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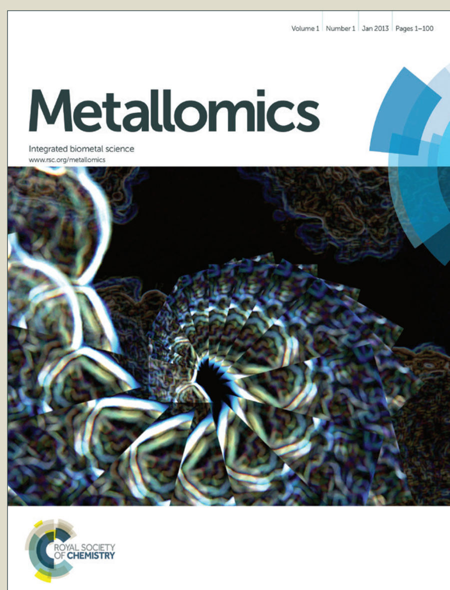

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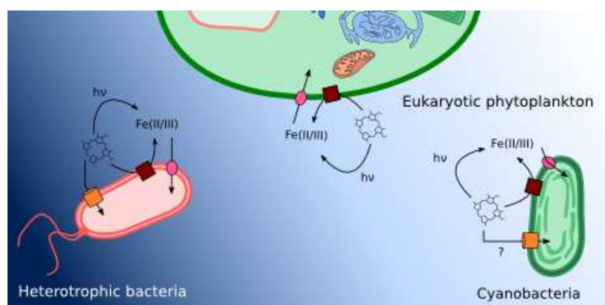

Heme is an essential enzyme cofactor in marine microbes as well as a dynamic and quantitatively significant component of the marine iron cycle.

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Heme in the marine environment: From cells to the iron cycle

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Abstract

Hemes are iron containing heterocyclic molecules important in many cellular processes. In the marine environment, hemes participate as enzymatic cofactors in biogeochemically significant processes like photosynthesis, respiration, and nitrate assimilation. Further, hemoproteins, hemes, and their analogs appear to be iron sources for some marine bacterioplankton under certain conditions. Current oceanographic analytical methodologies allow for the extraction and measurement of heme *b* from marine material, and a handful of studies have begun to examine the distribution of heme *b* in ocean basins. The study of heme in the marine environment is still in its infancy, but some trends can be gleaned from the work that has been published so far. In this review, we summarize what is known or might be inferred about the roles of heme in marine microbes as well as the few studies on heme in the marine environment that have been conducted to date. We conclude by presenting some future questions and challenges for the field.

Introduction

Iron is a critical micronutrient for planktonic organisms in the oceans. It is a cofactor or cofactor component in enzymes that facilitate photosynthesis, respiration, dinitrogen fixation, nitrate reduction, ammonia assimilation, and nitrite assimilation[1]. The large iron requirements of the enzymes that facilitate these fundamental biogeochemical processes directly link iron to the carbon and nitrogen cycles of the marine environment. Ecologically, iron is a limiting nutrient for marine phytoplankton and bacteria. Up to 33% of the surface ocean is classified as high-nutrient low-chlorophyll (HNLC) regimes where macronutrients are in excess and iron concentrations very low, typically picomolar. Mesoscale iron fertilization experiments have unequivocally demonstrated that iron influences initiation and development of phytoplankton blooms and thus has downstream effects on the cycling of carbon, nitrogen, and silicon as well as effects on trophic dynamics[2].

Fe is extremely scarce in seawater relative to its biological demand due to its thermodynamic propensity to hydrolyze into oxyhydroxide species with very low solubility. These oxyhydroxide species have further tendency to adsorb to sinking particulate matter or other iron hydroxide species resulting in significant iron export out of the euphotic zone where, paradoxically, biological demand is highest. The remaining dissolved iron in the surface ocean presumed to be bioavailable is almost exclusively (>99%) bound to largely unknown organic ligands. Traditionally, these ligands have been operationally partitioned into two classes, L_1 and L_2, based on their conditional stability constants – a measure of the iron binding affinity for each class[3–5]. L_1, with the largest constants, have a greater affinity for iron than ligands from the L_2 class. These Fe-binding ligands in seawater are hypothesized to be of biological origin, and have been shown to rapidly accumulate after inputs of iron from

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mesoscale fertilization experiments[4,6]. Very little is known of the structural diversity of the iron binding ligand pool, but some L_1 constituents have been identified using mass spectrometry techniques[7,8].

Dissolved organic matter (DOM) is a complex mixture of new and degraded biogenic molecules, some of which have an iron chelating capacity. It is likely that a proportion of the L_2 class is derived from the decomposition of complex DOM, of which the altered products have iron binding capabilities[9], such as recalcitrant and ancient humic compounds of a sedimentary origin[10]. Other weaker chelators may be fragments of large proteins picked over by heterotrophic bacteria or the liberated prosthetic groups from the metalloproteins of lysed phytoplankton cells. Early on it was proposed that iron porphyrin complexes, such as heme and hemoproteins, could be major components of the weak iron-binding ligand pool in seawater[11]. Heme-like molecules are probably released into the ocean water column upon the death and rupture of planktonic cells and the degradation of cellular proteins, however the fate of these molecules after cellular release has remained largely unknown. Recent advances in marine microbial genomics along with analytical improvements in the molecular study of iron speciation in marine systems are revealing heme to be a dynamic and quantitatively significant component of the marine biogenic iron pool. Here we review what is known about heme in the marine environment from the perspectives of how marine microbes acquire, transform, manage, and employ heme-based molecules. We also summarize recent biogeochemical studies regarding the analysis and distributions of heme in the ocean with an eye as to how these and future studies may inform our current knowledge of the marine biogeochemical iron cycle.

Intracellular roles for heme in marine microbes

Hemes, iron-porphyrin complexes, function as prosthetic groups in numerous proteins that perform diverse biological functions across all domains of life. The ubiquity of the heme group in enzymes is due to its abilities to readily function as an electron source/sink and small molecule binding site. Some of the roles for hemoproteins include facilitating mitochondrial and chloroplast electron transfer reactions, divalent gas transport and storage, organic substrate oxygenation, peroxide reduction, cellular signal transduction, and regulation of gene expression[12]. The heme molecule is a critical component in essential cellular processes that respond both directly and indirectly to the chemical and physical environments of marine phytoplankton and bacterioplankton. From this perspective, heme is a functional molecular link between the cellular level biology of phytoplankton and bacterioplankton and their resulting ecology. Here we summarize some of the major intracellular roles for heme and hemoproteins in the context of marine phytoplankton and bacterioplankton **(Fig. 1)**.

Electron transfer: Respiration and Photosynthesis

Heme is a central component of electron transport complexes including those participating in the processes of aerobic and anaerobic respiration, some forms of extended anaerobic respiration, and photosynthesis. The terminal portion of the electron transport chain in aerobic and anaerobic respiration utilizes a number of cytochromes, membrane-associated hemoproteins, for the generation of ATP[13,14]. Many of these processes occur at the inner membrane of the mitochondria in eukaryotes and the cytoplasmic membrane of prokaryotes[14,15]. However, extended respiratory electron transport systems have recently been discovered to transport electrons all the way to the outer membrane of gram negative bacteria thus utilizing extracellular oxidants in respiration[16]. These systems are often characterized by an abundance of c-type cytochromes[17,18]. Hemoproteins are also involved in the process of oxygenic photosynthesis with many functional similarities to those in the electron transport chain from respiration[19]. Photosystem II contains a heme complex in the D1 reaction center called cytochrome b_{559}, a heterodimer composed of one alpha subunit (PsbE) and one beta (PsbF) subunit[20].

