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Discovery, taxonomic distribution, and phenotypic characterization of a gene required for 3-methylhopanoid production

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H hopanoids are pentacyclic triterpenoid lipids produced by a variety of bacteria that are often utilized as geological proxies or biomarkers for certain bacterial species and their metabolisms. Among the various hopanoid structures produced by bacteria (1), those methylated at the C-3 position and those with a penta- and hexafunctionalized amino polar side group are thought to be primarily produced by Type I and Type X methanotrophs (Fig. 1A) (2). As such, the occurrence of these hopanoids in conjunction with their significant \(^{13}C\)-depletion in modern ecosystems are often utilized as an indicator of methanotrophic communities (3). In particular, environmental lipid analyses have uncovered the existence of aerobic methanotrophy in a variety of environments including, for example, the surface sediments of an active marine mud volcano in the Barents Sea and in theoxic-anoxic transition zone of the Black Sea water column (4, 5). Furthermore, the recalcitrant nature of hopanoid hydrocarbons allows for their preservation in ancient sediments, which may provide evidence for aerobic metabolisms deep in Earth’s history. Although the functionalized amino side group is lost over time, methylation of the A-ring is retained (6). Thus, the presence of C-3 methylated hopanes in sediments 2.5–2.7 billion years old has been used as one of several lines of molecular and isotopic evidence for Neoarchean aerobiciosis (7–11).

The effectiveness of these specific hopanoids as indicators for aerobic methanotrophy rests partly on the premise that the majority of C-3 methylated hopanoid producers are aerobic methanotrophs. However, the production of 3-methylhopanoids has also been demonstrated in the acetic acid bacteria (12) indicating that the taxonomic distribution of 3-methylhopanoids is not restricted to methanotrophs. Furthermore, recent studies utilizing molecular approaches to identify hopanoid biosynthesis genes in sequenced genomes have highlighted that the diversity of bacteria capable of producing a specific hopanoid structure could be underestimated (13–15). These studies have also shown that a more precise interpretation of hopane hydrocarbon signatures in both ancient and modern ecosystems requires not only a grasp of the taxonomic distribution of methylhopanoid producers but also a deeper understanding of their physiological function in extant bacteria (16, 17).

To this end, we employed a combination of microbial genetics, microbial physiology, and bioinformatics analysis to begin to understand the biosynthesis and function of C-3 methylated hopanoids in *Methylococcus capsulatus*. A genetic system for constructing unmarked in-frame deletion mutants was utilized to identify a methylase required for the production of 3-methylhopanoids. Bioinformatics analysis of this methylase revealed a diverse taxonomic distribution beyond the methanotrophic and acetic acid bacteria. Furthermore, phenotypic analysis of the C-3 methylase mutant uncovered a potential role for 3-methylhopanoids in late stationary phase survival. These studies highlight the power of combining gene discovery with bioinformatics and physiological analyses to potentially enhance our understanding of biomarker signatures in the rock record.

Results and Discussion

Identification of a C-3 Methylase in the *M. capsulatus* Genome. To identify a protein required for the methylation of hopanoids at the C-3 position, the genome of *M. capsulatus* was examined for possible C-3 methylase candidates utilizing search criteria based on two previous findings. First, bacterial feeding studies done with labeled methionine have posited that *S*-adenosylmethionine (AdoMet) is a potential methyl donor in the biosynthesis of both 2-methyl and 3-methylhopanoids (12). Second, it was recently discovered that a B-12 binding radical AdoMet protein, HpnP, is required for the production of 2-methylhopanoids in the α-Proteobacterium *Rhodopseudomonas palustris* (14). Accordingly, we hypothesized that the methylase responsible for 3-methylhopanoid production was also a radical AdoMet protein possibly containing a B-12 binding domain.

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Using InterPro ([http://www.ebi.ac.uk/interpro/](http://www.ebi.ac.uk/interpro/)), an integrated database of predictive protein signatures, 21 proteins with a radical AdoMet motif were identified in the *M. capsulatus* genome. These 21 proteins were queried against the acetic acid bacterial genomes. Although the MCA0738 gene was not surrounded by other hopanoid biosynthesis genes on the chromosome (Fig. 1), the occurrence of this particular gene in both *M. capsulatus* and all sequenced acetic acid bacterial genomes made it an attractive candidate for encoding the C-3 methylase.

