ANTIOXIDANTS & REDOX SIGNALING
Volume 6, Number 5, 2004
© Mary Ann Liebert, Inc.

Forum Review

Bacterial Heme Oxygenases

NICOLE FRANKENBERG-DINKEL

ABSTRACT

The importance of heme oxygenases in heme catabolism, iron utilization, and cellular signaling has been recognized for many years and is a well studied process in eukaryotes. Through the accessibility of an increasing number of bacterial genomes, it has become evident that heme oxygenases are also widespread in prokaryotes. In these organisms, the heme oxygenase reaction serves a similar function as in eukaryotes. A major role of bacterial heme oxygenases has been attributed to acquisition of iron in prokaryotic pathogens, but other functions, such as involvement in phytobilin biosynthesis, have been described. This review summarizes the current state of the art on bacterial heme oxygenase research. The various biological roles of this enzyme in prokaryotes and their biochemical properties will be discussed. *Antioxid. Redox Signal.* 6, 825–834.

INTRODUCTION

HEME OXYGENASES (HOs) (E.C. 1.14.99.3), first identified in eukaryotes, have key functions in the catabolism of heme and in the biosynthesis of phytobilins, in addition to their involvement in iron metabolism, oxidative stress, and pathogen responses (1, 11, 37, 39, 53). In an oxygen-dependent reaction, heme is cleaved to yield biliverdin (BV) IXα, Fe^{2+}, and carbon monoxide (CO) in equimolar amounts (Fig. 1). The reaction requires a total of three oxygen molecules and seven electrons for the cleavage of one heme molecule (34). Heme cleavage can result in four possible isomers of BV, *i.e.,* IXα, IXβ, IXγ, and IXδ, which reflect the eliminated carbon bridge position in the heme precursor (Fig. 1). The best characterized HOs are those with the α-meso carbon bridge specificity from mammals and plants. In mammalian heme catabolism, BV is further metabolized by biliverdin reductase (BVR) to yield bilirubin (BR) (28) (Fig. 2). In red algae and plants, the interconversion from heme to BV is catalyzed by ferredoxin (Fd)-dependent HOs that are homologues of the mammalian enzymes (13, 31, 32). Eukaryotic HOs have been studied in detail for many years (for a review, see 49), but only recently has the field of bacterial HOs become a focus of interest and the importance of these enzymes in bacterial pathogenesis, iron acquisition, and chromophore biosynthesis been recognized. This review focuses on the current state of research on bacterial HOs, and their biological role and biochemical properties will be discussed.

GENERAL CHARACTERISTICS AND PHYLOGENY OF BACTERIAL HEME OXYGENASES

Bacterial HOs are highly related among each other. Phylogenetic analysis revealed a single tree with five clades of bacterial HO proteins: cyanobacterial HO-1 and HO-2, Isd, HmuO, PigA, and BphO (Fig. 3). Each clade suggests its own family that can be underlined by their biological function. In contrast to their eukaryotic counterparts, bacterial HOs are not membrane-bound, but soluble enzymes. They all lack the C-terminal membrane anchor present in mammalian HOs. On average, the molecular mass of bacterial HOs is ~25,000 Da, and therefore they are slightly smaller than the eukaryotic enzymes (~36,000 Da). Interestingly, a new group of bacterial HOs (Isd) that has been recently identified in *Staphylococcus aureus* has a molecular weight of only ~13,000 (43). Some bacterial HOs (*e.g., Synechocystis* HO-1) show a very high identity in amino acid sequence to human HO-1 (38% identity, 67% similarity), whereas in others the homology is as low as 19% identity and 42% similarity (HemO from

Institute for Microbiology, Technical University Braunschweig, Braunschweig, Germany.