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Cytochrome b_6f, which participates in the shuttling of electrons from photosystem II to photosystem I, contains a Rieske [2Fe-2S] protein and four heme groups[21]. In copper deficient conditions, cyanobacteria and many algae utilize a heme-containing cytochrome c_6 complex instead of plastocyanin to transport electrons between the cytochrome b_6f complex and photosystem I[22].

Electron transfer: Management of Reactive oxygen species

In the marine environment, reactive oxygen species (ROS) are produced from the photochemical oxidation of DOM in waters penetrable by solar radiation, particularly that in the ultraviolet wavelengths[23]. However, recent field and laboratory studies have shown that the ROS superoxide is produced extracellularly and light-independently by marine bacteria and algae and may be the dominant source of superoxide in some marine waters[24–27]. In marine algae it has been inferred that extracellular superoxide is produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases[28], which are membrane bound enzyme complexes that produce superoxide through a reactive heme prosthetic group[29]. In particular, the genomes of the marine diatoms *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* contain putative homologs to human, plant, and fungal NADPH oxidases, which suggests algal extracellular superoxide production may be due to these hemoproteins[28]. A recent study has indicated that marine heterotrophic bacteria may produce extracellular superoxide by way of NADPH oxidases homologous to those in eukaryotes[26]. This evidence suggests that both marine heterotrophic bacteria and phytoplankton may produce extracellular superoxide through a heme-dependent reaction.

ROS have negative effects on marine microbes by inhibiting critical metabolic processes such as photosynthesis or by interfering with biological membranes. Excessive intracellular hydrogen peroxide and other ROS are harmful for almost all cell types and thus must be managed rapidly and efficiently. Heme is the prosthetic group in catalases, peroxidases, and catalase-peroxidases which are antioxidant enzymes involved in the degradation of hydrogen peroxide and other organic hydroperoxides[30–32]. Catalases and peroxidases both break oxygen–oxygen single bonds, but the nature of the electron donor in the two reaction mechanisms differs between the two enzyme families. In higher plants, catalase activity is concentrated in the peroxisome, a single membrane bound organelle that mediates a wide array of biochemical processes including fatty acid β-oxidation, photorespiration, metabolism of hydrogen peroxide, and synthesis of plant hormones[33]. Very little is known of peroxisomes in unicellular marine algae, although some work has been done with the model freshwater alga *Chlamydomonas reinhardtii*[34]. Peroxisomes are predicted to exist in *T. pseudonana* from analyses of its genome[35], but their general distribution in marine algae is largely unknown. Peroxidases in higher plant models are concentrated in the apoplast and vacuole, but little work has been done with model algae. Plant vacuolar peroxidases catalyze the oxidation and polymerization of a variety of phenolic compounds and other secondary metabolites while reducing hydrogen peroxide[36]. Extrapolating from these plant models, it is likely that peroxidase activity is also present in algal vacuoles.

Electron transfer: Other redox reactions

Heme-containing proteins in marine bacteria and phytoplankton are also involved in the reduction and oxidation of various endogenous and exogenous compounds. For example cytochrome P450 monooxygenase and cytochrome b_5 function (often in conjunction) in oxidizing a wide variety of substrates. Cytochrome b_5 functions in the anabolic metabolism of fats and steroids as well as in the catabolism of xenobiotics and compounds of endogenous metabolism[37]. Heme-proteins are also involved in processes of inorganic nutrient uptake which often requires reduction of the substrate to a usable form. In marine algae, reduction of nitrate to nitrite is accomplished by a NADPH nitrate

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reductase. This enzyme contains a molybdenum core as well as heme cofactors that facilitate electron transport between NADPH and nitrate[38]. In marine diatoms, the NADPH nitrate reductase enzyme is localized to the cytoplasm[39,40]. Diatom genomes also encode ferredoxin nitrite reductases that appear to be targeted to the chloroplast[41]. Ferredoxin nitrite reductases utilize a siroheme, a heme analog, and a [4Fe–4S] cluster as catalytic cofactors[42]. A recent study has convincingly argued that when rapidly relieved of iron stress diatoms appear to partition iron to nitrate and nitrite reductases and other nitrate assimilation proteins while continuing to utilize iron-free photosynthetic proteins[43]. In the same study, iron addition also induced a significant upregulation of the porphyrin biosynthesis pathway[43], further suggesting that diatoms rapidly partition iron into hemoproteins when relieved of iron stress. Sulfite reductases, which conduct the six electron reduction from sulfite to sulfide, also contain heme-like cofactors important in inorganic sulfur assimilation. Much like nitrite reductases, sulfite reductases utilize siroheme and iron-sulfur clusters as cofactors and are targeted to the plastid[44]. Finally, heme-containing ferric reductases are employed in the solubilization of extracellular complexed iron. Three types of heme-containing ferric reductases are employed in algae, NADPH oxidases (cytochrome b_{558} containing), cytochrome b_5 reductases, and cytochrome b_{561}, and all appear to be involved in iron mobilization in organelles and at the plasma membrane[45]. Heme-based ferric reductase genes have been identified in genomes of *P. tricornutum* (PtFRE1 – PtFRE4)[46] and *T. pseudonana* (TpFRE1 and TpFRE2)[35,47]. It is likely some of these putative diatom ferric reductases localize to the outer membrane and are involved in extracellular iron assimilation, although they may be involved in intracellular iron trafficking or other unrelated roles[45].

Signaling and sensing: O_2 and NO

In bacteria another major role for heme-containing proteins is in sensing. At the molecular level, heme sensor proteins act as bistable switches by binding to molecular oxygen (O_2), nitric oxide, or carbon monoxide and inducing a conformational change in the sensor. The activated sensor protein domain then interacts with a protein domain capable of a response, which leads to modulation of expression levels of specific proteins[48]. No biochemical studies have been done on signaling pathways in planktonic marine bacteria, but the findings uncovered in model terrestrial strains are likely applicable to these organisms. For example, certain rhizobial bacteria utilize a heme-based sensor, FixL, that regulates metabolic processes under aerobic and microaerobic conditions[49]. The ultimate down-stream effect of the protein is to prevent the expression of nitrogen fixation and denitrification systems when O_2 concentrations are high enough to become deleterious.

NO signaling has been extensively studied in vertebrate systems, but has only recently been acknowledged as an important signaling system in bacteria and plants[50,51]. Both NO binding proteins as well as NO synthases utilize heme cofactors. Recently, a functional heme-nitric oxide/oxygen binding domain was identified in *Shewanella oneidensis* and was shown to influence biofilm formation by modulating cyclic-di-GMP metabolism[52]. Many of these systems have been shown to exclusively bind NO, but in some bacterial strains they have equal affinity for both O_2 and NO[50]. Regardless, they appear to be important in regulating communal bacterial behavior such as in biofilm formation, dispersal, motility and symbiosis. NO signaling has also been implicated in regulatory mechanisms in plants, although numerous details as to the production of NO *in vivo* have yet to be elucidated[53]. NO production seems to be localized to the cytoplasm in higher plant cells, but cellular localization in algae remains unknown. Further, it appears that there are numerous NO enzymatic sources other than NO synthase in plants [51]. Recently, a NO synthase with significant homology to those in vertebrates has been identified in the marine green alga, *Ostreococcus tauri*[54]. The NO synthase identified in *O. tauri* was shown to be functional when heterologously expressed in *E. coli*, and its expression *in vivo* was

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dependent upon light irradience and growth phase on the alga. This suggests a link between NO production and algal physiology and points to a potential role for NO in regulating cellular processes in *O. tauri.* NO synthases may facilitate the development and demise of algal blooms in the marine environment as NO has been linked to reduced growth and photosynthesis and increased cell death in marine diatoms[55].

