Biosynthesis of 2-methylbacteriohopanepolyols by an anoxygenic phototroph

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Sedimentary 2-methyhopanes have been used as biomarker proxies for cyanobacteria, the only known bacterial clade capable of oxygenic photosynthesis and the only group of organisms found thus far to produce abundant 2-methylbacteriohopanepolyols (2-MeBHPs). Here, we report the identification of significant quantities of 2-MeBHP in two strains of the anoxygenic phototroph *Rhodopseudomonas palustris*. Biosynthesis of 2-MeBHP can occur in the absence of O_2, deriving the C-2 methyl group from methionine. The relative abundance of 2-MeBHP varies considerably with culture conditions, ranging from 13.3% of total bacteriohopanepolyol (BHP) to trace levels of methylation. Analysis of intact BHPs reveals the presence of methylated bacteriohopane-32,33,34,35-tetrol but no detectable methylation of 35-aminobacteriohopane-32,33,34-triol. Our results demonstrate that an anoxygenic photoautotroph is capable of generating 2-MeBHPs and show that the potential origins of sedimentary 2-methylhopanoids are more diverse than previously thought.

2-methyhopanes | biomarkers | cyanobacteria | *Rhodopseudomonas palustris*

Much of what we know about the evolution of life on Earth derives from the record of multicellular organisms that produce readily identifiable fossil remains. However, the vast majority of life's history has been dominated by microbes that do not leave diagnostic morphologic fossils. Their metabolic inventions profoundly changed the Earth's environment (1), yet it is difficult (if not impossible) to precisely identify them from their simple shapes, even in the rare cases when ancient intact fossils are found (ref. 2 and references therein). An alternative strategy for accessing the early history of microbial life is the study of "molecular fossils," organic compounds that can be traced to specific biomolecules produced by select groups of modern organisms and thus have the potential to serve as chemical fossils (3–6).

Pentacyclic hydrocarbon molecules known as hopanes are ubiquitous in both modern and ancient sedimentary rocks containing appreciable organic matter (7). Many possess distinctive carbon skeletons with extended side chains, from which they can be recognized as the chemical fossils of bacteriohopanepolyols (BHPs) (Fig. 1*a*), functionalized triterpenoid lipids found in many modern bacteria (8). Until now, BHPs methylated at C-2 (Fig. 1*a*) had been found in abundance only in cyanobacteria (9–11), and in "trace" quantities in several other bacteria cultured aerobically (12, 13). The accumulation of substantial quantities of 2-methylhopanes in sediments has thus been proposed as a marker for cyanobacteria (10), and the discovery of these biomarkers in 2.7-billion-year-old shales has been used as one line of evidence that cyanobacteria had evolved and were conducting oxygenic photosynthesis by that time (14–16). Similarly, the ratio of 2-methylhopanes to desmethylhopanes, expressed as the 2-methylhopane index (10), has been used as a means of estimating changes in the ecological significance of cyanobacteria in marine environments over specific time intervals. Examples include mass extinction (17) and ocean anoxic events (18–20) likely associated with photic zone euxinia (21, 22) and black shale units with anomalous depletions in $\delta^{15}N$ (23).

Bacteriohopanoids are often regarded as the bacterial equivalent of sterols because of both similarities in their biosynthesis and evidence that they can serve similar functions (24). Like sterols, BHPs have been found to exhibit membrane-condensing effects in lipid monolayer studies (25, 26). It has been further proposed that they may serve to enhance the stability or barrier function of membranes in a number of bacteria, including *Alicyclobacillus acidocaldarius* (27), *Zymomonas mobilis* (28), *Frankia* sp. (29), *Methylococcus capsulatus* (30), and *Streptomyces coelicolor* (31). There are limited data indicating that BHPs may be required for growth in purple nonsulfur bacteria (32), and some effort has been made to constrain their subcellular localization in cyanobacteria (33, 34). Nonetheless, there are still insufficient data to explain the functional role of bacteriohopanoids in photosynthetic bacteria. Furthermore, few studies to date have addressed changes in the ratio of 2-methylbacteriohopanepolyol (2-MeBHP) to total BHP (henceforth "2-MeBHP ratio") with varying culture conditions (e.g., ref. 35). Thus, the data needed to confidently interpret sedimentary 2-methylhopanes as biomarkers are incomplete. To begin to address these issues, we examine BHP methylation in the anoxygenic phototroph *Rhodopseudomonas palustris*.

