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Identification and characterization of *Rhodopseudomonas palustris* TIE-1 hopanoid biosynthesis mutants

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Abstract

Hopanes preserved in both modern and ancient sediments are recognized as the molecular fossils of bacteriohopanepolyols, pentacyclic hopanoid lipids. Based on the phylogenetic distribution of hopanoid production by extant bacteria, hopanes have been used as indicators of specific bacterial groups and/or their metabolisms. However, our ability to interpret them ultimately depends on understanding the physiological roles of hopanoids in modern bacteria. Towards this end, we set out to identify genes required for hopanoid biosynthesis in the anoxygenic phototroph *Rhodopseudomonas palustris* TIE-1 to enable selective control of hopanoid production. We attempted to delete seventeen genes within a putative hopanoid biosynthetic gene cluster to determine their role, if any, in hopanoid biosynthesis. Two genes, *hpnH* and *hpnG*, are required to produce both bacteriohopanetetrol and aminobacteriohopanetriol, whereas a third gene, *hpnO*, is required only for aminobacteriohopanetriol production. None of the genes in this cluster are required to exclusively synthesize bacteriohopanetetrol, indicating that at least one other hopanoid biosynthesis gene is located elsewhere on the chromosome. Physiological studies with the different deletion mutants demonstrated that unmethylated and C_{30} hopanoids are sufficient to maintain cytoplasmic but not outer membrane integrity. These results imply that hopanoid modifications, including methylation of the A-ring and the addition of a polar head group, may have biological functions beyond playing a role in membrane permeability.

INTRODUCTION

Hopanoids are pentacyclic triterpenoid molecules produced by a variety of bacteria that are readily preserved in both modern and ancient environments (Ourisson & Albrecht, 1992). Based on the distribution of hopanoids in modern microbes, hopane signatures detected in environmental samples and ancient sedimentary rocks can be used to link specific bacteria and their metabolisms to a particular ecosystem or a certain period in earth history (Brocks & Pearson, 2005; Brocks & Summons, 2004). As a result, hopanoid biomarkers have the potential to provide valuable information about modern and ancient microbial communities. However, recent studies of hopanoid distribution and function in bacteria have challenged

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the use of particular hopanoids as indicators of specific bacteria and/or their metabolisms (Doughty et al., 2009; Rashby et al., 2007; Welander et al., 2009). Indeed, it now seems likely that hopanoids may be better markers of cell biological processes than metabolisms (Doughty et al., 2011), although much remains to be done to understand their biological roles in detail.

A robust interpretation of hopanoids in modern and ancient settings requires knowledge of their phylogenetic distribution and cellular function. With one exception (Welander et al., 2010), our understanding of which organisms produce particular hopanoids comes from lipid analyses of cultured bacteria, usually tested under only one growth condition. These analyses have provided important information regarding both the diversity of hopanoid producing bacteria and the impressive range of structures they make (Rohmer et al., 1984; Talbot et al., 2008). Yet, because only a tiny fraction of the total number of microorganisms in nature has been cultured (Pace, 1997) and because hopanoid production varies under different growth conditions (Doughty et al., 2009; Rashby et al., 2007), it is likely that our assessment of the taxonomic distribution of these molecules is biased and incomplete. For these reasons, it would be useful if we could bypass culturing when inferring which organisms produce certain hopanoids. Recently, we demonstrated that it is possible to use genes involved in hopanoid biosynthesis for this purpose (Welander et al., 2010). While this study focused on using the *hpnP* gene as a proxy for 2-methylhopanoids to assess the capacity for production of these molecules amongst modern bacteria, if we knew the identity of other genes responsible for catalyzing the production of other hopanoid structures, we could similarly predict which organisms make different hopanoids.

While understanding the phylogenetic distribution of hopanoid producing bacteria has involved the efforts of many research groups (Pearson et al., 2007; Pearson et al., 2009; Rohmer et al., 1984; Talbot et al., 2008), understanding the biological function of these molecules has received comparatively less attention. Based on their structural and biosynthetic similarities to eukaryotic sterols, hopanoids have been proposed to serve as sterol surrogates in bacterial membranes (Kannenberg & Poralla, 1999; Ourisson et al., 1987). In support of this, several studies have shown that hopanoids localize to the cytoplasmic and outer membranes of bacteria (Doughty et al., 2009; Jahnke, 1992; Jurgens et al., 1992; Simonin et al., 1996) and that they play a role in enhancing membrane impermeability and stability (Berry et al., 1993; Horbach et al., 1991; Poralla et al., 1984; Poralla et al., 2000; Welander et al., 2009). Consistent with this, sporulenes—hopanoid-like molecules produced by *Bacillus subtilis*—have been shown to localize to spores, possibly protecting them from oxidative stress (Bosak et al., 2008). We have found that the lack of hopanoid production by *Rhodopseudomonas palustris* weakens its outer membrane integrity and makes it more sensitive to pH (Welander et al., 2009) and temperature stress (Doughty et al., 2011). Recently, we showed that hopanoid mislocalization in *R. palustris* causes defects in cell division under certain growth conditions (Doughty et al., 2011). However, all of these studies have explored the phenotypic landscape for strains defective in the production of all hopanoids or hopanoid-like molecules. To our knowledge, no studies have yet been done to determine whether specific hopanoids modifications have particular biological effects.

R. palustris is an attractive model organism in which to study hopanoid biology because it produces diverse triterpenoids, is genetically tractable and does not require hopanoids under all conditions (Jiao et al., 2005; Welander et al., 2009). It produces three classes of hopanoids: 1) C_{30} hopanoids, and the related gammacerane-type triterpenoid tetrahymanol, that do not have a polar head group, 2) extended C_{35} hopanoids that contain the polyhydroxylated side chain with or without a terminal amino group, and 3) both C_{30} and C_{35} hopanoids, including tetrahymanol, that are methylated at the C-2 position (Fig. 1a).

Previously, we identified a putative hopanoid biosynthetic gene cluster in the *R. palustris* genome (Welander et al., 2010) (Fig. 1b); four genes within this region are known to be involved in hopanoid biosynthesis (Kleemann et al., 1994; Welander et al., 2010; Bradley et al., 2010). The *shc* gene encodes the squalene hopene cyclase. It catalyzes the initial cyclization of squalene and is required to produce all hopanoids in addition to tetrahymanol (Fig. 2, reaction 1). The *hpnP* gene, located approximately seven kilobases upstream of *shc*, encodes a B-12 binding radical S-adenosylmethionine (SAM) protein that catalyzes the methylation of hopanoids at the C-2 position (Fig. 2, reactions 7, 8, 9) (Welander et al., 2010). Recently, it was shown in *Methylobacterium extorquens* that *hpnG*, a putative nucleoside phosphorylase, and *hpnH*, a B-12 binding radical SAM protein, are required for the addition of the hopanoid side chain (Fig. 2, reactions 2 and 3) (Bradley et al., 2010).

To both expand our ability to assess the potential for hopanoid production using culture-independent methods and to generate new mutants to explore the biological function of specific hopanoids, we undertook this study. Our results better define the hopanoid biosynthetic pathway in *R. palustris* TIE-1, and highlight the possibility that modified hopanoids have distinct biological roles. This work lays a foundation for future phylogenetic, biochemical and physiological studies that will inform the interpretation of hopane molecular fossils.

METHODS

Bacterial strains, media and growth conditions

Bacterial strains used in this study are listed in Table S1. *Escherichia coli* strains were grown in lysogeny broth (LB) at 37°C. *Rhodopseudomonas palustris* strains were grown chemoheterotrophically in YPS medium (0.3% yeast extract, 0.3% peptone, 10 mM succinate, 100 mM MOPS at pH 7.0) at 30°C while shaking at 250 RPM under ambient light. For growth on solid medium, LB or YPS was solidified with 1.5% agar and supplemented, if necessary, with gentamicin at 20 μg/ml (*E. coli*) or 800 μg/ml (*R. palustris)*, kanamycin at 400 μg/ml (*R. palustris*), or chloramphenicol at 30 μg/ml (*E. coli*).

For growth curves, a bile salt mixture prepared from fresh ox bile (Himedia, Mumbai, India) was added to YPS medium before autoclaving to a final concentration of 0.5%. Exponential growth phase cells were inoculated in triplicate into 5 ml of the appropriate YPS medium with a 1% inoculum and monitored for growth by following the increase in absorbance at 600 nm over time. Absorbance was measured by transferring a 100 μl aliquot to a 96-well plate and measuring the optical density on a Synergy 2 Microplate Reader (BioTek, Winooski, VT).

For cell envelope stress experiments, one colony of each strain was inoculated into 5 ml of YPS medium and incubated at 30°C for three days. Aliquots of each culture (250 μl) were transferred into four sterile microfuge tubes and pelleted at 14000 RPM for 1 minute. The supernatant was removed and cells were resuspended in 250 μl of YPS alone or 250 μl of YPS supplemented with 2 mM ethylenediaminetetraacetic acid (EDTA), 0.5% bile salts, or 2 mM EDTA plus 0.5% bile salts. Cells were incubated at 30°C for four hours in the dark. Cultures were serially diluted and colony forming units (CFU) were determined using the drop plate method (Herigstad et al., 2001).

DNA methods, plasmid construction and transformation

All plasmid constructs used in this study are described in Table S2. The sequences of oligonucleotide primers are listed in Table S3. QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) was used for isolation of plasmid DNA from *E. coli*. Genomic DNA from *R. palustris* strains was isolated using the DNeasy Blood and Tissue Kit (Qiagen). All PCR

products and digested cloning intermediates were gel purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI). DNA sequences of all cloning intermediates were confirmed by sequencing at the Biopolymers Laboratory in the MIT Center for Cancer Research or through the GENEWIZ Boston Laboratory. *E. coli* strains were transformed by electroporation using an Electroporator 2510 (Eppendorf, Hamburg, Germany) as recommended by the supplier. Plasmids were mobilized from *E. coli* S17-1 into *R. palustris* by conjugation on YPS agar plates that were incubated overnight at 30°C (Parales & Harwood, 1993; Welander et al., 2009).