Methods for heme acquisition in marine microbes

As outlined above, heme is required for the functioning of many essential enzymes. To fulfill their needs, marine organisms may synthesize their own heme from starting materials as well as utilize exogenous heme as an iron/heme source.

Heme biosynthesis

The biosynthesis of heme is a fundamental metabolic capability common to both prokaryotes and eukaryotes. Not only is the heme biosynthetic pathway necessary for the function of essential enzymes and proteins, but it is also involved in synthesizing other porphyrin-based molecules such as chlorophylls, bacteriochlorophylls, phycobillins, and the corrin center of vitamin B12. The essential roles of porphyrins in photosynthesis ensure that the pathway is conserved in marine phytoplankton although in eukaryotic algae the different enzymes in the pathway appear to have multiple evolutionary origins including from cyanobacteria, alphaproteobacteria, and heterotrophic eukaryotes[56]. The majority of all sequenced bacterial genomes also contain the necessary components for heme biosynthesis[57], but some notable exceptions lack genes necessary in the canonical heme biosynthetic pathway suggesting that they do not use heme, produce it through an unknown pathway, or rely strictly on heme from the external environment[58,59]. Of the genomes of marine organisms surveyed in this review (see methods), all contained the majority of components required for a full heme biosynthetic pathway.

Although the heme biosynthesis pathway is generally well conserved in prokaryotes and eukaryotes **(Fig. 2)** the initial part of the pathway differs between taxonomic groups[57,60]. In the first step the first universal heme precursor synthesized is δ-aminolevulinic acid (ALA). In marine photosynthetic eukaryotes as well as all prokaryotes excluding the alphaproteobacteria, ALA is synthesized by glutamyl-tRNA synthase through the C5 pathway using glutamate as the starting material. Marine alphaproteobacteria synthesize ALA by condensation of succinyl-CoA with glycine by means of aminolevulinate synthase. After the synthesis of ALA the remainder of the heme biosynthesis pathway is generally the same in all organisms (**Fig. 2**). However, there are two different forms of the enzymes converting coproporphyrinogen III to protoporphyrinogen IX (HemF or HemN) and protoporphyrinogen IX into protoporphyrin IX (HemY or HemG). The difference between the two is that of oxygen-dependence, and the oxygen-independent versions of the enzymes (HemN and HemG) appear to be restricted to prokaryotes. Ultimately, eight molecules of ALA are converted to protoporphyrin IX in a series of six enzymatic steps. In the final enzymatic reaction a ferrochelatase (HemH) inserts iron into the porphyrin ring generating the final heme molecule which can be used directly or modified further before insertion into hemoproteins[57,60,61].

In marine photoautotrophs, the terminus of the heme biosynthesis pathway merges with the chlorophyll biosynthesis pathway where magnesium is inserted into the porphyrin ring instead of iron. In marine phototrophic eukaryotes heme biosynthesis and catabolism are compartmentalized to the mitochondria and chloroplasts, while in marine prokaryotes these processes take place in the cytoplasm and/or

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periplasm. In the chloroplasts heme is utilized in the assembly of the cytochrome b_6f complex of the photosynthetic electron transport chain, while heme catabolic products are utilized as precursors in phycobilin pigments in phytoplankton with phycobilisomes and in the synthesis of the photoregulatory phytochromobilin apoprotein[62]. It appears now that in model phototrophic eukaryotes all of the genes coding for enzymes common to both heme and chlorophyll biosynthesis are expressed exclusively in the chloroplast **(Fig. 1)** [61,63]. However, isoforms of protoporphyrinogen oxidase (HemY) and ferrochelatase (HemH) have been shown to be dually targeted to the plastidal inner membrane and the mitochondrial inner membrane and may be involved in heme biosynthesis[64,65].

Exogenous heme uptake

Marine phytoplankton and bacterioplankton must extract iron from seawater to satisfy their metabolic requirements, and heme may represent a significant iron source for some species. One of the earliest studies investigating the uptake of heme in the marine environment, reported that heme was more bioavailable than siderophore iron-ligand complexes for two species of marine diatoms as well as for natural algal assemblages. In contrast, the study also reported that heme bioavailability was drastically lower than that of siderophore complexed iron for two marine *Synechococcus* species, suggesting that different groups of phytoplankton specialize in utilizing different classes of ligand-bound iron[66]. This work, as well as subsequent similar studies which employed radiolabelled iron porphyrin complexes presumed to be formed via seawater equilibration[67–71], is difficult to interpret mechanistically due to uncertainty regarding the chemical speciation of iron radiotracers added in association with porphyrins, which do not appear to effectively form complexes with iron under seawater conditions[72]. While results of radiotracer uptake studies can be ambiguous, the existence of iron uptake pathways and homeostasis mechanisms in marine microbial genomes and metagenomes can also be bioinformatically inferred from functional information derived from well studied model organisms. Recent evidence indicates that a number of marine heterotrophic bacteria can directly aquire intact heme from seawater and many have putative systems for heme uptake encoded in their genomes[73,74]. Although the ecological and biogeochemical impacts of this capability have yet to be explored, it does suggest that in certain marine microenvironments heme may be a relatively abundant form of iron. As of this writing the direct heme uptake system[73–75] and hemophore-mediated uptake systems are the only forms that have been identified in the genomes of sequenced marine bacteria although other systems exist in terrestrial organisms[59]. Further, no genomes of Gram-positive bacteria isolated from the marine environment contained homologs to any heme uptake system, although they are generally less-well represented in sequence databases. Here we will review the mechanisms of uptake for the two heme uptake systems identified in Gram-negative marine bacterial genomes.

Direct heme uptake systems bind heme or hemoproteins to a TonB dependent transporter (TBDT) at the outer membrane (OM) and transport heme to the periplasm **(Fig. 3)**. Once a heme substrate is recognized at the extracellular surface of the TBDT, conformation changes in the protein transmit a signal to a complex consisting of TonB, ExbB, and ExbD proteins located in the periplasmic space[58,59]. The TonB complex energizes the TBDT to unidirectionally shuttle heme through the outer membrane. Once in the periplasm, heme is intercepted by a periplasmic binding protein and shuttled to an ATP binding cassette (ABC) transporter at the inner membrane (IM) after which it is moved to the cytoplasm. Heme ABC transporters consist of IM-spanning permease domains that are energized by ATP binding domains which catalyze ATP hydrolysis on the cytoplasmic side of the IM. Once in the cytoplasm free heme must be dismantled or sequestered due to its reactivity. In many gram negative bacteria, cytoplasmic heme is degraded by heme oxygenases (HO, PFAM01126) with structural similarities to mammalian HOs[76,77]. However, some bacterial genomes lack any homologs to

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mammalian HOs, and in a pathogenic *Escherichia coli* strain, heme degrading activity is accomplished by a protein, ChuS, lacking structural similarity to any known HOs[78]. However, another study has shown that a ChuS homolog in *Pseudomonas aeruginosa* does not have HO activity and is instead responsible for delivering heme to an already identified heme oxygenase[79]. Further, the ChuS family (PFAM05171) contains sequence similarity to a different protein family implicated in heme utilization, HutX (PFAM06228). Even though the exact function of ChuS (PFAM05171) and HutX (PFAM06228) remains unclear, most putative direct heme uptake operons contain a gene encoding one of the two protein families.