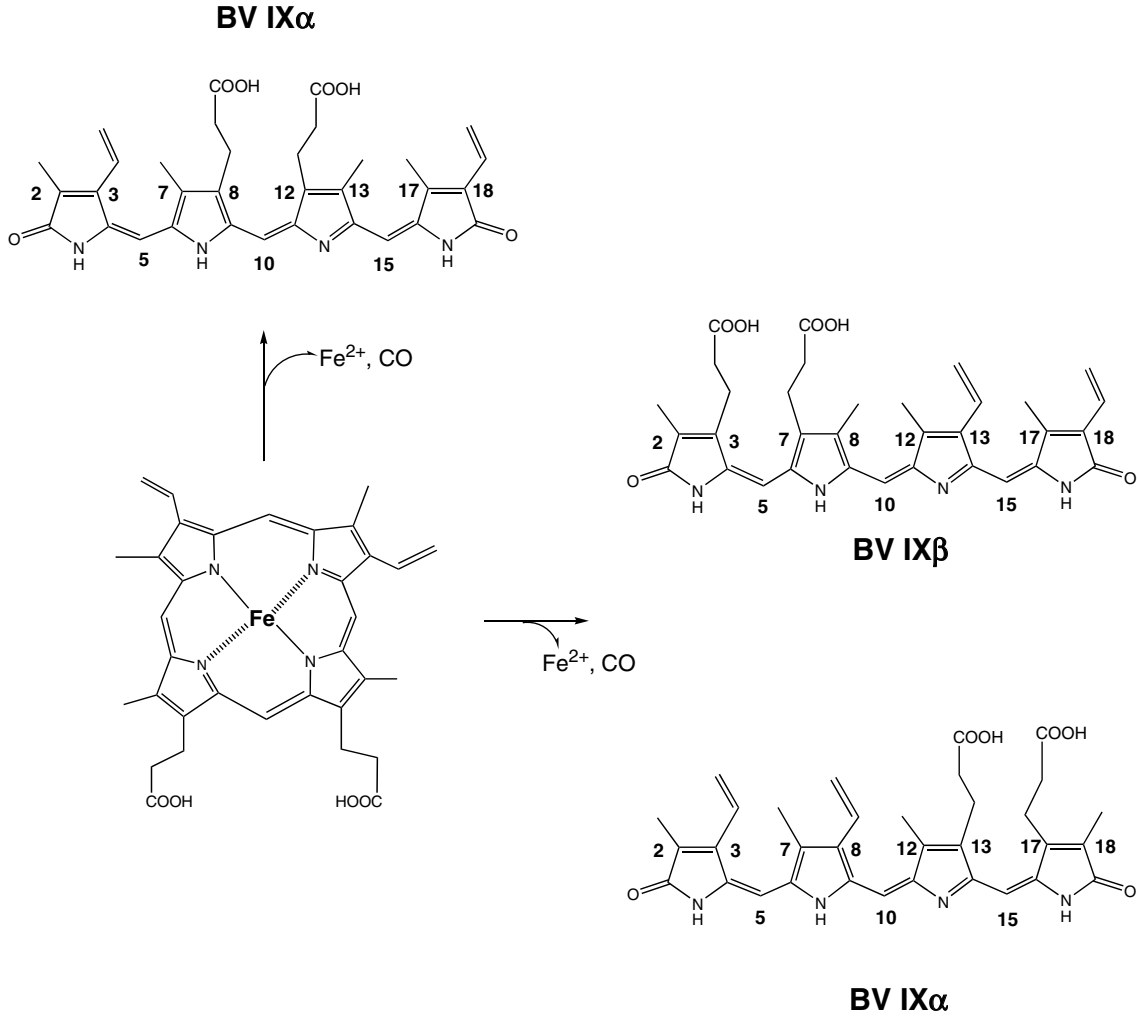

FIG. 1. Heme oxygenase reaction of bacterial HOs. Two types of bacterial HOs are known so far that show different regiospecificities toward the cleavage of the heme macrocycle. Most HOs cleave the heme at the α-meso carbon position yielding BV IXα. The only HO showing a different regiospecificity is PigA from *P. aeruginosa*, which displays a β- and δ-carbon-specific HO.

Neisseria meningitidis) or even lower (BphO from *Pseudomonas aeruginosa*) (30).

BIOLOGICAL ROLE OF BACTERIAL HEME OXYGENASES

HOs involved in phytobilin biosynthesis

Phytobilins are linear tetrapyrrole molecules that are synthesized in plants, algae, and cyanobacteria and that are the precursors of the chromophores for the light-harvesting phycobiliproteins and the photoreceptor phytochrome (16). In the course of their biosynthesis, the product of the HO reaction, BV IXα, is subsequently converted to one of the major bilins present in cyanobacteria, phycocyanobilin and phycoerythrobilin (Fig. 2). This conversion is catalyzed by a recently discovered family of related Fd-dependent bilin reductases (17). Bilins are usually bound to apo-proteins through single or double covalent linkages and can be further modified during this process. This reaction is autocatalytic for the photoreceptor phytochrome, but requires special lyases for phycobiliproteins. The binding to the latter results in a great diversity of bilin chromophores that completely span the visible light spectrum.

One large group of bacteria that contain HOs are the cyanobacteria. The HOs from these organisms are among the best characterized bacterial HOs. In these organisms, the HO reaction is the first step during the biosynthesis of phytobilins (Fig. 2). The first cyanobacterial HO to be cloned and characterized was HO-1 from *Synechocystis* sp. PCC6803 (11). Interestingly, *Synechocystis* sp. HO-1 is only slightly inhibited by Sn-protoporphyrinIX, a potent inhibitor of mammalian and algal HOs (11). The genome of *Synechocystis* sp. (22) contains two genes, *ho1* and *ho2*, encoding potential HOs (11). However, so far only the recombinant HO-1 protein was shown to catalyze the conversion from heme to BV IXα. The lack of biochemical data on HO-2 is due mainly to insolubility issues and therefore is not conclusive at this point. The exact role for the occurrence of two HOs in this organism