Construction of *R. palustris* hopanoid biosynthesis deletion mutants

All deletion strains were constructed in *R. palustris* as previously described (Welander et al., 2009). Briefly, a deletion plasmid construct was made by fusing via PCR one kilobase of the upstream region with one kilobase of the downstream region of the gene of interest in the suicide vector pJQ200SK (Quandt & Hynes, 1993). The plasmid was integrated onto the *R. palustris* chromosome through homologous recombination and the resulting merodiploid was both gentamicin resistant and sucrose sensitive. To remove the plasmid from the chromosome and generate the deletion mutant, one gentamicin resistant colony was grown in YPS broth without any antibiotic selection for two days and serial dilutions were plated on YPS agar supplemented with 10% sucrose. Sucrose resistant colonies were screened by PCR. Genomic DNA was isolated from putative deletion mutants and verified by PCR as bona fide deletion mutants (Figure S1).

Disruption of *hpnC, hpnD*, and *hpnE* was also attempted by insertion of a kanamycin resistance cassette via the TargeTron Gene Knockout System (Sigma-Aldrich, St. Louis, MO). This system utilizes an RNA-protein complex re-targeted to the specific gene of interest to permanently insert a group II intron carrying a kanamycin resistance marker. Primers designed to re-target the intron were selected on the TargeTron Design Web site and used to amplify a 350-bp re-targeted intron product for each gene. Each intron was digested with *HindIII* and *BsrGI* and ligated into the TargeTron vector pACD4K-C according to the TargeTron User Guide to generate pPVW72 (*hpnC*), pPVW73 (*hpnD*), and pPVW74 (*hpnE*). Because the parent plasmid (pACD4K-C) did not contain the necessary elements to be transferred into *R. palustris* via conjugation, the re-targeted intron for each gene together with the functional components of the TargeTron system were subcloned from pPVW72, pPVW73, and pPVW74 into pSRK-Gm, which placed the intron under the expression of an IPTG inducible promoter (Khan et al., 2008). To do so, each TargeTron vector was digested with *HindIII* and *PshAI* and the 5.4-kb intron fragment was blunted with T4 DNA Polymerase (New England Biolabs, Ipswich, MA). The pSRK-Gm vector was digested with *NdeI* and *NheI*, blunted, and dephosphorylated with calf intestinal alkaline phosphatase (New England Biolabs). The intron fragment for each gene was ligated into the digested pSRK-Gm to produce pPVW75 (*hpnC*), pPVW76 (*hpnD*), and pPVW77 (*hpnE*). Each plasmid was transferred into *R. palustris* and plated on YPS plates supplemented with gentamicin. To express the intron, gentamicin resistant colonies were inoculated into YPS plus gentamicin and grown to stationary phase. Cultures were diluted 1:100 (1 ml final volume) in YPS plus gentamicin supplemented with 1 mM IPTG to induce expression of the intron and incubated overnight at 30°C with shaking. Cells were pelleted, resuspended in 0.1 ml, and plated on YPS agar plus kanamycin to select for target site insertion.

Complementation of *ΔhpnG, ΔhpnH,* and *ΔhpnO* deletion mutants

Primers were designed to amplify *hpnG, hpnH*, and *hpnO* with the addition of an *NheI* site at the 3' end and fusing an *NdeI* site with the start codon at the 5' end. Each gene was amplified, digested, and ligated into *NdeI/NheI* cut pSRK-Gm. The resulting plasmids were mated into the appropriate *R. palustris* mutant. For lipid analysis, mutant strains carrying the

complementation plasmid were grown at 30°C for 3 days in 100 ml of YPS supplemented with gentamicin and 1 mM IPTG to induce expression of the complementing gene.

Analysis of hopanoid production

For analysis of hopanoids in each mutant, cells were grown in 100 ml YPS under aerobic conditions at 30°C to stationary phase (3 days). Cells were harvested by centrifugation at 5000 × *g* for 10 minutes at 4°C and stored at −20°C. Lipids were extracted by sonicating the cells in Teflon centrifuge tubes (VWR, Bridgeport, NJ) for 15 minutes at room temperature in 10 ml of 10:5:4 (v:v:v) methanol (MeOH): dichloromethane (DCM):water (Bligh & Dyer, 1959). Samples were centrifuged for 10 minutes at 3000 × *g* and the supernatant was transferred to a new tube. Cell pellets were sonicated again in 10 ml of MeOH:DCM:water (10:5:4, v/v/v), centrifuged, and the supernatant was combined with the first extraction. The samples were separated into two phases by adding 20 ml 1:1 (v/v) DCM: water, centrifuged for 10 minutes at 3000 × *g*, and the organic phase was transferred to a new vial. To the remaining aqueous phase, 10 ml of DCM: water (1:1, v/v) was added again, centrifuged, and the organic phase was combined with the previous extract. The organic solvents were evaporated under N_2 and the total lipid extract (TLE) was redissolved in 2 ml DCM. The TLE was divided into two 1 ml aliquots. One aliquot was separated by chromatography on a silica gel column. Six fractions were eluted: F1: hexane; F2: hexane: DCM (4:1, v/v); F3: DCM; F4: DCM: ethyl acetate (EtOAC) (4:1, v/v); F5: EtOAc; F6: MeOH. Separation of the TLE facilitated the detection of diplopterol in fraction 4. Fractions 4, 5, 6 and the remaining TLEs were incubated in 100 μl of acetic anhydride: pyridine (1:1, v/v) for 1 hour at 70°C to derivatize alcohols into acetate esters.

The hydrocarbon fractions (F1 and F2), the acetylated fractions (F4, F5, and F6), and the acetylated TLEs were analyzed by high temperature gas chromatography-mass spectrometry (GC-MS) as previously described (Welander et al., 2009). The acetylated TLEs were also analyzed by liquid chromatography-mass spectrometry (LC-MS). The LC-MS system comprises a 1200 Series HPLC (Agilent Technologies, Santa Clara, CA) equipped with an autosampler and a binary pump linked to a Q-TOF 6520 mass spectrometer (Agilent Technologies) via an atmospheric pressure chemical ionization (APCI) interface (Agilent Technologies) operated in positive ion mode. The analytical procedure was adapted from Talbot et al. (Talbot et al., 2001). A Poroshell 120 EC-C18 column (2.1 × 150 mm, 2.7 μm; Agilent Technologies), set at 30°C, was eluted isocratically first with MeOH/water (95:5, v:v) for 2 min at a flow rate of 0.15 ml/min, then using a linear gradient up to 20% (v) of isopropyl alcohol (IPA) over 18 min at a flow rate of 0.19 ml/min, and isocratic for 10 min. The linear gradient was then set to 30% (v) of IPA at 0.19 ml/min over 10 min, and maintained for 5 min. The column was subsequently eluted using a linear gradient up to 80% IPA (v) over 1 min at a flow rate of 0.15 ml/min and isocratic for 14 min. Finally the column was eluted with MeOH/water (95:5, v:v) at 0.15 ml/min for 5 min. The APCI parameters were as follows: gas temperature 325°C, vaporizer temperature 350°C, drying gas (N_2) flow 6 l/min, nebulizer (N_2) flow 30 l/min, capillary voltage 1200 V, corona needle 4 μA, fragmentor 150 V. Data were recorded by scanning from m/z 100 to 1600. Identification of the hopanoids was done using their exact mass and by comparison of the retention time and the mass spectra with published data (Talbot et al., 2007; Talbot et al., 2003b).

Bioinformatic analysis

Homologs of *R. palustris* HpnH and HpnO were identified in the Kyoto Encyclopedia of Genes and Genomes (KEGG) by TBLASTN (Altschul et al., 1997) and aligned using MUSCLE (Edgar, 2004). Maximum likelihood trees were constructed by PhyML (Guindon & Gascuel, 2003) using the LG+gamma model, six gamma rate categories, ten random

starting trees, SPR+NNI branch swapping, and substitution parameters estimated from the data. The HpnO tree was generated and edited by importing the resulting PhyML tree into iTOL: http://itol.embl.de/ (Letunic & Bork, 2007).

RESULTS

Deletion analysis of genes surrounding the *shc* locus

To identify genes that are required for hopanoid biosynthesis, we generated markerless, in-frame deletions of the genes surrounding the *shc* locus (Fig. 1b). Each resulting mutant was grown under heterotrophic conditions and harvested at early stationary phase for lipid extraction. Identification of all *R. palustris* hopanoids and any biosynthetic intermediates was performed by high temperature GC-MS and/or LC-MS of the acetylated total lipid extract (TLE). The one exception was the C_{30} alcohol hopanoid diplopterol, which was converted to a mixture of hopene isomers during the derivatization procedure.. To verify whether a gene was involved in diplopterol biosynthesis, the TLE from each mutant had to be separated over a silica gel column as described in the methods section. Analysis of the separated hydrocarbon and alcohol fractions by GC-MS enabled us to test for the production of diplopterol. A summary of the GC-MS and LC-MS data for all mutants is shown in the supplemental material (Table S4).

In total, we attempted to delete seventeen genes, ten of which are downstream of *shc* and seven of which are upstream (Fig. 1b), limiting our deletion analysis at Rpal_4270 (*eutB*) and Rpal_4250 for two reasons. First, these genes are not required for hopanoid biosynthesis (Table S4). Second, the gene upstream of Rpal_4270 is predicted to encode a chemotaxis sensory protein, while the gene downstream of Rpal_4250 is predicted to encode a carbohydrate-selective porin. Based on these annotations and the proposed hopanoid biosynthesis pathway shown in Figure 2, it seemed unlikely that these genes would be involved in hopanoid biosynthesis.