Direct heme uptake systems have been identified by homology and conservation of gene order in a number of sequenced marine bacteria. Hopkinson and colleagues[73] showed that *Microscilla marina,* a member of the *Cytophagia* group known to be associated with marine particulate matter, can sustain growth on heme as the sole iron source. They also demonstrated that heme uptake genes identified by homology to those in human pathogens were upregulated under iron stress and during growth on heme. A subsequent study[74] demonstrated that *Ruegeria* sp. TrichCH4B, a member of the Roseobacter clade isolated from the marine nitrogen fixing cyanobacterium *Trichodesmium erythraeum*, could sustain growth on heme and had a putative heme uptake genomic locus similar to that found in *M. marina.* Further, *R.* sp. TrichCH4B could utilize a variety of other iron-porphyrin complexes in addition to heme, and its putative heme uptake locus was upregulated under iron stress. The authors of this study also demonstrated the presence of putative heme uptake loci in roughly half of all Roseobacter genomes sequenced to date, suggesting that this capability might be generally common in the clade. They were also able to amplify genes homologous to the putative cytoplasmic heme utilization gene (ChuS, PFAM05171) of *R.* sp. TrichCH4B from a variety of coastal and open ocean waters demonstrating its presence in diverse marine environments. Of all the marine bacterial genomes in the Integrated Microbial Genomes[80] (IMG) database, nearly 24% have at least one type of complete putative heme uptake system with the vast majority being direct uptake systems. This abundance of putative uptake systems may be indicative of the significance of heme as an iron source for marine bacteria. The marine direct uptake systems appear to utilize heme cytoplasmic proteins of both ChuS and HutX families, and in roughly equal proportion **(Fig. 4)**. The most recent published study[75] examining general iron uptake systems in marine bacterial genomes found heme uptake systems to be common in isolate marine bacterial genomes but uncommon in the Global Ocean Sampling[81] (GOS) marine metagenomes. Two additional studies examining GOS reported a similar lack of heme uptake systems[82,83]. However, the lack of heme uptake genes in the GOS dataset is likely due to the dominance in the dataset of picocyanonbacteria and *Pelagibacter* species[81] whose genomes almost entirely lack heme uptake systems[75]. Targeted metagenomics of marine microzones, such as particles, where heme is more likely to be a component of the bioavailable iron pool may yield a greater diversity and abundance of heme uptake genes.

Hemophore-mediated heme uptake systems utilize a protein secreted outside the cell to bind and mobilize heme to a TBDT at the OM. Generally, the hemophore-mediated heme uptake operon encodes a heme-binding hemophore (HasA), an IM complex for exporting apo-hemophores outside the cell (HasDEF), a TBDT hemophore receptor (HasR), two regulatory proteins (HasI and HasS), a TonB-like protein, but no IM heme ABC transporter **(Fig. 3)** [58]. In addition to binding HasA, HasR has affinity for heme and hemoglobin but is most efficient when a hemophore is used. The HasA hemophore-mediated heme uptake system has been identified in *Serratia marcescens*, *P. aeruginosa*, *Pseudomonas fluorescens*, and *Yersinia pestis*[59]. In this study we have identified previously unreported putative HasA-like hemophore-mediated heme uptake systems in the genomes of the Gram-negative marine

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bacteria *Pseudoalteromonas luteoviolacea* 2ta16 (isolated from a tropical coral), *Thalassospira profundimaris* WP0211 (from deep sea sediment), *Thalassospira xiamenensis* M-5 (from oil-contaminated surface seawater), *Alcanivorax dieselolei* B5 (oil-contaminated seawater), and *Pseudovibrio* sp. JE062 (from a Caribbean marine sponge) (**Fig 3B**). Although direct heme uptake systems appear to be the most common in the marine environment, it appears that hemophore-mediated uptake may be useful under certain conditions. Further sequenced marine bacterial genomes will aid in assessing the prevalence of HasA-like heme uptake in the marine environment.

Even though little is known of heme uptake in marine heterotrophic bacteria, even less is known in marine phytoplankton. The genomes of *Synechococcus* sp. PCC 7002, a strain from brackish water, and *Prochlorococcus marinus* str. MIT9202, isolated from the tropical South Pacific Ocean, are the only marine cyanobacterial genomes that have TBDT with significant homology to those in well-characterized heme uptake operons[75]. However, the genomic regions around the TBDT in each strain have little synteny to classical heme uptake operons and lack the presence of genes coding HOs or the ChuS/HutX protein. To our knowledge heme uptake experiments have not been performed with either strain. Recently, a transcriptomic study of the marine diatom *P. tricornutum* grown under iron limitation reported an increase in the number of transcripts coding for a putative HO (PFAM01126)[46]. Although the cellular location of this HO was not determined, the authors postulated that if localized to the outer membrane it could be responsible for the apparent diatom heme utilization reported in Ref. 66[66]. However, the genomes of many marine cyanobacteria also contain HOs of the same family as in *P. tricornutum*. Thus, if the observed disparity of heme uptake between diatoms and cyanobacteria in Ref. 66[66] is due to known HOs, it must be due to differential regulation and/or cellular localization of a similar gene product in the two phytoplankton classes. Greater insight into heme utilization by marine phytoplankton will likely be gained from further genome sequencing and the genetic manipulation of model strains[84].

Heme in the marine environment

Undoubtedly, heme-like molecules and hemoproteins are abundant in marine organisms and play important roles in their cellular biology. However, marine scientists are only just beginning to apply what is known of heme at the cellular/molecular level to the scales of ecology and biogeochemistry. Converging evidence is beginning to suggest that heme and hemoproteins persist long enough in seawater and on marine particulates to be considered relevant players in iron biogeochemical cycling. Further, field measurements of intracellular heme and hemoprotein content in marine phytoplankton may be indicative of nutritional status as well as community function. The paucity of measurements in these areas leaves much to be explored in heme biogeochemistry. Here we review what is currently known about the aqueous chemistry and distributions of extracellular heme and hemoproteins in the ocean and in marine phytoplankton.

Aqueous chemistry of heme and hemoproteins

The aqueous chemistries of hemes are strongly influenced by their tetrapyrrole structure (**Fig. 5**). The porphyrin ring makes the complexes inherently insoluble in water[85], however this insolubility is tempered by the presence of different substituted side chains. Consequently, the long hydrophobic side chain of heme *a* decreases this compound's solubility in water at neutral pH, while the presence of the multiple carboxylic acid side chains increases the solubility of siroheme. However, when bound to a protein, the solubilities of hemes are controlled by the protein structure, so that heme *b* proteins such as peroxidases, catalases and cytochrome b_5 are readily dissolved in water, while cytochromes such as b_6

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and b_{559}, which are membrane associated, are much less soluble. Unmetallated porphyrins are unlikely to form complexes with iron(III) in seawater because incorporation of iron(III) into porphyrin rings is generally not favoured as a result of steric hindrance[85]. The most likely mechanism for early reports of iron(III) complexation by porphyrins in seawater[11,86] is perhaps via prior reduction of iron(III) to iron (II)[72]. However even incorporation of iron (II) is kinetically slow, with reported yield of only 10% heme after one hour in ideal (reducing) conditions[87], and added porphyrins have been found to have little effect on the solubility of Fe(III) in seawater[88]. Once formed, iron porphyrins are relatively stable complexes, and the most common processes for removing iron require the breakdown of the porphyrin ring via oxidation[89]. Consequently, an equilibrium stability constant determined under classical conditions has yet to be reported for heme or any other iron porphyrin (to the best of our knowledge). Hemes are rapidly oxidised in aqueous solution at pH 8, and the coupled oxidation of hemes results in breakdown of the methene bridges between the porphyrin rings producing biliverdins[90]. The process appears to involve coordination between oxygen and the unoccupied iron ligand sites, so that oxidation is slowed by the presence of ions that can compete with or shield iron from oxygen[90,91]. In contrast, iron(III) porphyrins are chemically less reactive, although they form insoluble μ-oxo bridged dimers[85,92]. Iron porphyrins are known to have a rich photochemistry due to their characteristic ring structure of conjugated double bonds[93], and have been commonly employed in chemical studies as photosensitizers for their ability to absorb light and transfer energy to desired reactants[94]. In seawater, ferrous complexes of protoporphyrin IX have been hypothesized to act as photosensitizing producers of superoxide, increasing the dark production of iron(II) from iron(III) following irradiation[72].

