Oxidation of Fe(II) leads to increased C-2 methylation of pentacyclic triterpenoids in the anoxygenic phototrophic bacterium *Rhodopseudomonas palustris* strain TIE-1

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ABSTRACT

Hopanoids are among the most widespread biomarkers of bacteria that are used as indicators for past and present bacterial activity. Our understanding of the production, function, and distribution of hopanoids in bacteria has improved greatly, partly due to genetic, culture-independent studies. Culture-based studies are important to determine hopanoid function and the environmental conditions under which these compounds are produced. This study compares the lipid inventory of *Rhodopseudomonas palustris* strain TIE-1 under anoxic photoautotrophic conditions using either H₂ or Fe(II) as electron donor. The high amount to which adenosylhopane is produced irrespective of the used electron donor suggests a specific function of this compound rather than its exclusive role as an intermediate in bacteriohopanepolyol biosynthesis. C-2 methylated hopanoids and tetrahymanol account for as much as 59% of the respective C-2 methylated/non-methylated homologs during growth with Fe(II) as electron donor, as compared with 24% C-2 methylation for growth with H₂. This observation reveals that C-2 methylated hopanoids have a specific function and are preferentially synthesized in response to elevated Fe(II) concentrations. The presence of C-2 methylated pentacyclic triterpenoids has commonly been used as a biosignature for the interpretation of paleoenvironments. These new findings suggest that increased C-2 methylation may indicate anoxic ferrous conditions, in addition to other environmental stressors that have been previously reported.

INTRODUCTION

Microbes have thrived on Earth for at least 3.5 billion years. As these organisms do not—with very few exceptions—leave behind diagnostic morphological fossils, other methods are needed to reconstruct ancient life and former environmental conditions. Among the most potent indicators for past prokaryotic life are molecular fossils, i.e. lipid biomarkers (e.g., Summons *et al.*, 1999; Brocks *et al.*, 2003; Brocks & Schaeffer, 2008). Some molecular fossils can be assigned to specific groups of organisms based on the lipid inventory of modern organisms. Among the most widespread bacterial molecular fossils are hopanoids (e.g., Peters *et al.*, 2005).

Hopanoids are pentacyclic triterpenoids produced by bacteria and are believed to play a functional role in membrane stability and integrity, equivalent to sterols in eukaryotes (e.g., Ourisson *et al.*, 1979, 1987; Jahnke *et al.*, 1992; Berry *et al.*, 1993; Kannenberg & Poralla, 1999; Welander *et al.*, 2009). They are ubiquitous in soils and sediments (e.g., Ourisson *et al.*, 1979; Ourisson & Albrecht, 1992; Farrimond *et al.*, 2000; Talbot & Farrimond, 2007; Cooke *et al.*, 2008a; Talbot *et al.*, 2008; Pearson *et al.*, 2009; Zhu *et al.*, 2011). Because their pentacyclic
ring structure is highly resistant to degradation, hopanoid hydrocarbons (geohopanoids) are among the oldest and most abundant organic compounds found in the rock record (e.g., Brocks et al., 1999, 2003). Culture-independent genetic studies have revealed that hopanoids are confined in their phylogenetic distribution to only about 10% of the bacteria (Pearson et al., 2007, 2009). Moreover, hopanoid biosynthesis varies greatly with culture conditions (e.g., Poralla et al., 1984; Rohmer et al., 1984; Rashby et al., 2007; Welander et al., 2009), and the actual functions of specific hopanoids have yet to be discovered.

