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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 H_2 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 H_2. 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.

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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 C_{30} hopanoids, as well as extended C_{35} bacteriohopanepolyols (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 C_{30} hopanoids were already known to exist in *Methylobacterium*, *Beijerinckia*, *Bradyrhizobium*, and *Rhodopseudomonas* (Bisseret *et al.*, 1985; Vilchèze *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 marine cyanobacteria are known to date that synthesize 2-methyl-hopanoids (Talbot *et al.*, 2008; Sáenz *et al.*, 2012); all known producers of 2-methyl-hopanoids among the cyanobacteria are either freshwater or soil bacteria. In a modern stratified cyanobacterial mat from a hypersaline lake on Kiritimati, 2-methyl-BHT was only found in deeper mat layers, where Alpha- and Deltaproteobacteria are thriving, but not in the uppermost layers dominated by cyanobacteria (Blumenberg *et al.*, 2013).

Hopanoid production in TIE-1 has already been studied under various growth conditions ranging from aerobic chemoheterotrophic growth in YP (yeast, peptone) medium, to anaerobic photoheterotrophic growth with acetate, to photoautotrophic growth with H_2 or thiosulfate (Rashby *et al.*, 2007; Welander *et al.*, 2009, 2010, 2012; Doughty *et al.*, 2011). However, even though isolated as an Fe(II)-oxidizing bacterium (Jiao *et al.*, 2005), hopanoid production has not been monitored during anaerobic phototrophic growth coupled to Fe(II) oxidation. The reasons for not cultivating TIE-1 with Fe(II) anaerobically are most likely the difficulties and inconveniences resulting from these culture conditions, especially slow growth and low cell numbers compared with H_2 cultures. Growth under Fe(II)-oxidizing conditions is particularly relevant for an anoxic, iron-rich Precambrian ocean, in which this metabolism may have been widespread and common (Garrels *et al.*, 1973; Hartman, 1984; Canfield, 2005). Phototrophic Fe(II)-oxidizing bacteria have been suggested as a mediator in the deposition of Precambrian banded iron formations under anoxic conditions (Widdel *et al.*, 1993; Kappler *et al.*, 2005; Posth *et al.*, 2008) and are therefore of particular interest to geobiologists. In this study, hopanoid production was analyzed under the environmentally relevant growth condition with Fe(II) as electron donor in comparison with H_2. Significant variations in the abundance of 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 H_2 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 0.3 g L^{-1} NH_4Cl, 0.14 g L^{-1} KH_2PO_4, 0.27 g L^{-1} NaCl, 0.1 g L^{-1} $CaCl_2*2H_2O$, 0.5 g L^{-1} $MgSO_4*7H_2O$. 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 $FeCl_2*4H_2O$ (final concentration in the medium ~10 mM Fe(II)). For cultures using H_2 as electron donor, the headspace was exchanged with H_2/CO_2 (80/20) once 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 H_2. 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 H_2, 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 (1:1 v/v) for 1 h at 60 °C and subsequent incubation at room temperature overnight. Other aliquots of the TLE were derivatized to trimethylsilyl (TMS)-ethers by reaction with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and pyridine (1:1 v/v) for 1 h at 70 °C. Further aliquots of the TLEs were saponified with 6% KOH in methanol for 3 h at 80 °C. After saponification, neutral lipids were extracted from the saponification extract four times with hexane. Neutral lipids were separated into three fractions according to their polarity by column chromatography (Supelco LC-NH_2 glass cartridges; 500 mg sorbed): hydrocarbons (F1) were eluted with hexane, ketones and esters (F2) with hexane/DCM (3:1 v/v), and alcohols (F3) were eluted with DCM/acetone (9:1 v/v). One aliquot of the alcohol fraction was derivatized to acetates by reaction with acetic anhydride and pyridine (1:1 v/v) for 1 h at 60 °C and subsequent incubation at room temperature overnight, another aliquot was derivatized to TMS-ethers by reaction with BSTFA and pyridine (1:1 v/v) 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 UI 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 °C to 150 °C at 10 °C/min; from 150 °C to 325 °C at 4 °C/min, 35 min isothermal for analyzing C_{30} 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-) bacteriohopane-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 C_{30}-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

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

at Newcastle University as described previously (van Winden *et al.*, 2012). Compounds were separated on a Thermo Finnigan Surveyor high-performance liquid chromatography (HPLC) system, equipped with a Phenomenex Gemini C_{18} 5 μm HPLC column (150 mm, 3.0 mm i.d.) and a security guard column of the same material. The program used was as follows: 0.5 mL/min at 30 °C with 90% A and 10% B (starting at 0 min); 59% A, 1% B, and 40% C (at 25 min); isocratic (to 40 min), returning to starting conditions over 5 min and stabilizing for 15 min, with A = methanol, B = water, C = propan-2-ol. LC/MS was performed on a Thermo Finnigan LCQ ion trap mass spectrometer (MS) equipped with an atmospheric pressure chemical ionization (APCI) source operated in positive ion mode. LC/MS was performed in data-dependent mode with two scan events: SCAN 1 was performed over the whole mass spectrum with a range of *m/z* 500-1300; with SCAN 2, a data-dependent MS^2 spectrum was recorded of the most abundant ion from SCAN 1.