As shown in Table 1, fifteen genes were deleted successfully, and three in addition to the squalene hopene cyclase and the *hpnP* methylase are relevant for hopanoid production in *R. palustris*. Although eleven of the deleted genes did not appear to affect hopanoid biosynthesis, we note that our hopanoid analysis did not involve quantifying the hopanoids produced or their localization in the cell. Therefore, we cannot exclude the possibility that some of these proteins play a role in hopanoid regulation or transport.

Attempts to delete the three genes located immediately upstream of *shc* were unsuccessful. Based on their annotation, they are most likely involved in the biosynthesis or modification of squalene, the biosynthetic precursor to hopanoids. Both *hpnC* (Rpal_4264) and *hpnD* (Rpal_4263) are thought to encode putative squalene synthases whereas *hpnE* (Rpal_4262) is annotated as a squalene associated flavin adenine dinucleotide (FAD) dependent desaturase. Several attempts were made to delete each of these genes individually and as double or triple mutants. We were able to verify that the deletion plasmid recombined into the chromosome at the correct locus (data not shown). However, when the deletion construct was excised from the chromosome, only colonies that restored the wild type locus were viable. A second method to insert a kanamycin resistance cassette in each of these genes via the TrageTron Gene Knockout System was also attempted. This system relies on site-specific insertion of a kanamycin cassette to the locus of interest and is thought to be more efficient than insertion by homologous recombination (Yao & Lambowitz, 2007). Nonetheless, we were unable to generate insertion mutants at these loci with this alternative method either.

hpnG and *hpnH* are required to produce both bacteriohopanetetrol and aminobacteriohopanetriol

The *hpnH* (Rpal_4256) and *hpnG* (Rpal_4260) genes were recently shown to be required for the formation of functionalized hopanoids in *Methylobacterium extorquens* (Bradley et al., 2010) and this role is conserved in *R. palustris.* The *hpnH* gene encodes a B-12 binding radical SAM protein and its deletion results in the exclusive production of the C_{30} hopanoids diploptene and diplopterol as well as tetrahymanol (Fig. 3). The *hpnG* gene encodes a putative nucleoside hydrolase and its deletion results in blocked production of both the bacteriohopanetetrol and the aminobacteriohopanetriol and the accumulation of an intermediate, identified by LC-MS as adenosyl hopane (Fig. 4). Complementation of these strains was accomplished by placing a copy of the respective gene on a self-replicating plasmid under the control of an IPTG inducible promoter (Khan et al., 2008). As can be seen in Figures 3 and 4, this restored production of aminobacteriohopanetriol and bacteriohopanetetrol in each deletion mutant.

Because *hpnH* is required to produce C_{35} hopanoids, this gene has the potential to identify bacteria that can make functionalized hopanoids. We used a bioinformatic approach to ascertain what percentage of hopanoid producing bacterial genomes (those with a copy of the squalene hopene cyclase, *shc*) also contains a copy of *hpnH*. Of the 1077 completed bacterial genomes in the Kyoto Encyclopedia of Genes and Genomes (KEGG) available at the time of this study, 119 harbor at least one copy of the squalene hopene cyclase. BLAST analysis of these *shc*-containing genomes with the *R. palustris* HpnH protein as a query resulted in 117 hits, indicating that a majority of hopanoid producers are capable of adding the polar head group to the hopene backbone. The only two bacteria that do not have an HpnH homolog in their genomes are the β-Proteobacterium *Rhizobium sp.* NGR234 and the Chloroflexus bacterium *Sphaerobacter thermophilus*. These strains have yet to be tested for hopanoid production but the presence of *shc* and lack of *hpnH* suggests they may only produce C_{30} hopanoids such as diplopterol and diploptene. Because the majority of bacterial genomes that harbor a copy of *shc* also contain *hpnH*, this indicates that most hopanoid-producing bacteria can produce C_{35} hopanoids.

Biosynthesis of aminobacteriohopanetriol

The enzymatic steps leading to the production of bacteriohopanetetrol and aminobacteriohopanetriol after the removal of adenosine from adenosyl hopane by HpnG are currently unknown. It has been proposed that aminobacteriohopanetriol could be generated by the reductive amination of formyl hopane (Rohmer, 1993) in a reaction similar to the aminotransfer reactions seen in the biosynthesis of many amino acids (Cunin et al., 1986; Ledwidge & Blanchard, 1999). The *hpnO* gene (Rpal_4253) is located adjacent to a recently identified hopanoid transporter (Doughty et al., 2011) within the hopanoid biosynthetic gene cluster and is annotated as a putative ornithine: oxo-acid aminotransferase. Deletion of the *hpnO* gene resulted in the loss of production of aminobacteriohopanetriol but not bacteriohopanetetrol (Fig. 5). Aminobacteriohopanetriol biosynthesis was restored when a copy of *hpnO* was re-introduced (Fig. 5). We did not detect the accumulation of the putative intermediates formyl or ribosyl hopane in the extracts of the *ΔhpnO* mutant. Because *ΔhpnO* has the enzyme(s) required to produce bacteriohopanetetrol and because we propose that formyl hopane is the branching point between the biosynthesis of these two hopanoids, we presume that all formyl hopane is converted to bacteriohopanetetrol in the *ΔhpnO* strain. Alternatively, the lack of formyl hopane accumulation could indicate that HpnO transfers the amino group to bacteriohopanetetrol rather than formyl hopane. However, this seems unlikely given that some bacteria, such as *M. capsulatus*, contain *hpnO* in their genome and are known to produce aminohopanoids but do not accumulate bacteriohopanetetrol (Cvejic et al., 2000; Talbot et al., 2003a; Zundel & Rohmer, 1985).

Furthermore, HpnO bears homology to the *N*-acetylornithine aminotransferase ArgD, a protein involved in arginine biosynthesis (Cunin et al., 1986). Biochemical analysis of ArgD has demonstrated that it acts on an aldehyde rather than a hydroxyl group supporting the idea that the formyl hopane is more likely the correct substrate (Ledwidge & Blanchard, 1999). Although we did not detect any formyl or ribosyl hopane in *ΔhpnO*, we did detect the presence of the lactone ribonyl hopane in this mutant, which could result from the oxidation of the ribosyl hopane intermediate during lipid extraction (Seemann et al., 1999).

In addition to *R. palustris*, many bacteria including *Bradyrhizobium, Streptomyces*, and *Methylococcus* species produce aminobacteriohopanepolyols (aminoBHPs), hopanoids with a C_{35} side chain containing a terminal amino group (Bravo et al., 2001; Cvejic et al., 2000; Poralla et al., 2000). To predict whether other bacteria have the ability to produce these hopanoids, we queried the *R. palustris* HpnO sequence against the KEGG database of completed bacterial genomes using BLAST. Because HpnO is homologous to ArgD, putative HpnO sequences cannot be differentiated from ArgD sequences based on their e-values alone. To identify true HpnO homologs, we constructed a maximum likelihood phylogenetic tree of the 88 HpnO homologs with the lowest e-values (cut off e^{-55}). A distinct clade of 28 bacteria is evident on the tree, the members of which all contain a copy of the *shc* gene and several of which have been shown to produce amino hopanoids (Fig. 6). Moreover, all putative HpnO homologs that lie outside of this clade occur in organisms that do not produce hopanoids (*Bacillus*) or that do not have a copy of the *shc* gene, indicating that true HpnO homologs cluster within this specific branch of the tree. Bioinformatic analysis of HpnO also identified several species containing an HpnO homolog that have not yet been tested for aminoBHP production, including the acidophilic methanotrophic bacterium *Methyloacidophilum infernorum* and the obligate anaerobe *Pelobacter carbinolicus.*

Hopanoids play a role in maintaining outer and cytoplasmic membrane integrity

One of our primary motivations for identifying specific hopanoid biosynthesis genes is to gain insight into the biological function(s) of different hopanoids by way of mutant analysis. Our previous studies of the *R. palustris Δshc* mutant demonstrated that a lack of all hopanoids prevented growth in the presence of bile salts (Welander et al., 2009). Most Gram-negative bacteria are resistant to bile salts because these molecules are incapable of crossing their asymmetric outer membrane. However, damage to the outer membrane often results in increased outer membrane permeability. Under these conditions, bile salts are able to enter the periplasm and penetrate the cytoplasmic membrane, resulting in cell death (Ruiz et al., 2006). Sensitivity to bile salts by Gram negative bacteria is thus interpreted as an indicator of a damaged and permeable outer membrane (Ruiz et al., 2006). To gauge whether specific hopanoids account for the general membrane sensitivity phenotype we had observed for the *Δshc* mutant, we tested the following mutants for bile salt sensitivity: 1) *ΔhpnH,* which only produces the C_{30} hopanoids diploptene and diplopterol, and tetrahymanol; 2) *ΔhpnO,* which does not produce the aminobacteriohopanetriol; 3) *ΔhpnP,* which does not produce any C-2 methylated hopanoids; and 4) *Δshc*, which does not produce any hopanoids.

Figure 7a shows that the mutants grow similarly to the wild type in the absence of bile salts. In the presence of 0.5% bile salts, the resistance level of the mutants groups into three categories (Fig. 7b). The first group is completely sensitive to bile salts; *Δshc*, which lacks all hopanoids, is the only strain in this group. The second group contains mutants that are completely resistant to bile salts; this group includes *ΔhpnP,* which does not produce any C-2 methylated hopanoids as well as *ΔhpnO,* which lacks the aminobacteriohopanetriol. The ability of these strains to grow in the presence of bile salts suggests that, under these conditions, neither methylation at the C-2 position nor the addition of the amino group to the

polar head significantly enhances outer membrane integrity. The third and final group is partially resistant to bile salts but grows slower than the wild type; the *ΔhpnH* mutant, which does not produce C_{35} hopanoids, displays this phenotype indicating that C_{35} hopanoids are needed to fully confer outer membrane integrity.

