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Composite Bacterial Hopanoids and Their Microbial Producers across Oxygen Gradients in the Water Column of the California Current

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Hopanoids are pentacyclic triterpenoid lipids produced by many prokaryotes as cell membrane components. The structural variations of composite hopanoids, or bacteriohopanepolyols (BHPs), produced by various bacterial genera make them potentially useful molecular biomarkers of bacterial communities and metabolic processes in both modern and ancient environments. Building on previous work suggesting that organisms in low-oxygen environments are important contributors to BHP production in the marine water column and that there may be physiological roles for BHPs specific to these environments, this study investigated the relationship between trends in BHP structural diversity and abundance and the genetic diversity of BHP producers for the first time in a low-oxygen environment of the Eastern Tropical North Pacific. Amplification of the hopanoid biosynthesis gene for squalene hopene cyclase (*sqhC*) indicated far greater genetic diversity than would be predicted by examining BHP structural diversity alone and that greater *sqhC* genetic diversity exists in the marine environment than is represented by cultured representatives and most marine metagenomes. In addition, the genetic relationships in this data set suggest microaerophilic environments as potential "hot spots" of BHP production. Finally, structural analysis of BHPs showed that an isomer of the commonly observed BHP bacteriohopanetetrol may be linked to a producer that is more abundant in low-oxygen environments. Results of this study increase the known diversity of BHP producers and provide a detailed phylogeny with implications for the role of hopanoids in modern bacteria, as well as the evolutionary history of hopanoid biosynthesis, both of which are important considerations for future interpretations of the marine sedimentary record.

Hopanoids, including the extended, side chain-containing bacteriohopanepolyols (BHPs), are pentacyclic isoprenoid lipids produced by some prokaryotes as cell membrane components (see Fig. S1 in the supplemental material or reference 1 for structure examples). Once described as the most abundant natural products on earth (2), BHPs and their degradation products are found almost ubiquitously across Earth's surface environments and in the fossil record, up to 2.7 billion years ago (3). The hopanoid ring structure is resistant to degradation and is well preserved in soils, rocks, and petroleum deposits and is frequently used as a biomarker indicating the presence of bacteria in ancient environments. Most famously, hopanoids methylated at the C-2 position in the sedimentary record were hypothesized to represent the presence of cyanobacteria and thus potentially the rise of oxygenic photosynthesis, since this structural modification is commonly observed in many modern freshwater cyanobacteria (4). Subsequent studies demonstrating the production of 2-methylbacteriohopanepolyols by an anoxygenic phototroph under anaerobic conditions, however, indicated that the potential origins of sedimentary 2-methylhopanes cannot be restricted to cyanobacteria (5). In addition, the gene responsible for C-2 methylation has been identified in alphaproteobacteria and freshwater cyanobacteria but not in marine cyanobacteria (6), raising the possibility that 2-methylhopanes were deposited under anaerobic conditions by anoxygenic organisms in addition to ancient cyanobacteria.

Accurate interpretation of the presence of hopane biomarkers in the sedimentary record, therefore, may require a more detailed knowledge of their distribution and function in modern microorganisms. Similar to the physiological role of sterols in eukaryotic membranes, BHPs appear to be involved primarily in membrane stabilization and were recently shown to have the same membrane-ordering properties as sterols (7). Unlike sterols in eukaryotic organisms, BHPs do not appear to be essential for overall cell growth and survival in bacteria (8, 9), presumably because those that lack BHPs produce other membrane lipids to compensate. BHPs are also important in other membrane functions such as pH tolerance and antibiotic resistance (8, 10), lipid raft formation (11), and late stationary-phase survival (12). However, most of these physiological roles were identified in cultured organisms, and in many cases it seems that roles important in one organism are not necessarily important in another. Furthermore, very little is known about the functions these BHPs might have in the natural environment, particularly in the marine environment.

BHPs exhibit considerable structural diversity, differing in the number, position, and nature of the functional groups in the side chain (1). The most common BHPs identified are tetrafunctionalized, usually with hydroxyl groups at C-32, C-33, and C-34, with the C-35 position (see Fig. S1 in the supplemental material) occupied by another hydroxyl, amino, or more complex structure such as an amino sugar or acid. These more complex structures are referred to as "composite BHPs." Common variations observed within the pentacyclic ring structure include methylation at C-2 or C-3 or unsaturation at C-6 or C-11 (see Fig. S1). In some cases,

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these structural variations appear to be associated with certain groups of bacteria, making BHPs possible molecular biomarkers of bacterial communities and metabolic processes. For example, type I methanotrophs contain abundant hexafunctionalized BHPs, while type II methanotrophs contain mostly tetra- and pentafunctionalized compounds (13, 14). However, currently the use of BHPs to fingerprint microbial communities is limited by a lack of knowledge of modern hopanoid distribution and abundance in natural environments, as well as how environmental conditions may influence the source and magnitude of hopanoid production.

BHP sources and distribution in the pelagic environment of the ocean are poorly understood. Only a few studies have directly measured BHPs in the water column (15–17). In these studies, the highest concentration of BHPs was observed in regions of the water column that were suboxic to anoxic, suggesting that organisms living in these transitional environments may be important contributors to BHP production in the water column. Initial culture studies seemed to restrict hopanoid biosynthesis to aerobic organisms (14), but it is now clear that many facultative and some strict anaerobes can also produce BHPs (5, 18, 19), including those methylated at the C-2 position.

The identities of these important BHP producers in low-oxygen environments are unknown. Studies targeting the gene (*sqhC*) for squalene hopene cyclase (SHC), which catalyzes the cyclization of the hopanoid ring structure (20), provide a culture-independent method of investigating the many questions regarding their phylogenetic associations and metabolic capabilities. An examination of the metagenomic Global Ocean Survey (GOS) data set estimated that ≤4% of the marine bacteria in surface waters are capable of hopanoid production and that these bacteria likely belong to uncharacterized taxa (21). Studies directly amplifying the *sqhC* gene from marine environments have also demonstrated considerable phylogenetic distance between environmental and sequenced organisms, as well as significant diversity even within a single environmental setting (22, 23).