To determine if MCA0738 did encode for a C-3 methylase, an unmarked in-frame deletion of MCA0738 was attempted by adapting a counter selection protocol that has been used in a variety of bacterial species (18, 19). This allelic exchange method involves integrating a suicide plasmid at the locus of interest by homologous recombination and subsequently excising the plasmid from the chromosome, which can result in the deletion of the gene of interest (Fig. S1). To delete MCA0738, a deletion plasmid containing a replacement allele missing the MCA0738 gene was transferred into *M. capsulatus* via conjugation and integrated onto the chromosome by homologous recombination. The plasmid was forced to excise from the chromosome through nonselective growth and several potential deletion colonies were screened by PCR for deletion of MCA0738. One strain was found to be devoid of this gene and was picked for further characterization (Fig. S1).

To verify that MCA0738 was required for C-3 methylation, a total lipid extract (TLE) was isolated from the MCA0738 deletion mutant and analyzed for its complement of bacteriohopanepolyols. As shown in Fig. 2, the MCA0738 deletion mutant is able to produce both the desmethyl aminobacteriohopanepentol and aminobacteriohopanetetrol but not their C-3 methylated counterparts as confirmed by detailed mass spectral analysis (Fig. S2). Furthermore, introduction of a copy of the MCA0738 gene on a self-replicating plasmid into the deletion strain restores production of the methylated hopanoids (Fig. 2). These data indicate that MCA0738 is the only gene required for C-3 methylation of hopanoids in *M. capsulatus* and we propose to rename this locus *hpnR* based on a previously established nomenclature in *Zymomonas mobilis* (20).

**Identification of Putative HpnR Homologues.** The radical AdoMet protein family encompasses a diverse set of proteins that catalyze a variety of biochemical reactions. The proteins in this family are primarily identified by the short amino acid sequence motif CxxxCxxC. As a result, BLAST analyses of *HpnR* return a variety of radical AdoMet proteins that may or may not be involved in 3-methylhopanoid biosynthesis. To determine which of these...
radical AdoMet proteins are genuine C-3 methylases, we con-
structed an unrooted maximum likelihood tree of 192 radical
AdoMet proteins retrieved through a protein BLAST search of the
M. capsulatus HpnR sequence against the Kyoto Encyclo-
dpedia of Genes and Genomes (KEGG) and National Center
for Biotechnology Information (NCBI) databases (e-value cut-off <
$e^{-100}$). This analysis shows that those radical AdoMet proteins
with an e-value lower than $e^{-100}$ cluster together (Fig. S3). Within
this clade, we find HpnR from M. capsulatus and homologues
from the acetate acid bacteria, the only currently known producers
of 3-methylhopanoids (Fig. 3). Further, all of the strains in this
clad also contain a copy of the squalene hopene cyclase gene,
which is required for hopenoid biosynthesis, as well as several
other hopanoid biosynthesis genes in their genomes (17, 21).
Therefore, it seems reasonable to propose that the cutoff for a
bona fide C-3 methylase is a value lower than $e^{-100}$.

Using this criteria, there are 52 putative homologues of HpnR
in the genomic and metagenomic databases (Fig. S3). The species
that contain these HpnR homologues are from a diverse set of
bacterial phyla: 33 strains of Proteobacteria (22 α, 3 β, and 8
γ-Proteobacteria), 11 Actinobacteria, 3 Nitrospira, 1 Acidobact-
erium, 1 candidate NC10 phylum organism, and 3 metagenomic
γ bacterial phyla: 33 strains of Proteobacteria (22
that contain these HpnR homologues are from a diverse set of
in the genomic and metagenomic databases (Fig. 3). The species
of Genes and Genomes (KEGG) and National Center for
M. capsulatus
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Therefore, it seems reasonable to propose that the cutoff for a
biphenyl Pseudomonas sp. DSM 6345s. Therefore, the distribution of
Methylobacterium nodulans and Nitrococcus mobilis HpnR
sequences tend to cluster outside their expected species phyl-
yplane phylogeny. Thus, the evolutionary history of this protein
remains unclear and more robust analyses are needed to better resolve it.

