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Hopanoid lipids may facilitate aerobic nitrogen fixation in the ocean

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Cyanobacterial diazotrophs are considered to be the most important source of fixed N_2 in the open ocean. Biological N_2 fixation is catalyzed by the extremely O_2-sensitive nitrogenase enzyme. In cyanobacteria without specialized N_2-fixing cells (heterocysts), mechanisms such as decoupling photosynthesis from N_2 fixation in space or time are involved in protecting nitrogenase from the intracellular O_2 evolved by photosynthesis. However, it is not known how cyanobacterial cells limit O_2 diffusion across their membranes to protect nitrogenase in ambient O_2-saturated surface ocean waters. Here, we explored all known genomes of the major marine cyanobacterial lineages for the presence of hopanoid synthesis genes, since hopanoids are a class of lipids that might act as an O_2 diffusion barrier. We found that, whereas all non–heterocyst-forming cyanobacterial diazotrophs had hopanoid synthesis genes, none of the marine *Synechococcus*, *Prochlorococcus* (non–N_2-fixing), and marine heterocyst-forming (N_2-fixing) cyanobacteria did. Finally, we conclude that hopanoid-enriched membranes are a conserved trait in non–heterocyst-forming cyanobacterial diazotrophs that might lower the permeability to extracellular O_2. This membrane property coupled with high respiration rates to decrease intracellular O_2 concentration may therefore explain how non–heterocyst-forming cyanobacterial diazotrophs can fix N_2 in the fully oxic surface ocean.

oxygen diffusion barrier | hopanoid lipids | nitrogen fixation | marine cyanobacteria

Marine cyanobacterial diazotrophs, i.e., those capable of reducing dissolved dinitrogen gas (N_2) into ammonia through N_2 fixation, are key suppliers of bioavailable N, a limiting nutrient for primary production in the ocean (1). Biological N_2 fixation is solely performed by the O_2-sensitive nitrogenase enzyme (2), and understanding how low intracellular O_2 concentrations are maintained in fully oxic open waters is a long-standing question that has attracted much interest (3–6).

Although, a priori, it would seem that N_2 fixation is incompatible with the O_2-evolving photosynthetic lifestyle of cyanobacteria, it is known that these microorganisms have evolved a variety of strategies to protect nitrogenase from O_2 inactivation. For example, some filamentous cyanobacteria, including the symbionts of marine diatoms, form specialized cells called heterocysts (7). A microaerobic environment is created inside heterocysts by inactivating oxygenic photosynthesis, by maintaining or enhancing respiration, and by the formation of an extra glycolipid cell envelope outside the cell wall (8). In contrast, non–heterocyst-forming cyanobacteria such as the filamentous *Trichodesmium* or the free-living unicellular *Crocosphaera* must separate photosynthesis and N_2 fixation either spatially or temporally to avoid exposing nitrogenase to the O_2 that they produce during the light hours (9, 10). In the unicellular cyanobacterial symbiont UCYN-A, all of the genes for the synthesis of the O_2-evolving photosystem II (PSII) apparatus have been lost and so UCYN-A doesn't generate O_2 (11). None of the aforementioned strategies, however, can protect nitrogenase of non–heterocyst-forming cyanobacterial diazotrophs from the O_2 that diffuses across cell membranes from the environment (including host photosynthesis in the case of UCYN-A). Mechanisms such as respiration, the Mehler reaction, and/or other O_2 scavenging strategies have been proposed as potential ways to overcome this problem (12), but whether these mechanisms are sufficient to lower the O_2 concentration in the inner cell while N_2 fixation takes place remains unknown.

We have discovered a consistent pattern of distribution of hopanoid synthesis genes among marine cyanobacteria that suggests that they may play an important role in marine N_2 fixation. Hopanoids are a class of membrane lipids that have been shown to confer special properties to cell membranes (13). Hopanoids can intercalate into lipid bilayers of membranes due to their planar and hydrophobic structure and might decrease their permeability to O_2 (14). Approximately 10% of bacteria, including plant-associated diazotrophs, have the gene for the synthesis of hopanoids (the squalene–hopene cyclase gene *shc*) (13). Interestingly, the only direct evidence showing that hopanoids facilitate N_2 fixation comes from studies of the terrestrial N_2-fixing heterotrophic bacteria *Frankia*. In *Frankia* sp., hopanoids might serve as an O_2 diffusion barrier in their N_2-fixing vesicles (15), with the thickness of the vesicle envelope directly correlated to the external O_2 concentration (16). However, this linkage was later questioned based on the observation of high proportions of hopanoids in membranes regardless of the N status in *Frankia* sp. (17).

