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

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Hopanoids methylated at the C-3 position are a subset of bacterial triterpenoids that are readily preserved in modern and ancient sediments and in petroleum. The production of 3-methylhopanoids by extant aerobic methanotrophs and their common occurrence in modern and fossil methane seep communities, in conjunction with carbon isotope analysis, has led to their use as biomarker proxies for aerobic methanotrophy. In addition, these lipids are also produced by aerobic acetic acid bacteria and, lacking carbon isotope analysis, are more generally used as indicators for aerobicism in ancient ecosystems. However, recent genetic studies have brought into question our current understanding of the taxonomic diversity of methylhopanoid-producing bacteria and have highlighted that a proper interpretation of methylhopanones in the rock record requires a deeper understanding of their cellular function. In this study, we identified and deleted a gene, hpnR, required for methylation of hopanoids at the C-3 position in the obligate methanotroph *Methylcoccus capsulatus* strain Bath. Bioinformatics analysis revealed that the taxonomic distribution of HpnR extends beyond methanotrophic and acetic acid bacteria. Phenotypic analysis of the *M. capsulatus* hpnR deletion mutant demonstrated a potential physiological role for 3-methylhopanoids; they appear to be required for the maintenance of intracytoplasmic membranes and cell survival in late stationary phase. Therefore, 3-methylhopanoids may prove more useful as proxies for specific environmental conditions encountered during stationary phase rather than a particular bacterial group.

**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*-adenosyl-methionine (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.

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 *Methylcoccus 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.

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### References

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Using 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 _Acetobacter pasteurianus_ genome, an acetic acid bacterium known to produce 3-methylhopanoids (12). Of these 21 proteins, MCA0738 was the only protein annotated as a B-12 binding radical AdoMet that also had a homologue in _A. pasteurianus_ (e-value of 0). A Basic Local Alignment Search Tool (BLAST) search of MCA0738 revealed a homologue in other acetic acid bacterial genomes. Although the MCA0738 gene was not surrounded by other hopanoid biosynthesis genes on the chromosome (Fig. 1B), 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 through 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 bacteriohopanepolys. As shown in Fig. 2, the MCA0738 deletion mutant is able to produce both the desmethyl aminobacteriohopanepentol and aminobacteriohopanepentol 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 constructed 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 Encyclopedia of Genes and Genomes (KEGG) and National Center for Biotechnology Information (NCBI) databases (e-value cut-off < $e^{-10}$). 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 clade also contain a copy of the squalene hopene cyclase gene, which is required for hopenoid biosynthesis, as well as several other hopenoid 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. 3). 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 Nitrospirae, 1 Acidobacterium, 1 candidate NC10 phylum organism, and 3 metagenomic sequences. In agreement with our current understanding of 3-methylhopanoid distribution in bacteria, all 21 of the partially or completely acetate acid bacterial genomes have an HpnR homologue. However, only three of the nine methanotrophic genomes sequenced to date (two complete and seven incomplete) have a copy of this protein suggesting that only a subset of methanotrophs may be capable of producing 3-methylhopanoids. The inconsistent distribution of HpnR among methanotrophs is also observed in the genomes of other hopanoid-producing bacterial genera. For example, 38 Burkholderia, 43 Streptomyces, and 8 Methylobacterium genomes have been sequenced to date. All of these genomes, except for Burkholderia pseudomallei MSHR346, contain a copy of the squalene hopene cyclase gene and at least one species from each of these groups has been shown to produce hopanoids. Yet, only three Burkholderia, eight Streptomyces, and one Methylobacterium contain the HpnR methylase suggesting that only a subset of species from certain genera may be able to produce 3-methylhopanoids. This observation is particularly critical when we consider that our current understanding of which bacteria produce certain hopanoid molecules is often based on lipid analysis of a few cultivable species of a certain genus.

The sporadic phylogenetic distribution of HpnR also suggests a potentially complex evolutionary history of this methylase. Two evolutionary scenarios seem plausible: either HpnR was present in the ancestor of all HpnR-containing bacteria and was repeatedly lost or it could have been acquired through horizontal gene transfer (HGT). For most of these taxa, the HpnR phylogeny is congruent with that of the species phylogeny based on 16S rRNA sequence (22). This consistency between the HpnR phylogeny and species phylogeny is evident for the acetate acid bacterial clade suggesting a vertical descent within this group. However, the Methylobacterium nodulans and Nitrococcus mobilis HpnR clades tend to cluster outside their expected species phylogeny clade suggesting acquisition through HGT in these organisms. 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-methylhopanoids 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 genome utilize a form of aerobic metabolism. Thus, it seems reasonable to continue to employ 3-methylhopanones in the rock record as indicators for the existence of aerobic metabolisms in deep time. The one organism that might be considered an exception to this rule is Candidatus Methylomirabilis oxyfera which was isolated 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 dissimulation of nitrite. It subsequently uses oxygen for the oxidation of methane through the same methanotrophic pathway utilized by other aerobic methanotrophs (23). Whereas M. oxyfera is encountered in anaerobic environments, it still requires oxygen for its metabolism. Therefore, the distribution of the C-3 methylase in oxygen-demanding bacteria seems robust for now and can be seen as further evidence for the use of 3-methylhopanones as proxies for the occurrence 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-methylhopanones in the environment. As a result, a proper analysis of the presence of 3-methylhopanones 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-methylhopanoids 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 to identify any potential phenotype(s) associated with the loss of 3-methylhopanone production.

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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 lower than $e^{-100}$ when queried against the M. capsulatus HpnR sequence. 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 mono-oxygenase 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-methylhopanoids 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 conditions 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 *Methyllobacterium* 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 this 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 (Kn), 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 hpnR 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.

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 (BLASTN) (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).

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