The relative abundances of BHPs were corrected for differences in compound response factors. N-containing BHPs showed a response 1.5 times that of non-N-containing BHPs. No internal standard was added for the BHPs, so BHP quantification was carried out indirectly by correlating GC-MS and HPLC/APCI-MS data for each sample, using GC-amenable BHT and aminotriol as the scale, which were quantified with an internal standard (5α-cholestane). It was assumed that the contents quantified by GC equal contents in HPLC, although it recently has been shown that the BHP contents on the HPLC-MS are higher by at least a factor of 2 (Sessions *et al.*, 2013). The content of adenosylhopane was then calculated from its relative abundance in the HPLC runs. An error of >20% has to be taken into account by applying this method (cf. van Winden *et al.*, 2012 and references therein). It has to be stressed that ionization efficiency varies for each molecule analyzed by HPLC/APCI-MS according to the analytical procedure, and without standards for each hopanoid, the large potential error in the quantification calls for caution when comparing these values with other values in the literature.

Quantification of lipids per g carbon

To compare the amounts of lipid compounds of cells grown on H_2 (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 H_2 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 phototrophic 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}. CH_2O 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.

$$4Fe^{2+} + HCO_3^- + 10H_2O + h\nu \rightarrow 4Fe(OH)_3 + (CH_2O) + 7H^+.$$

RESULTS AND DISCUSSION

Pentacyclic triterpenoid inventory of TIE-1 grown with H_2 vs. Fe(II)

TIE-1 grown with either H_2 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 C_{30} 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 H_2 or 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 H_2, these compounds were produced only in very small amounts when grown with Fe(II) (Fig. 2; Table 1). 2-Methyl-BHT **VIb** was produced by both cultures, but in minor amounts compared with the other BHPs. Both cultures produced the C_{30} pentacyclic triterpenoids hop-22(29)-ene (diploptene) **Ia,** hop-21-ene **IIa**, hopan-22-ol (diplopterol) **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-dimethyltetrahymanol **Vc** in low abundance, which has been reported in TIE-1 before (Rashby *et al.*, 2007), but was not present in the cultures grown with H_2. Moreover, underivatized diplopterol **IIIa**, as well as its C-2 methylated homolog, underivatized 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

Table 1 Production and C-2 methylation of C_{30} pentacyclic triterpenoids and C_{35} bacteriohopanepolyols by *Rhodopseudomonas palustris* TIE-1 grown with either H_2 or Fe(II) as electron donor

Compound		TIE-1 on H_2		TIE-1 on Fe(II)	
		Content (%)	2-Me (%)	Content (%)	2-Me (%)
C_{30} pentacyclic triterpenoids		42.9		50.5	
I	Diploptene	5.5	8	0.6	11
II	Hop-21-ene	0.4	14	0.2	37
III	Underivatized diplopterol	9.0	24	4.7	58
IV	Diplopterol	12.6	22	37.7	59
V	Tetrahymanol	15.4	24	5.9	50
Vc	2,20-Dimethyl-tetrahymanol	n.d.		1.4	
C_{35} bacteriohopanepolyols		57.1		49.5	
VI	Bacteriohopanetetrol	10.6	1	1.4	25
VII	Aminobacteriohopanetriol	11.4	n.d.	0.2	n.d.
VIII	Adenosylhopane	35.1	n.d.	47.9	n.d.
Total amount of pentacyclic triterpenoids (mg g^{-1} C)		42		14	

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.