Results

While investigating 2-MeBHP production by cyanobacteria, we grew and analyzed the anoxygenic phototroph *R. palustris* CGA009 as a negative control. Using standard analytical methodology (9), we were surprised to find significant production by *R. palustris* of hopanoids methylated at C-2. Production of 2-MeBHP was subsequently reproduced in a recent environmental isolate of *R. palustris*, strain TIE-1 (36). TIE-1 is capable of conducting anoxygenic photosynthesis in the absence of oxygen, using either an organic substrate during photoheterotrophic growth or an inorganic electron donor, such as H_2, thiosulfate, or Fe(II), during photoautotrophic growth (36). In the presence of oxygen, the synthesis of photosynthetic machinery is repressed in *R. palustris*, and aerobic heterotrophy or chemoautotrophy is used. Like many other α-Proteobacteria, TIE-1 is capable of nitrogen fixation. Because of its ability to use a broader range of electron donors than CGA009, TIE-1 was selected as a model organism for further experiments.

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Abbreviations: 2-MeBHP, 2-methylbacteriohopanepolyol; BHP, bacteriohopanepolyol; GC-MS, gas chromatography-mass spectrometry.

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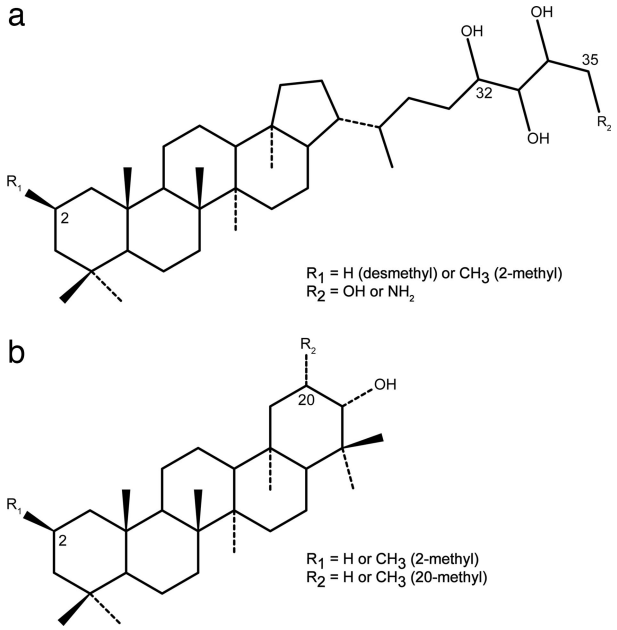

Fig. 1. Cyclic triterpenoids identified in *R. palustris* TIE-1. (*a*) Generalized BHP structure. For 32,33,34,35-bacteriohopanetetrol, R_2 = OH; for 35-amino-32,33,34-bacteriohopanetriol, R_2 = NH_2. Samples analyzed by GC-MS have shortened side chains (see *Results*). (*b*) Tetrahymanol and analogs methylated at C-2 and C-20.