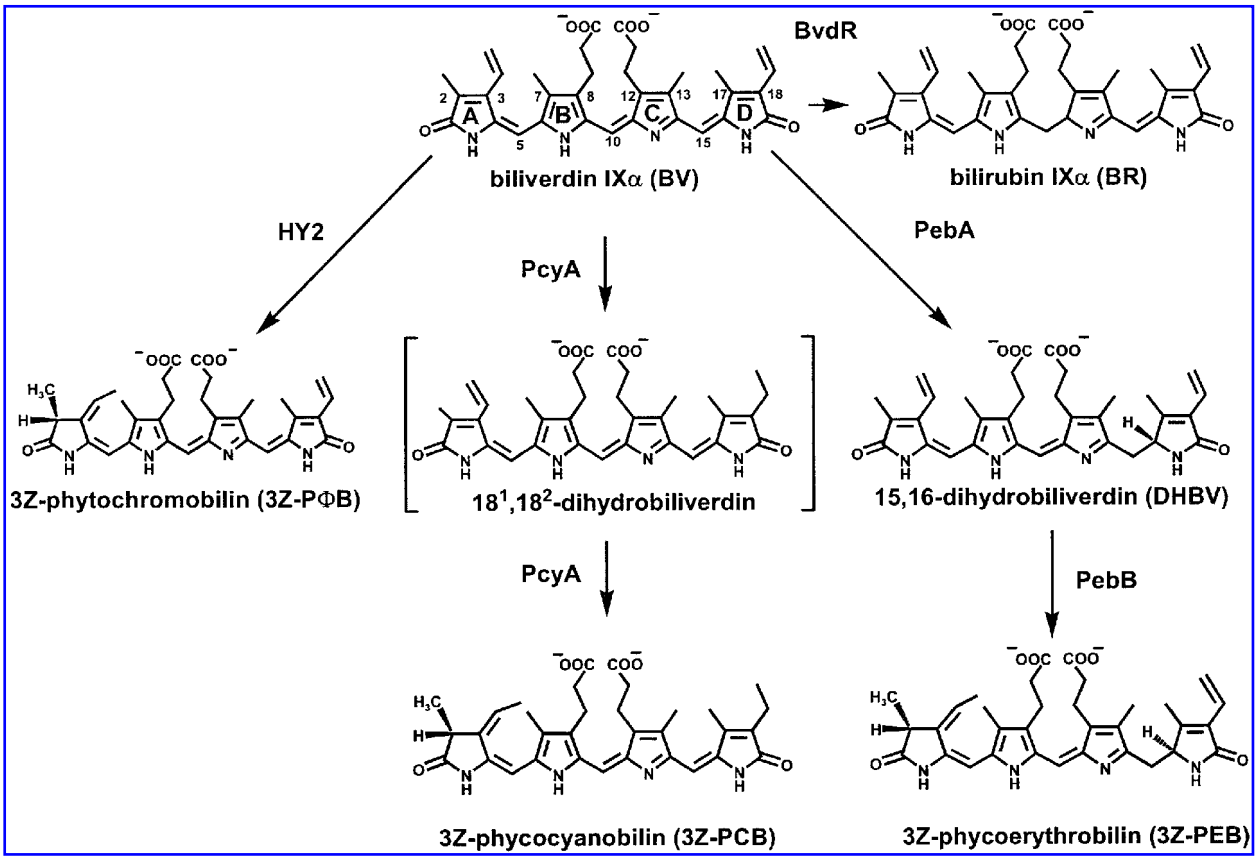

FIG. 2. Pathway of the biosynthesis of phytobilins in plants and cyanobacteria. The HO reaction is the common biosynthetic step for the biosynthesis of phytobilins in plants and cyanobacteria yielding the precursor BV IXα. BV IXα is subsequently reduced by Fd-dependent bilin reductases or NADPH-dependent BVR, which among prokaryotes has so far been only identified in *Synechocystis* sp. Enzyme abbreviations used are: HY2, phytochromobilin synthase or 3Z-phytochromobilin:ferredoxin oxidoreductase; PcyA, 3Z-phycocyanobilin:ferredoxin oxidoreductase; PebA, 15,16-dihydrobiliverdin:ferredoxin oxidoreductase; PebB, 3Z-phycoerythrobilin:ferredoxin oxidoreductase; BvdR, biliverdin IXα:NAD(P)H oxidoreductase.