Most of the genetic and metagenomic data surveyed so far, however, originated from the upper few meters of the ocean or from primarily aerobic environments. Whether BHP producers in low-oxygen environments exhibit similar patterns is unknown. In this study, we investigated the relationship between trends in BHP structural diversity and abundance and the genetic diversity of BHP producers in low-oxygen environments. On the basis of previous work, we hypothesized that BHP concentrations and structural diversity should increase with decreasing oxygen concentrations and that more rare or unique structures might be identified in the most anoxic samples. In addition, we expected that examining the genetic diversity of the *sqhC* gene would provide insight into the identities and metabolic strategies of BHP producers, including whether there are BHP producers that are unique to low-oxygen environments. Therefore, we chose to sample at two stations, one inside and one outside the Santa Barbara Basin (Fig. 1). Station 1 (34°17.3′N, 120°2.1′W, 585-m water depth) was located near the deepest part of the Santa Barbara Basin, which is only intermittently ventilated because of the presence of a sill that restricts water circulation below a depth of about 470 m (24). Here, bottom waters become seasonally suboxic to anoxic and commonly observed water column deficits in nitrate relative to phosphate and elevated nitrite concentrations have been attributed to sedimentary denitrification (25). For comparison to station 1, we chose station 2 (33°51.0′N, 119°47.6′W, 1,395-m water depth), which is located outside the basin in normally ventilated waters of the California Current system.

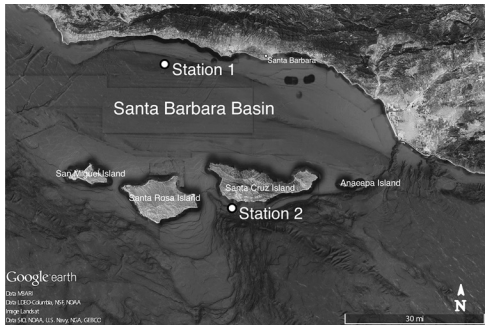


FIG 1 Map showing sampling locations for the present study.

MATERIALS AND METHODS

Sample collection. All samples were collected during the Cal-Echoes cruise on the R/V *Melville* in September 2010. Depth profiles of temperature and salinity were obtained via conductivity, temperature, and depth (CTD) measurements, and dissolved oxygen concentrations were measured at both stations with a Sea-Bird Electronics oxygen probe validated by Winkler titrations. Particulate organic matter (POM) samples were collected by *in situ* filtration with two battery-powered large-volume pumps (WTS-LV; McLane Research Laboratories, Falmouth, MA). An acid-washed Nitex screen with a 45-μm pore size was used as a prefilter, and POM was collected on a precombusted 0.7-μm glass fiber filter (GF/F). Multiple deployments were carried out in order to obtain "replicate" samples from each depth at both stations (see Table S1 in the supplemental material). Sampling volumes were determined with both the pump control software and a mechanical flow meter attached to the pump's outlet, which generally agreed to within 10%. Surface POM samples were collected with a diaphragm pump fitted with a 0.7-μm GF/F but no prefilter. Surface water was filtered for 45 min to 1 h per sample, but because of changing flow rates during filtration, the total volume filtered could not be accurately determined. All GF/Fs collected were wrapped in precombusted aluminum foil and immediately frozen to −80°C until extraction in the laboratory.

Lipid extraction. Lipids were extracted according to previously described methods (26). Total lipid extracts (TLEs) were redissolved in dichloromethane (DCM) to a concentration of 10 mg/ml and stored at −20°C until further analysis. A sediment sample from station 1 in the Santa Barbara Basin was Soxhlet extracted for 48 h in DCM-methanol (MeOH) (2:1, vol/vol) rather than sonicated, and the TLE was then isolated according to reference 26.

Derivatization and high-performance liquid chromatography (HPLC)-atmospheric pressure chemical ionization (APCI)-mass spectrometry (MS) analysis of BHPs. BHPs were analyzed according to previously described methods (27), with a few modifications. TLEs were acetylated in 1:1 pyridine-acetic anhydride for 20 min at 70°C, dried, and then redissolved in a known volume of 60:40:20 MeOH-isopropanol-DCM. A 10-μl volume of each TLE, adjusted to an approximate concentration of 10 mg/ml, was injected onto a Zorbax Eclipse XDB (Agilent Technologies, Santa Barbara, CA) 5-μm C_{18} column (150 by 4.6 mm [inside diameter]) at room temperature. Analyses were performed with an Agilent 1200 HPLC system coupled to a Thermo-Finnegan LTQ-XL linear ion trap mass spectrometer equipped with an APCI source and running in positive mode. The instrument was tuned to the molecular ion of acetylated bacteriohopanetetrol (BHT) at *m/z* 655 from an infused TLE of *Rhodopseudomonas palustris* TIE-1 obtained from D. Newman, Caltech (28). MS instrument parameters were as follows: capillary temperature,

200°C; APCI vaporizer temperature, 400°C; source current, 6 μA; sheath gas, 40; auxiliary gas flow, 7 (arbitrary units). Data were acquired in three scan events, MS^1, MS^2, and MS^3. Scan 1 was a full MS scan from 200 to 2,000 *m*/*z*. Scan 2 used a dynamic exclusion procedure to isolate ions from scan 1 for collision-induced dissociation (CID) fragmentation by using a mass list of known BHP parent masses. Similarly, scan 3 was programmed to isolate the most abundant ion from MS^2 for CID. Fragmentation was achieved with CID set to an intensity of 35 (arbitrary units).

Because of the lack of authentic BHP standards, quantification of BHPs should be considered only semiquantitative; however, for comparison, all BHPs were quantified by using a standard curve generated for acetylated BHT. This was accomplished by first quantifying the amount of BHT present in an acetylated TLE of *R. palustris* TIE-1 by gas chromatography-MS and then using the same TLE to create a standard curve on the LC-MS. The response was linear over 3 orders of magnitude. Total BHP concentrations were then reported relative to the concentration of BHT in each sample.

DNA extraction and analysis. A subsample of each GF/F was extracted for DNA analysis by a previously described chloroform-phenol extraction procedure (29), excluding the cetyltrimethylammonium bromide purification step. In addition, two extractions of phenol-chloroform-isoamyl alcohol (25:24:1), followed by one extraction with chloroform-isoamyl alcohol (24:1), were substituted for the aqueous phenol and phenol-chloroform extractions in the original procedure, respectively. DNA was concentrated by ethanol precipitation, resuspended in a small volume of TE buffer, and then stored at −20°C.

16S rRNA gene PCR was used to assess bacterial community diversity at each sampling depth. DNA extracts from multiple samples from the same station and depth were combined before amplification to reduce any bias associated with temporal differences in sampling. The primers used were universal 16S rRNA gene bacterial primers 27F (5′ AGA GTT TGA TCC TGG CTC AG 3′) and 1522R (5′ AAG GAG GTG ATC CAG CCG CA 3′).