The bile salt sensitivity of the *Δshc* mutant indicate that hopanoids play a protective role in the outer membrane. However, *Δshc* does not contain any hopanoids in the cytoplasmic membrane either. Given that bile salts act by disrupting the cytoplasmic membrane after bypassing the outer membrane (Begley et al., 2005; Merritt & Donaldson, 2009), hopanoids might also play a protective role in the cytoplasmic membrane. To test this, we measured the survival of different strains in the presence of ethylenediaminetetraacetic acid (EDTA) and 0.5% bile salts. EDTA is a chelator that destabilizes the outer membrane of Gram-negative bacteria, rendering it more porous to detergents and other molecules (Leive, 1965; Nikaido, 2003). Therefore, EDTA exposure results in outer membrane permeability that permits the passage of bile salts into the periplasm, making the cytoplasmic membrane susceptible to bile salt damage (Fig. 8a). If hopanoids were to play a protective role in the cytoplasmic membrane, we would expect the wild type strain to withstand exposure to bile salts upon treatment with EDTA and *Δshc* to be susceptible.

Each strain was grown to late exponential phase and resuspended in YPS alone (control) or YPS supplemented with 0.5% bile salts only, 2 mM EDTA only or 0.5% bile salts and 2 mM EDTA together. As shown in Figure 8b, the survival of the wild type strain under all stress conditions was similar to the negative control, indicating that neither EDTA nor bile salts alone inhibits the survival of *R. palustris*. Further, the weakening of the outer membrane by the addition of EDTA does not make the wild type more susceptible to bile salts. In contrast, the survival of *Δshc* dropped ~2 orders of magnitude when treated with bile salts alone. Survival decreased further when the cells were treated with both EDTA and bile salts, suggesting that EDTA treatment increases the influx of bile salts into *Δshc,* resulting in damage to the cytoplasmic membrane. The other hopanoid mutants tested behaved similarly to the wild type (Fig. 8b). The most resistant mutants were *ΔhpnO* and *ΔhpnP*, suggesting that neither aminobacteriohopanetriol nor 2-methylhopanoids are required for outer- or cytoplasmic membrane protection. To further test whether hopanoids play a protective role in the cytoplasmic membrane, we measured the bile salt sensitivity and cell envelope stress response of the *R. palustris ΔhpnN* mutant. *hpnN* encodes an RND-like transporter that helps localize hopanoids to the outer membrane (Doughty et al., 2011). Although this mutant still produces an equivalent amount of hopanoids compared to the wild type, they are restricted to the cytoplasmic and inner-cytoplasmic membranes. Given that the lack of hopanoids appears to increase outer membrane permeability, bile salts should be able to enter the periplasm of *ΔhpnN.* If hopanoids help protect the cytoplasmic membrane, we would expect *ΔhpnN* to resist bile salts even though hopanoids are not in its outer membrane. As seen in Figure 7b, this is the case. *ΔhpnN* showed a slight decrease in survival when exposed to bile salts alone (Fig. 8b). However, unlike *Δshc*, treatment with both EDTA and bile salts did not decrease the survival of *ΔhpnN* (Fig. 8b). Together, these results suggest that hopanoids help protect both the outer- and cytoplasmic membranes.

DISCUSSION

A nuanced interpretation of hopanes in the environment is limited by inadequate information regarding hopanoid distribution and function in modern organisms. To help achieve this, we are undertaking a variety of studies in the genetically tractable bacterium *R. palustris* TIE-1. Here we have identified genes required for hopanoid biosynthesis and characterized the phenotypes of specific hopanoid-deficient strains.

Our deletion analysis of a putative hopanoid biosynthetic cluster in *R. palustris* revealed that only five of the genes in this region are required for hopanoid biosynthesis. However, more genes may be necessary for production of its different hopanoids. We were unable to identify genes that are required solely (*i.e.* diagnostic) for the production of diplopterol, tetrahymanol, or bacteriohopanetetrol. Both diplopterol and tetrahymanol have been proposed to be produced directly by the squalene hopene cyclase (Hoshino & Sato, 2002; Kannenberg & Poralla, 1999). In agreement with this hypothesis, heterologous expression of various squalene hopene cyclases in *E. coli* has been found to result in the production of both diploptene and diplopterol (Kleemann et al., 1994; Perzl et al., 1997; Tippelt et al., 1998; Reipen et al., 1995). Furthermore, one in vitro study has shown the production of tetrahymanol from certain squalene analogs in cell free extracts of *E. coli* expressing squalene hopene cyclase from *Alicylcobacillus acidocaldarius* (Hoshino & Kondo, 1999). Therefore, it is possible that no additional proteins are required for diplopterol or tetrahymanol biosynthesis. .

However, it seems that at least one more protein is required for the formation of bacteriohopanetetrol. The enzymatic steps leading to the production of bacteriohopanetetrol after the removal of adenosine from adenosyl hopane by HpnG are currently unknown. Previous studies have identified a lactone hopanoid, ribonyl hopane, in the α-proteobacterium *Nitrosomonas europaea* as an intermediate in the biosynthesis of C_{35} hopanoids (Seemann et al., 1999). The presence of ribonyl hopane in some of the *R. palustris* mutant extracts (Fig. 5) led us to initially propose that the biosynthesis of the C_{35} hopanoids would require an oxidation of the ribosyl hopane produced by HpnG to form ribonyl hopane. The lactone ring of this hopanoid could then be opened via a lactone hydrolase in a reaction similar to the hydrolysis of the quorum sensing molecule acylhomoserine lactone (AHL) by the acylhomoserine lactonase (Wang et al., 2010). A potential candidate to catalyze the lactone hydrolysis was an alpha/beta hydrolase (Rpal_4252) located within the putative hopanoid biosynthesis gene cluster shown in Figure 1. However, deletion of this gene did not impair bacteriohopanetetrol biosynthesis.

This result, along with the fact that many simple sugars such as ribose and glucose exist in aqueous solution as an equilibrium mixture of open-chain aldehydes and cyclic alcohols (McMurry & Begley, 2005), raised the possibility that an enzyme might not be required to open the lactone ring. This mechanism has been proposed previously in the literature (Rohmer, 1993) and it seems reasonable to suppose that the ribosyl hopane generated by HpnG is in equilibrium with formyl hopane, an open chain aldehyde hopanoid, as shown in reaction 4 of Figure 2. If formyl hopane is an intermediate in the biosynthesis of bacteriohopanetetrol, then only a reduction of the terminal aldehyde group would be required to form the bacteriohopanetetrol (Fig. 2, reaction 5). Initially we hypothesized that a putative glucose-methanol-choline oxidoreductase (Rpal_4251) located downstream of *hpnO* might catalyze this conversion. However, deletion of this gene did not block bacteriohopanetetrol biosynthesis implying that its specific biosynthesis gene is located elsewhere on the chromosome and/or there is functional redundancy between genes in this region and others elsewhere on the chromosome.

Our inability to delete *hpnC, hpnD*, and *hpnE*, three genes located immediately upstream of *shc*, prevented us from assigning them a role in hopanoid biosynthesis. However, their annotation suggests they are involved in the biosynthesis or modification of squalene or phytoene (Table 1), the biosynthetic precursors of hopanoids and carotenoids, respectively (Maresca et al., 2008). A previous study demonstrated that heterologous expression of the HpnC protein from *Zymomonas mobilis* and *Bradyrhizobium japonicum* resulted in squalene production (Perzl et al., 1998). In this study, it was proposed that HpnD may be involved in the formation of dehydrosqualene, a precursor to carotenoid biosynthesis, while

no conclusions could be drawn about the functional role of HpnE (if any) in hopanoid or carotenoid biosynthesis. Our inability to delete these genes in *R. palustris* suggests that squalene and/or carotenoids may be required for its growth. A few studies have proposed a role for squalene in maintaining the impermeability of membranes to protons and sodium ions as well as possibly playing a role in alleviating pH stress (Aono & Ohtani, 1990; Haines, 2001; Hauss et al., 2002). Furthermore, C_{30} triterpenoids such as squalene were recently shown to have a potential role in the assembly of putative lipid rafts in bacterial membranes (Lopez & Kolter, 2010). However, whether these compounds play similar roles in *R. palustris* remains to be determined.

Of the gene products that were identified as being involved in hopanoid biosynthesis in *R. palustris* TIE-1, HpnH and HpnG are necessary to produce both bacteriohopanetetrol and aminobacteriohopanetriol, whereas HpnO is uniquely required to produce aminobacteriohopanetriol. Thus, the *hpnO* gene can be used to predict which organisms are capable of making aminobacteriohopanepolyols (aminoBHPs), assuming HpnO exclusively catalyzes the amino transfer step in all organisms. Our analysis of HpnO phylogenetic distribution indicates that aminoBHP production is found in a variety of bacterial species. AminoBHPs are thus unlikely to be useful biomarkers for a specific species or metabolism. Interestingly, bacteria that contain HpnO are known to produce different aminoBHPs. For example, *R. palustris* produces aminobacteriohopanetriol, which contains three hydroxyl groups in addition to the amine group on its side chain. *Methylococcus capsulatus* does not produce aminobacteriohopanetriol but does make aminobacteriohopanetetrol and -pentol, which contain four and five hydroxyl groups respectively (Cvejic et al., 2000). Both of these species contain HpnO, suggesting that the addition of the hydroxyl groups follows the amino transfer. Therefore, while HpnO may enable us to predict which organisms can produce aminoBHPs, it does not allow us to distinguish between those that have three, four, or five hydroxyl groups. Because certain polyhydroxylated aminoBHPs are often used as biomarkers for methane oxidizing bacteria in modern environments (Cooke et al., 2008), it is important to not only identify a genetic marker that can distinguish between the potential to produce these different polyhydroxylated aminoBHPs but also to investigate the function of these different amino hopanoids.