We compiled data on hopanoid production and mined the publicly available genomes of marine cyanobacteria to provide an exploration of the presence of hopanoid biosynthetic and modification genes across all of the major marine cyanobacterial lineages, including both diazotrophs and non-diazotrophs (Fig. 1). We found that the *shc* gene for synthesizing hopanoids was consistently present in all of the non–heterocyst-forming cyanobacterial diazotrophs, including unicellular cyanobacterial symbionts with extremely reduced genomes such as UCYN-A. In contrast, none of the non-diazotrophic marine *Synechococcus* and *Prochlorococcus*, which are the dominant cyanobacteria in the ocean (18, 19), nor the heterocyst-forming marine cyanobacteria *Calothrix rhizosoleniae* SC01 and *Richelia intracellularis* HH01 had the *shc* gene in their genomes. The same pattern was observed for almost all of the hopanoid modification genes except for the *hpnK* and *hpnP* genes (Fig. 1). These observations suggest that, whereas the capacity of allocating hopanoids into cell membranes may be universal across all marine non–heterocyst-forming diazotrophic cyanobacteria, it is absent from all marine *Synechococcus* and *Prochlorococcus* (which do not fix N_2) and from heterocyst-forming marine cyanobacteria (which already protect nitrogenase from O_2 by heterocysts). Furthermore, in *Crocosphaera* and *Cyanothece*, the transcription of the *shc* gene peaks right before the nitrogenase-encoding gene (*nifH*) starts increasing its expression level (data collected from ref. 20), and simultaneous expression of both markers has also been

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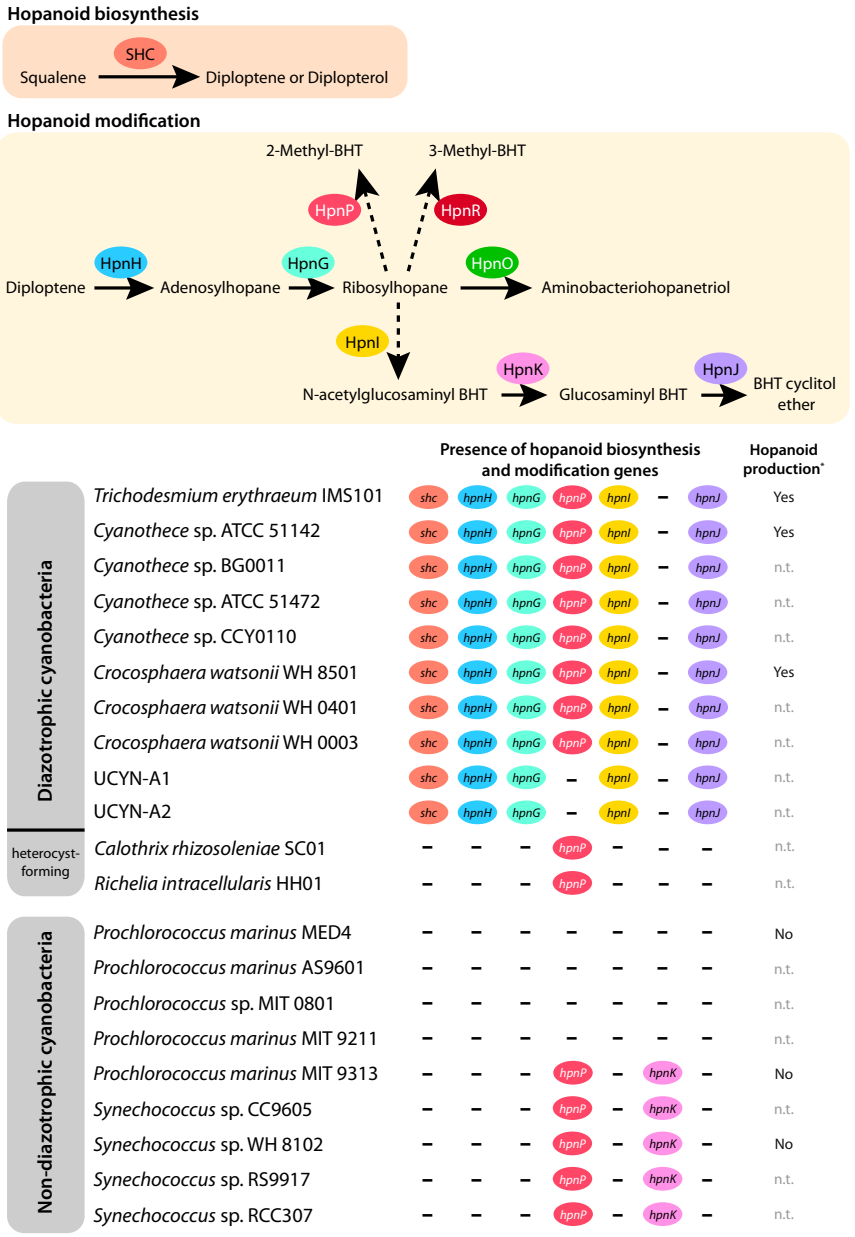