PCR was also used to amplify the gene for SHC (*sqhC*), which is the enzyme responsible for the cyclization of squalene to form the pentacyclic hopanoid ring structure (17). Degenerate primers targeted conserved regions from an alignment of known SHC amino acid sequences (19) to frame the catalytic site of protonation conserved in all SHCs, a DxDD motif. The forward primer, SHC_F, had the sequence 5′ TCN CCN RTN TGG GAY AC 3′ and targets the conserved amino acid sequence SP(V/I)WDT (19). The reverse primer, SHC_JKR, had the sequence 5′ GCC CCA NCK NCC RWA CCA 3′ and targets the amino acid sequence W(F/W/Y)GRWG, about 185 amino acids downstream of the forward primer site. On the basis of the bacterial genomes available in databases, we expect this degenerate primer set to cover all bacterial genera with a sequenced representative containing an *sqhC* gene, with the exception of certain planctomycetes that did not contain the conserved sites used for primer design. For detailed PCR methods and temperature program information, see the supplemental material.

PCR products were cloned with the Invitrogen TOPO TA cloning kit for sequencing and the Thermo Scientific CloneJET PCR cloning kit for 16S rRNA gene and *sqhC* libraries, respectively, with One Shot TOP10 chemically competent *Escherichia coli* (Invitrogen). Colonies were picked into the wells of 96-well plates containing 10% glycerol in LB medium (50 μg/ml kanamycin) and grown for exactly 12 h at 37°C before submission for sequencing by Beckman Coulter Genomics.

Bioinformatic analysis. Classification using only the forward 16S rRNA gene reads was done with the Ribosomal Database Project classifier (30). The "marine microbial communities from the eastern subtropical North Pacific Ocean, expanding oxygen minimum zones" metagenome (NCBI no. 408172; GOLD no. Gm00303) was searched through the Joint Genome Institute online portal by using the partial SHC sequence of *Alicyclobacillus acidocaldarius*, which was trimmed at the forward and reverse primer sites. Only hits containing both the catalytic site and either the forward or the reverse primer sites were retained for further analysis.

Tree construction. In order to compare sequence distributions and classifications between samples, a reference *sqhC* phylogeny was generated from an amino acid multiple-sequence alignment of the full SHC protein obtained from 67 sequenced genomes. The multiple-sequence alignment was constructed with the program T-Coffee (31) run under default parameters and was trimmed with TrimAl (32) to remove columns containing only gaps and to remove poorly aligned regions in the -automated1 mode. A reference maximum-likelihood phylogenetic tree was then constructed in RAxML v7.4.2 (33) under the gamma distribution for rate heterogeneity and using the Whelan and Goldman amino acid substitution matrix. Robustness of inference was assessed from 500 bootstrap resamplings. For the phylogenetic analysis of environmental *sqhC* sequences, the translated sequence fragments were added to the full-length SHC reference tree alignment with MAFFT (34), and pplacer v1.1 (35) was used to locate those sequence fragments within the phylogeny of the reference tree. pplacer infers the taxonomic classification of unknown sequence fragments by contextualizing them within a phylogeny where the taxonomic topology is known. In doing so, pplacer finds an attachment location and pendant branch length that maximize the likelihood of the tree with that new branch attached. A taxonomic rank is then derived from the lowest common ancestor of the leaves of the given clade from which the read is placed. Taxonomic ranks were collected in tabular format with the guppy to_csv command, and the class level ranking was used in subsequent analyses regarding the taxonomic distribution of SHCs.

A traditional phylogenetic tree was also generated with partial amino acid sequences corresponding to the region amplified by PCR. The same reference set of sequences (including the outgroups) used in the full amino acid sequence alignment were trimmed to match the forward and reverse primer sites and aligned with the California Current system amplicons, previously published sequences (reference 19, accession numbers EF030658 to EF030691; reference 20, accession numbers EU500771, EU500772, EU500776, EU500783 to EU500822, and EU500824), and metagenomic hits with MAFFT (34) with the L-INS-i option. The alignment was then trimmed with TrimAl (32) with the gappyout option to give a final alignment length of 179 amino acids. Phylogenetic trees were generated with RaxML (33) on Cipres (36) with the LG+G+I model and 2,000 bootstraps. Clades were assigned so that the median pairwise patristic distances (branch lengths) between leaf nodes in the phylogeny were smaller than the distances computed randomly among 90% of the total sequence population. Specifically, a median pairwise patristic distance cutoff of 0.74 resulted in clusters at this percentage level. The algorithm is essentially as outlined previously (37), except that internal nodes in the tree were not accounted for in our implementation. The algorithm was implemented in R (clustertree.R code for clustering phylogenetic trees, Glenn Lawyer, figshare [2012], http://dx.doi.org/10.6084/m9.figshare.97225).

Nucleotide sequence accession numbers. California Current system *sqhC* nucleotide sequences were deposited in GenBank under accession numbers KF310107 to KF310134, KF310136 to KF310140, KF310142 to KF310431, KF310433 to KF310439, KF310441 to KF310479, and KF310481 to KF310526.

RESULTS

Biogeochemical characteristics of Santa Barbara Basin POM. Measured nutrient and oxygen concentrations from CTD casts at stations 1 and 2 are plotted along with profiles of these variables from the Fall 2010 CalCOFI cruise at CalCOFI station 81.8 46.9 (34.27°N, 120.02°W) in the Santa Barbara Basin and CalCOFI station 83.3 55.0 (33.74°N, 120.4°W), near our station 2 (Fig. 2). The oxygen profiles at both stations followed similar patterns with depth, and station 1 was consistently more oxygen depleted than station 2. The oxygen concentrations at a 475-m depth, as measured by modified Winkler titration, were 0.07 and 0.4 ml/liter at

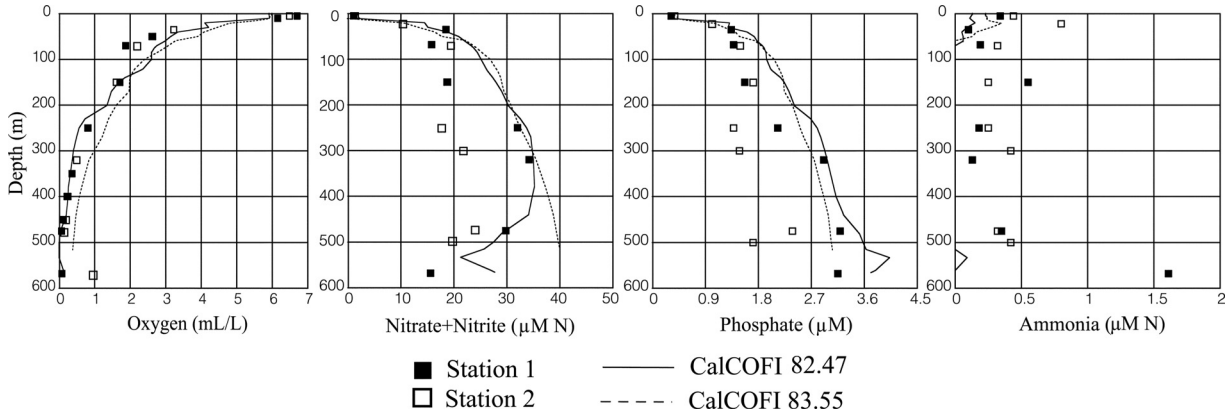


FIG 2 Nutrient and oxygen measurements at stations 1 and 2 during the CalEchoes cruise, plotted with nutrient profiles from the Fall 2010 CalCOFI cruise from CalCOFI stations 82.47 (34.27°N, 120.02°W) and 83.55 (33.74°N, 120.4°W), closest to stations 1 and 2, respectively.