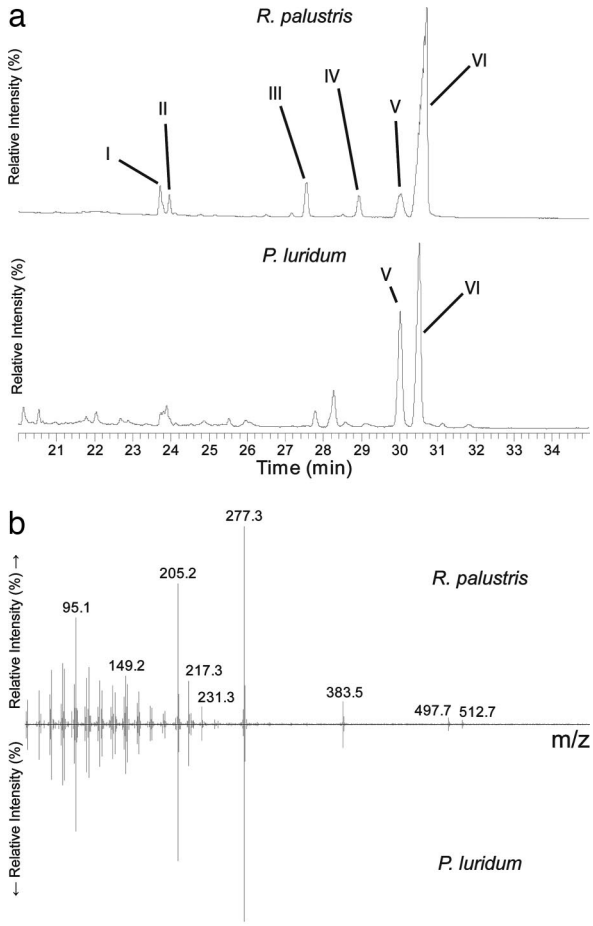

Fig. 2. Compounds identified by GC-MS as acetate derivatives. (*a*) Chromatograms of cleaved lipid extracts from TIE-1 and the cyanobacterium *P. luridum*. Numbered compounds: I, 2-methyltetrahymanol (tentative); II, tetrahymanol; III, C_{31} hopanol; IV, $C_{32:1}$ hopanol; V, 2-methyl C_{32} hopanol; VI, desmethyl C_{32} hopanol. The *y* axis is flame ionization detector signal intensity. (*b*) Mass spectra of 2-methyl C_{32} hopanol from TIE-1 (*Upper*) and *P. luridum* (*Lower*). *y* axis is relative abundance. Data are representative of multiple samples (>30 from TIE-1 and CGA009).

For analysis by gas chromatography-mass spectrometry (GC-MS), functionalized BHP side chains are oxidatively cleaved between vicinal hydroxyl groups and the resultant aldehydes reduced back to hopanols according to the technique developed by Rohmer *et al.* (9). Using this method, we identified a C_{32} 2-methylhopanol in lipid extracts from *R. palustris* CGA-009 and TIE-1 by comparison of retention times (Fig. 2*a*, compound V) and electron impact mass spectral fragmentation pattern (Fig. 2*b*) with a standard from the cyanobacterium *Phormidium luridum*. The desmethyl analog (Fig. 2*a*, compound VI) identified in *R. palustris* (9) is present in all samples. We do not observe these hopanols in lipid extracts that are not subjected to side chain cleavage [supporting information (SI) Fig. 5], indicating that the parent compound is a polyol. In addition, we note the presence after cleavage and reduction of a C_{31} hopanol (Fig. 2*a*, compound III) and a $C_{32:1}$ hopanol with an unsaturated side chain (Fig. 2*a*, compound IV), possibly similar to that observed by Fischer *et al.* (37). Both compounds are methylated at C-2 in the same relative proportion as the C_{32} hopanol (data not shown).

To examine BHPs with intact side chains, we used atmospheric pressure chemical ionization-liquid chromatography-ion trap MS (38–40). Extracted ion chromatograms indicate that bacteriohopane-32,33,34,35-tetrol, 2-methylbacteriohopane-32,33,34,35-tetrol, and 35-aminobacteriohopane-32,33,34-triol account for the most abundant ions in the base peak chromatogram (Fig. 3), and secondary fragmentation (MS^2) data for these compounds (SI Fig. 6) are in agreement with published spectra (40). We did not detect methylation of 35-aminobacteriohopane-32,33,34-triol. Adenosylhopane, recently reported from *R. palustris* using similar methodology (13), was not observed, although it may have been retained in the nonpolar fraction during acetone precipitation (see *Methods*). Both the tetrol and aminotriol are expected to yield C_{32} hopanols after cleavage and reduction. Pentol, aminotetrol, and other complex BHPs capable of producing C_{31} or $C_{32:1}$ hopanols upon cleavage and reduction were not detected. Consequently, the origin of these hopanols in *R. palustris* remains unknown.

Based on its appearance under phase microscopy and as single colonies on solid media, TIE-1 remained axenic throughout our experiments. Likewise, no procedural blanks contained any detectable BHP or 2-MeBHP (see *Methods*). Nevertheless, to confirm that 2-MeBHPs were produced by *R. palustris* TIE-1 and not the result of cyanobacterial contamination during culturing or processing, we conducted an anaerobic incubation of TIE-1 in the presence of [*methyl*-^{13}C,$^{2}H_3$]methionine in conjunction with small-subunit rRNA gene PCR. It has been suggested that *S*-adenosylmethionine acts as the donor for methylation at the C-2 position in other bacteria (9, 41, 42). TIE-1 cultured in the presence of labeled methionine produced 2-MeBHPs shifted by 4 mass units (Fig. 4) relative to the control, confirming biosynthesis of 2-MeBHP in culture. We performed PCR on an aliquot of this methyl-labeled culture, using cyanobacterial 16S rRNA gene primers, and detected no product after 40 amplification cycles (SI Fig. 7). As a positive control, the same sample was spiked with *Synechocystis* sp. PCC 6803 at a concentration below that which would be expected to produce detectable BHP based on prior experiments with this organism (data not shown), resulting in a PCR product of the expected length under the same