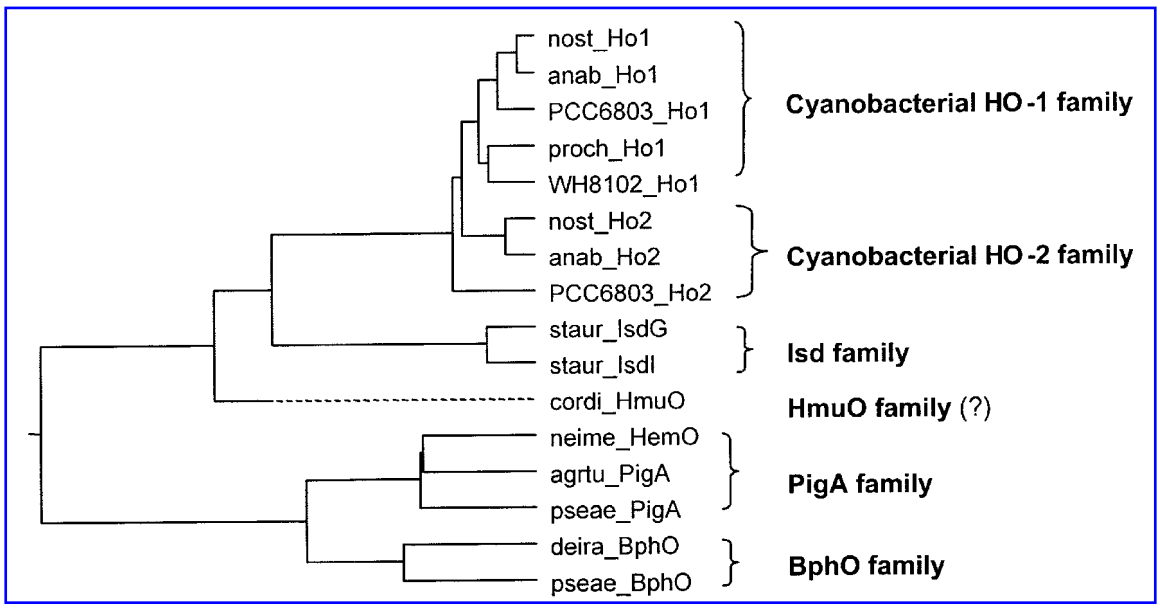

FIG. 3. Phylogenetic tree of bacterial HOs. A multiple sequence alignment using Clustal W was used to calculate a phylogram. nost, *Nostoc punctiforme*; anab, *Anabaena* sp. PCC7120; PCC6803, *Synechocystis* sp. PCC6803; WH8102, *Synechococcus* sp. WH8102; staur, *Staphylococcus aureus*; cordi, *Corynebacterium diphtheriae*; neime, *Neisseria meningitidis*; agrtu, *Agrobacterium tumefaciens*; pseae, *Pseudomonas aeruginosa*; deira, *Deinococcus radiodurans*.

remains unclear, but it has been speculated that the two HOs might have different affinities for oxygen and therefore are active at various oxygen tensions, a speculation that still needs to be tested. Genes encoding putative HO-1s and HO-2s can be found in the available genomes of other sequenced cyanobacteria, including *Anabaena* sp. PCC7120, *Thermosynechococcus elongatus*, and *Nostoc punctiforme*. Phylogenetically, they are all very closely related to the HO-1s and HO-2s, building separate clades within the cyanobacteria (Fig. 3). Interestingly, under iron-deficient conditions where many other bacterial HOs are up-regulated (see later section), *ho1* from *Synechocystis* sp. is down-regulated whereas *ho2* levels remain unchanged (42). This observation is consistent with a decrease in phycobiliproteins and synthesis of new chlororphyll-containing light-harvesting structures under iron deficiency (4).

Besides those HOs found in cyanobacteria, HOs have been identified in the genomes of the smallest known oxygen-evolving autotrophs, such as *Prochlorococcus marinus* (19). In contrast to cyanobacteria, this phytoplankton does not possess light-harvesting phycobiliprotein structures, but uses divinyl-chlorophylls instead. Interestingly, all available genomes of *Prochlorococcus* species contain a full set of genes encoding enzymes for phytobilin biosynthesis, which are mostly organized in operons consisting of *ho1*, *pebA,* and *pebB* genes (the latter encoding bilin reductases) (17, 19). Although some strains contain a degenerated phycoerythrin gene, its function is not yet fully understood. As *Prochlorococcus* sp. have very compact genomes most likely lacking any silent, nonfunctional genes, the function of this HO might be indeed in phytobilin biosynthesis (19). Although this assumption has already been proven biochemically through functional recombinant protein (N. Frankenberg-Dinkel, unpublished observations), its *in vivo* function still needs to be confirmed. Whether HO-1 serves additional functions in *Prochlorococcus*, such as iron utilization, remains to be tested.

The use of external heme as iron source

Iron is a limiting factor for bacterial growth and survival and is particularly essential for pathogens to cause disease. Bacteria have developed sophisticated systems to deal with the low supply of free extracellular iron. The most abundant source of iron in a host is usually heme, and therefore it is not surprising that many pathogenic prokaryotes can use heme as an iron source. Two mechanisms for the acquisition of iron from heme have been described in prokaryotic organisms: (a) the cleavage of the heme macrocycle through the action of HOs (39) and (b) the removal of iron from heme through a reversed ferrochelatase reaction (27). Some bacteria (*e.g., Bacteroides fragilis*) even have an absolute requirement for exogenous supplies of heme (or its precursor, protoporphyrin IX) because they lack the enzymes required for heme biosynthesis (35). For extracellular pathogens, access to host heme requires the liberation of heme and hemoglobin from red blood cells by proteases and hemolysins. Bacteria are able to use heme or hemoglobin as direct sources of iron. In Gram-negative bacteria, this involves binding of heme or heme complexes to outer membrane (OM) receptors followed by transport of the extracted heme group across the OM in a TonB-ExbB-ExbD-dependent fashion. Some bacteria secrete hemophores that bind extracellular heme and hemoglobin, and mediate their delivery to the OM receptors (10). Transport of heme across the cytoplasmic membrane seems to require an ABC transporter (2). Once in the cytoplasm, the heme is degraded by HOs releasing the complexed iron. Most of these systems are regulated by the ferric uptake repressor (Fur). Fur is a classical apo-repressor that requires iron in order to bind to target DNA sequences (Fur-boxes) in the promoter regions of iron-regulated genes. If the intracellular iron level (Fe^{2+}) reaches a certain threshold, expression of Fur-regulated genes is repressed. On the other hand, under iron-deficient conditions transcription is derepressed (48).

In Gram-positive bacteria, genes encoding proteins involved in heme or hemoprotein uptake have only been described in a few species, including *Corynebacterium* and *Streptococcus* species (14, 39, 46). In *C. diphtheriae,* three genes, *i.e., hmuT*, *hmuU,* and *hmuV,* are required for heme and hemoglobin uptake. These genes appear to be part of an ABC transport system and have significant sequence homology to genes from Gram-negative bacteria involved in the utilization of heme and hemoglobin as iron sources. Heme is first bound to a membrane-associated lipoprotein HmuT followed by transport of heme to the cytoplasm via the permease HmuU using energy supplied by the ATPase HmuV (14). Recently, iron-regulated surface determinants (*isd* genes) have been identified in the Gram-positive pathogen *Staphylococcus aureus* and were shown to encode factors for hemoglobin binding and transfer of heme iron to the cytoplasm. Furthermore, the *isd* operon encodes two putative HOs and is under the control of Fur (29, 43). Other Gram-positive bacteria appear to be able to utilize heme compounds as well. Very early studies on the utilization of heme have measured the production of CO, the by-product of the HO reaction in the Gram-positive bacteria *Bacillus cereus* and *Streptococcus mitis* (15).