Given the taxonomic diversity of potential 3-methylhopanoid
producers uncovered by our analysis, the detection of 3-methyl-
hopanoids in both modern and ancient sediments cannot be
attributed specifically to aerobic methanotrophic bacteria without
other lines of evidence (e.g., carbon isotope data). However, all of
the bacterial species that contain an HpnR homologue in their
genomes utilize a form of aerobic metabolism. Thus, it seems
reasonable to continue to employ 3-methylhopanes in the rock
record as indicators for the existence of aerobic metabolisms deep
in time. One organism that might be considered an exception to
this rule is Candidatus Methylomirabilis oxyfera which was iso-
lated from anoxic sediments and grows anaerobically by coupling
nitrite reduction to methane oxidation (23). Although M. oxyfera
is an anaerobe, it is also an oxygenic organism as it produces its
own supply of oxygen through the dismutation of nitrite. It sub-
sequently uses this oxygen for the oxidation of methane through
the same methanotrophic pathway utilized by other aerobic
methanotrophs (23). Whereas M. oxyfera is encountered in ana-
erobic environments, it still requires oxygen for its metabolism.
Therefore, the distribution of the C-3 methylase in oxygen-de-
manding bacteria seems robust for now and can be seen as further
evidence for the use of 3-methylhopanes as proxies for the oc-
currence of aerobic metabolisms in ancient environments.

3-Methylhopanoids Play a Role in Late Stationary Phase Survival. The
diverse and sporadic distribution of HpnR introduces further
ambiguity in our ability to correlate specific bacterial taxa to
3-methylhopanoids in the environment. As a result, a proper ana-
lysis of the presence of 3-methylhopanes in the rock record needs
to move beyond simply understanding which organisms produce
these molecules. A more nuanced interpretation may be achieved
if we better understand the physiological function of 3-methyt-
hopanoids in bacteria as well as the environmental factors that
induce their production in the cell. To this end, we have begun
physiological characterization of the M. capsulatus hpnR mutant

Fig. 3. Maximum likelihood phylogenetic tree of putative HpnR sequences.
A total of 192 radical AdoMet proteins were used to generate the tree: Fifty-
two HpnR sequences plus 140 radical AdoMet proteins with an e-value less than $e^{-10}$. The tree was rooted by using the 140 sequences as an out group to the 52 HpnR sequences shown. The full unrooted tree is shown in Fig. S3.
A previous study in *M. capsulatus* demonstrated that 3-methylhopanoids accumulate preferentially in stationary phase cells (24). To test whether 3-methylhopanoids play a role in stationary phase physiology, we grew both the wild type and Δ*hpnR* strains in batch culture for fourteen days. Cultures were supplemented with methane only at the point of inoculation (day 0) to ensure that they would become nutrient-limited and enter stationary phase. Cell growth was monitored by following the OD at 600 nm. Under these growth conditions, it was determined that the cells were entering stationary phase and ceased oxidizing methane on day 2 of growth (Fig. S4). Given that the methane in the headspace was not depleted (Fig. S4), we presumed that the cessation of growth on day 2 resulted from the depletion of oxygen. These experiments also demonstrated that Δ*hpnR* cells exhibited similar growth characteristics to the wild type strain during exponential growth. However, upon entering stationary phase, the methylase mutant seemed to experience a larger drop in OD than the wild type indicating a potential loss in viability under these conditions.

To better assess cell viability in stationary phase, the number of cfu on day 2, 7, and 14 of growth were determined. As shown in Fig. 4, the wild type strain maintains the same number of cfu throughout stationary phase. The Δ*hpnR* strain sustains a similar number of viable cells on day 2 and 7, even though the observed drop in OD occurs immediately upon entering stationary phase at day 2. However, the number of cfu drops approximately six orders of magnitude on day 14 suggesting a role for 3-methylhopanoids in late stationary phase (Fig. 4). To more directly show that the lack of 3-methylhopanoids was responsible for this reduction in viability, we also determined the cfu values for the Δ*hpnR* strain complemented with a copy of the methylase gene (Fig. 4; Δ*hpnR* + pPVW100). The overall cfu values for the complemented strain were approximately two orders of magnitude lower than the wild type on each day tested. This reduction in cfu was most likely a result of the sluggish growth observed in the presence of the kanamycin antibiotic necessary to maintain the complementing plasmid. Nevertheless, reinstating 3-methylhopanoid production in the Δ*hpnR* strain did result in sustained cell viability through day 14.