The bacterium *Rhodopseudomonas palustris* strain TIE-1 has emerged as an excellent model organism to study hopanoid biosynthesis and function (Rashby et al., 2007; Welander et al., 2009, 2010, 2012; Doughty et al., 2011). It produces several C30 hopanoids, as well as extended C35 bacteriohopanepolys (BHPs) with a functionalized side chain. TIE-1 is capable of methylating hopanoids at C-2 (Rashby et al., 2007; Welander et al., 2009, 2010, 2012), which is rare among hopanoid producers (Welander et al., 2010). In fact, while C-2 methylated C30 hopanoids were already known to exist in *Methyllobacterium*, *Beijerinckia*, *Bradyrhizobium*, and *Rhodopseudomonas* (Bisseret et al., 1985; Vichéze et al., 1994; Bravo et al., 2001; Rashby et al., 2007), C-2 methylated BHPs were found in high abundances at first only in cyanobacteria (Rohmer et al., 1984; Summons et al., 1999). Only later, they were also recognized in other bacteria, including TIE-1 (Rashby et al., 2007; Talbot et al., 2007; Welander et al., 2010). Despite the fact that C-2 methylated hopanoids are not only found in cyanobacteria, other potential source organisms are often not considered in paleoenvironmental studies (e.g., Jia et al., 2012). In many instances, other bacteria (e.g., anoxygenic phototrophs) may well have been the producers of 2-methyl-hopanoids. In fact, no cultured bacteria (e.g., anoxygenic phototrophs) may well have been recognized in other bacteria, including TIE-1 (Rashby et al., 2009, 2010, 2012). In many instances, C-2 methylation of hopanoids are reported for the two growth conditions, which has significant implications for the interpretation of the sedimentary record.

**MATERIALS AND METHODS**

**Bacterial strain and growth conditions**

*Rhodopseudomonas palustris* strain TIE-1 (Jiao et al., 2005) is a purple non-sulfur bacterium belonging to the Alphaproteobacteria. It grows photoautotrophically by oxidizing Fe(II) to Fe(III) or by using H2 as electron donor. TIE-1 was originally isolated from a freshwater iron-rich mat in Woods Hole, MA, USA. The strain was cultivated in anoxic mineral medium with the phosphate content reduced to 1.0 mM (Hohmann et al., 2010). Addition of NaCl compensated for the ionic strength lost by reducing the phosphate content. The freshwater medium contained 0.5 g L\(^{-1}\) NH\(_4\)Cl, 0.14 g L\(^{-1}\) KH\(_2\)PO\(_4\), 0.27 g L\(^{-1}\) NaCl, 0.1 g L\(^{-1}\) CaCl\(_2\)\(\cdot\)2H\(_2\)O, 0.5 g L\(^{-1}\) MgSO\(_4\)\(\cdot\)7H\(_2\)O. After autoclaving and cooling under N\(_2\)/CO\(_2\) (90/10) gas, 22 mM bicarbonate buffer was added, which was autoclaved separately under a N\(_2\)/CO\(_2\) atmosphere. Then, the following solutions were added from sterile stocks: 1 mL L\(^{-1}\) trace element solution, 1 mL L\(^{-1}\) vitamin B\(_{12}\) solution and 10 mL L\(^{-1}\) of a modified vitamin solution (Ehrenreich & Widdel, 1994). This vitamin solution was ten times diluted and amended with 50 mg L\(^{-1}\) riboflavin. The pH of the final medium was adjusted to 6.9. For cultures using Fe(II) as electron donor, FeCl\(_2\) was added to the medium from a sterile stock of anoxic 1 M FeCl\(_2\)\(\cdot\)4H\(_2\)O (final concentration in the medium ~10 mM Fe(II)). For cultures using H2 as electron donor, the headspace was exchanged with H\(_2\)/CO\(_2\) (80/20) over every 3 days. Inoculation of cultures was from a fresh preculture grown with the respective electron donor. Cultures were grown in 500 ml medium in 1 L Schott bottles for cultures on H\(_2\), or in 100 ml medium in 200 ml serum.
bottles for cultures on Fe(II). Incubation was at 30 °C and at >600 lux under a tungsten light bulb. Fe(II) oxidation was quantified and monitored over time by using the spectrophotometric ferrozine assay (see below). Cultures were harvested in late exponential growth phase after approximately 85% Fe(II) had been oxidized to Fe(III) (after 19 days), or after 14 days of incubation in exponential growth phase during growth on H₂. Cells were harvested by centrifugation at 10,000 g under anoxic conditions (Herolab, HiCen 21), frozen and freeze-dried (Christ, Alpha 1–4). For cultures grown on H₂, the material of three different cultures, 500 mL each, was combined, while for cultures grown on Fe(II), the material of eight different cultures, 100 mL each, was combined to obtain the amount of biomass required for lipid analysis. Therefore, cultures were not analyzed separately; hence, no standard deviations can be given, and the data shown in this study represent an average of these pooled multiple cultures.