HOs involved in iron acquisition

As described in the last paragraph, pathogenic bacteria that are able to use heme as iron source contain very efficient heme receptors and heme uptake systems. All these organisms also encode at least one gene encoding a HO. This whole heme-iron acquisition system is tightly regulated through global iron regulators.

The first bacterial gene with homologies to eukaryotic HOs from a pathogen was the gene *hmuO* from the Gram-positive bacteria *C. diphtheriae* and *C. ulcerans* (39). The promoter region of the *hmuO* genes shows consensus binding sequences for the global iron repressor DtxR (diphtheria toxin repressor) (38). DtxR, similar to the regulator Fur in Gram-negative bacteria, controls many iron-dependent processes in *C. diphtheriae,* including the expression of the *tox* gene, encoding the diphtheria exotoxin that causes severe tissue damage throughout the body (38). Therefore, the *hmuO* gene is repressed by iron and DtxR and activated in the presence of heme (38).

The first identified HO from a Gram-negative bacterium was the HO from *Neisseria* sp. encoded by the *hemO* gene (52). As expected, the *hemO* genes contain upstream Fur binding sites for iron regulation (52). Chromosomal knockout

mutants confirmed the involvement of HemO in heme utilization as these mutants were unable to use heme or hemoglobin as sources of iron, but were fully capable of using inorganic iron and iron-transferrin as iron supply (52).

One of the most exceptional HOs from a bacterial pathogen is found in the Gram-negative *Pseudomonas aeruginosa*. The *pigA* gene, originally identified in a screen for iron-regulated genes is located in a polycistronic operon of a total of five genes, *pigA–E* (33). Some of these genes encoding proteins involved in iron utilization include an alternative σ factor (*pigE*) and a ferripyoverdin receptor (*i.e.,* siderophore receptor; *pigD*). The expression of these genes is again regulated by Fur (33). The *pigA* gene product, which has homologies to the neisserial HemO, is the most unusual bacterial HO characterized so far. Unlike all other eukaryotic and prokaryotic HOs that have a regiospecificity for cleavage toward the α-meso carbon position, PigA targets the β- and δ-meso carbons of the heme macrocycle (36). This cleavage yields the unusual BV isomers BV IXβ and BV IXδ in a 30:70 ratio and is due to an unusual seating of the substrate heme in the active site pocket (Fig. 1) (6). The purpose for the production of these BV isomers is currently unknown, but it might be related to the fact the *P. aeruginosa* possesses a second HO (BphO) with an α-meso carbon specificity. Each HO seems to have a distinct function in *P. aeruginosa* that might be defined through the produced BV isomers. It is intriguing that phylogenetically the neisserial HemO protein falls into the PigA family although it has an α-meso carbon specificity (Fig. 3).

Two other interesting HOs have recently been identified and biochemically characterized in the Gram-positive pathogen *S. aureus* (43). The HOs IsdG and IsdI are unusually small proteins with a molecular weight of ~13,000. Overall their homology to other known bacterial HOs is low because BLAST homology searches using known bacterial HOs as queries do not reveal the *S. aureus* proteins (43). Only other Gram-positive pathogens like *Staphylococcus epidermidis*, *Listeria monocytogenes,* and *Bacillus anthracis* have homologues of IsdG and IsdI (43). Similar to other HOs from pathogenic bacteria, *S. aureus* IsdG and IsdI are involved in iron utilization, and therefore their transcription is regulated through Fur (29). The *isd* locus of *S. aureus* encodes a whole set of proteins for heme utilization as iron source, including genes encoding surface protein, lipoproteins, membrane transporter, and cytoplasmic proteins (29).

In addition to their role in iron acquisition, HOs might also protect prokaryotic cells against heme toxicity (7).

HOs involved in phytochrome chromophore biosynthesis in nonphotosynthetic bacteria

Another group of HOs besides those involved in phytobilin biosynthesis and iron utilization from heme have been identified in mostly nonphotosynthetic bacteria (3). The genes usually occur in operons with a gene encoding a bacterial phytochrome and are designated *bphO* (for bacterial phytochrome heme oxygenase).

Phytochromes, first identified in plants, algae, and cyanobacteria, are photoreversible light-signal transducing photoreceptors. They can exist in two spectrally stable forms: the red light-absorbing Pr form and the far-red light-aborbing Pfr form. Both forms are interconvertable by light. In plants, phytochromes control processes such as seed germination, shade avoidance, and flowering (44). Much less is known about the function of bacterial phytochromes, first identified in species such as *Deinococcus radiodurans* and *P. aeruginosa* (13). On the molecular level, they show characteristics of a typical sensor kinase of a bacterial two-component system. Therefore, a gene encoding a response regulator is often in close genomic proximity (13). All bacterial phytochromes studied so far show typical photoactivity *in vitro* using a BV chromophore, but their function *in vivo* remains to be elucidated (3, 18, 23, 24). The use of BV as chromophore is in agreement with the fact that these organisms lack genes encoding bilin reductases, the enzymes involved in phytobilin biosynthesis (see first section) (17). Furthermore, bacterial phytochromes lack a conserved cysteine residue that has been shown to be the site of covalent attachment of the phytochromobilin chromophore in plants (13). Due to their close genomic localization with the *bphO* genes, it is postulated that their gene products are important for the biosynthesis of a functional phytochrome (3).