stations 1 and 2, respectively. Nitrite was also detectable in bottom waters of the Santa Barbara Basin at station 1.

The C/N ratio and isotopic analysis of bulk POM, as described in the supplemental material, distinguished the physical and biological settings of the two stations (see Fig. S2 in the supplemental material). The C/N ratio of POM was low at the surface at both stations but increased at a 475-m depth at station 1 (see Fig. S2A in the supplemental material), and the C/N ratio of the SBB sediment sample was elevated relative to the suspended POM in the upper water column. The resuspension of these sediments at station 1 must be responsible in part for the increased variability in the observed C/N ratio of POM at 475 m. Lower POM-δ^{15}N values at the surface at station 1 suggested production fueled by recent local upwelling, whereas the higher value of surface POM at station 2 was more consistent with horizontal advection of nitrate to fuel production at this location (see Fig. S2B). Higher δ^{13}C values at station 1 also reflected increased dissolved inorganic carbon utilization and thus greater primary production at the surface than at station 2. This signal was present throughout the water column at station 1, while at station 2 δ^{13}C-POM levels increased with depth, identifying either diagenetic processing during transit through the water column or subsurface advection of POM from a different location (see Fig. S2C).

BHP diversity and abundance. A total of six distinct BHP structures were identified in water column POM samples, as summarized in Fig. 3. For the complete identification and quantification of BHPs in each individual sample, see Table S1 in the supplemental material. Both the structural diversity and abundance of BHPs increased with depth and with greater suboxia (Fig. 4), and the most suboxic sample at station 1 contained more than five times the amount of total BHP found at the same depth at station 2.

The distribution patterns of some individual BHPs also appear to be correlated with depth and oxygen concentration. Consistent with its reputation as the most cosmopolitan BHP in natural samples, BHT (Fig. 3A) was present in all samples except those from the surface, in which no BHPs were detected. BHT II, an apparent stereoisomer of BHT I, was identified only in the station 1 samples from a 475-m depth. The lack of BHT II in samples from station 2 at the same depth supports its association with low oxygen concentrations rather than the depth or environmental setting. This is consistent with previous observations suggesting that this compound is found only in the water column where oxygen levels approach anoxia (15). BH-aminotriol (Fig. 3B) was detected in all nonsurface samples except at a 250-m depth at station 1, and a putative unsaturated BH-aminotriol (Fig. 3C) was detected in all nonsurface samples except at a 250-m depth at station 2.

Samples from repeated deployments to the same depth generally agreed with each other in terms of the BHPs detected, with one exception. In one filter from a 475-m depth at station 1, two additional BHPs, (5′-D-ribonyl)hopane (Fig. 3D) and adenosylhopane (Fig. 3E), were identified that were not present in other samples from 475 m. Because of the presence of these additional structures and also disproportionately higher BHP concentrations, we treated this as a separate sample in further quantitative BHP analysis (475′m here).

The identification of adenosylhopane, a hopanoid normally found in sediments and used as a tracer of terrestrially derived bacterial organic matter, combined with total BHP concentrations that were 10 times higher than the other three samples from 475 m at station 1, led to the hypothesis that sediment may have been incorporated into filter 475′m. Although we sampled well above the bottom of the basin at station 1, it is possible that the sediment coring activities during the CalEchoes cruise resulted in the resuspension of sedimentary material. Therefore, one surface sediment sample from station 1 was analyzed in order to compare the BHP composition of sediments with that of the water column. Four of the five hopanoids identified in sample 475′m were identified in the sediment sample: BHT I, BH-aminotriol, unsaturated BH-aminotriol, and adenosylhopane. In addition, the sediment sample had a higher concentration of BHPs per gram of carbon than did water column samples (data not shown). These data suggest that incorporation of sedimentary organic matter likely altered the composition of this 475′m filter.

Interestingly, BHT II was not detected in the sediment sample. A comparison of BHT I/BHT II ratios in three water column samples from 475 m at station 1 gave an average value of 3:1. However, the 475′m filter, which had a BHP profile resembling that of the underlying sediment, had a much lower ratio. This further supports the hypothesis that BHT II may be produced only in the water column under anoxic conditions.

16S rRNA gene and *sqhC* phylogenetic analysis. 16S rRNA gene analysis of samples from each depth demonstrated that *Proteobacteria* dominate the suspended POM (0.7 to 43 μm) at both