Quantification of iron (ferrozine assay)

Fe(II) and Fe(total) were quantified by the spectrophotometric ferrozine assay modified from Stookey (1970) and described by Hegler et al. (2008). Quantification of Fe(II) and Fe(total) of all samples was carried out before inoculation and during Fe(II) oxidation by the cultures to determine the degree of Fe(II) oxidation.

Extraction and derivatization of lipids

All glassware was baked at 400 °C for 4 h before use to remove traces of organic compounds. The freeze-dried material was extracted three times with dichloromethane (DCM)/methanol (3:1 v/v) by sonication for 15 min at room temperature. Three internal standards (2-methyl-octadecanoic acid, 1-nonadecanol, and 5α-cholestane) were added to all samples before extraction. The extracts were combined and washed with DCM-extracted water. The organic phases were collected, combined and dried with sodium sulfate. Excess solvent was removed under reduced pressure. Aliquots of the total lipid extracts (TLEs) were acetylated by reaction with acetic anhydride and pyridine at room temperature overnight, another aliquot was derivatized to TMS-ethers by reaction with BSTFA and pyridine for 1 h at 70 °C.

GC analysis

Lipids in the TLEs and in fractions F1–F3 were identified and quantified by coupled gas chromatography–mass spectrometry (GC–MS) with an Agilent 7890 A GC system coupled to an Agilent 5975 C inert MSD mass spectrometer at the University of Vienna. Two different GC columns were used for analysis: a 30 m HP-5 MS U1 fused silica capillary column (0.25 mm i.d., 0.25-μm film thickness) with the following temperature program: 60 °C (1 min); from 60 to 150 °C at 10 °C/min; from 150 °C to 325 °C at 4 °C/min, 35 min isothermal for analyzing C30 hopanoids in F1 and F3; and a 15 m DB-5HT fused silica capillary column (0.25 mm i.d., 0.10 μm film thickness) with the following temperature program: 80 °C (3 min); from 80 °C to 200 °C at 15 °C/min; from 200 °C to 250 °C at 10 °C/min; from 250 °C to 340 °C at 15 °C/min, 20 min isothermal for analyzing fractions F1, F3, and the silylated and acetylated TLEs. The carrier gas was helium. All samples were run parallel in full scan (m/z 50–800) and selected ion monitoring mode (m/z 191, 205, 367, 369, 381, 383) for identification of pentacyclic triterpenoids, including the BHPs (2-methyl-) bacteriohopan-32,33,34,35-tetrol (BHT) and 35-aminobacteriohopane-32,33,34-triol (aminotriol). Identification of individual compounds was based on comparison of retention times and mass spectra with published data and reference compounds. The C30-pentacyclic triterpenoids were quantified with an internal standard in the F3 fraction (1-nonadecanol); the F1 fraction and the TLE were quantified with 5α-cholestane as internal standard. It was assumed that the response factors of the various compounds on the GC-MS were identical. This may have resulted in an over- (e.g., diplopterol) or underestimation (e.g., BHT, aminotriol) of the contents of specific hopanoids in quantifications based on the total ion current (TIC) (see Sessions et al., 2013). The 2-methyl percentages of all pentacyclic triterpenoids were calculated by extracting the m/z 191 and m/z 205 of the coeluting 2-methyl- and non-methylated hopanoids of the TIC on the DB-5 HT column.

HPLC/APCI-MS analysis

Bacteriohopanepolysols were also analyzed from the acetylated TLE dissolved in methanol/propan-2-ol (60:40 v/v)
RESULTS AND DISCUSSION

Pentacyclic triterpenoid inventory of TIE-1 grown with H2 vs. Fe(II)