To date, only one study has reported concentrations of iron porphyrins in natural waters[95]. Nanomolar equivalents of iron(III) protophorphyrin IX like compounds, which could include hemes or hemoproteins, were detected in estuarine waters. The aqueous chemistry of hemes outlined above suggests that, if released intact from cells in the reduced form into oxygenated seawater at a pH of around 8, oxidation and dimerization processes will dominate the marine chemistry of heme and concentrations of heme in solution would be negligible. However, it is possible that more heavily substituted hemes such as siroheme and soluble heme proteins like peroxidase, catalase and soluble cytochrome c may survive cell lysis and remain in solution, potentially contributing to the dissolved iron pool. The limited investigations into hemes in natural waters coupled with the lack of knowledge of the intracellular abundance of siroheme and the soluble hemoproteins in marine microbes means that the significance of a dissolved iron porphyrin pool in seawater is still largely unknown.

Methods for the chemical analysis of heme in particulate marine samples

The most common and efficient method for extracting heme from plants and other biological materials is via acidified acetone[96]. Heme has traditionally been quantified using the pyridine hemochrome method using the strong secondary adsorption bands resulting from coordination of pyridine to the fifth and sixth ligand binding sites of the reduced iron porphyrin[97]. However, this method requires dilution or manipulation of samples to bring them into aqueous solution[96], which increases both the risks of sample degradation and the quantity of starting material required for analysis. Furthermore, direct spectrophotometric determination of heme in marine samples is likely to be problematic as result of the presence of many potential interfering algal pigments. Gledhill (2007) reported an extraction technique for marine samples that utilizes detergents and ammonia rather than acidified acetone. Gledhill (2007) was also able to successfully separate extracts by high performance chromatography and then detect heme *b* spectrophotometrically. Ammoniacal detergent standards of iron(III) protoporphyrin IX are stable for at least six months if kept in the dark at 4°C, but heme in extracted samples is not so stable and should be kept at 4°C and analyzed within 24 hours[98]. The loss of heme from extracts on prolonged

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storage possibly results from degradation via oxidation. To date losses of heme in samples have been successfully minimized by reducing the time and temperature between extraction and analysis[99,100]. Analysis of phytoplankton cultures indicates that the relative standard deviation in heme concentrations between experimental triplicates is typically of the order of 25 ± 20 % (n = 21)[99]. The detergent extraction technique does not completely liberate heme from all proteins[99,100]. Therefore the extraction technique is operational rather than fully quantitative, and expressed concentrations of heme *b* are thus underestimates of total cellular heme *b* content. Comparison of acid acetone extraction with ammoniacal detergent extraction suggests that approximately 80 % of heme *b* is extracted using the latter method from two species of marine phytoplankton[99] and recovery of a major part of the heme *b* fraction present in phytoplankton appears to be consistent with comparisons with the cellular iron concentrations (see below).

The determination of heme *b* is not contamination prone as is the determination of particulate iron and sampling is carried out using the same filtration equipment as for chlorophyll *a* with no specialist trace metal free sampling equipment required. Samples can therefore be obtained on cruises where such facilities are unavailable[99], potentially shedding light on an important fraction of the iron pool over a broader temporal and spatial scale than is currently possible. Sensitive chemiluminescence and fluorescence techniques for the detection of heme *b* in plants and algae have also been described[95,101,102] and these offer the potential for ship-board analysis or even sensor development, although, as with spectrophotometry, there may be issues relating to the specificity of the analysis[95]. Recent work in Gledhill's laboratory has applied electrospray ionization-mass spectrometry (ESI-MS) to the detection of heme *b* after separation by HPLC[103]. The use of characteristic collision induced fragmentation patterns resulted in a highly specific detection method that confirmed the identity of heme b in marine particulate samples, supporting the previously published data. Detection by mass spectrometry was found to be more sensitive and overcame the potential for interferences that can be an issue in the spectrophotometric determination of heme b[98]. Furthermore, comparison of heme b concentrations determined by the two methods indicated good agreement between spectrophotometric results and those obtained by ESI-MS, in the absence of interferences[103]. A selective mass spectrometric detection technique will clearly be useful for comparison with any future developments of ship-board techniques. However further analytical work is clearly required on optimizing suitable extraction protocols and the determination of hemes other than heme *b*. The tendency for heme in extracts to degrade has so far frustrated the development of such a protocol as many rigorous extraction and digestion techniques require incubation at temperatures higher than 4°C. Further efforts in this regard are still ongoing, in particular with respect to understanding the exact mechanism of heme degradation. A robust total extraction method would enhance our understanding of the overall significance of this iron pool, and possibly lead, amongst other things, to a useful biogenic iron proxy. A further strategy for investigation of hemes in marine organisms is through determination of individual proteins via proteomics[104]. Such approaches have been used to gain detailed insight into the metabolic responses of marine organisms to particular environmental stresses[105,106]. However analysis of proteomes is not trivial[107] and such approaches have yet to be applied to the open ocean environment.

Distributions of particulate heme in the marine environment

The usefulness of heme as a prosthetic group in proteins has made it an abundant component of the biogenic iron pool and in theory, the total heme pool in phytoplankton could be of the order of 40 % of the total "active" iron pool[98]. Picomolar concentrations of heme *b* in particulate material have been reported for several regions in the Atlantic Ocean, and the distributions of heme b were found to be

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oceanographically consistent[99,100]. Reported concentrations are considerably lower than particulate iron concentrations reported for the same regions (**Table 1**). However, this likely results from the non-biogenic origin of much of the particulate iron observed in the ocean[108–110]. Biogenic iron concentrations in the ocean are difficult to estimate, although progress has been made with the introduction of washing techniques designed to eliminate non-biogenic particulate iron[111–114], and in the determination of the abundance of iron in individual cells[115–118]. Heme *b* has been found to make up between 6 and 26 % of the total cell associated iron for phytoplankton in nutrient and light replete laboratory conditions. Further, extrapolating from dissolved iron content and heme b concentrations at stationary phase for iron limited cultures (0.5 nmol L^{-1} – similar to oceanic dissolved iron concentrations) resulted in 14 – 26% of the total iron inventory being incorporated as heme b[99]. This suggests that concentrations of heme *b* determined using ammoniacal detergent extraction represent a significant component of the biogenic iron pool in the marine environment, although the use of heme *b* as a proxy for biogenic iron is limited by the wide range of heme *b*: particulate iron values. The range of heme b: particulate iron values observed between species and growth conditions could arise from variability in the proportion of iron allocated to hemoproteins between individual species. For example, theoretical calculations indicate that the heme b content of the electron transport chain can vary between 14 and 23 % of the total iron content[98]. However, further uncertainty is introduced as a result of the operational nature of the extraction protocol, as interspecies variability in extraction efficiencies has received only limited attention to date[99]. Nevertheless, the consistent oceanographic trends observed for heme b concentrations coupled with the broad compatibility observed with biogenic iron concentrations in the Southern Ocean (**Table 1**) suggest that any errors are likely to be systematic rather than random, increasing the potential interpretative power of the analysis. Thus, while caution should be exercised when interpreting heme b distributions due to uncertainties discussed above and the operational nature of the analysis, determination of heme b has the potential to provide valuable information on iron biogeochemistry and its impacts on microbes in the ocean.