Finally, our preliminary physiological studies suggest different hopanoids have distinct cellular roles. The extreme bile salt sensitivity of the *Δshc* mutant reveals that some hopanoids play an important role in enhancing membrane integrity. However, the resistance of the *ΔhpnO* and *ΔhpnP* mutants to bile salts implies that aminobacteriohopanetriol and those hopanoids methylated at the C-2 position do not contribute significantly to this function. The *ΔhpnH* mutant, which only produces C_{30} hopanoids, grows more poorly than the wild type in the presence of bile salts but better than *Δshc*, suggesting that C_{35} and C_{30} hopanoids may both contribute to outer membrane integrity. While we were unable to test whether bacteriohopanetetrol enhances outer membrane integrity for lack of a specific bacteriohopanetetrol mutant, we cannot rule this out. Indeed, given the abundance of bacteriohopanetetrol in the outer membrane of *R. palustris* (Doughty et al., 2011) and previous *in vitro* studies demonstrating bacteriohopanetetrol promotes membrane condensation (Ourisson & Rohmer, 1992), it would be surprising if this C_{35} hopanoid did not play a role. Finally, our results suggest that hopanoids may provide a protective effect in the cytoplasmic membrane but biophysical studies with model membranes are needed to confirm these preliminary phenotypic interpretations.

CONCLUSION

Now that we have defined the majority of the genes in the hopanoid biosynthetic pathway in *R. palustris*, and have begun to test different hopanoid biosynthesis mutants with respect to

membrane permeability and other phenotypic attributes (Doughty et al., 2011), the way is open to identify novel and specific functions that must exist for methylated as well as the more complex functionalized hopanoids. It is our expectation that a deeper understanding of hopanoid biosynthesis and function will provide a better basis for interpreting the meaning of these geologically important molecules. For example, rather than evaluating the presence of a single biosynthetic gene in organisms and environmental samples, we can now test for the presence of multiple genes to more precisely identify those taxa that are the most significant contributors to geohopane production. The gene HpnH is a case in point: adenosyl hopane is widely proposed as a soil-specific hopanoid marker and tracer for continental organic matter in marine sediments (Cooke et al., 2008; Handley et al., 2010); this suggests that the bacterial sources of this compound possess HpnH and, therefore, the ability to convert diploptene to adenosyl hopane. Whether they would be expected to also possess genes that catalyze downstream steps in extended BHP biosynthesis is less clear, but patterns of hopanoid gene expression by soil bacteria may help resolve the specific source(s) of this extended BHP. Knowledge of hopanoid-specific biosynthetic genes will also permit searches for the presence of analogous genes in other hopanoid-producing bacteria and, through analysis of sequence similarities, allow us to develop an improved understanding of their distributions across the bacterial domain with a view to elucidating their evolutionary histories. Beyond using genes to infer ecological and evolutionary relationships, future studies of mutant strains coupled to biochemical and biophysical studies of purified hopanoids will provide insight into their biological functions, and thus, equip us to better interpret what their molecular fossils are telling us about the history of life.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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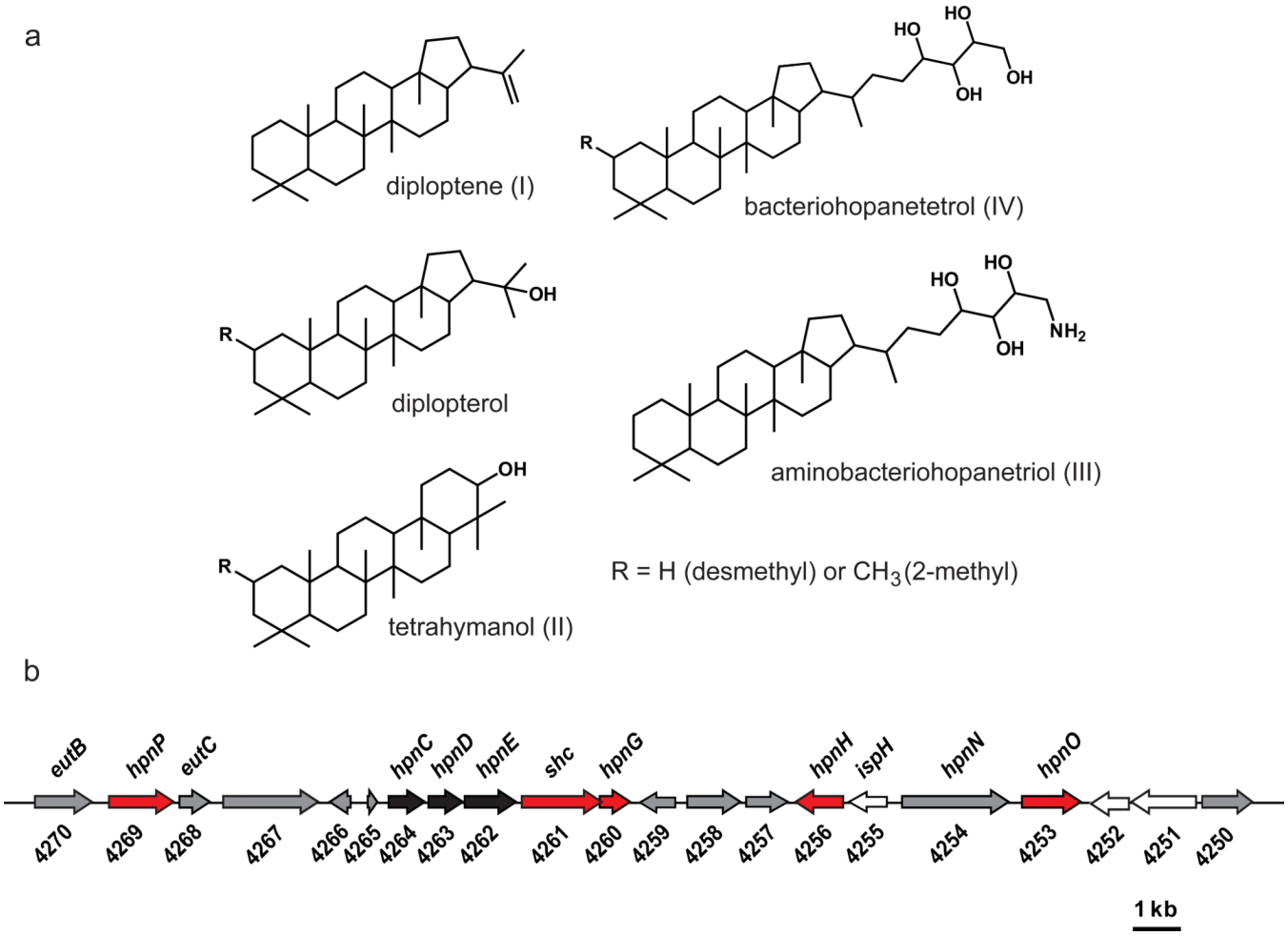

Figure 1.

(a) Hopanoid lipids produced by *R. palustris*. The C_{30} hopanoids include diploptene (I), diplopterol and the gammacerene molecule tetrahymanol (II) while the functionalized hopanoids include the bacteriohopanetetrol (IV) and the aminobacteriohopanetriol (III). Only three of the five molecules can be methylated at the C-2 position. Roman numerals correspond to peaks in Figures 3, 4, and 5. (b) A putative hopanoid biosynthetic gene cluster identified in the *R. palustris* genome. Red arrows represent genes that were shown to be involved in hopanoid biosynthesis, grey arrows represent genes not involved in hopanoid biosynthesis, black arrows represent genes that could not be deleted, and white arrows are genes with putative redundant copies elsewhere on the chromosome. The numbers under the arrows represent the gene numbers corresponding to the genome annotation while the labels above the arrows are the gene names.