At this point, it is worth mentioning that *P. aeruginosa* represents a very unusual scenario as this organism contains two different genes encoding a HO that show different regiospecificity. As described earlier, PigA is involved in iron reutilization from heme under iron-limiting conditions and produces the BV isomers IXβ and IXδ (Fig. 1). On the other hand, *P. aeruginosa* also contains a *bphO* gene, located upstream of *bphP* (bacterial phytochrome). We were able to demonstrate that BphO is indeed a HO with a regiospecificity toward the α-meso carbon yielding BV IXα (R. Wegele, and N. Frankenberg-Dinkel, unpublished observations). Furthermore, the produced BV is able to bind to BphP yielding a photoactive phytochrome (R. Tasler, and N. Frankenberg-Dinkel, unpublished observations). It remains to be elucidated if the PigA reaction products are also able to bind to BphP and yield a functional phytochrome. In this regard, it seems more likely that this is not the case and that *P. aeruginosa* has developed a sophisticated method to discriminate between BV produced during iron starvation and BV produced for phytochrome chromophore biosynthesis. This mechanism might be important to prevent a downstream signal transduction if a "wrong" chromophore is bound. Another interesting question in this matter is the origin of the substrate heme for the two HOs. It is almost certain that the source of heme for PigA is extracellular heme, most likely provided by the host cell. As heme is only taken up under iron-limiting conditions, the source for BphO might rather be intracellular heme from the bacteria's own biosynthesis.

At this point, no uniform nomenclature for bacterial HO genes has been obtained. To provide the reader with an overview, Table 1 summarizes all bacterial HOs characterized so far, their encoding genes, transcriptional regulation, and bacterial origin.

Function of the products of the HO reaction

Although it becomes more and more evident that the major function of heme cleavage in bacterial pathogens is the

Table 1. Characterized Bacterial HOs, Their Encoding Genes, and Transcriptional Regulation

Organism	*Gene*	*Promoter region regulator binding site*
Synechocystis sp. PCC6803	*ho1*	Unknown
Synechocystis sp. PCC6803	*ho2*	Unknown
Prochlorococcus marinus	*ho1*	Unknown
Corynebacterium diphtheriae	*hmuO*	DtxR-box
Staphylococcus aureus	*isdI/isdG*	Fur-box
Neisseria meningitidis	*hemO*	Fur-box
Pseudomonas aeruginosa	*bphO*	Unknown
Pseudomonas aeruginosa	*pigA*	Fur-box
Deinococcus radiodurans	*bphO*	Unknown

acquisition of iron, the fate of the other products of the HO reaction, BV and CO, remains unresolved. Due to the lack of genes encoding homologues of mammalian BVRs, it seems unlikely that BV is further metabolized to BR in bacteria. In pathogens, the production of BV might only be a waste product and transported out of the cell (49). On the other hand, BV is also known to have antioxidant function and could serve a second purpose in these organisms as well. CO, the third reaction product obtained during HO catalysis, has been shown to have signaling functions in eukaryotes. Whether it is able to serve a similar purpose in prokaryotes remains unclear.

BIOCHEMICAL PROPERTIES OF BACTERIAL HEME OXYGENASES

Mechanism of bacterial HOs

The oxygenolytic cleavage of heme by HOs seems to proceed via a quite similar mechanism in all organisms (Fig. 4; for detailed mechanism, see 34, 49). The reaction involves several spectroscopic distinct intermediates (see Table 2). It is postulated that the reaction starts with the binding of one equivalent of heme to form a ferric (Fe^{3+})-heme-HO complex. The formation of this complex is followed by the first electron transfer from the reducing system yielding a ferrous (Fe^{2+})-heme-HO complex. This complex subsequently binds molecular oxygen through coordination to the iron to generate a metastable oxy-complex. In a following electron transfer reaction, a ferric hydroperoxy is produced that, after self-hydroxylation of the α-meso carbon, forms ferric α-meso-hydroxyheme. The next step in the reaction is the conversion of ferric α-meso hydroxyheme to ferrous verdoheme and involves the input of another molecule of oxygen and an electron. The following transformation of ferrous-verdoheme to ferric-BV requires both oxygen and reducing equivalents and is the least understood part of the mechanism. For the liberation of free iron and BV, the iron of the ferric-BV is further reduced to the ferrous state by the reducing system.

Spectral properties of bacterial HOs

All HOs form a distinct complex with heme that gives an optical absorption spectrum with a Soret band around 402 nm that is highly distinguishable from the Soret band of free heme (Table 2). By utilizing difference spectroscopy, the stoichiometry of the heme binding reaction can be examined. All characterized bacterial HOs display a 1:1 ratio of heme to protein, which is consistent with mammalian and plant HOs (8, 30, 32, 47, 53). In general, all bacterial HOs display similar spectroscopic properties for their ferric-, ferrous-, oxy-, and CO-heme-HO complexes. The largest difference is seen in the ferric state of the heme-HO complex where the position of the Soret band varies between 402 nm for the *Synechocystis* sp. HO-1 and 412 nm for the newly identified HOs IsdI and IsdG from *S. aureus* (Table 2).