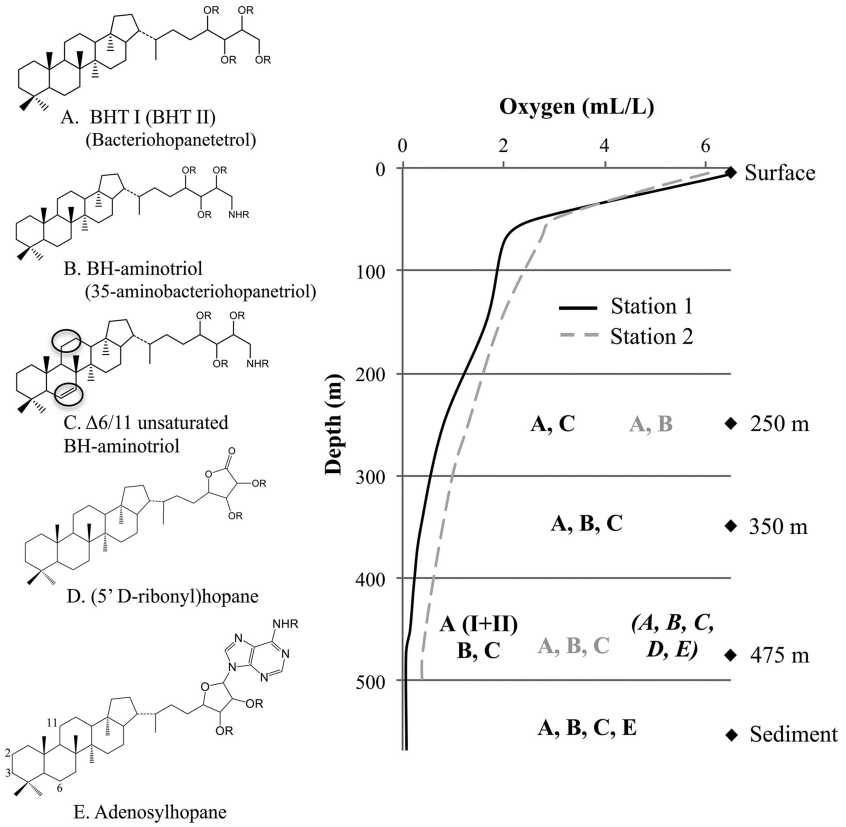


FIG 3 Structures of BHPs identified at the sampled depths represented by the black diamonds. The 475′m sample, in which all five structures were identified, is in italics.

stations, and there was little difference between the two stations, except in surface samples (Fig. 5). However, there is a shift from *Alphaproteobacteria* to *Gammaproteobacteria* with depth, which mirrors previous observations in the free-living bacterial commu-

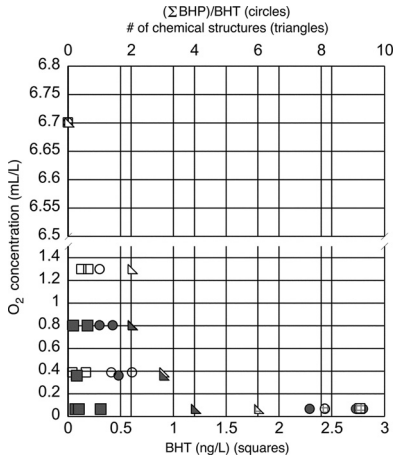


FIG 4 Relationship between oxygen concentration and BHP diversity and abundance. Filled and open symbols represent stations 1 and 2, respectively. The concentration of BHT in ng/liter of water filtered (squares) and the total BHPs relative to the BHT concentration (circles) are shown for multiple samples from each depth. The numbers of structures identified at different depths are represented by triangles. Crosshatched shapes represent sample 475′m from station 1.

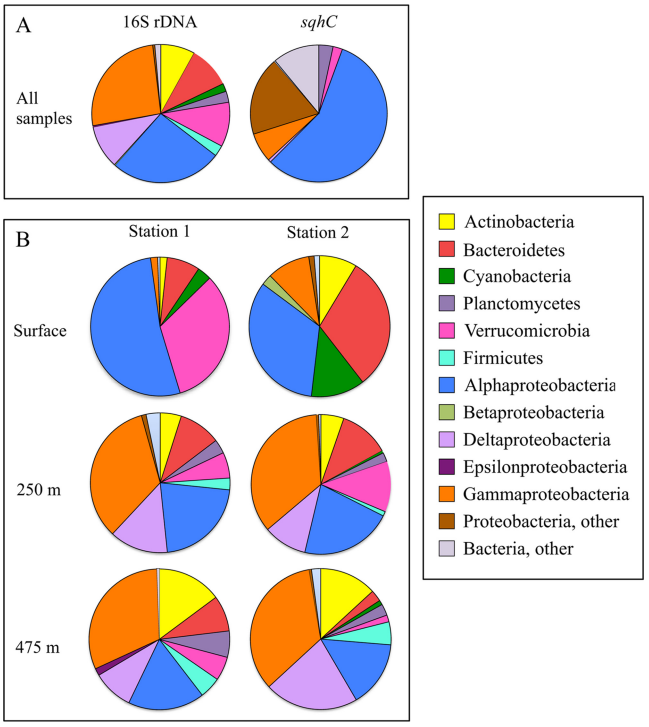


FIG 5 Comparison of overall 16S rRNA gene distribution with *sqhC* sequence classification inferred with pplacer (A) and classification of 16S rRNA gene sequences by station and depth by using the Ribosomal Database Project (B).

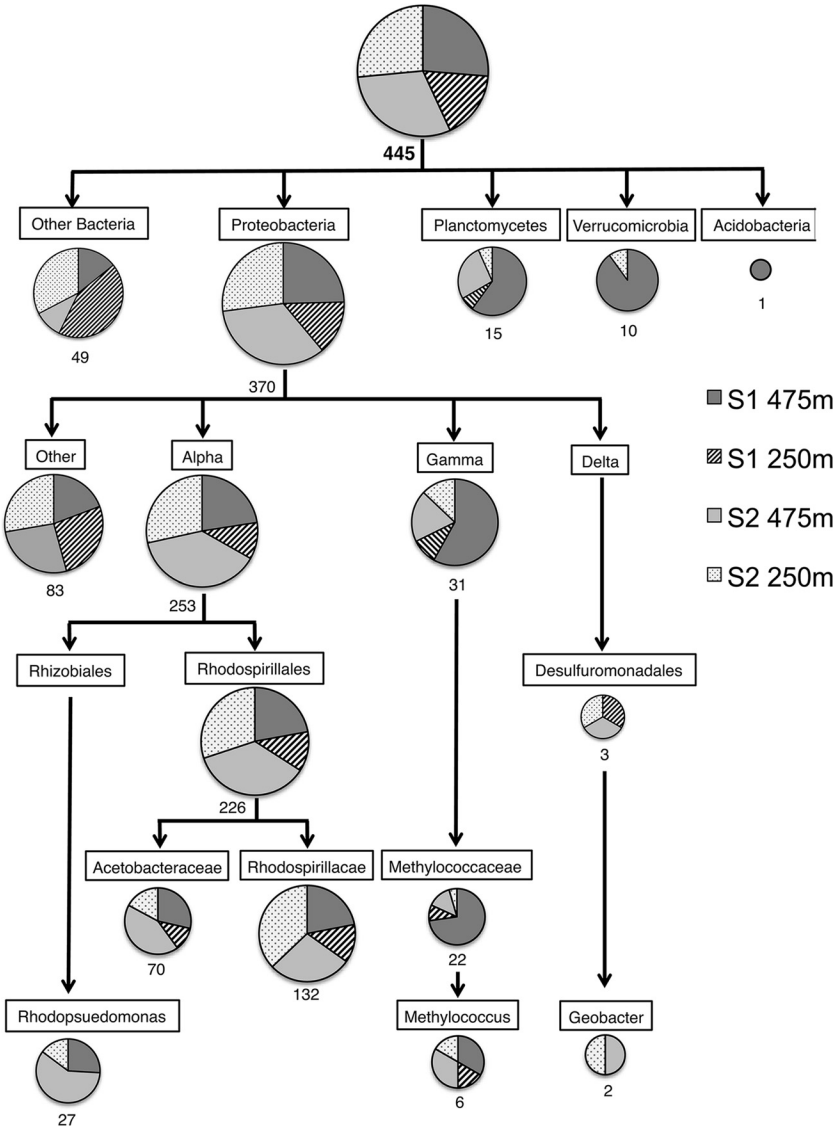


FIG 6 Phylogenetic affiliations of SHC sequences with pplacer. Pie charts are scaled to the log + 1 of the number of sequences (indicated next to each chart) that could be reliably assigned to that classification level. Note that the number of sequences in each successive level does not necessarily sum to the number of sequences depicted by the pie chart in the level above.