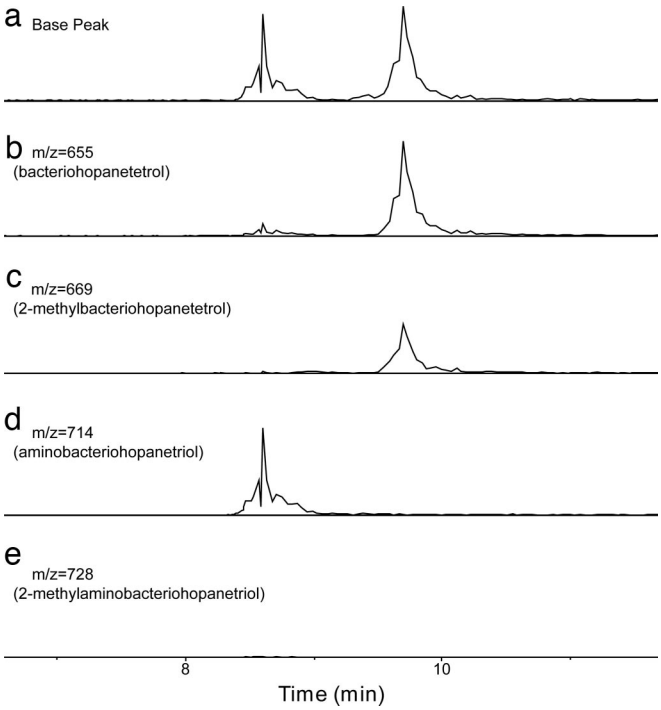

Fig. 3. Liquid chromatography-atmospheric pressure chemical ionization-ion trap MS chromatograms of intact BHPs. (*a*) Base beak trace (*m/z* = 200–800) of acetylated BHPs. (*b–e*) Extracted ion chromatograms correspond to bacteriohopanetetrol (*m/z* = 655) (*b*), 2-methylbacteriohopanetetrol (*c*), aminobacteriohopanetriol (714) (*d*), and 2-methylaminobacteriohopanetriol (728) (*e*). The *y* axis (signal intensity) scale is identical for all plots. Data are representative of four samples.

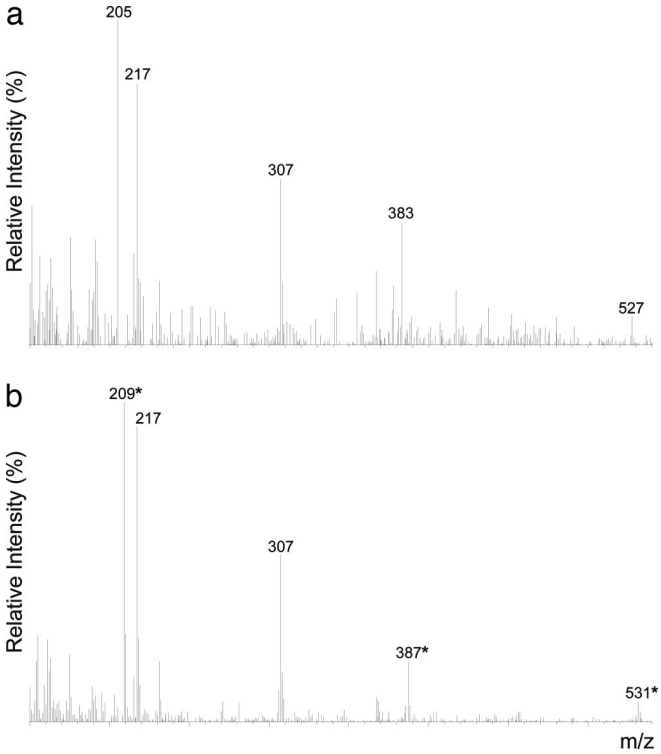

Fig. 4. Mass spectra from trimethylsilyl ethers of 2-methyl C_{32} hopanol from TIE-1 cultured with normal (*a*) or $^{2}H_{3}{}^{13}C$-labeled (*b*) methionine. Fragments containing ring A are shifted by 4 mass units (*).