TIE-1 grown with either H2 or Fe(II) as electron donor contained numerous pentacyclic triterpenoids. The inventory shown in Table 1 is in good agreement with previous findings, although TIE-1 was grown under different conditions (Welander et al., 2010). About 50% of the pentacyclic triterpenoids found were C30 compounds, the other were BHPs (Table 1). In general, TIE-1 produced similar compounds with either electron donor, only the relative abundances varied. The dominant pentacyclic triterpenoid under both growth conditions was adenosylhopane VIIIa (see Fig. 1 for structures of compounds; Table 1) with 35% and 48% of all triterpenoids for cultures grown on H2 and Fe(II), respectively. While TIE-1 produced relatively high amounts of bacteriohopane-32,33,34,35-tetrol (BHT) VIa and 35-aminobacteriohopane-32,33,34-triol (aminotriol) VIIa when grown with H2, these compounds were produced only in very small amounts when grown with Fe(II) (Fig. 2; Table 1). 2-Methyl-BHT VIIb was produced by both cultures, but in minor amounts compared with the other BHPs. Both cultures produced the C30 pentacyclic triterpenoids hop-22(29)-ene (diploptene) Ia, hop-21-ene Ila, hopan-22-ol (diplopteron) IVa, and tetrahymanol Va, as well as their respective C-2 methylated homologs (Figs 3, S1 and S2; Table 1). TIE-1 grown with Fe(II) also contained tentatively identified 2,20-dimethylhopane Ic and 2,20-dimethylhopane Id in low abundance, which has been reported in TIE-1 before (Rashby et al., 2007), but was not present in the cultures grown with H2. Moreover, undervatisated diplopterol IIIa, as well as its C-2 methylated homolog, undervatisated 2-methyl-diplopterol IIIb, were found (Fig. S3; see Jeng et al. (2003) for mass spectra).

While mostly the same pentacyclic triterpenoids were present in cultures with the two different electron donors, the relative abundances of the C-2 methylated homologs

Quantification of lipids per g carbon

To compare the amounts of lipid compounds of cells grown on H2 (producing only cells) and cells grown on Fe(II) (producing cell–mineral aggregates), amounts of lipids obtained per weight of sample were back-calculated to amounts per g carbon. For cultures with H2 as electron donor, we assumed the ratio of carbon weight per dry weight to equal 0.5 (Bratbak & Dundas, 1984) and divided the amounts obtained per weight of sample by 2. For cultures with Fe(II) as electron donor, we referred to the stoichiometric reaction equation for anoxygenic photosynthetic Fe(II) oxidation (Widdel et al., 1993; Kappler & Newman, 2004) as in the equation below. The biogenic Fe mineral precipitate was assumed to be Fe(OH)3 with a molecular weight of 107 g mol–1. CH2O is a proxy for biomass with a molecular weight of 30 g mol–1. The ratio of Fe mineral to biomass is 4:1. The cell–mineral aggregates therefore have a molecular weight of 458 g mol–1 (4*107 g mol–1 + 1*30 g mol–1). The percentage of carbon is 2.6% (12 g mol–1/458 g mol–1). The amount of compounds per g carbon consequently corresponds to 2.6% of the amount of compounds per g dry sample.

$$4Fe^{2+} + HCO_3^- + 10H_2O \rightarrow 4Fe(OH)_3 + (CH_2O) + 7H^+.$$
varied significantly (Table 1). C-2 methylation was observed for all compounds except for aminotriol VII and adenosylhopane VIII. In both cultures, pentacyclic triterpenoid alcohols (diplopterol IV and tetrahymanol V, as well as the underivatized diplopterol III), showed the highest degree of methylation, while hopenes (diploptene I and hop-21-ene II) were less methylated. The diplopterol III and tetrahymanol V showed the highest degree of methylation in the Fe(II)-grown cultures, with 58% and 50% methylation, respectively. The highest degree of methylation in the H2-grown cultures was observed for diplopterol III, with 59% methylation. The underivatized diplopterol III showed the highest degree of methylation in both cultures, with 58% and 50% methylation, respectively.

### Table 1

<table>
<thead>
<tr>
<th>Compound Description</th>
<th>TIE-1 on H2</th>
<th>TIE-1 on Fe(II)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C30 pentacyclic triterpenoids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I Diploptene</td>
<td>5.5</td>
<td>0.6</td>
</tr>
<tr>
<td>II Hop-21-ene</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>III Underivatized diplopterol</td>
<td>9.0</td>
<td>4.7</td>
</tr>
<tr>
<td>IV Diplopterol</td>
<td>12.6</td>
<td>37.7</td>
</tr>
<tr>
<td>V Tetrahymanol</td>
<td>15.4</td>
<td>5.9</td>
</tr>
<tr>
<td><strong>C35 bacteriohopanepolyols</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VI Bacteriohopanetetrol</td>
<td>10.6</td>
<td>1.4</td>
</tr>
<tr>
<td>VII Aminobacteriohopanetriol</td>
<td>11.4</td>
<td>0.2</td>
</tr>
<tr>
<td>VIII Adenosylhopane</td>
<td>35.1</td>
<td>47.9</td>
</tr>
<tr>
<td><strong>Total amount of pentacyclic triterpenoids (mg g⁻¹ C)</strong></td>
<td>42</td>
<td>14</td>
</tr>
</tbody>
</table>