### 3-Methylhopanoids and Intracytoplasmic Membrane Formation in Late Stationary Phase

The decreased viability of the methylase mutant suggests that it may be deficient in mechanisms necessary to cope with the stresses encountered in stationary phase. *M. capsulatus* is one of several methanotrophs that are capable of forming Azotobacter-like cyst structures. Cyst formation has been shown to be important in enhancing the survival of a bacterium under adverse environmental conditions such as those experienced in stationary phase (25). Accordingly, we hypothesized that the lack of 3-methylhopanoids in the methylase mutant may result in inadequate cyst formation which, in turn, compromises viability during prolonged stationary phase incubation.

To test this hypothesis, transmission electron microscopy (TEM) of both wild type and mutant cells on day 2 and day 14 of growth were analyzed for the formation of cyst-like structures. As shown in Fig. 5, no cyst-like cells were found to be produced either by the wild type or the methylase mutant throughout stationary phase suggesting that 3-methylhopanoids play a role in stationary phase survival independent of cyst formation. On the other hand, the images revealed formation of extensive intracytoplasmic membranes (ICM) in late stationary phase. In particular, both the wild type and Δ*hpnR* were capable of forming these membranes in early stationary phase (day 2). But by day 14, the wild type had significantly more ICM present than on day 2 whereas the 3-methylhopanoid deletion mutant was no longer producing these membranes. The ability of the methylase mutant to produce lamellar membranes upon entering stationary phase but not deep into stationary phase suggests that 3-methylhopanoids play a role in maintaining these membranes rather than a role in forming them. Complementation of the deletion strain with the *hpnR* gene partially restored the production of ICM on day 14 further indicating that 3-methylhopanoids may be important in maintaining ICM formation during stationary phase.

The decreased viability of the methylase mutant along with its inability to maintain ICM formation leads us to speculate that ICM maintenance is necessary for survival in late stationary phase. Based on our physiological data, we presume that the cells in our cultures are limited for oxygen rather than methane (Fig. S4). Previous studies have shown that ICM formation and methane oxidation rates in methanotrophs increase under similar high methane/low oxygen growth conditions (26). Because the methane monooxygenase is a membrane-bound enzyme localized to the ICM it is thought that the increase in ICM formation allows for increased methane oxidation at low oxygen levels. This strategy may then aid survival in low oxygen environments encountered in nature.

Interestingly, these high methane/low oxygen growth conditions are reminiscent of the modern and ancient seafloor methane seeps in which 3-methylhopananes and 3-methylhopanoid-producing methanotrophs have been detected (4, 27). The physical conditions at these seafloor methane seeps are quite transitory, particularly in terms of the availability of methane and oxygen (28). The methanotrophs in these communities must be adapted to survive persistent low oxygen levels as well as to respond rapidly to methane pulses (28). Thus, our late stationary phase studies could be demonstrating a role for ICMs and 3-methylhopanoids in the persistence of certain methanotrophic communities in their natural environments. This hypothesis is particularly appealing when we consider a recent study on the fate of spilled methane from the 2010 Deepwater Horizon oil spill (29). In this study, the idea was put forward that aerobic methanotrophic communities may act as dynamic biofilters of large-scale methane inputs into the ocean (29). These methanotrophic communities persist for the most part in nutrient-limited environments yet seem poised to respond to sudden influxes of methane. Some of the methanotrophs identified from this potential methanotrophic bloom after the Deepwater Horizon disaster were *γ*-Proteobacteria of the *Methylococcales* family that is known to contain 3-methylhopanoid-producing methanotrophic species such as *M. capsulatus*. Therefore, it is possible that the ability to survive in these transient methanotrophic environments may be linked to 3-methylhopanoid production.