PCR conditions. Given that we have not used *methyl*-^{13}C,$^{2}H_{3}$-labeled compounds in any prior experiments, contamination by cyanobacteria can confidently be excluded.

To assess the extent to which the 2-MeBHP ratio in *R. palustris* might vary, we grew TIE-1, using a range of conditions. Over all conditions tested, methylation of C_{32} hopanols from individual cultures ranged from 0.4% to 13.3%, with a mean of 4.2% (Table 1). 2-MeBHP ratios were lowest in aerobic and log-phase photoheterotrophic cultures. Higher ratios were observed in photoautotrophic cultures and in postexponential photoheterotrophic cultures, which may have been experiencing organic substrate limitation and thus growing autotrophically at harvest. 2-MeBHPs were observed in cultures grown in anaerobically prepared sealed flasks in an anaerobic chamber at room temperature and harvested by using centrifuge bottles designed to recover oxygen-sensitive enzymes in their active form. In addition to confirming that O_2 is not required for the biosynthesis of 2-MeBHPs, this experiment yielded the highest 2-MeBHP ratio observed in TIE-1 to date.

Tetrahymanol (Fig. 1*b*) was present in all lipid extracts from TIE-1, consistent with the first report of prokaryote-derived tetrahymanol in *R. palustris* (43). In addition, three methylated analogs were observed. They are tentatively identified as 2-methyltetrahymanol, 20-methyltetrahymanol, and 2,20-dimethyltetrahymanol on the basis of published mass spectra from compounds reported in *Bradyrhizobium japonicum* (42). Methylation of tetrahymanol was consistently greater at C-2 than at C-20, comprising nearly 70% of all tetrahymanol analogs in some samples (Table 1). In samples subjected to BHP cleavage, total tetrahymanols were on average 25-fold less abundant than BHP. However, tetrahymanol was present in greater concentration in raw lipid extracts (SI Fig. 5), indicating its loss during the BHP cleavage protocol. Because BHPs cannot be easily quantified by GC-MS without side-chain cleavage, the ratio of tetrahymanols to BHP *in vivo* remains uncertain.

Discussion

Previous work demonstrated that BHPs can be synthesized under anaerobic conditions (37, 44–46). Our data now provide evidence that methylation at C-2 can occur in the total absence of molecular oxygen as well. Based on our limited survey of growth conditions, several patterns emerge regarding this structural modification. First, methylation is minimal during log-phase heterotrophic growth. This trend suggests that BHP methylation may be specifically repressed when growth is not dependent on carbon fixation, implying a functional link between autotrophy and 2-MeBHPs. Second, the higher degree of methylation in cultures grown at low temperature may suggest a role for BHP methylation in the maintenance of membrane fluidity under these conditions, possibly by affecting the conformation of the A-ring (35). Both hypotheses merit further investigation.

Regardless of the mechanism, the potential for variability in BHP methylation based on growth condition has not been widely appreciated, which may explain why 2-MeBHPs have not been detected previously in *R. palustris* (9, 13, 47, 48) and suggests that 2-MeBHPs in other organisms could have been missed where multiple culture conditions were not tested. C-2 methylation of bacteriohopanoids has been observed in other α-Proteobacteria, including the closely related *B. japonicum* (13), and has been shown to incorporate methyl groups originally obtained from methionine, probably through the intermediate *S*-adenosylmethionine (9, 41, 42). Transfer of the intact methyl group from methionine in TIE-1 thus suggests a conserved role for *S*-adenosylmethionine in the biosynthesis of BHPs. Ultimately, identification of the enzyme or set of enzymes responsible for catalyzing this reaction may permit

Table 1. BHP and tetrahymanol production and methylation by *R. palustris* TIE-1 with varying culture conditions