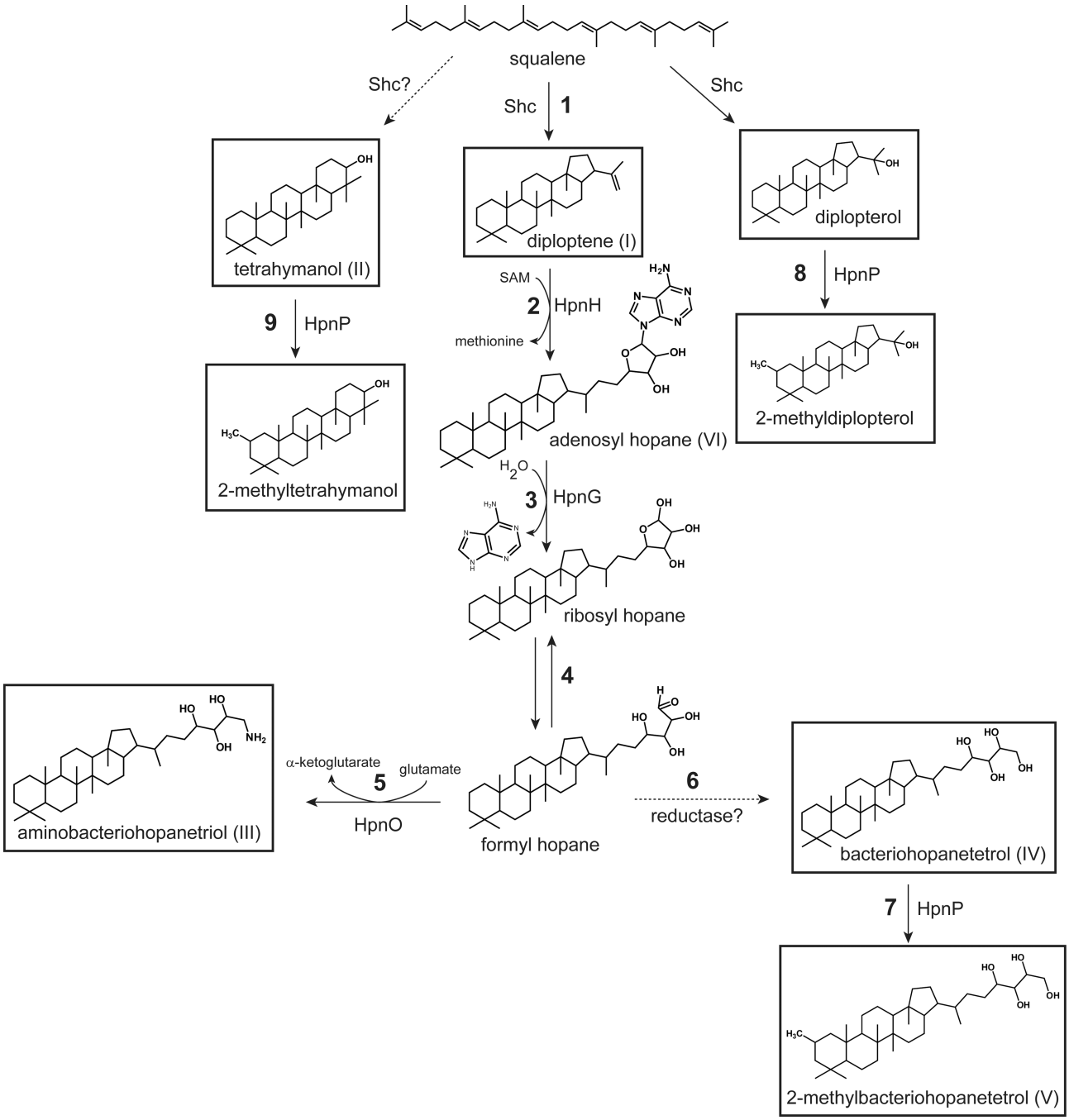

Figure 2.

Proposed hopanoid biosynthetic pathway. Solid arrows indicate steps for which we have genetic data and broken arrows are proposed reactions. Boxed hopanoids represent the end products produced by *R. palustris*. Roman numerals correspond to peaks in Figures 3, 4, and 5.

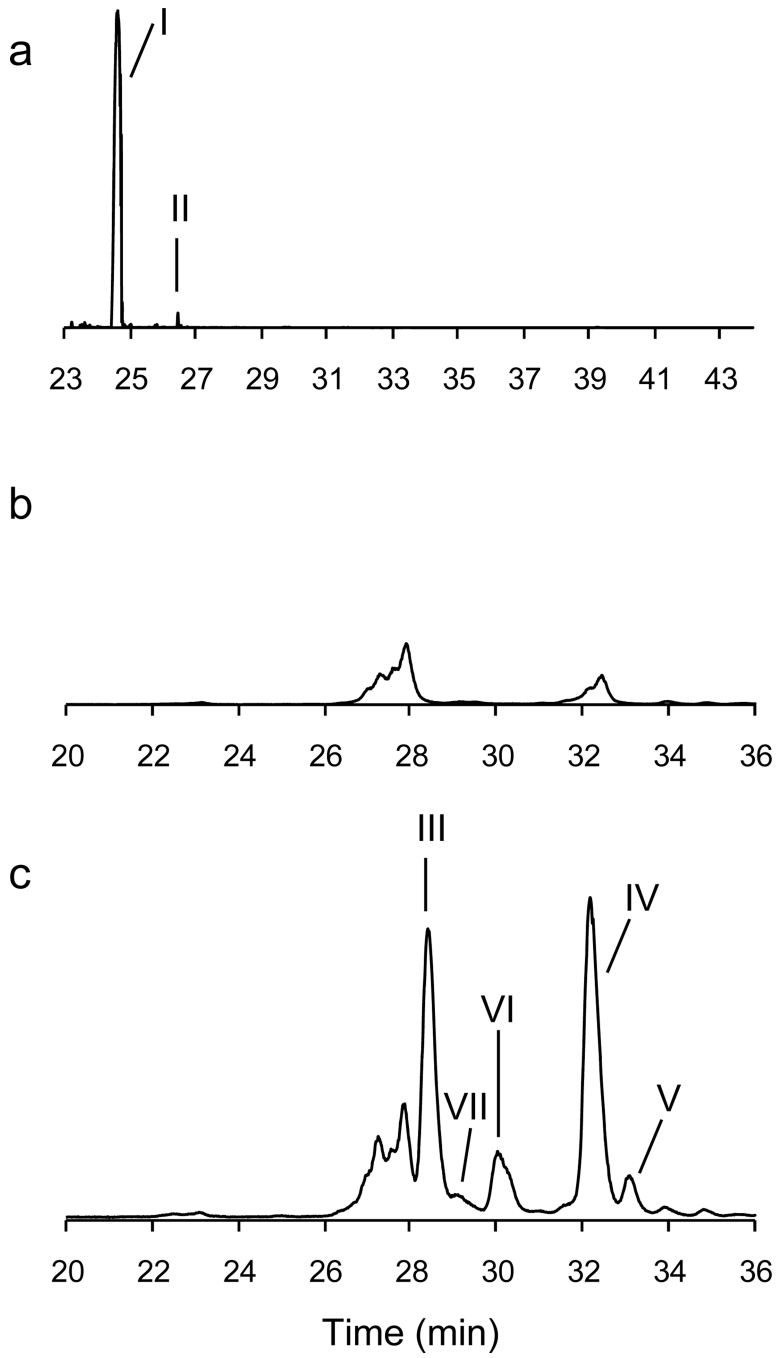

Figure 3.

Chromatograms of acetylated total lipid extract from *hpnH* demonstrating the lack of extended hopanoids in this mutant. (a) *ΔhpnH* GC-MS 191 ion chromatogram showing the production of diploptene and tetrahymanol. (b) *ΔhpnH* LC-MS total ion chromatogram demonstrating no production of the aminobacteriohopanetriol or bacteriohopanetetrol. Peaks at 28 and 32 minutes are acetylated diacylglycerols. (c) LC-MS total ion chromatogram of *ΔhpnH* complemented with a self-replicating plasmid containing a copy of the *hpnH* gene (pPVW80). I, diploptene; II, tetrahymanol; III, aminobacteriohopanetriol; IV, bacteriohopanetetrol; V, 2-methylbacteriohopanetetrol; VI, adenosyl hopane; VII, ribonyl

hopane. Hopanoids were identified base on their mass spectra shown in Fig. S2 and the production of diplopterol was verified by GC-MS in the alcohol fraction (data not shown).

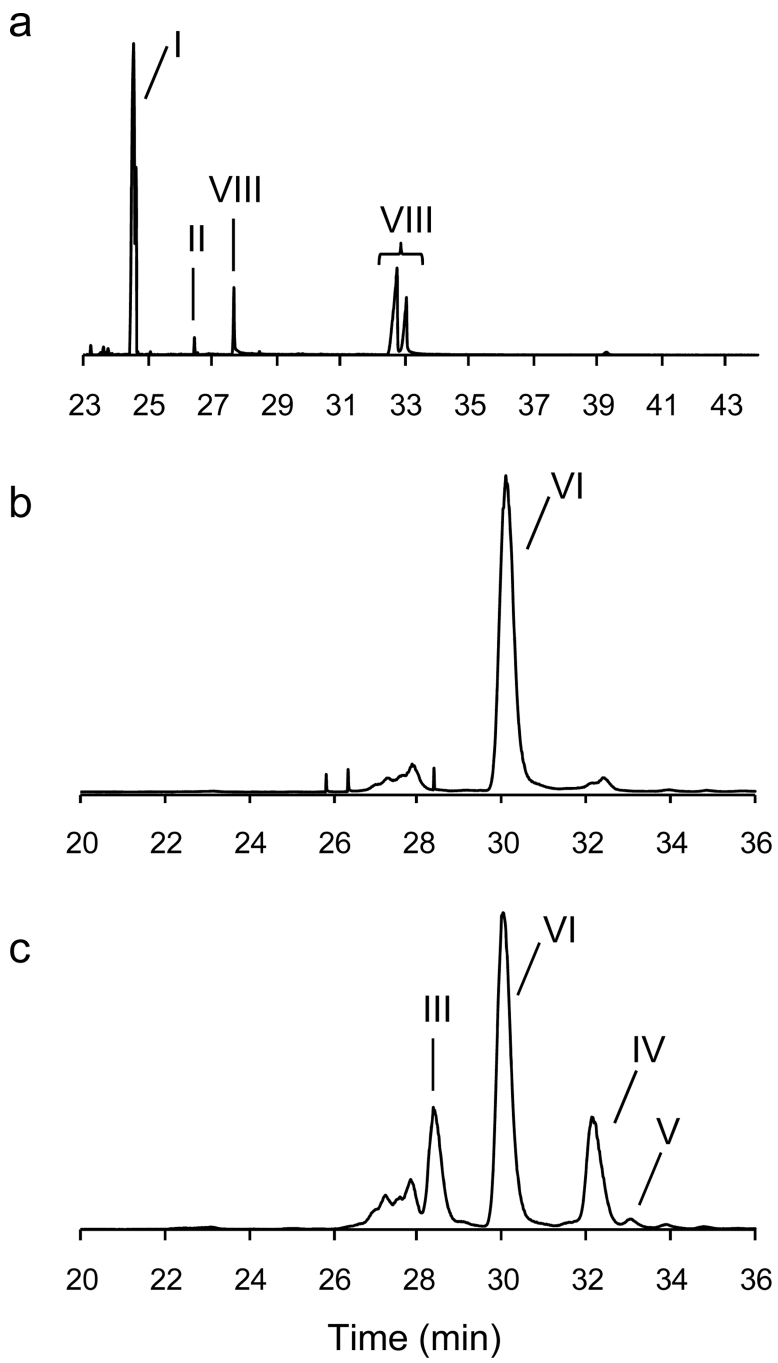

Figure 4.