Contents are given in percent of total triterpenoids; C-2 methylation is given in percent of the combined non-methylated and C-2 methylated homologs. Structures of compounds: see Fig. 1. n.d., not detected.
and hop-21-ene II) as well as BHT VI revealed much less methylation. With Fe(II) as electron donor, C-2 methylation was generally more abundant for all compounds than in cultures grown with H₂. 22-24% of pentacyclic triterpenoid alcohols of the respective C-2 methylated/non-methylated couplets, and 8-14% of hopenes were C-2 methylated with H₂ as electron donor, compared with a C-2 methylation of 50-59% for alcohols and 11-37% for hopenes with Fe(II) as electron donor. Thus, for growth with Fe(II), C-2 methylation was more abundant, particularly for diplopterol. To exclude the possibility that 2-methyl-hopenes formed during the analytical procedure such as the acetylation (cf. Doughty et al., 2011), the hydrocarbon fraction was additionally analyzed (F1; Fig. S1). In this fraction, C-2 methylated hopenes were also found to be prevalent, confirming their authenticity in the

![Fig. 2 HPLC/APCI-MS partial mass chromatograms showing BHPs in the acetylated total lipid extracts of cultures of Rhodopseudomonas palustris TIE-1 grown with H₂ (A) or Fe(II) (B) as electron donor. Numbered compounds: VI, bacteriohopanetetrol; VII, aminotriol; VIII, adenosylhopane, a non-methylated homolog; b, C-2 methylated homolog. Structures of compounds: see Fig. 1.](image)

![Fig. 3 GC-MS ion chromatograms of acetylated total lipid extracts of Rhodopseudomonas palustris TIE-1 cultures grown on H₂ (left) or Fe(II) (right) as electron donor. In the total ion current (TIC) chromatograms (A and D), all compounds are apparent. In the single ion chromatograms with m/z 191 (B, E) and m/z 205 (C, F), only pentacyclic triterpenoids are apparent. Non-methylated triterpenoids with their major fragment of m/z 191 are distinguished from C-2 methylated triterpenoids with their major fragment of m/z 205. The authenticity of C-2 methylated hopenes was confirmed by analysis of the F1 fraction (Fig. S1). Numbered compounds: I, diploptene; II, hop-21-ene; III, underivatized diplopterol, IV, diplopterol, V, tetrahymanol; VI, bacteriohopanetetrol; VII, aminotriol; a, non-methylated homolog; b, C-2 methylated homolog; c; 2,20-dimethylated homolog; Structures of compounds: see Fig. 1.](image)
acetylated TLE. Interestingly, C-2 methylation in BHPs was observed as well, but was minor compared with C30 hopanoids. Only traces of 2-methyl-BHT VIb were detected with H2 as electron donor. With Fe(II), BHT VI was produced only in very small amounts, but methylation at C-2 was prominent with 25%.

It needs to be mentioned that artifacts were produced either during the derivatization procedure or when injecting the samples on the split-splitless injector, which are shown in more detail in the Supporting Information and are only briefly summarized here. Acetylation of F3 and the TLE led to the formation of C30 hopanoids from diplopterol. This became obvious by extremely high abundances of hopanoids in the acetylated F3 compared with F1 (Table S1). Likewise, highly increased abundances of hopanoids were found in the acetylated TLE compared with F1 (Figs 3 and S1; Table S1). Additionally, there was a decrease in hopanols in the acetylated TLE or acetylated F3 compared with the TMS-derivatized F3 fractions (Table S1). To exclude the artifacts produced during derivatization, the relative abundances given in Table 1 for hopanes derive from fraction F1, and the values for the hopanols derive from TMS-derivatives of fraction F3. Only the relative abundances of BHPs were obtained from the acetylated TLE, needed for relating abundances measured on the GC-MS and the HPLC/APCI-MS.