Fig. 1. Hopanids in marine cyanobacteria. (*Upper*) A schematic representation of the hopanoid biosynthesis and modification pathways, including enzymes and products. (*Lower*) Summary of the presence/absence of the genes involved in the synthesis and modification of hopanoids across a selection of the major marine cyanobacterial lineages. All of the available marine cyanobacterial genomes in NCBI (May 2019) were screened for this analysis, yet only 21 are shown, for simplification. Asterisk (*), experimentally tested in refs. 20 and 21 (n.t., not tested). Enzymes participating in hopanoid pathways: squalene–hopene cyclase (SHC), hopanoid biosynthesis-associated radical SAM protein (HpnH), hopanoid-associated phosphorylase (HpnG), hopanoid biosynthesis-associated glycosyltransferase protein (HpnI), hopanoid biosynthesis-associated protein (HpnK), hopanoid biosynthesis-associated radical SAM protein (HpnJ), aminotransferase (HpnO), hopanoid 2-methyltransferase (HpnP), and hopanoid C3 methylase (HpnR); 3-methylhopanoid production has never been found in marine cyanobacteria (28); *hpnO* was absent in all of the screened strains. Dashed arrows indicate that enzymes driving intermediate steps are unknown. See ref. 13 for further details on hopanoid biosynthesis.

detected in UCYN-A (21). These patterns are further supported by previous observations of hopanoid production in the cyanobacterium *Crocosphaera watsonii* WH8501 in the context of N_2 fixation (22, 23). However, the role of hopanoids in N_2 fixation was discarded because *C. watsonii* WH8501 showed constant levels of hopanoids regardless of light–dark periods or the availability of fixed N (23).

We thus propose that the presence of hopanoids in the whole-cell membrane is a conserved trait in marine non–heterocyst-forming cyanobacterial diazotrophs that might confer protection to nitrogenase by reducing the rate of diffusion of extracellular O_2 into the cell. In parallel, as shown for *Cyanothece* (24), increases in respiration rates can presumably lower the intracellular O_2 concentration to levels suitable for nitrogenase activity while fulfilling the adenosine 5′-triphosphate (ATP) demand required for N_2 fixation. Although the constant levels of hopanoids to total lipids has previously been argued to discount a role of hopanoids in marine N_2 fixation (23), we believe that hopanoids reduce O_2 membrane permeability that limits the diffusion rate and facilitates respiratory protection of nitrogenase. It is also possible that hopanoids can form rafts, i.e., membrane microdomains with high hopanoid content that promote dynamic changes in membrane permeability based on redistributions of hopanoid molecules in

the membrane (13). Hopanoid rafts have been detected in *C. watsonii* (25), which suggests that *Crocosphaera* might have such dynamic changes in membrane permeability.

Since members of non–heterocyst-forming freshwater cyanobacteria (e.g., *Aphanothece*, *Pleurocapsa*, endosymbionts of the diatoms *Rhopalodia gibberula* and *Epithemia turgida*) and noncyanobacterial diazotrophs (e.g., *Azotobacter*) also have the *shc* gene, we believe that our hypothesis, which provides a mechanism that restricts O_2 diffusion analogous to the heterocyst, may provide an important research direction for future studies devoted to understanding N_2 fixation in different environments (marine, freshwater, terrestrial) as well as other O_2-sensitive processes (e.g., methanogenesis) when happening in well-oxygenated environments (26, 27).

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