nity (38, 39). This 16S rRNA gene data set, in theory, represents the particle-associated microbial community, but because the GF/Fs used for sample collection have only a nominal pore size of 0.7 μm, these filters could potentially isolate up to 50% of the free-living community as well (40). Therefore, it is difficult to partition these 16S rRNA gene data into free-living and particle-attached communities. However, comparing the basin station (station 1) to the open-ocean station demonstrates that changes in class level community structure are broadly related to depth rather than to the oxygen concentration (Fig. 5).

The *sqhC* gene was amplified from samples from all depths except the surface. The absence of this gene from surface water samples may explain why no BHP structures were identified in these samples. Classification of translated sequences with a reference tree of full amino acid sequences from cultured organisms shows that the phylogenetic associations of amplified SHC sequences appear to broadly follow the same distribution as 16S rRNA gene classification, in that the majority of the sequences map to representatives of the *Alphaproteobacteria* and *Gammaproteobacteria* (Fig. 6). As with the 16S rRNA gene distribution, there appear to be few clear differences in the community composition of hopanoid producers between stations 1 and 2. One detectable difference is that more *sqhC* gene sequences map to the *Gammaproteobacteria* at station 1 while station 2 contains more sequences from the *Alphaproteobacteria*. In addition, most of the nonproteobacterial sequences are found at station 1 (Fig. 6).

The taxonomic associations discussed above must be treated with caution, however, as the majority of the amplified SHCs are not closely related to their closest sequenced relatives, averaging 60% amino acid identity with their best matches. For context, SHCs are generally >90% identical within a genus, >75% identical within a subgroup classification such as *Alphaproteobacteria*,

50 to 60% identical between subgroups such as *Gammaproteobacteria* and *Alphaproteobacteria*, and 40 to 55% identical between major groups such as *Cyanobacteria* and *Alphaproteobacteria* (22). None of the California Current SHCs exceeded the 75% similarity threshold and therefore cannot necessarily be assigned to a specific class subdivision like *Alphaproteobacteria* or *Gammaproteobacteria*. However, it has been previously reported that identities of >60% may be sufficient to identify environmental SHC sequences that are affiliated with a particular phylogenetic group (20). Most of the California Current SHCs fall into the 55 to 74% identity range, indicating that they are at least classifiable within a major group such as *Proteobacteria*. About 12% of California Current SHCs fall below 55% similarity and likely represent major groups that are not yet included in genomic databases.

To view these potentially novel groups in a phylogenetic context, a tree was constructed by a partial amino acid alignment (179 amino acids in length) of cultured representative SHCs, California Current system (CC) SHC amplicons, and SHC sequences obtained from other sampling sites, including a freshwater lake, Pacific Ocean surface waters, and soil and aquatic samples from the Bahamas (22, 23) (Fig. 7). Also included in the tree are positive hits to SHCs from the newly assembled metagenome project, "marine microbial communities from expanding oxygen minimum zones (OMZs) in the northeastern subarctic Pacific Ocean" (expanding oxygen minimums [EOM] metagenome). Genomic sequences in the tree include at least one species of each genus known to contain an *sqhC* gene. Clades consisting of closely related sequences have been collapsed to simplify the tree; however, a list of collapsed sequences originating from each sampling depth and station is shown in Table S2 in the supplemental material. The tree was further divided into clades representing the top 10% of the most closely related sequences at a given internal node with median pairwise distance thresholding. For the distribution of sequences in each clade, see Fig. S3 in the supplemental material. The colored bars provide a crude metabolic context for uncultured hopanoid producers based on the metabolic capabilities of the closest cultured organisms in the tree.