\textbf{C30 pentacyclic triterpenoid vs. BHP abundances: implications for hopanoid function in TIE-1 grown with H2 or Fe(II)}

The major difference between the hopanoid inventories of the two TIE-1 cultures is the lower abundance of BHT and aminotriol during growth with Fe(II). BHT and aminotriol belong to the most commonly found hopanoids in bacterial cultures and the environment, but are therefore of rather low source specificity. Adenosylhopane, the dominant hopanoid in the two TIE-1 cultures, has been considered as an intermediate in the production of BHPs including BHT and aminotriol (cf. Neunlist et al., 1988; Bradley et al., 2010). Other than that, adenosylhopane has not been ascribed a specific function in bacterial cells. It has been detected in pure cultures of \textit{Rhodoblastus acidophilus} (formerly \textit{Rhodopseudomonas acidophila}; Neunlist et al., 1988), the ammonium-oxidizer \textit{Nitrosononas europaea} (Seemann et al., 1999), the nitrogen-fixing bacterium \textit{Bradyrhizobium japonicum} (Bravo et al., 2001), and the purple non-sulfur bacteria \textit{R. palustris} and \textit{Rhodomicrobium vaniellii} (Talbot et al., 2007; Welander et al., 2012). In contrast to our results, adenosylhopane was mostly present in minor amounts in previous studies, agreeing with its putative status as an intermediate in BHP biosynthesis. In our cultures of TIE-1 grown on Fe(II), BHT and aminotriol were produced in very low abundance, either because they were not required or could not be synthesized under these conditions. The enzyme encoded by the \textit{hpnG} gene, transforming adenosylhopane to ribosylhopane (Bradley et al., 2010; Welander et al., 2012), might consequently not be as active under Fe(II)-oxidizing conditions. The greater abundance of adenosylhopane in the Fe(II) cultures compared with the H2 cultures could potentially reflect the accumulation of this putative intermediate. Although adenosylhopane was relatively less abundant in the cultures grown on H2, it was still a major compound. A specific function of adenosylhopane in TIE-1 might, thus, be an alternative explanation for its overall abundance rather than representing solely an intermediate in BHP production.

Adenosylhopane has so far only rarely been found in marine sediments (Blumenberg et al., 2010), but it is among the most abundant BHPs in soils (Talbot & Farrimond, 2007; Cooke et al., 2008a). High contents of adenosylhopane and related compounds, such as 2-methyl-adenosylhopane or compounds in which the adenine group is replaced by a yet unidentified group, in soils led to the suggestion that adenosylhopane can be used as an indicator of terrestrial input into lacustrine and marine environments (Talbot & Farrimond, 2007; Cooke et al., 2008a,b; Reitmeyer et al., 2010). Purple non-sulfur bacteria like TIE-1 are common in both terrestrial and aquatic environments and the production of high amounts of adenosylhopane under the chosen growth conditions challenges the concept of adenosylhopane as a specific biomarker for soil bacteria (see also Zhu et al., 2011).

Several studies aimed at elucidating the function of hopanoids in bacteria, as for example their possible role in nitrogen fixation (Blumenberg et al., 2012; Sánchez et al., 2012). However, so far, it is only known that hopanoids are localized in the cytoplasmic and outer cell membranes, where they enhance membrane stability and integrity (Ourisson et al., 1987; Jahne et al., 1992; Simonin et al., 1996; Kannenberg & Poralla, 1999; Doughty et al., 2009; Welander et al., 2009; Sánchez et al., 2012), but their exact function is still enigmatic. Consequently, specific functions can neither be allocated to C30 hopanoids nor to BHPs. While most previous studies focused on the putative function of BHPs, C30 hopanoids gained little attention and were commonly interpreted as precursors of BHPs. Likewise, diploptene is thought to be the precursor of adenosylhopane (Bradley et al., 2010; Welander et al., 2012). Its low concentration in both cultures of this study agrees with diploptene being a precursor in BHP biosynthesis. However, the comparably high abundance of diplopterin and its C-2 methylated homolog rather suggest a specific function for these compounds, especially because diplopterol does not seem to act as an intermediate in BHP synthesis (Bradley et al., 2010; Welander et al., 2012). Only little is known about the functions of specific hopanoids. Welander et al. (2012) were the first to propose distinct
functions for individual hopanoids. These authors have shown that aminotriol and C-2 methylated hopanoids do not contribute significantly to membrane integrity in the presence of bile salts, while C₃₀ and other C₃₅ hopanoids do contribute. Interestingly, in our study, TIE-1 produced aminotriol when grown with H₂, but hardly any aminotriol when grown with Fe(II), also suggesting a specific function for this BHP.