Reducing partner for bacterial HOs

Most biochemical characterization and enzymology of bacterial HOs have been carried out using mammalian cytochrome P450 reductase and NADPH to generate reducing

Table 2. Spectroscopic Data of Characterized Bacterial Heme-HO Complexes

	HO-1 (30) Synechocystis *sp.*	*HmuO (8)* C. diphtheriae	*HemO (52)* N. meningitidis	*PigA (36)* P. aeruginosa	*BphO** P. aeruginosa	*IsdG (43)* S. aureus	*IsdI (43)* S. aureus
Ferric							
Soret (λ_{max})	402	404	406	406	409	412	412
Visible (λ_{max})	498, 630	500, 630		632	504, 638	532, 567	532, 567
Ferrous							
Soret (λ_{max})	427	434	n.d.a.†	434	n.d.a.	n.d.a.	n.d.a.
Visible (λ_{max})	555			550			
Oxy							
Soret (λ_{max})	410	410	n.d.a.	410	412	414	414
β, α (λ_{max})	537, 574	540, 570		540, 570	545, 579		
CO							
Soret (λ_{max})	418	421	421	419	n.d.a.	n.d.a.	n.d.a.
Visible (λ_{max})	536, 566	538, 568	538, 568	537, 567			

*R. Wegele and N. Frankenberg-Dinkel, unpublished observations.
†n.d.a., no data available.

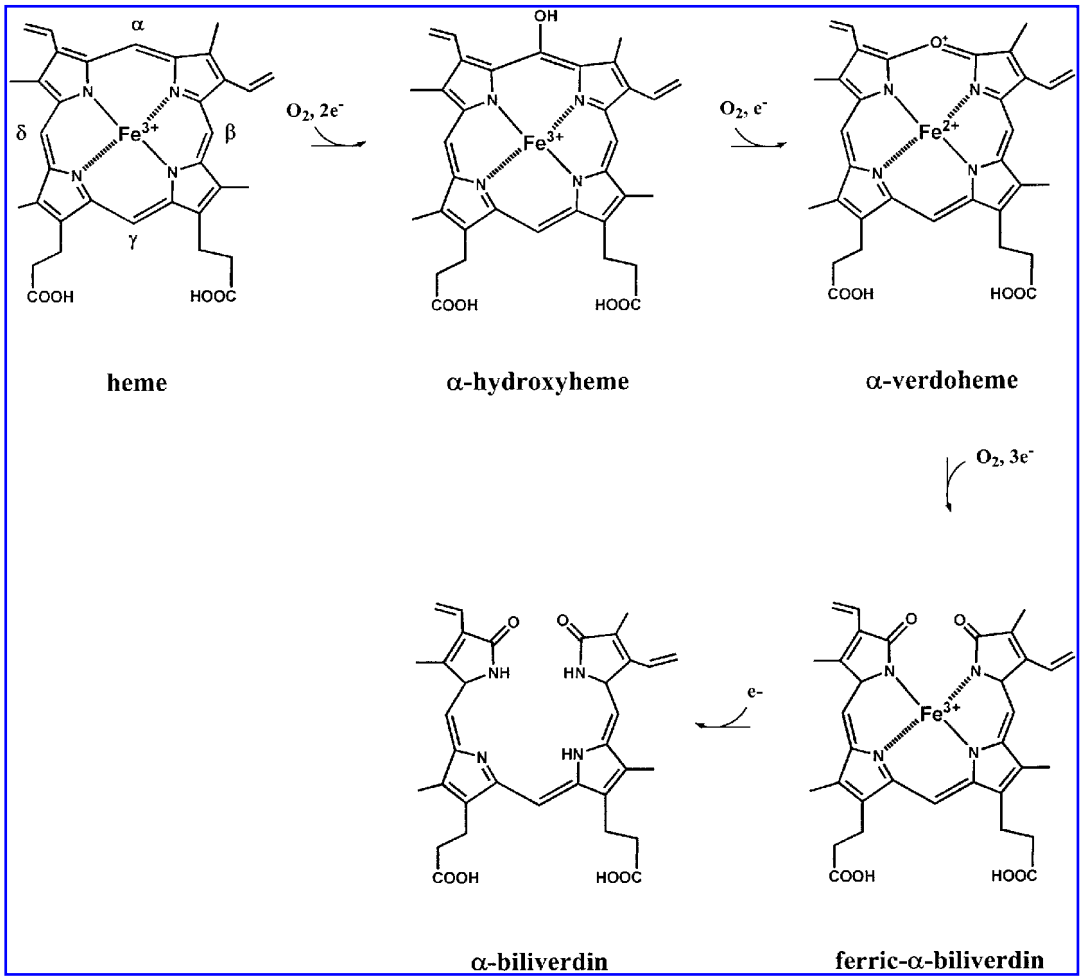

FIG. 4. Mechanism of HO. Chemical structures of heme and the intermediates during the HO reaction.