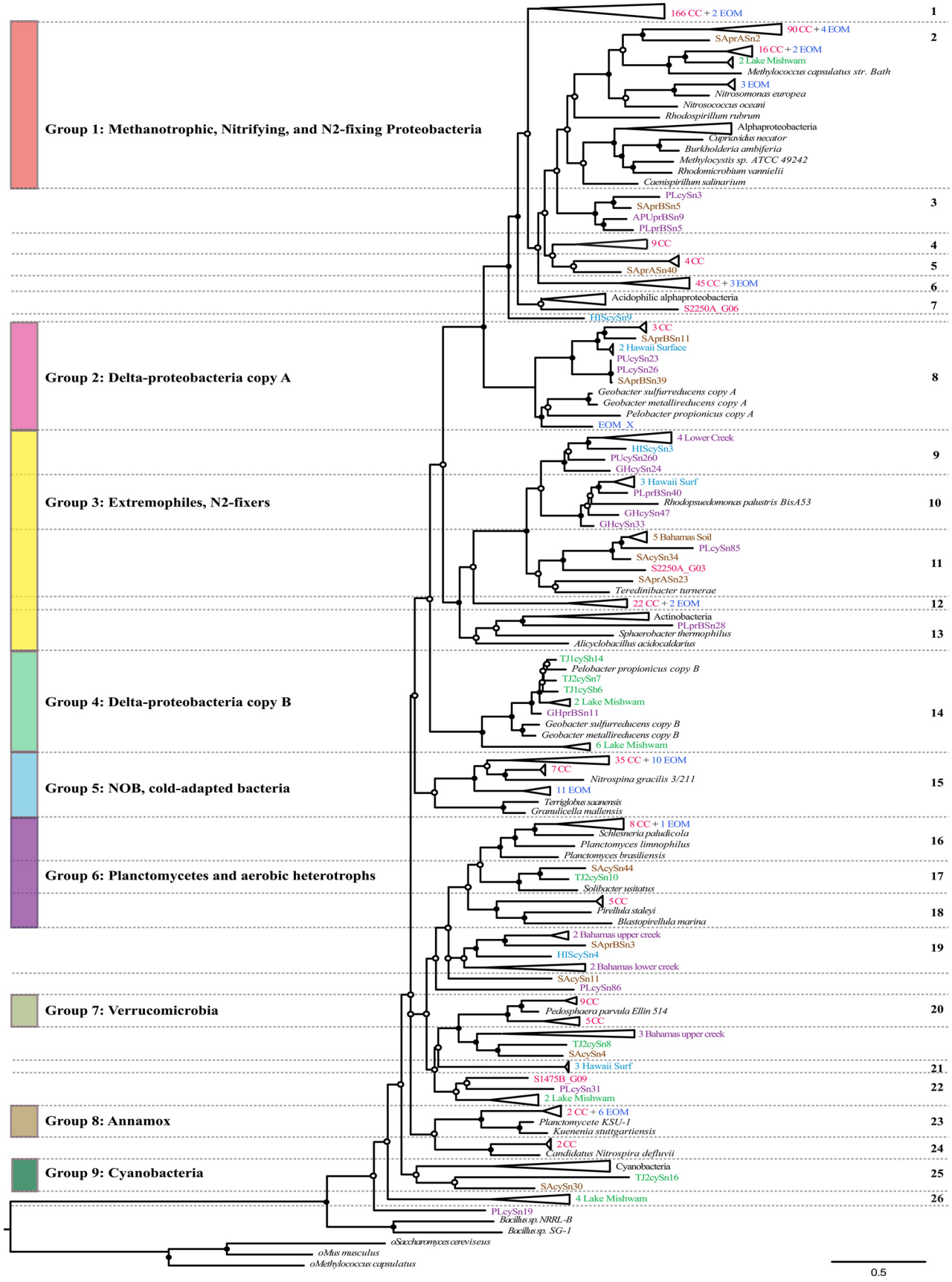
DISCUSSION

Our analysis demonstrates that both BHP abundance and structural diversity increase with decreased water column oxygen concentrations, in agreement with previous observations (15). This finding suggests that bacteria living under these conditions are important contributors to BHP production in the marine water column and that there may be physiological roles for hopanoids associated with these low-oxygen environments. This is supported by the phylogenetic distribution of these environmental SHCs in relation to cultured organisms, which suggests that hopanoid producers in this data set most likely possess what we might call "fringe" metabolic strategies in order to grow under transitional environmental conditions. Many of these are thought to be ancient survival strategies that originated early in microbial evolution, before the permanent oxygenation of the oceans and atmosphere. Thus, greater SHC diversity may exist in low-oxygen environments, particularly within oxic-anoxic transition zones, which require specialized metabolic strategies relative to normally ventilated environments. Alternatively, extant hopanoid producers sampled here may be relics from a time when oxygenation and deoxygenation of the oceans were spatially and temporally widespread, such that these organisms can now thrive only in specialized environments such as the transitional environments captured in this study. Along with the fact that so far none of the major oceanic phylotypes such as the SAR11 or *Roseobacter* clade have been found to contain an *sqhC* gene, the present findings may explain why relatively few *sqhC* sequences were obtained from the GOS data set (21), which included exclusively aerobic environments in ocean surface waters.

Recent work on BHPs has been focused on understanding the distribution of BHP side chain diversity in various environments, with the goal of using such distributions to fingerprint hopanoid-producing bacterial communities and processes (41–43). However, while there appear to be a number of structures that can be associated with specific bacterial groups or metabolisms (13, 14), the cosmopolitan nature of the structures observed in this study makes it challenging to link a chemical structure with a biological source in the marine environment. For example, we did not identify any methylated BHPs even in the most anoxic samples, despite the identification of a group of *sqhC* sequences that appears to cluster with a cultured representative of methanotrophic bacteria that are thought to be the major source of 3-methylbacteriohopanepolyols in the environment (Fig. 7, clade 2). In fact, none of the structures observed here are exclusive to particular bacterial groups or to marine environments, with the possible exception of BHT II. The identification of BHT II only in anoxic samples suggests that this isomer may be one of the few marine BHP structures with an environmental pattern and has the potential to be developed as a biomarker for water column hypoxia/anoxia that could be used in studies of past environments, as well as present ecosystems. This utility would be enhanced should BHT II prove to be the same compound as a similarly eluting BHT isomer observed previously (51), which may be a C22 isomer on the basis of the continued presence of the second isomer even after cleavage of the side chain by periodic acid oxidation.

With regard to interpretation of the geologic record, the increase in the total relative abundance of BHPs with decreasing oxygen concentrations may be the more interesting trend. This trend has been observed consistently in other low-oxygen regions, including the Arabian Sea, the Peru Margin, and the Cariaco Basin (15), as well as the Black Sea (17). In these studies, nearly all profiles of BHPs display a 5- to 7-fold increase in concentration, with maxima near the top of or within the anoxic zone. Assuming that a significant fraction of the BHP flux to sediments originates from the water column, the varying concentration of hopanoids in marine sediments could potentially reflect oxygen concentration changes in the overlying water column. In combination with other biomarkers, as well as isotope measurements, such variations in overall hopanoid abundance could be useful for identifying past anoxic events. The SBB would be an ideal place to investigate whether these variations in total hopanoids would be large and responsive enough to be observed in sediment cores. The distribution pattern of hopanoids in ancient sediments might also give insight into the past extent of OMZs, a topic that is becoming more relevant because of recent observations that modern OMZs appear to be expanding (44).

The diversity of the amplified SHC sequences reflects the observation that low oxygen gradients harbor a kind of metabolic diversity different from that of normally ventilated environments (45). The CC SHCs (CC sequences in Fig. 7) are not closely related to the SHC sequences previously amplified from other environments, and few clades in the phylogenetic tree contain both.



Rather, the CC SHCs group mostly with each other in several large clades and/or with cultured representatives. Notably, the previously identified "unknown marine group" (UMG) of SHC sequences (20), consisting of clades 9 and 10 (Fig. 7), contains no CC SHCs. However, the environments previously sampled (22, 23) are distinct from the low-oxygen environments of the eastern North Pacific Ocean sampled here, and this may explain the limited homology between our sequences and previously sequenced environmental SHCs. In fact, some CC and EOM sequences (clade 12) fall near clades 9 and 10 but do not cluster directly with previous sequences assigned to the UMG.