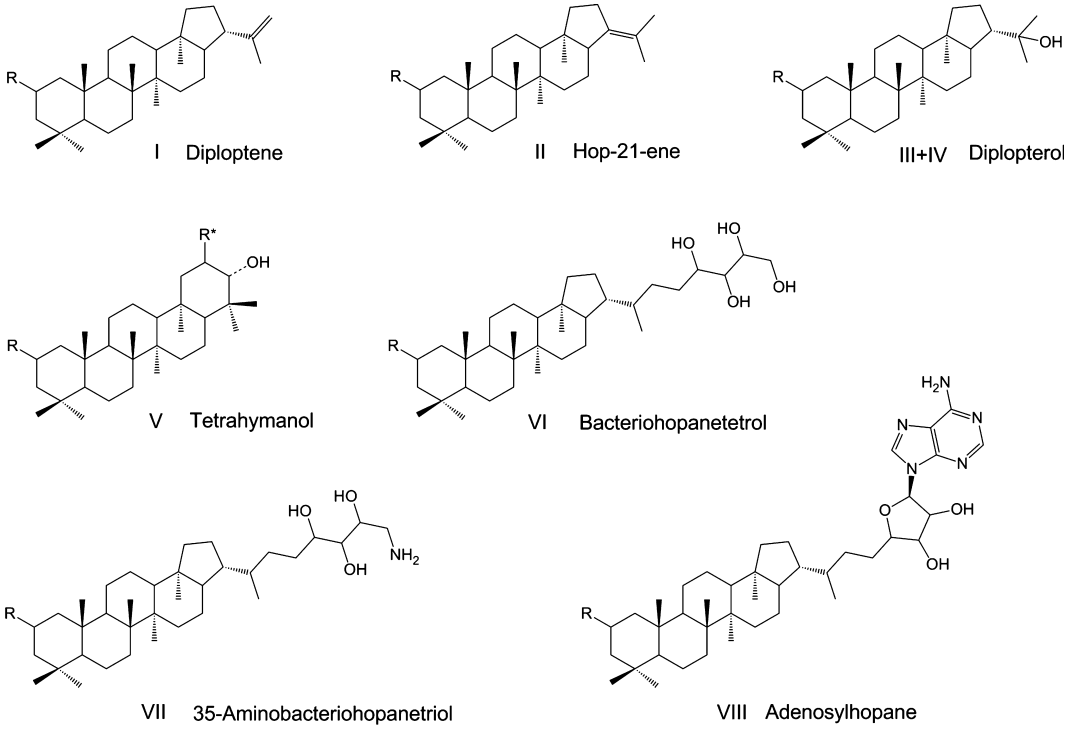

Fig. 1 Structures of pentacyclic triterpenoids detected in extracts of *Rhodopseudomonas palustris* strain TIE-1. The lipids were derivatized and analyzed as acetates or trimethylsilyl-ethers. Diplopterol was present in the extracts in its derivatized as well as its underivatized form, referred to in the text with the numbers IV and III, respectively.

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**

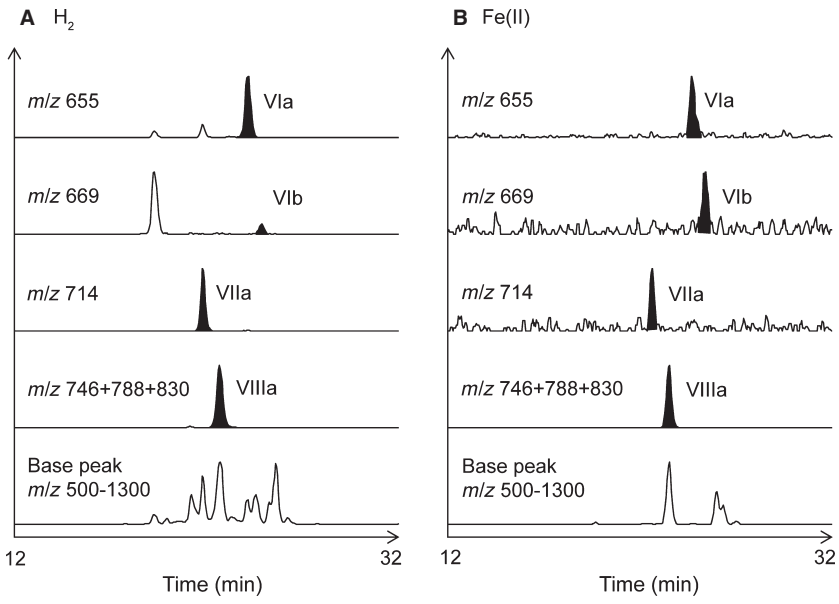

Fig. 2 HPLC/APCI-MS partial mass chromatograms showing BHPs in the acetylated total lipid extracts of cultures of *Rhodopseudomonas palustris* TIE-1 grown with H_2 (A) or Fe(II) (B) as electron donor. Numbered compounds: VI, bacteriohopanetetrol; VII, aminotriol; VIII, adenosylhopane, a non-methylated homolog; b, C-2 methylated homolog. Structures of compounds: see Fig. 1.