Culture condition*	Total C32 hopanol, μg/mg	2-MeC32 hopanol, %	Total tetrahymanol, μg/mg	2-Methyltetrahymanol, %
Aerobic	724 (391)	0.9 (0.1)	ND	ND
Photoheterotrophic	580 (217)	0.5 (0.1)†	4.5 (3.0)	18.5 (6.5)
Photoautotrophic (thiosulfate)	478 (159)	2.8 (2.2)	20.8 (20.8)	40.8 (23.7)
Photoautotrophic (H_2)	681 (691)	8.5 (2.5)	10.9 (11.3)	54.8 (4.1)
Photoheterotrophic; stationary phase	640 (264)	4.9 (1.8)	16.5 (8.3)	65.5 (1.5)
Photoautotrophic (thiosulfate); stationary phase	567 (200)	2.0 (0.9)	27.1 (5.8)	42.9 (10.5)
Photoautotrophic (H_2); stationary phase	552 (146)	1.5 (1.4)	27.1 (14.5)	32.1 (1.4)
Anaerobic chamber; stationary phase	1,475 (775)	12.1 (1.6)‡	78.8 (28.1)	52.0 (15.8)

Each value represents the mean ($\pm$ 1σ) of three replicate cultures grown under identical conditions. ND, not determined.
*See *Methods* for detailed culture conditions.
†Lowest value in an individual culture = 0.4%.
‡Highest value in an individual culture = 13.3%.

the use of genomic data in constraining the range of organisms capable of producing 2-MeBHPs (37).

The production of 2-MeBHP by an anaerobic photoautotroph whose ancestors might well have flourished in Precambrian oceans may refine our interpretation of the biomarker record of sedimentary 2-methylhopanes. Two specific issues arise from our characterization of 2-MeBHP production in *R. palustris*.

First, because 2-MeBHPs may be produced by organisms that do not engage in oxygenic photosynthesis and because their biosynthesis does not require molecular oxygen, 2-methylhopanes cannot be used as de facto evidence for oxygenic photosynthesis. It is possible that 2-MeBHPs will be shown to play a direct role in another physiological process; however, their interpretation as biomarkers for oxygenic photosynthesis must remain contingent on additional lines of evidence.

Second, the case for cyanobacteria as the prime source of sedimentary 2-MeBHPs remains unresolved. We have detected 2-MeBHPs in only one species of anoxygenic phototroph thus far, and it is not a marine organism. Although no marine cyanobacteria in pure culture have yet been found to produce 2-MeBHPs, enrichment cultures of marine cyanobacteria (11) and cyanobacterial mats from marginal marine environments (49) have yielded 2-MeBHPs. Testing natural communities of anoxygenic photosynthetic bacteria for 2-MeBHPs is an obvious next step. 2-MeBHP ratios are often much higher in cyanobacteria than in *R. palustris* (10), and the 2-methylhopane index observed in the rock record in some cases exceeds the maximum value observed in our experiments (10). However, we do not know the upper limit of methylation in *R. palustris* or what constraints impose this limit. Furthermore, because we observe methylation of bacteriohopanetetrol but not aminobacteriohopanetriol, any preferential preservation of BHPs bearing particular side chain functionalities could significantly alter the 2-methylhopane index observed in the rock record.

Further study of the biosynthesis of 2-MeBHP and their production in modern environments may help constrain likely sources of ancient 2-methylhopanes. Because carbon fixation in *R. palustris* and cyanobacteria is likely to impart a similar carbon-isotopic signature (50), the use of compound-specific ^{13}C analyses may not be helpful. Here, the analysis of intact BHP from environmental samples may be advantageous. α-Proteobacteria, for instance, generally do not produce methylated (or desmethyl) pentols, so these may help to identify depositional settings in which cyanobacteria have been the primary contributors (assuming that this pattern holds in natural environments). Conversely, the identification of 2-methylbacteriohopanetetrol and methylated gammaceranes in *R. palustris* may help to explain cases in which the presence of these compounds is otherwise difficult to interpret.

In summary, our results indicate that the production of substantial quantities of polyhydroxylated 2-methylhopanoids occurs in phototrophs other than cyanobacteria and that their biosynthesis does not require molecular oxygen. We have demonstrated that 2-MeBHP ratios can vary substantially for a given organism and that the chemical identity of hopanoid side chains can be closely associated with A-ring methylation. Because *R. palustris* is metabolically versatile, fast-growing, and genetically tractable (36), it holds promise as a model system for advancing our understanding of the biosynthesis and physiological role of these important biomarkers.