Chromatograms of acetylated total lipid extract from *hpnG* demonstrating the lack of aminobacteriohopanetriol and bacteriohopanetetrol production in this mutant. (a) *ΔhpnG* GC-MS 191 ion chromatogram showing the production of diploptene, tetrahymanol and possible degradation products of adenosyl hopane. (b) *ΔhpnG* LC-MS total ion chromatogram demonstrating the accumulation of the adenosyl hopane biosynthetic intermediate. Peaks at 28 and 32 minutes are acetylated diacylglycerols. (c) LC-MS total ion chromatogram of *ΔhpnG* complemented with a self-replicating plasmid containing a copy of the *ΔhpnG* gene (pPVW81). I, diploptene; II, tetrahymanol; III, aminobacteriohopanetriol; IV, bacteriohopanetetrol; V, 2-methylbacteriohopanetetrol; VI, adenosyl hopane; VIII,

putative adenosyl hopane degradation products. Hopanoids were identified based on their mass spectra shown in Fig. S2 and the production of diplopterol was verified by GC-MS in the alcohol fraction (data not shown).

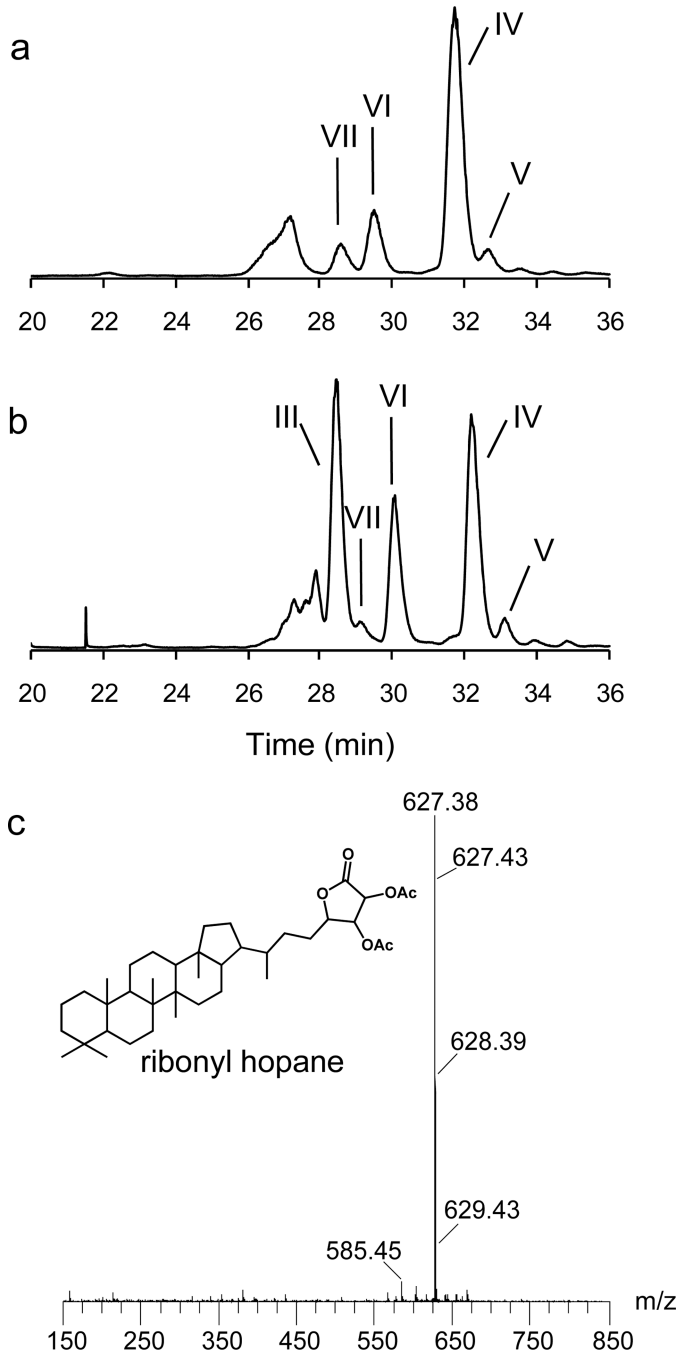

Figure 5.

LC-MS total ion chromatogram of acetylated total lipid extract from *ΔhpnO* demonstrating production of the biosynthetic intermediate ribonyl hopane and blocked production of aminobacteriohopanetriol. (a) *ΔhpnO*, (b) *ΔhpnO* complemented with a copy of *hpnO* on a self-replicating plasmid, (c) an APCI-Q-TOF mass spectrum of acetylated ribonyl hopane showing the pseudomolecular ion at 627 Da. III, aminobacteriohopanetriol; IV, bacteriohopanetetrol; V, 2-methylbacteriohopanetetrol; VI, adenosyl hopane; VII, ribonyl hopane.

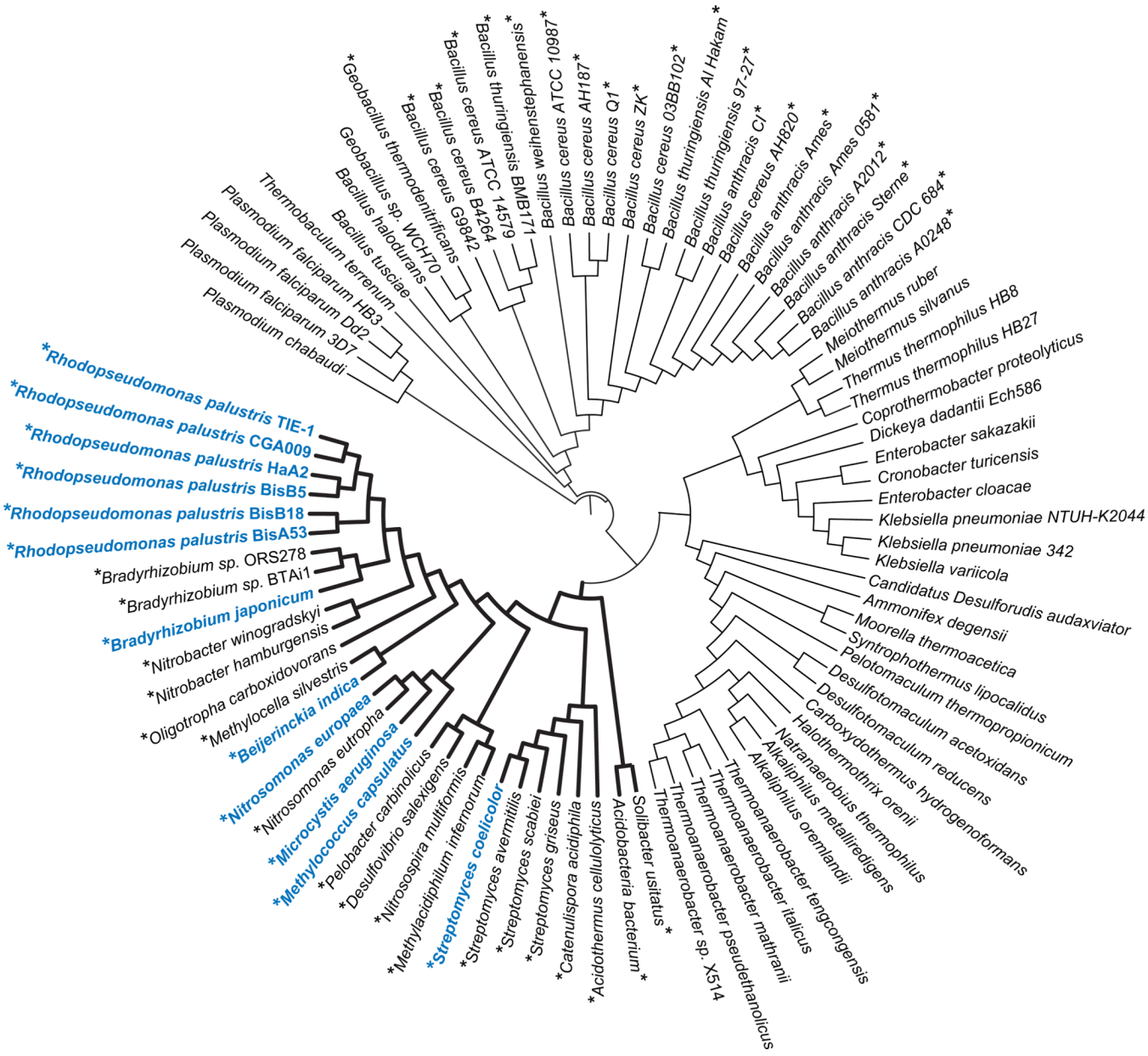

Figure 6.

Maximum likelihood phylogenetic tree of HpnO in sequenced genomes. Sequences found within the bold clade represent true HpnO homologs. Organisms whose names are in blue have been shown to produce aminoBHPs and those with an asterisk are organisms that have a copy of *shc* in their genome.