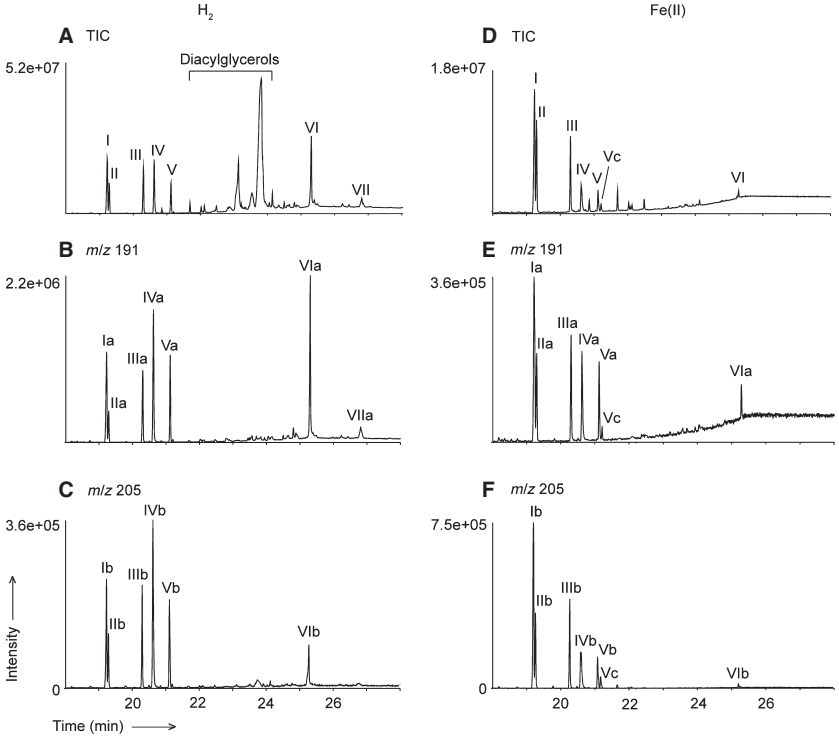

Fig. 3 GC-MS ion chromatograms of acetylated total lipid extracts of *Rhodopseudomonas palustris* TIE-1 cultures grown on H_2 (left) or Fe(II) (right) as electron donor. In the total ion current (TIC) chromatograms (A and D), all compounds are apparent. In the single ion chromatograms with *m/z* 191 (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.

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 H_2. 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 H_2 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

acetylated TLE. Interestingly, C-2 methylation in BHPs was observed as well, but was minor compared with C_{30} hopanoids. Only traces of 2-methyl-BHT **VIb** were detected with H_2 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 C_{30} hopenes from diplopterol. This became obvious by extremely high abundances of hopenes in the acetylated F3 compared with F1 (Table S1). Likewise, highly increased abundances of hopenes 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 hopenes 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.

C_{30} pentacyclic triterpenoid vs. BHP abundances: implications for hopanoid function in TIE-1 grown with H_2 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 *Rhodoblastus acidophilus* (formerly *Rhodopseudomonas acidophila*; Neunlist *et al.*, 1988), the ammonium-oxidizer *Nitrosomas europaea* (Seemann *et al.*, 1999), the nitrogen-fixing bacterium *Bradyrhizobium japonicum* (Bravo *et al.*, 2001), and the purple non-sulfur bacteria *R. palustris* and *Rhodomicrobium vanniellii* (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 of 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 *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 H_2 cultures could potentially reflect the accumulation of this putative intermediate. Although adenosylhopane was relatively less abundant in the cultures grown on H_2, 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; Rethemeyer *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; Sáenz *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; Jahnke *et al.*, 1992; Simonin *et al.*, 1996; Kannenberg & Poralla, 1999; Doughty *et al.*, 2009; Welander *et al.*, 2009; Sáenz *et al.*, 2012), but their exact function is still enigmatic. Consequently, specific functions can neither be allocated to C_{30} hopanoids nor to BHPs. While most previous studies focused on the putative function of BHPs, C_{30} 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 diplopterol 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 C_{30} and other C_{35} hopanoids do contribute. Interestingly, in our study, TIE-1 produced aminotriol when grown with H_2, 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 C_{30} 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 C_{30} 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; Vilchèze *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 dehydration of diplopterol and 2-methyl-diplopterol to hopenes 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; Sáenz *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 *Nitrobacter* (Bisseret *et al.*, 1985; Vilchèze *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 N_2-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. Anoxygenic 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, anoxygenic 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 degradation products of BHPs and C_{30} hopanoids).

CONCLUSIONS

An observed variation of the pentacyclic triterpenoid inventories of *Rhodopseudomonas palustris* TIE-1 grown photoautotrophically with either H_2 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-aminobacteriohopanetriol, 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 anoxygenic 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 anoxygenic 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 hopenes and hopanols and their C-2 methylated homologs in bacterial cultures of *Rhodopseudomonas palustris* TIE-1 grown with H_2 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 H_2 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 H_2 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).