The primary control on heme *b* distributions in the ocean appears to be biomass[99,100]. Heme *b* to particulate organic carbon (POC) ratios in particulate material reported to date vary by an order of magnitude 0.06 - 1.0 μmol mol^{-1}, with the lowest values observed in the iron limited regions of the high latitude North Atlantic (HLNA). However, this variability is not due solely to the intracellular heme b contents of marine phytoplankton because bulk POC contains carbon from heterotrophs as well as detritus. Indeed, comparison of carbon content from POC and that estimated from phytoplankton cell counts indicates that heterotrophic and detrital carbon contribute more to total POC in the HLNA[119] than in other lower latitude areas[120]. This disparity in POC source composition may partially explain low heme *b*:POC ratios in the HLNA, although low heme *b*:POC ratios obtained exclusively from phytoplankton biomass in the HLNA suggest that POC source composition cannot account for all of the variability in heme *b*:POC ratios[100]. Thus, variability in heme *b*:POC observed within biogeographically similar regions may reflect variability in overall nutrient concentrations, as seen in phytoplankton laboratory cultures[99,100]. Still, further data points that also account for phytoplankton community composition are needed to support such a conclusion. Ratios of heme *b* to phytoplankton carbon were reported to vary between 0.07 and 0.78 μmol mol^{-1} in the Celtic Sea and the high latitude North Atlantic[99,100]. Interestingly, the range of these ratios appears to be consistent with the lower end of total Fe:C quotas reported for some field data[118,121,122].

Although heme *b* distributions are primarily controlled by biomass, overall correlations with chlorophyll are quite poor, especially considering that the two compounds are both strongly associated with photosynthesis. Thus, heme *b* concentrations do not appear to increase with depth to the same

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4 extent as chlorophyll *a* concentrations[99,100]. The differences in the abundance of heme *b* and chlorophyll *a* may relate to the way in which the tetrapyrrole biosynthetic pathway is regulated[60,63,123,124]. Low heme content has been shown to increase the production of tetrapyrroles[125] while magnesium chelatase has a higher affinity for protoporphyrin IX than ferrochelatase[126]. Consequently, low heme content could potentially increase protoporhyrin IX production, while lack of iron would inhibit ferrochelatase activity, leading to a higher production of chlorophyll relative to heme. Unfortunately, published studies have not been able to differentiate between intracellular heme pools. Different hemoproteins have different labilities towards the extraction method[100], and thus, particularly labile intracellular heme pools may be preferentially extracted over others. Decreases in the total cellular abundance of heme *b* and chlorophyll *a* would, therefore, disproportionately be influenced by reductions in abundance of specific hemoproteins with particularly labile heme *b* prosthetic groups. Further research and optimization in this area have the potential to improve our knowledge of intracellular heme b resource allocation under various nutrient limiting conditions.

Conclusion/Future Directions

Currently, one of the grand challenges in chemical oceanography is integrating and reconciling measurements at the molecular/mechanistic level with large scale bulk chemical measurements taken in the field. A comprehensive perspective of the marine iron biogeochemical cycle should ideally integrate geochemical, biochemical, physiological, and genomic/transcriptomic/proteomic information. In this review we have presented the current state of heme biogeochemistry science and what is known or can be inferred about the physiological functions of heme in marine phytoplankton and bacterioplankton. Numerous challenges still remain in the field.

At the cellular scale, we need a better understanding of the allocation of hemes between intracellular pools related to photosynthesis, respiration, nutrient acquisition, ROS management, and cellular signaling and how those allocations may or may not vary with respect to ecological conditions. Proteomics approaches are likely the most promising techniques in this area[104]. Although some progress has been made in marine heterotrophic bacteria, we need a better understanding of direct heme uptake including the genes involved, their regulation, and mechanisms. The same inroads need to be made looking at heme bioavailability in marine phytoplankton. In the analytical realm, improved extraction techniques for heme (ideally a universal extraction) are required from marine material as well as advances in separation and detection methodologies. Chemiluminescence and fluorescence techniques are promising technologies in this area as well as the potential for utilizing genetically modified marine organisms as bioreporters[127]. In the field, more data is needed to tease out trends in heme *b* concentrations with respect to biogenic iron and other bulk biogenic properties. The chemical research into heme abundance in marine phytoplankton currently suggests that heme *b* makes up a significant component of the particulate biogenic iron pool, and that heme *b* is depleted relative to chlorophyll *a* and POC in nutrient, particularly iron, limited regions of the ocean. However, further ocean basin-scale measurements will provide new insights into heme biogeochemistry, and the development of simple, field-ready measurement techniques will expedite this process.

Methods

Marine microbial genomes with the habitat metadata tag "Marine" were searched using the Joint Genome Institute Integrated Microbial Genomes (IMG) database[128]. Heme biosynthesis pathways were considered present if genomes contained the components specific to heme biosynthesis in the KEGG metabolic pathway "Porphyrin and chlorophyll metabolism" map00860[129]. PFAM[130] protein families mentioned in the text were searched using the IMG function search. Marine bacterial genomes were

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considered to have a full heme uptake operon based on the colocalization (within a 10 gene neighborhood) of genes coding for a ChuS or HutX protein, a heme outer membrane TBDT, and the three heme ABC IM transporter components (periplasmic binding protein, permease, ATPase). See Fig. 3D for an example. The marine bacterial 16S rRNA phylogeny was constructed from IMG marine genomes that had a corresponding 16S rRNA sequence available in the Greengenes database[131]. These 16S rRNA genes were extracted from the total Greengenes alignment and used with RAxML[132] v8.0.0 to generate a maximum likelihood tree under the gamma distriution, using the general time-reversible model for DNA evolution and utilizing 500 random sampling bootstraps.

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Figures and Tables

Fig. 1. Distribution of the major proteins containing heme-like cofactors in a hypothetical diatom. Question marks indicate hypothetical/unconfirmed presence in the cell. The major cellular organelles are listed in boldface and the proteins with heme or siroheme cofactors (and the heme biosynthesis pathway) are listed beneath them. In bacteria these proteins may be either in the cytosol, periplasm, or inner and outer membranes. In cyanobacteria, photosynthetic and respiratory cytochromes reside in the thylakoid membrane.

Fig. 2. Biosynthetic pathway for heme and other tetrapyroles in marine eukaryotic algae and bacteria. 5-aminolevulinic acid (ALA) is the universal precursor in all organisms. Marine Alphaproteobacteria use Glycine as a starting product while all other marine bacteria and eukaryotic algae use L-glutamate. Protoporphyrin IX is synthesized from ALA and then can be further modified into heme, phytochromobilin, phycobilins or Chlorophyll a. Siroheme, a heme-like cofactor, and Vitamin B12 are synthesized from Uroporphyrinogen III. Dashed lines indicate multiple enzymatic steps. ALAD, ALA dehydratase: ALAS, ALA synthase, CoprOX, coproporphyrinogen oxidase; FeChe, ferrochelatase; GluRS, glutamyl-tRNA synthetase; GluTR, glutamyl-tRNA reductase; GSAT, glutamate-1-semialdehyde aminotransferase; HBS, hydroxymethylbilane synthase; PPOX, protoporphyrinogen IX oxidase; UroDeC, uroporphyrinogen decarboxylase; UroSyn, uroporphyrinogen III synthase.