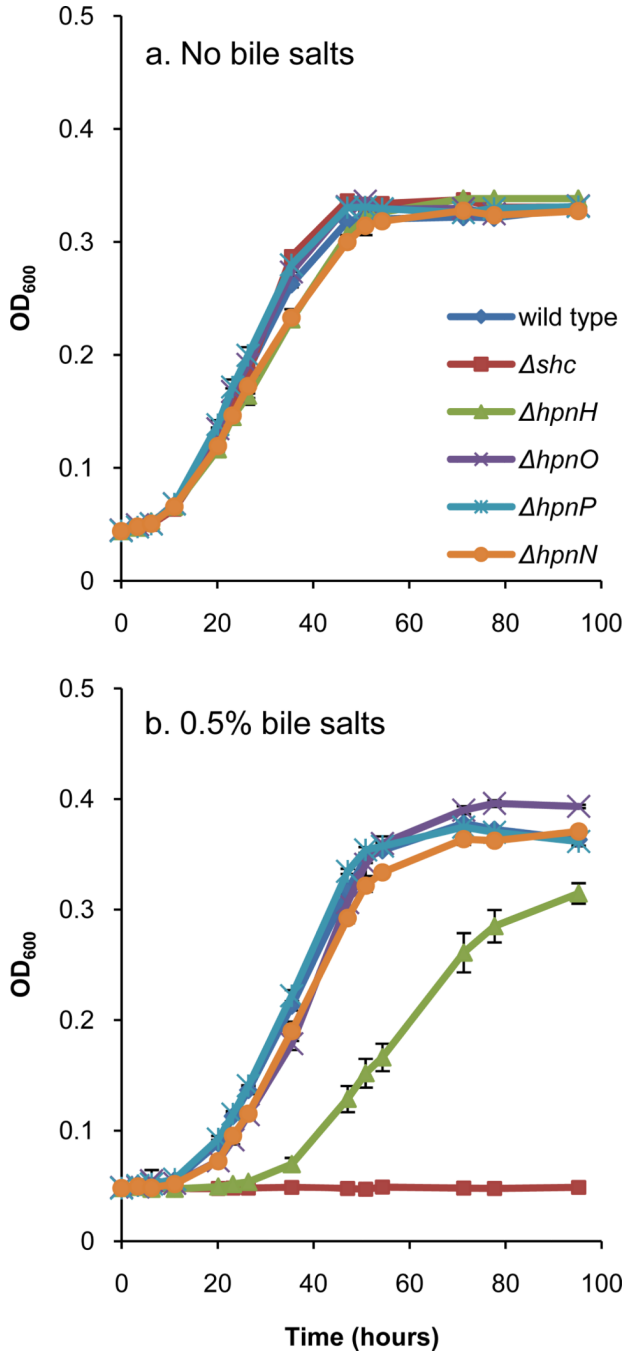

Figure 7.
Growth of hopanoid biosynthesis mutants under (a) standard chemoheterotrophic conditions and (b) standard chemoheterotrophic conditions plus 0.5% bile salts. Each time point represents the average of three replicates (the error bars represent standard deviations and may not be visible beneath the data point markers). Each growth curve was repeated at least three times and representative growth curves are shown.

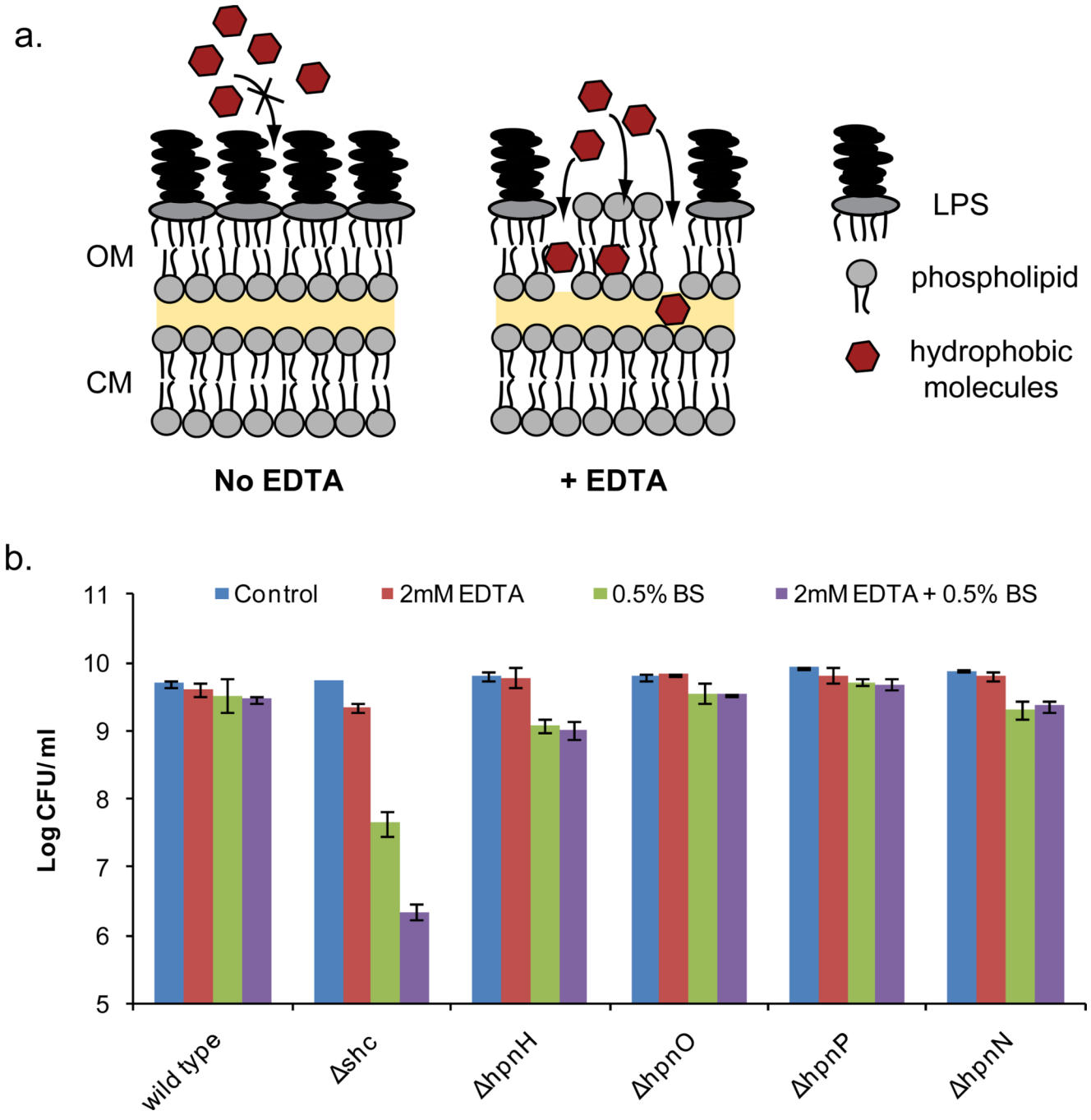

Figure 8.

Survival of cell envelope stress induced by EDTA in *R. palustris*. (a) EDTA treatment of Gram-negative bacteria results in the loss of LPS, flipping of phospholipid bilayers to the outer leaflet, and formation of transient "cracks". This allows for the passage of hydrophobic molecules (blue ovals) by diffusion and flip-flopping through the phospholipid patches. Less hydrophobic molecules (red hexagons) are able to pass through the cracks between the LPS and the phospholipid patch. (b) Survival of the different *hpn* mutants to cell envelope stress. Each strain was treated for four hours with YPS alone (control) or with YPS supplemented with 2 mM EDTA, 0.5% bile salts, or 2 mM EDTA plus 0.5% bile salts. Viability was assessed by determining CFUs for each treatment and the log CFU for each condition is

shown. Error bars represent the standard deviation for three experiments. LPS: lipopolysaccharide; OM: outer membrane; CM: cytoplasmic membrane; BS: bile salts.

Table 1

Putative hopanoid biosynthesis genes deleted in *R. palustris* TIE-1

Gene	ORF#	Involved in hopanoid biosynthesis?	Annotation	Function
eutB	**4270**	**no**	**ethanolamine ammonia-lyase large subunit**	**not established**
hpnP	4269	yes[2]	B-12 binding radical SAM	methyl transfer to A ring at C-2
eutC	**4268**	**no**	**ethanolamine ammonia-lyase small subunit**	**not established**
-------	4267	no[2]	putative RND superfamily transporter	not established
-------	**4266**	**no**	**hypothetical protein**	**not established**
-------	**4265**	**no**	**hypothetical protein**	**not established**
hpnC	**4264**	**unknown[1]**	**squalene synthase**	**not established**
hpnD	**4263**	**unknown[1]**	**squalene synthase**	**not established**
hpnE	**4262**	**unknown[1]**	**squalene associated FAD-dependent desaturase**	**not established**
hpnF	4261	yes[2]	squalene-hopene cyclase	cyclization of squalene
hpnG	**4260**	**yes**	**nucleosidase**	**removal of adenine from adenosyl hopane**
-------	**4259**	**no**	**lysR transcriptional regulator**	**not established**
-------	**4258**	**no**	**phosphonoacetate hydrolase**	**not established**
-------	**4257**	**no**	**phosphate binding periplasmic protein**	**not established**
hpnH	**4256**	**yes**	**B-12 binding radical SAM**	**addition of adenosine to hopane skeleton**
ispH	**4255**	**no**	**4-hydroxy-3-methylbut-2-enyl diphosphate reductase**	**not established**
hpnN	4254	no[2]	hopanoid associated RND transporter	transport of hopanoids to the outer membrane
hpnO	**4253**	**yes**	**ornithine-oxo-acid transaminase**	**production of aminoBHT**
-------	**4252**	**no**	**alpha/beta hydrolase fold hypothetical protein**	**not established**
-------	**4251**	**no**	**glucose-methanol-choline oxidoreductase**	**not established**
-------	**4250**	**no**	**amidohydrolase**	**not established**

Genes deleted in this study are shown in bold

[1] Unable to construct these gene deletions

[2] Previously published data (Welander et al., 2010; Welander et al., 2009; Doughty et al., 2011)