Increased C-2 methylation of hopanoids during growth on Fe(II): implications for the rock record

C-2 methylation occurred in TIE-1 cultures grown with either electron donor, but especially in C₃₀ pentacyclic triterpenoids and only to a lesser degree in BHPs. The suggested functions of 2-methyl-hopanoids in bacteria are manifold, such as governing membrane fluidity (Bisseret et al., 1985), or protection from pH or temperature stress (Rashby et al., 2007; Welander et al., 2009), but a specific function for the C-2 methylation has not been proven so far. Our results reveal significant variations in the relative abundance of C-2 methylation for C₃₀ pentacyclic triterpenoids and BHPs with methylation being highly increased in the Fe(II) cultures. It seems that the C-2 methylation was chiefly dependent on the different growth conditions TIE-1 was subjected to, which in turn indicates that the degree of C-2 methylation is a response to changing environmental conditions.

Varying C-2 methylation in response to culture conditions such as composition of medium, carbon source, incubation temperature or growth phase has been proposed before (Bisseret et al., 1985; Vilchêze et al., 1994; Rashby et al., 2007; Welander et al., 2009). Previous studies of TIE-1 cultures reported 2-methyl-tetrahymanol and 2-methyl-BHT (Rashby et al., 2007; Welander et al., 2009). Like in this study, aminotriol was detected only without C-2 methylation. Interestingly, in these earlier studies on TIE-1 (Rashby et al., 2007; Welander et al., 2009), diplopterol and 2-methyl-diplopterol were not found. In our study, diplopterol was prominent and showed the highest degree of C-2 methylation. In another recent study, Welander et al. (2012) observed the dehydrogenation of diplopterol and 2-methyl-diplopterol to hopanes upon acetylation of the TLE, confirming that diplopterol and 2-methyl-diplopterol were indeed produced by TIE-1 by analyzing the alcohol fractions of the extracts. In the earlier studies on TIE-1 (Rashby et al., 2007; Welander et al., 2009), these compounds were probably produced as well, but could not be identified in the non-purified TLE. While all studies on TIE-1 reported high abundances of C-2 methylation (up to 65% of total hopanoids), it is difficult to compare previous results with those found here, because the degree of C-2 methylation was not given for single hopanoids, but only on aggregate, either for the total hopanoids or the tetrafunctionalized BHPs only.

The increased C-2 methylation of pentacyclic triterpenoids observed in this study for Fe(II) cultures, especially of diplopterol and tetrahymanol, may well represent a response to stress arising from growth on Fe(II). Bacteria performing Fe(II) oxidation at neutral pH experience stress because their metabolic products are poorly soluble Fe(III) minerals (Miot et al., 2009; Schädler et al., 2009). It has been proposed that the enzyme oxidizing Fe(II) is located in the periplasm (Kappler & Newman, 2004; Croal et al., 2007; Saraiva et al., 2012) and that an acidic microenvironment around the cell may prevent the cells from becoming encrusted by minerals in otherwise neutral pH environments (Hegler et al., 2010; Saraiva et al., 2012).

Adaptation to these for the organism harsh conditions is necessary, and varying the composition of diplopterol and tetrahymanol by increasing the degree of C-2 methylation might be the strategy of TIE-1 to maintain membrane integrity in acidic microenvironments. Our observations are in accordance with earlier studies, indicating that a modification of the composition of hopanoids (e.g., C-2 methylation) rather than a change of overall hopanoid abundance occurs in response to stress induced by pH (Welander et al., 2009).