Methods

Strains and Growth Conditions. CGA-009 was grown photoautotrophically on H_2 in batch culture at 30°C, with 2,000-lux illumination provided by tungsten bulbs. TIE-1 was cultured under conditions summarized in Table 1. All TIE-1 cultures were inoculated after five consecutive 10-ml transfers at exponential phase under conditions otherwise consistent with the final batch culture condition [transfers were conducted in an anaerobic glove box (Coy) unless the final batch culture was aerobic]. Glassware was baked at 450°C for 8 h before use. Growth phase was monitored daily by optical density at 660 nm. Most phototrophic TIE-1 cultures (conditions 2–7) were grown in 250 ml of minimal freshwater medium (51) at 30°C with 2,000-lux illumination. All media were prepared anaerobically, and cultures were inoculated in a Coy anaerobic glove box. Flasks and neoprene stoppers were placed in the glove box at least 1 day before use. Cultures were incubated with a 250-ml head space (flask void volume) of gas, consisting of pure N_2 for acetate and thiosulfate cultures and 4:1 H_2:CO_2 for hydrogen cultures. For growth conditions 1–4, cells were harvested during the log phase. For all other experiments, cells were harvested on day 2 of the stationary phase. pH was buffered to 6.8 using 20 mM bicarbonate for conditions 2–4 and 2 mM bicarbonate plus 20 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid for conditions 5–7. Normally, acetate (conditions 2 and 5) and thiosulfate (conditions 3 and 6) were provided at 20 mM concentration. One set of thiosulfate cultures (Table 1, condition 8) was inoculated and incubated entirely in a Coy anaerobic glove box to eliminate the possibility of incidental oxygen exposure. In this case, incubation was at room temperature (≈22°C), and 10 mM thiosulfate was provided, initially followed by a second addition of the same amount after 1 week. Aerobic batch cultures (condition 1) (250 ml) were serially transferred and grown on YP medium [0.3% yeast extract/0.3% Bacto Peptone (Difco, Detroit, MI)] in a shaking incubator at 30°C and 250 rpm. No illumination was provided for aerobic cultures, and final aerobic cultures were grown in complete darkness.

GC-MS Analysis. Cells were harvested by centrifugation at ≈12,000 × *g* in solvent-washed spin bottles, frozen, and lyophilized overnight in an organic-clean freeze dryer (VirTis K-series; VirTis, Gardiner, NY). Dry samples were sonicated in 1:2:0.8 dichloromethane (DCM):methanol:water for 30 min at room temperature and separated into two phases by the addition of 1:1 DCM:water to obtain lipid extracts. To cleave functionalized side chains, extracts were treated with H_5IO_6 (300 mg) in 8:1 tetrahydrofuran:water (3 ml) for 1 h at room temperature, recovered, dried over $NaSO_4$, and reduced with $NaBH_4$ (100 mg) in methanol (3 ml) for 4 h at room temperature according to the procedure of Rohmer *et al.* (9). The resulting hopanols were separated from other compound classes by solid-phase extraction on a Sepra aminopropyl stationary phase (Phenomenex, Torrance, CA). All samples were derivatized as acetate esters for GC-MS analysis by reaction with 1:1 acetic anhydride:pyridine for 20 min at 70°C. An internal standard (10 μg of cholesterol acetate or squalane) was added to each sample. To test for background contamination, growth media and Teflon spin bottles were extracted repeatedly with DCM, which was subsequently evaporated under a stream of N_2. The residue was processed and analyzed according to the standard analytical procedure described above. No hopanoids were observed in any of the procedural blanks.

Hopanols were separated on a ThermoFinnigan Trace GC (Thermo Scientific, Waltham, MA) equipped with a DB-5ms or ZB-5-ms capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) programmed to 320°C for 30 min. A sample injection was from a programmable temperature vaporizing injector programmed to 350°C and operated in splitless mode. Column effluent was split (4:1) between a ThermoFinniganDSQ mass spectrometer and a flame ionization detector. The C_{32} hopanols were identified from their electron impact mass spectra and by comparison of retention times to a hopanoid extract of known composition derived from *P. luridum* (Fig. 2). C_{31} analogs were determined from electron impact mass spectra and comparison with an extract from *Nostoc punctiforme* PCC 73102 (data not shown). $C_{32:1}$ analogs were identified by the substitution of an m/z = 215 C + D + E + side chain fragment for the normal m/z = 217 fragment and the presence of an m/z = 369 fragment, indicating a saturated ring system. Tetrahymanol was identified based on comparison with a standard from *Tetrahymena pyriformis*. Quantitation was based on flame ionization detector peak areas by comparison with the internal standard. The minimum detection limit was defined independently for each sample by a 20:1 signal:noise ratio based on peak area and was below 1 μg per g of dry mass for all samples.