Fig. 3. Representative schematic of protein machinery utilized in A) hemophore-mediated heme uptake and C) direct heme uptake. Organization of putative hemophore uptake operon B) in *Pseudovibrio* sp. JE062 and direct heme uptake operon in *Ruegeria* sp. TM1040. Gene symbols are colored as the corresponding proteins in A) and C). ExbB, TBDT energy transduction component; ExbD, TBDT energy transduction component; *fecR*, Iron senstive regulatory element ; HasA, Hemophore; HasR, TBDT - heme/hemophore; HlyD, HasA secretion protein; HmuR, TBDT - heme; HmuS, Heme utilization protein – ChuS family; HmuT, Periplasmic heme binding protein; HmuU, IM-spanning ABC permease; HmuV, ABC ATPase; hyp, Hypothetical; HrtD, HasA secretion protein – ATPase; *rpoE*, Iron senstive regulatory element ; TonB, TBDT energy transduction component. Question marks indicate hypothetical/unconfirmed pathways for export/import.

Fig. 4. An unrooted 16S rRNA maximum likelihood phylogeny of marine bacterial genomes indexed in the Integrated Microbial Genomes (IMG) database. Bootstrap values of >80% are shown as red symbols for 500 resamplings. Scale bar equals 0.1 substitution per site. The outer ring is colored by bacterial taxonomic grouping (Phylum or lower). The inner ring represents genomes that contain a full

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direct heme uptake locus and is colored by the family of the heme utilization protein present in each locus. See methods for further description.

Fig. 5. Structures of A) the hydrophobic hemes *a-c* and B), the more soluble siroheme

Table 1. Range of values reported for biogenic iron and heme *b* in the Southern Ocean

List of abbreviations

HNLC	High-nutrient low-chlorophyll
L_1	"Strong" operationally defined iron-binding ligand class
L_2	"Weak" operationally defined iron-binding ligand class
DOM	Dissolved organic matter
ATP	adenosine triphosphate
ROS	reactive oxygen species
NADPH	nicotinamide adenine dinucleotide phosphate
ALA	δ-aminolevulinic acid
TBDT	TonB dependent transporter
ABC	ATP binding cassette
IM	Gram-negative bacterial inner membrane
OM	Gram-negative bacterial outer membrane
HO	Heme oxygenase
HLNA	High latitude North Atlantic
GOS	Global Ocean Sampling
POC	Particulate organic Carbon

Table 1. Range of values reported for biogenic iron and heme *b* in the Southern Ocean

Region	Biogenic iron (pM)	Heme *b* (pM)	Size fraction	Method/reference
SE New Zealand (46.24 S, 178.72 E)	40 - 310		>0.2 µm	Oxalate wash*[108]
S Australia (46 – 60 S, 139 – 140 E)	100 - 380		>0.2 µm	Oxalate wash[#112]
Scotia Sea (52 – 60 S, 38 – 45 E		0.6 - 21	>0.7 µm	Heme *b* direct determination[100]

*only samples where unwashed and washed fractions were obtained are used
estimated from Fig. 4 of reference 112

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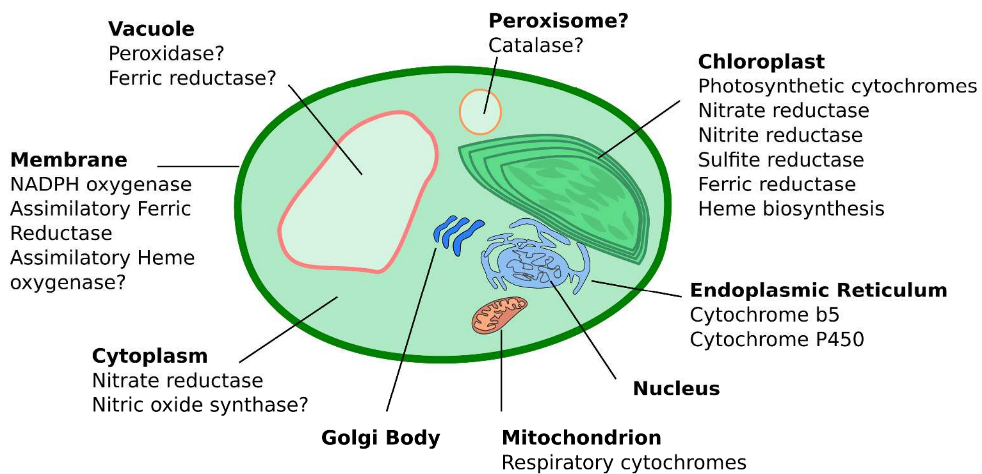

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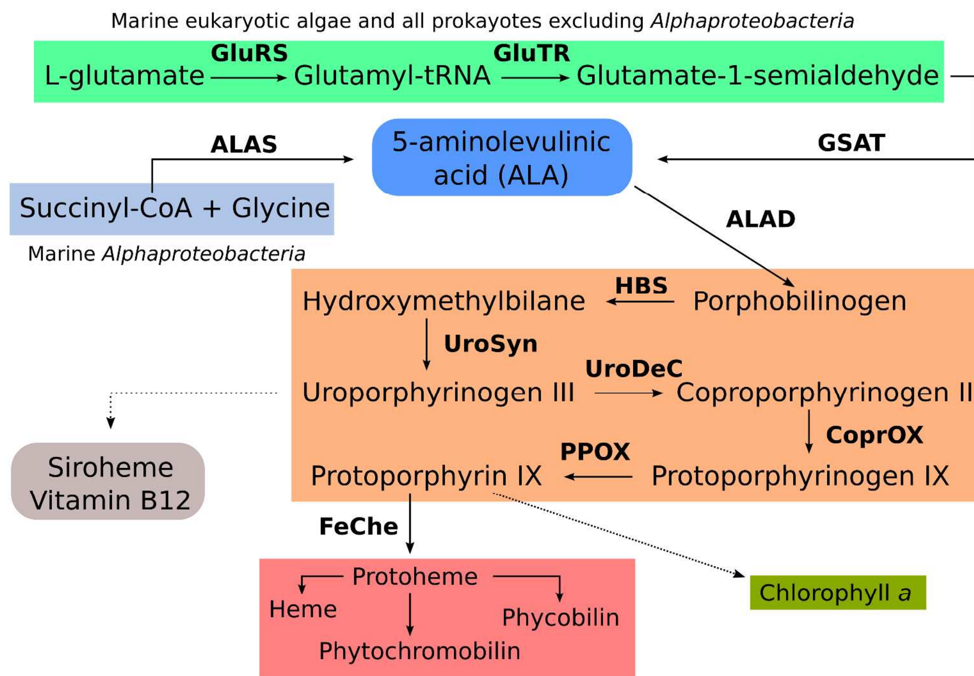