In contrast, many of the SHC sequences obtained from the EOM metagenome do show close similarity to sequences amplified from the California Current system, with an average of 83% similarity. This suggests that primer bias is not responsible for the dissimilarity between CC sequences and those obtained in previous studies. The EOM SHCs are represented in the largest clades in the tree, made up primarily of CC sequences, such as clades 1, 5, and 6 (Fig. 7). Furthermore, while SHCs from low-oxygen waters are found in nearly every part of the phylogenetic tree in Fig. 7, the large clades contain sequences from all of the California Current system samples analyzed, as well as EOM metagenomic sequences. Therefore, the individuals identified in these clades likely include groups of hopanoid producers that are cosmopolitan in regions with relatively low oxygen levels like the eastern North Pacific Ocean. Focusing on BHP producers in these large clades to examine environmental variability and abundance may enable a more accurate estimate of their contribution to the pelagic microbial community. Examination of other low-oxygen metagenomes may reveal whether these groups can be found outside the Eastern Pacific region as well.

Overall, the genetic diversity of the *sqhC* gene provides additional insight into the metabolic capabilities and evolutionary history of uncultured hopanoid producers in the environment, information not provided by the identification of BHP structures themselves. The broad phylogenetic distribution of SHCs from the California Current system (Fig. 7) supports the idea that a wide range of bacterial groups is capable of hopanoid production and suggests an ancient lineage with little evidence of frequent lateral transfer of the *sqhC* gene, as the trees of *sqhC* sequences are similar in topology to trees based on 16S rRNA gene sequences. These results are consistent with the conclusions of previous work (20). We interpret the cooccurrence of *sqhC* with unusual bacterial metabolic strategies that are in many cases linked to low-oxygen environments as reflecting a connection between *sqhC* evolution and metabolic strategies within reducing environments (Fig. 7). This is discussed in more detail below.

Many of the characterized hopanoid producers possess multiple metabolic strategies and adaptations consistent with their presence in challenging environments. These organisms include those living at extreme temperatures or pHs (e.g., *Streptococcus thermophilus*, *A. acidocaldarius*), in polluted environments (*Cupriavidus necator* N1), in anoxic sediments (*Geobacter* sp.), and in soils where gas concentrations, pH, and salinity often vary and where metabolic plasticity is common (*Rhizobiales*). In addition, many of the metabolically versatile organisms identified in Fig. 7 also possess multiple nitrogen metabolisms and even those organisms considered to be primarily aerobic have evolved to tolerate low-oxygen conditions. Examples among the *Proteobacteria* include *Methylococcus capsulatus* (46), *Nitrobacter* sp. (47), and *R. palustris* (48). Similarly, "*Candidatus* Nitrospira defluvii" is a nitrite-oxidizing bacterium (NOB) that has key genes that function in nitrite oxidation, electron transport, and respiration in common with the annamox organism "*Candidatus* Kuenenia stuttgartiensis" (49), suggesting that these organisms may have coevolved under low-oxygen conditions. Members of the phylum *Nitrospirae* are also related to the major marine NOB *Nitrospina gracilis*, which lacks classical reactive oxygen defense mechanisms and uses the reductive tricarboxylic acid cycle for carbon fixation (50), indicating that it likely evolved under microaerophilic conditions as well.

Although the interpretation of metabolic associations is somewhat limited by the phylogenetic distance between cultured and environmental sequences and the low bootstrap support for deeper nodes in the tree, the overall distribution of CC SHC sequences in relation to these potential metabolic groups supports the observation that hopanoid biosynthesis is phylogenetically widespread but uncommon among the majority of modern prokaryotes and suggests that hopanoid production may be associated with ancient metabolic strategies that are found in a limited number of modern environments. The distribution of SHCs from the low-oxygen environments of the eastern Pacific further suggests that extant producers are likely related to early hopanoid-producing organisms that evolved alongside microaerophilic metabolic strategies necessary to survive the varying redox conditions that characterized the early Earth.

Clearly, the abundance of hopanoids in the geological record suggests that their biosynthesis was once more common. We could imagine a scenario on the early Earth where hopanoids were important components of ancient bacterial membranes but became less important as oxygen concentrations increased and new metabolic strategies emerged to fill new environmental niches. Over evolutionary time scales, this would have resulted in the loss of the biosynthetic pathway from most bacterial genomes and only those organisms that continued to use metabolic strategies allowing them to occupy biogeochemically challenging, specialized niches retained the ability to synthesize hopanoids. This is a possible explanation for the absence of *sqhC* from the genomes of today's major marine clades, which for the most part occupy aerobic, mesophilic environments in the upper ocean rather than the microaerophilic environments to which extant hopanoid producers appear to be adapted.

A major goal of examining the diversity of composite BHPs in modern-day marine environments is to facilitate the link between the sedimentary inventory of these compounds and their water column source. An extension of this goal is to understand why BHPs are produced, which requires an understanding of the metabolic characteristics that require BHP biosynthesis and whether

FIG 7 Maximum-likelihood tree of SHC and oxidosqualene cyclase sequences from characterized bacterial species (italics) and alignable sequences from the California Current (pink), previous studies including freshwater and marine samples from the Bahamas (purple), soil from the Bahamas (brown), Hawaii surface waters (light blue), and Lake Mishwam, a methanogenic freshwater lake (green). Hits from the EOM metagenome are in dark blue. Nodes marked with solid circles indicate bootstrap equivalents of 0.5 or greater, while empty circles indicate values of <0.5.

BHPs are essential for the survival of these organisms in particular environments. Our data and those of others (15) confirm that structural characterization of composite BHP structures in the water column with the goal of linking structural diversity to source and metabolism is challenging and perhaps prohibitive because of both the cosmopolitan nature of certain BHPs and the low concentrations of individual, more rare or unique BHPs. However, our genetic survey of *sqhC*, as in previous work (20), confirms that this approach is more successful at identifying the potential metabolic strategies of hopanoid producers and brings us a step closer to understanding their evolutionary history and the ecological niches they occupy in the modern ocean. It also identifies a heretofore undocumented diversity of BHP producers in low-oxygen environments and confirms hypotheses concerning the "fringe" nature and potential environmental plasticity of these hopanoid producers.

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Composite Bacterial Hopanoids and Their Microbial Producers across Oxygen Gradients in the Water Column of the California Current

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Page 7493, column 1, paragraph 5, line 4: "(19)" should read "(22)."

Page 7493, column 1, paragraph 5, line 8: "(19)" should read "(22)."

Page 7493, column 2, paragraph 2, line 6: "reference 19" should read "reference 22."

Page 7493, column 2, paragraph 2, line 7: "reference 20" should read "reference 23."

Page 7497, column 1, paragraph 1, line 9: "(20)" should read "(23)."

Page 7499, column 1, paragraph 1, line 4: "(20)" should read "(23)."

Page 7499, column 1, paragraph 3, line 12: "(20)" should read "(23)."

Page 7500, column 1, line 8: "(20)" should read "(23)."

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