Methionine Labeling and Cyanobacterial 16S PCR Amplification. TIE-1 was cultured anaerobically at 30°C and 2,000-lux illumination, with 10 mM acetate, 20 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (pH 7.0), and either 1 mM L-methionine or 1 mM L-[*methyl*-^{13}C,$^{2}H_3$]methionine (Sigma–Aldrich, St. Louis, MO). PCR was performed with cyanobacterial 16S PCR primers CYA106F and a 1:1 mixture of CYA718R(a) and CYA718R(b) (52) and a FailSafe PCR kit (Epicentre, Madison, WI), using buffer D. PCR program steps were as follows: 10 min at 95°C; 40 cycles of 30 sec at 95°C, 30 sec at 53°C, and 1 min at 72°C; and a 5-min final extension at 72°C. Template consisted of TIE-1 culture sampled before harvesting (≈10^9 cells per ml). A positive control template was created by spiking 0.5-ml TIE-1 template with 5 μl of *Synechocystis* sp. PCC 6803 culture (≈10^7 cells per ml). For qualitative GC-MS analysis, cells were harvested and processed as above. Hopanols were derivatized as trimethylsilyl ethers with 5:2 BSTFA:pyridine. Qualitative analysis for methionine labeling was conducted on an HP 6890 GC (Agilent, Palo Alto, CA) with an Agilent HP-5MS capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) programmed to 320°C as in *GC-MS Analysis* and injected by a splitless programmable temperature vaporizing injector set to 325°C. Electron impact MS was carried out on an HP 5973 mass selective detector.

Liquid Chromatography-Atmospheric Pressure Chemical Ionization-MS Analysis. Dry cells from postexponential acetate-grown cultures (condition 5) were extracted as above with dichloromethane/MeOH/H_2O. Lipid extracts were separated by precipitation in cold acetone and centrifugation at 2,000 × *g* into polar (pellet) and nonpolar (supernatant) fractions (53). The polar fraction was saponified by heating at 70°C overnight in 0.5 M NaOH in 1:1 H_2O:methanol, extracted without acidification into MTBE, and acetylated at 70°C in 1:1 acetic anhydride:pyridine. Separation was performed on an Agilent 1100 Series HPLC, using a Waters (Milford, MA) Nova-Pak C_{18} 3.9 × 150 mm column and the following gradient: 100% A to 1 min, linear gradient to 100% B at 8 min, then isocratic to 12 min; A = 90% methanol, 10% water; B = 59% methanol, 1% water, 40% isopropyl alcohol. All organic solvents were HPLC grade or higher; water was purified by using a Nanopure water purifier (Barnstead, Dubuque, IA). Positive-mode atmospheric pressure chemical ionization was performed by using an Agilent ion trap mass spectrometer. Ionization parameters were optimized by direct infusion of an acetylated preparation of hexane-1,2,3-triol. Vaporizer temperature was 300°C, dry gas flow was 10 liters/min, and corona current was 3 μA. MS^2 was performed at 35% collision energy.

With profound sadness we record that Sky Rashby passed away on August 25, 2007, while this manuscript was in press. This is his work and his first scientific publication. We mourn the tragic loss of this talented young scientist and compassionate individual. We thank members of the O.K.N. and A.L.S. laboratories, Chad Vecitis, and Nathan Dalleska for technical assistance; Helen Talbot and Tsege Embaye for helpful discussion; Jack Meeks (University of California, Davis, CA) and Arthur Grossman (Stanford University, Stanford, CA) for providing cyanobacterial strains; and Linda Jahnke (National Aeronautics and Space Administration Ames Research Center, Moffett Field, CA) for advice and cyanobacterial samples. This work was supported by grants from the Packard Foundation and Howard Hughes Medical Institute (to D.K.N.) and the National Science Foundation (to A.L.S.) and the National Aeronautics and Space Administration Exobiology Program (to R.E.S.).

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