known since 1974, a second isolate of this organism has until now not been reported. In field studies carried out by two of us (MHM and MNP), a spring was discovered in Lassen National Park (California, USA) that seemed geochemically similar to the warm acidic spring that yielded *Rpi. globiformis* (Pfennig 1974) and which contained a microbial mat of the strongly acidophilic green alga *Cyanidium* overlying a purple-red layer. Cells from the latter appeared similar to those of *Rps. globiformis* (large weakly motile cocci) and so cultures were pursued and eventually obtained. Considering its habitat, pigments, physiology, and cell morphology, the Lassen purple bacterium was thought to be a new strain of *Rhodopila globiformis* and thus was tentatively designated *Rhodopila* strain LVNP.

A 16S rRNA gene phylogenetic tree (Fig. 2) revealed that the Lassen and Yellowstone *Rhodopila* isolates were closely related yet phylogenetically distinct. Moreover, the genome of the Lassen isolate (8.1 Mb)

was significantly larger than that of *Rpi. globiformis* (7.2 Mb, Imhoff et al. 2018) and the average nucleotide identity between the two genomes was only 93.1%. Thus, the two strains may be separate *Rhodopila* species rather than strains of the same species. *Rhodopila* is the most acidophilic PNS bacterium known (Madigan and Imhoff, 2005) and produces unique purple-red carotenoids (Fig. 1c) (Schmidt and Liaaen-Jensen 1973) closely related to okenone, a carotenoid detected in 1.6 Gyr-old rocks from Northern Australia (Brocks et al. 2005, Brocks and Schaeffer 2008). *Rhodopila* is also phylogenetically distinct from other PNS bacteria and the only anaerobic and acidophilic phototroph that groups with the *Acetobacteraceae*, a bacterial family that includes acetic acid-producing bacteria and other aerobic and acidophilic bacteria (Kerstens et al. 2006); this can be seen clearly in Fig. 2.

Hopanoid analyses

Lipid analyses of pure cultures of both strain LVNP and the type strain of *Rpi. globiformis* (strain DSM 161^T), grown either photosynthetically or aerobically in the dark (Pfennig 1974), revealed 3-MeBHP production by both strains. Gas chromatographic–mass spectrometric analyses (Zundel and Rohmer 1985) confirmed that the hopanoids were indeed 3-MeBHPs (Fig. 3a) and not 2-MeBHPs (Fig. 3b). Strain LVNP grown phototrophically at pH 5 synthesized 0.9 µg BHP/mg total fatty acid and 0.03 µg 3-MeBHP/mg total fatty acid. By contrast, phototrophic cultures of the mildly acidophilic PNS bacterium *Rhodoblastus acidophilus* and the neutrophilic species *Rhodopseudomonas palustris* (Fig. 3) did not produce 3-MeBHPs; *Rps. palustris* did, however, contain 2-MeBHPs as was previously reported (Welander et al. 2010). Moreover, liquid chromatography–mass spectrometry analyses of cells of *Rhodopila* strain LVNP showed that it produced not only 3-MeBHP but a suite of BHPs. Although not quantified, LC-MS identified several extended BHPs, including bacteriohopanetetrols and bacteriohopanetetrol cyclitol ethers (Table 1).

Genomic evidence for 3-MeBHP production in *Rhodopila* species

To further explore the production of 3-MeBHP production in *Rhodopila*, the genome sequence of strain LVNP was determined and compared with the previously published genome of *Rpi. globiformis* DSM 161^T (Imhoff et al. 2018). Genomic analyses confirmed that both organisms were genetically equipped to produce 3-MeBHPs (Table 2). The C-3 BHP methylase HpnR is encoded in both genomes, and a phylogenetic tree constructed from HpnR sequences (Fig. 4) mirrored the 16S rRNA gene tree (Fig. 2). Specifically, HpnR from the *Rhodopila* species was related to HpnR from species of *Acetobacteraceae* and distinct from that produced by methanotrophic *Methylococcaceae* (Fig. 4); the latter are well-known producers of 3-MeBHPs but are only distant relatives of *Acetobacteraceae* (Fig. 2). Genes encoding several other hopanoid biosynthesis enzymes (Belin et al. 2018) were identified in the genomes of both *Rhodopila* strains (Table 2) consistent with the production of several related BHPs identified from cells of *Rhodopila* strain LVNP (Table 1).

Discussion

Our results are the first to show the production of 3-MeBHPs in bacteria grown anaerobically, thus refuting the contention that these lipids are only produced by obligately aerobic bacteria. It is thus possible, and perhaps even likely, that these lipids are produced by various other anaerobic species of *Bacteria*—however, still no species of *Archaea* have been shown to contain hopanoids (Sahm et al. 1993).