The observations presented here point to a potential role of 3-methylhopanoids (and ICM formation) in cell viability under nutrient-limited conditions. These findings are pertinent given that low nutrients and harsh conditions are known to be ubiquitous in natural environments and as a result, microbes in nature are thought to persist in a type of stationary phase.
(30). We are currently pursuing molecular and physiological studies to better pinpoint the factors that induce ICM formation in stationary phase, the potential role of 3-methylhopanoids in ICM maintenance, and how 3-methylhopanoids might aid cell viability. If a relationship between 3-methylhopanoids and cell viability during stationary phase can be further established, then 3-methylhopanoids have the potential to be proxies for the particular environmental stressors (e.g., low oxygen) encountered during stationary phase.

Materials and Methods

Bacterial Strains, Media, and Growth Conditions. Bacterial strains used in this study are listed in Table S1. Escherichia coli strains were grown in lysogeny broth (LB) and M. capsulatus strains were grown in nitrate minimal salts (NMS) medium supplemented with 10 μM CuSO4 (31) at 37 °C while shaking at 250 rpm. M. capsulatus batch cultures were sealed in serum vials without removing the ambient air and given methane: carbon dioxide mix (95:5) at 60 kPa over ambient pressure. For growth on solid medium, LB or NMS was solidified with 1.5% agar and supplemented, if necessary, with 15 μg/mL gentamicin (Gm), 50 μg/mL kanamycin (Km), 600 μM diaminopimelic acid (DAP), or 5% sucrose. M. capsulatus plates were incubated in Vacu-Quik Jars (Almore International, Inc.) and supplied with methane: carbon dioxide mix at 20 kPa over ambient pressure. Additional details are described in SI Materials and Methods.

DNA Methods, Transformation, and Mutant Construction. All plasmid constructs and the sequences of oligonucleotide primers used in this study are described in Table S1. Construction of the ΔhpnR deletion mutant and complemented strain is described in SI Materials and Methods. DNA sequences of all cloning intermediates were confirmed by sequencing at the GENEWIZ Boston Laboratory. E. coli strains were transformed by electroporation. Plasmids were mobilized from E. coli BW20767 into M. capsulatus by conjugation as described in SI Materials and Methods.

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Analysis of Hopanoid Production. M. capsulatus strains were grown in 50 mL NMS at 37 °C for 3 d. Lipids were extracted and analyzed by liquid chromatography–mass spectrometry (LC–MS) as previously described (17, 21). The LC–MS system comprises a 1200 Series HPLC (Agilent Technologies) equipped with an autosampler and a binary pump linked to a Q-TOF 6520 mass spectrometer (Agilent Technologies) via an atmospheric pressure chemical ionization interface (Agilent Technologies). Hopanoids were identified on the basis of accurate mass measurements of their protonated molecular ions, fragmentation patterns in MS–MS mode, and by comparison of relative retention time and the mass spectra with published data (32).

Bioinformatics Analysis. InterPro (http://www.ebi.ac.uk/interpro) was used to identify putative radical AdoMet proteins in the M. capsulatus genome. HpnR homologues were identified in the KEGG and the NCBI databases by a translated Basic Local Assignment Search Tool (TBLASTN) (33) and were aligned using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) program (34). Maximum likelihood trees were constructed by phylogenetic estimation using maximum likelihood (PhyML) (35) using the LG + γ model, four gamma rate categories, ten random starting trees, Nearest Neighbor Interchange (NNI) branch swapping, and substitution parameters estimated from the data. The HpnR tree was generated and edited by importing the resulting PhyML tree into the Interactive Tree of Life tool (ITOL) (http://itol.embl.de) (36).

Fig. 5. Deletion of 3-methylhopanoid production results in reduced intracytoplasmic membranes during stationary phase. TEM images show ICM formation (black arrows) by all cells on day 2 of growth. On day 14 of growth the ΔhpnR mutant has significantly lower ICM formation than the wild type and complemented strains (ΔhpnR + pPVW100). (Black scale, 0.5 μ.)