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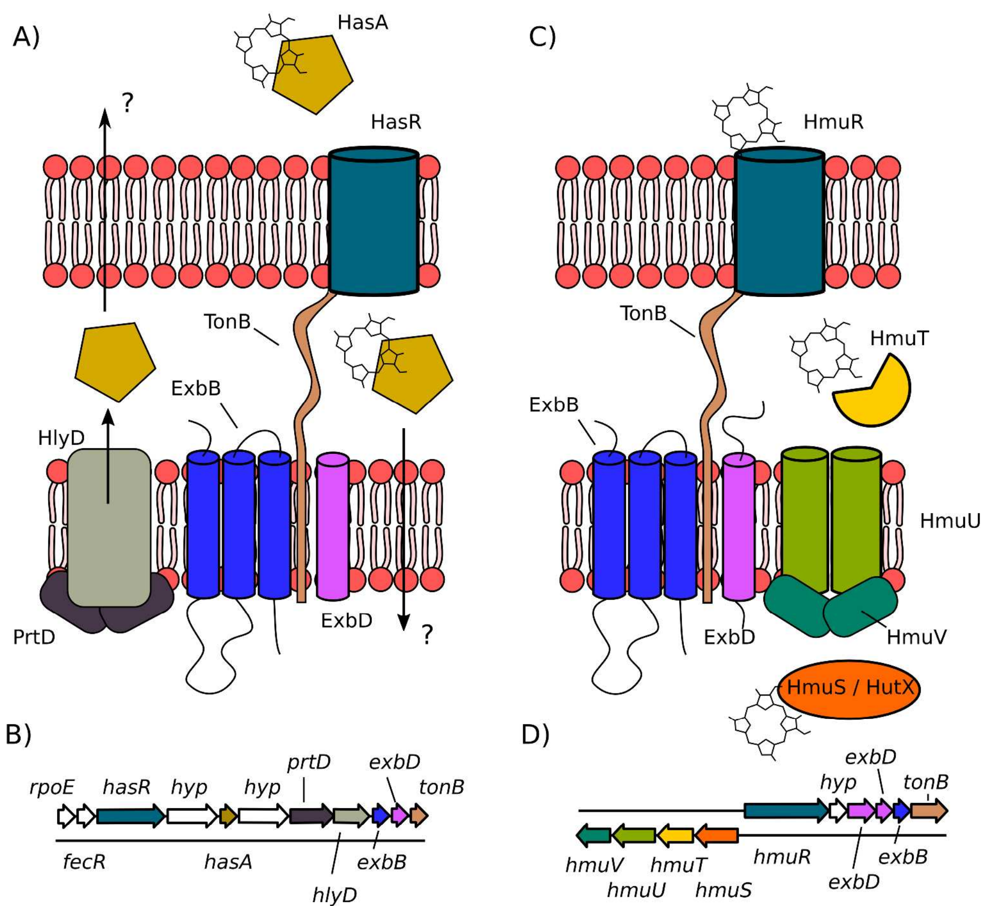

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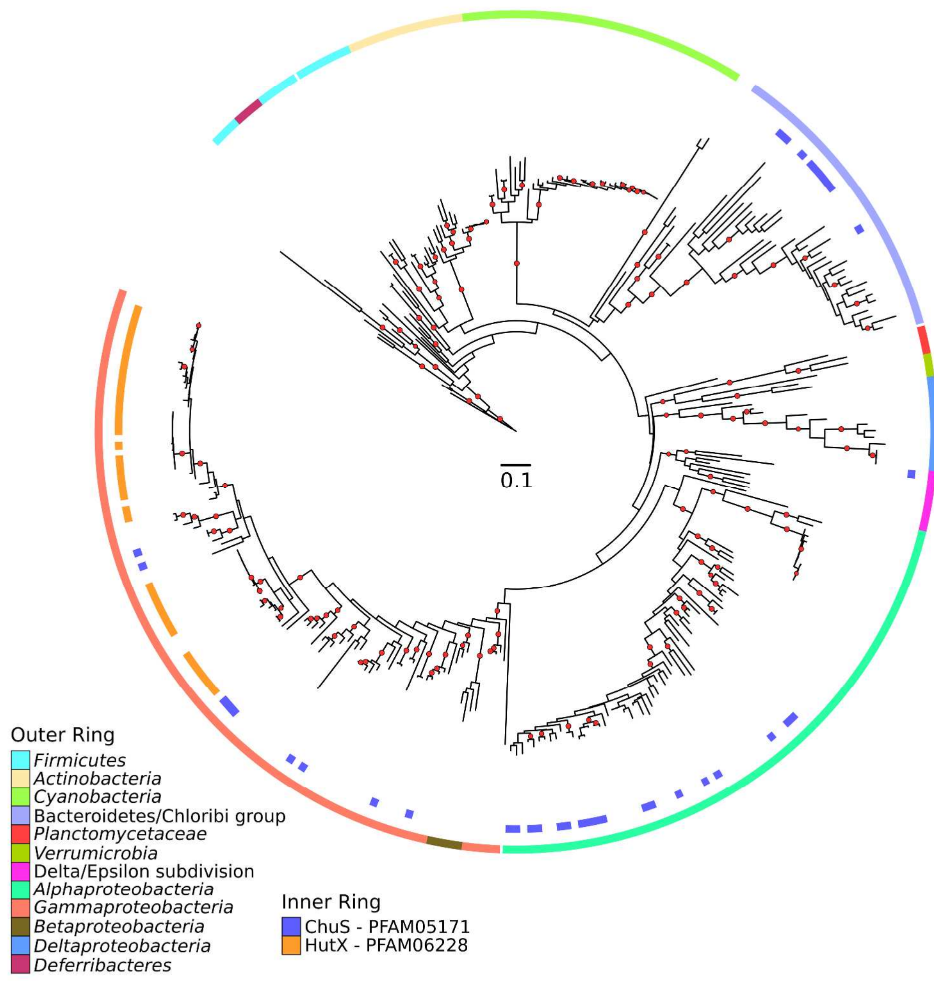

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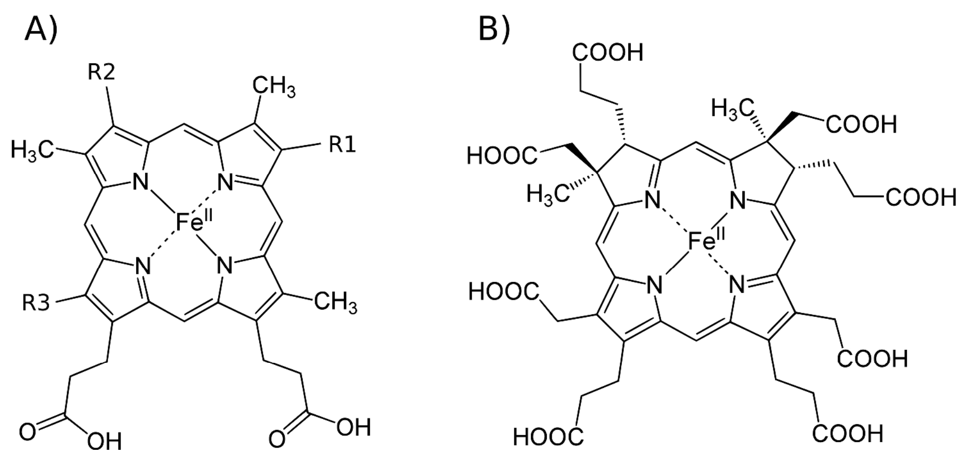

Heme	R1	R2	R3
a	$-CH=CH_2$	-HFAR	-CHO
b	$-CH=CH_2$	$-CH=CH_2$	$-CH_3$
c	$-CH(C\text{-}S\text{-}yl)CH_3$	$-CH(C\text{-}S\text{-}yl)CH_3$	$-CH_3$

HFAR - hydroxyethylfarnesyl group
C-S-yl - Thioether linkage to apoprotein

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