The physiological link between acetic-acid bacteria and *Rhodopila* does not revolve around energy metabolism but instead the ability of both organisms to thrive in strongly acidic habitats. Whether such a lifestyle requires these unusual lipids is unknown, but the fact that 3-MeBHPs are produced by many neutrophilic methanotrophic bacteria and have not been reported from some other potentially acidophilic bacteria, such as *Thiobacillus* (Rohmer et al. 1979), or other acidic habitats (Talbot et al. 2016), leaves this question unanswered. Nevertheless, 3-MeBHPs obviously play some role in the physiology of *Rhodopila* species, and the genetic links between this phototroph and acidophilic bacteria (Fig. 2) and the fact that 3-MeBHPs are membrane integrated, suggest that 3-MeBHPs may help maintain membrane function in their acidic habitats. Indeed, a function for hopanoids in maintaining membrane integrity and surviving general environmental stressors has been shown in the cyanobacterium *Nostoc* (Ricci et al. 2017). In addition, it has been shown that extended hopanoids aid the chemotrophic bacterium *Bradyrhizobium* in withstanding hypoxic/low O₂ growth conditions and various other physiological stressors (Kulkarni et al. 2015). Hence, if there exists a widespread link between 3-MeBHPs and microbes that inhabit extreme environments, it is possible that these lipids are produced by anoxygenic phototrophs that thrive in hypersaline, hyperalkaline, and permanently hot or cold environments; all of these habitats contain a diversity of purple bacteria (Madigan and Jung 2009; Jahnke et al. 2014).

Production of 3-MeBHPs by *Rhodopila* highlights the potential importance of anoxygenic phototrophs in the geological rock record and has at least two major geochronological implications. First, the fact that anaerobically grown *Rhodopila* species can produce 3-MeBHPs—lipids heretofore observed only in bacteria whose metabolism requires O₂—indicates that the presence of 3-methylhopanes (the degradation product of 3-MeBHP) in ancient rocks can no longer be used as *prima facie* evidence that oxic conditions existed at the time of deposition. Consequently, linking 3-methylhopanes to O₂-dependent bacterial metabolisms, such as aerobic methanotrophy (Brocks et al. 2005; Farrimond et al. 2004; Waldbauer et al. 2009), should be done cautiously and only with corroborating evidence.

Second, the relatively high abundance of 3-methylhopanes in some ancient sediments has been used to infer that freshwater was present in these past aquatic bodies (Brocks et al. 2005; French et al. 2020). This is because when sulfate is limiting, such as is typical in a freshwater lake, methanogenic *Archaea* outcompete sulfate-reducing bacteria for available electron donors (Hoehler et al. 1998). Thus, in ancient sediments containing 3-methylhopanes it has been assumed that the overlying body of water was fresh and that methanogenic microbes produced the methane that fed 3-Me BHP-containing methanotrophs. However, because our results show that 3-methylhopanes can no longer be unambiguously connected to methanotrophic (or any other obligately aerobic) bacteria, concluding that a given sedimentary rock

containing 3-methylhopanes must have formed in a freshwater environment (Brocks et al. 2005) may be erroneous.

Declarations

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Conflicts of interest/competing interests: The authors declare they have no financial or other conflicts of interests.

Availability of data and material: Data *Rhodopila* strain LVNP are available from MNP upon request.

Code availability: Not applicable

Authors' contributions: MNP, MHM, and MLK generated the data. MTM drafted the manuscript and all authors contributed to editing the manuscript.

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Tables

Due to technical limitations, table 1 and 2 is only available as a download in the Supplemental Files section.

Figures

Figure 1

a Acidic (pH 3.9) and sulfidic warm spring in Lassen Volcanic National Park. b Sampling area designated by black arrow in a. The microbial mat contains the red alga *Cyanidium* (Cyn) overlying a red layer from which *Rhodospirillum rubrum* strain LVNP was isolated; the white area is elemental sulfur. c Phase-contrast photomicrograph of a dense suspension of cells of strain LVNP

Figure 2

Phylogenetic tree of 16S rRNA genes from some phototrophic purple bacteria and 3-MeBHP-producing bacteria. Taxa shown in red are anoxygenic phototrophs and all are PNS bacteria except for the purple sulfur bacterium *Thermochromatium tepidum*. All Acetobacteraceae shown and the two methanotrophic bacteria are known producers of 3-MeBHPs. The labels “Alpha,” “Beta” and “Gamma” refer to classes of the phylum Proteobacteria, and numbers at the nodes are bootstrap percentages based on 1000 replications

Figure 3

a Gas chromatographic–mass spectrometric analyses of 3-methylbacteriohopanepolyols (3-MeBHPs). The compounds, extracted from cultures of *Rhodopila* strain LVNP grown photosynthetically at pH 5, are separated and analyzed as their acetate derivatives. The mass fragment 191 is diagnostic of unmethylated BHPs (blue chromatogram) while the 205 fragment is diagnostic of their methylated equivalent (red chromatogram). The 3-MeBHPs elute after their unmethylated equivalents. b Analysis of *Rhodopseudomonas palustris* as a control. This PNS bacterium produces 2-MeBHPs (Rashby et al. 2007), which elute before their unmethylated equivalents. I.S., internal standard

Figure 4

Protein tree of HpnR, the C-3 methylase that methylates BHPs to form 3-MeBHP. Both *Rhodopila* strain LVNP and *Rhodopila globiformis* strain DSM 161T genomes encode HpnR, and the amino acid sequences of this protein cluster with those from their close phylogenetic relatives, the acetic acid bacteria. C-2 BHP methylase (HpnP) from *Rhodopseudomonas palustris* was used as the outgroup in the tree. Numbers at the nodes are bootstrap percentages based on 1000 replications. * denote strains that have confirmed 3-Me BHP production in cultured lipids

Supplementary Files

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