In recent sediments, 2-methyl-hopanoids have rarely been found, and, if found, have mostly been interpreted as biomarkers of cyanobacteria (e.g., Farrimond et al., 2004; Talbot & Farrimond, 2007; Pearson et al., 2009). Cyanobacteria thrive in the oxic photic zone of lakes and oceans, while anoxygenic phototrophs are present in the sediment surface, in the lower photic zone, or in microbial mats. Some strains, such as TIE-1, are also able to grow under oxic conditions, switching to a chemotrophic metabolism. Both cyanobacteria and anoxygenic phototrophs produce hopanoids that may end up in the sediment. Discrimination of these two groups based on hopanoids will, thus, require diagnostic hopanoids other than 2-methyl-hopanoids. Cyanobacteria produce pentafunctionalized BHPs (Talbot et al., 2008), which are not known to be produced by anoxygenic phototrophs. Marine cyanobacteria produce BHT-cyclitol ether as major BHP (Talbot et al., 2008; Saúnez et al., 2012). TIE-1 is not known to produce BHT-cyclitol ether or pentafunctionalized BHPs, but a combination of non- and C-2 methylated diplopterol, non- and C-2 methylated tetrahymanol, and non- and C-2 methylated BHT as well as non-methylated adenosylhopane. C-2 methylated hopanoids can also be produced by other bacteria, such as Methylobacterium, Beijerinckia, Bradyrhizobium, or Nitrobrochacter (Bisseret et al., 1985; Vilchêze et al., 1994; Bravo et al., 2001; Welander et al., 2010). These bacteria are either associated with roots or plants (Beijerinckia; Bradyrhizobium), are typically involved in N₂-fixation (Beijerinckia; Bradyrhizobium), or are
facultative aerobes (*Nitrobacter*) or obligate aerobes (*Methylobacterium*).

The results on the influence of different growth conditions on biomarker patterns of TIE-1 may also be relevant for older and more mature sediments. The extreme abundance of C-2 methylated hopanoids and tetrahymanol in TIE-1 grown under anoxic photoautotrophic Fe(II)-oxidizing conditions is of great significance for the interpretation of ancient iron-rich environments. Anoxicogenic phototrophs were suggested as mediators in the deposition of banded iron formations (Widdel *et al.*, 1993; Kappler *et al.*, 2005; Posth *et al.*, 2008). The growth conditions with Fe(II) as electron donor in our experiments mimic the environment of the Precambrian anoxic ferrous oceans. Sadly, the detection of specific biosignatures in mature sediments is limited by biodegradation and thermal maturity. However, anoxicogenic phototrophs may be identified even in rather mature sediments based on the presence of C-2 methylated and regular gammacerane (the derivative of tetrahymanol) in addition to 2-methyl-hopanes (the degragation products of BHPs and C30 hopanoids).

**CONCLUSIONS**

An observed variation of the pentacyclic triterpenoid inventories of *Rhodopseudomonas palustris* TIE-1 grown photo-autotrophically with either H2 or Fe(II) as electron donors are apparently the result of a response to changing environmental conditions. This variation is reflected by the lower abundance of bacteriohopanetetrol (BHT) and 35-aminobacteriohopanetetrol, and the significantly higher degree of C-2 methylation of various pentacyclic triterpenoids during growth with Fe(II). The increased C-2 methylation of hopanoids during growth on Fe(II) possibly reflects a response to the impending encrustation of cells by iron minerals and the resultant pH stress in the microenvironment of the cell. Yet, the effective function of C-2 methylation of pentacyclic triterpenoids is not understood with certainty to date. When interpreting biomarker patterns of recent sediments or mature sedimentary rocks that include C-2 methylated pentacyclic triterpenoids, a possible contribution from anoxicogenic phototrophs should be taken into account. The combination of (2-methyl-) diplopterol, (2-methyl-) tetrahymanol, (2-methyl-) BHT and non-methylated adenosylhopane, or their diagenetic products is a specific biomarker pattern that can indicate the presence of TIE-1 and potentially other TIE-1-like anoxicogenic phototrophs.

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### SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Effect of different derivatization techniques on the quantification of hopanes and hopanols and their C-2 methylated homologs in bacterial cultures of *Rhodopseudomonas palustris* TIE-1 grown with H2 or Fe(II) as electron donor. Annotation to Table S1.

**Fig. S1** GC-MS ion chromatograms of the hydrocarbon fractions F1 of *Rhodopseudomonas palustris* TIE-1 cultures grown on H2 or Fe(II) as electron donor.

**Fig. S2** GC-MS ion chromatograms of the silylated alcohol fractions F3 of *Rhodopseudomonas palustris* TIE-1 cultures grown on H2 or Fe(II) as electron donor.

**Fig. S3** Mass spectra of (A) underivatized diplopterol and (B) underivatized 2-methyl-diplopterol, identified based on a mass spectrum of this compound published by Jeng et al. (2003).