power. Ascorbate has also been shown to serve as the electron donor. For most bacterial HOs, it has been described that the final product of the reaction with ascorbate is Fe^{3+}-BV. One exception is HO-1 from *Synechocystis* sp. where the final product of the ascorbate-supported reaction is free BV (30). HemO and PigA enzymes can use cytochrome P450 reductase as a reductant that also yields Fe^{3+}-BV as a product. This product is not released from the active site using iron chelators or acidification, indicating that the release of the product is the rate-limiting step in bacterial HO reactions. The same step is also rate-limiting in mammalian HOs, but the reaction is driven forward by BVR (26). As bacteria in general lack genes encoding BVR, another protein seems to be required to fulfill this function. Interestingly, for recombinant HmuO protein, controversial results have been obtained concerning the BV produced. Reports using the natural gene expressed in *E. coli* and cytochrome P450 reductase yielded the Fe^{3+}-BV (50); on the other hand, expression of HmuO from a synthetic gene resulted in iron-free BV (8). Whether the differences in these results are due to the origin of the gene or rather to the experimental nature is unclear at this point. But it is noteworthy that this is the first observation of an iron-free BV produced in a cytochrome P450 reductase-driven bacterial HO reaction. In contrast to the bacterial HOs from pathogens that seem to obtain their reducing equivalents from NADPH via cytochrome P450 reductase, cyanobacterial HOs are soluble enzymes that seem to use Fd as their reducing partner. Interestingly, it was shown that catalysis is more efficient if a second reductant, such as Trolox or ascorbate, is present (11). The Fd dependence is in agreement with the HOs found in plants where Fd is the electron donor and which seem to be the closest eukaryotic relatives. This was further confirmed by the ability of *Synechocystis* sp. HO-1 to complement an *Arabidopsis HY1* (deficient in one HO) mutant (51). Interestingly, *Synechocystis* sp. HO-1 can also use cytochrome P450 reductase for the HO reaction, but the reaction is arrested at the oxy-complex stage, indicating that cytochrome P450 reductase might not be the right reducing partner for this bacterial HO (30). The observed Fd dependence of cyanobacterial HOs led us to test Fd as a potential reducing partner for the two HOs from *P. aeruginosa*, PigA and BphO. In both cases, Fd is able to deliver the electrons (R. Wegele and N. Frankenberg-Dinkel, unpublished observations).

In vivo *reducing partner of bacterial HOs*

Escherichia coli expressing recombinant bacterial HOs usually show a dark green color after induction. In general, the purification of recombinant protein yields a very tight HO-BV (not Fe^{3+}-BV!) complex. These results strongly indicate that almost all bacterial HOs are functional in *E. coli* and can use an *E. coli* reducing system as the electron donor. Bacterial HOs were shown to use either a cytochrome P450 reductase system or Fd as a reducing partner *in vitro*. *E. coli* cells possess several possible reductants. *E. coli* has a Fd of the adrenodoxin type [2Fe-2S], which has been shown by genetic analyses to perform an essential role in the maturation of various iron-sulfur proteins (25). Indeed, *E. coli* Fd is more structurally related to the adrenodoxin-type Fds, *i.e.,* bovine adrenodoxin and *P. putida* putidaredoxin, than to plant-type Fds (21). Other than this adrenodoxin-type Fd, the *E. coli* genome possesses two flavodoxin genes and a flavorubredoxin gene (5). As no cytochrome P450 reductase has been described for *E. coli*, it is fair to assume that during recombinant production of HOs in *E. coli*, a flavodoxin serves as the reductant. Similar to the last electron transfer step in cytochrome P450 reductase, flavin mononucleotide is the redox active cofactor in flavodoxin. Many other bacteria that carry HOs exhibit a similar scenario to *E. coli* in terms of reducing systems. Usually there are more than one potential interacting reductants present. At this point, the identification of the endogenous electron donor for any of the bacterial HOs remains to be elucidated.

Regiospecificity of bacterial HOs

With one exception, all characterized HOs, including those from mammals and plants, display an α-meso carbon specificity for cleavage of the heme macrocycle. The only HOs displaying a different regiospecificity is the PigA HO from *P. aeruginosa*. Interestingly, homologues of PigA can also be found in the genomic database of other pathogenic bacteria (*i.e., Agrobacterium tumefaciens*; see Fig. 3), but for those the regiospecificity remains to be determined. ^{1}C and ^{13}C NMR studies on the unique HO from *P. aeruginosa* have revealed that an unusual seating of the heme is responsible for the varied regiospecificity (6). The heme propionates are positioned so that they are pointing toward the direction of the solvent-exposed heme edge. This seems to be due to the lack of stabilizing interactions between the protein and the propionate side chains of the heme that are usually present in HOs with α-hydroxylating activity. As a result, the heme group in PigA is rotated ~110° in plane, consequently placing the δ-meso carbon in a position where the other HOs typically place their α-meso heme carbon. Interestingly, the mutation of two amino acid residues corresponding to those that are usually involved in these interactions in the other HOs (*i.e.,* K16, Y112 of *N. meningitidis* HemO) changed the regioselectivity of the PigA enzyme toward producing BV IXα (55%), BV IXβ (10%), and BV IXδ (35%) (6). It was found that these data were due to a mixture of PigA molecules displaying two distinct heme seatings. A recently presented crystal structure of this enzyme (J. Friedmann, L. Lad, H. Li, A. Wilks, and T.L. Poulos. Structural basis for novel delta-regioselective heme oxygenation in the opportunistic pathogen *Pseudomonas aeruginosa. Biochemistry* 43: 5239–5245, 2004) will give more insight into this phenomenon.

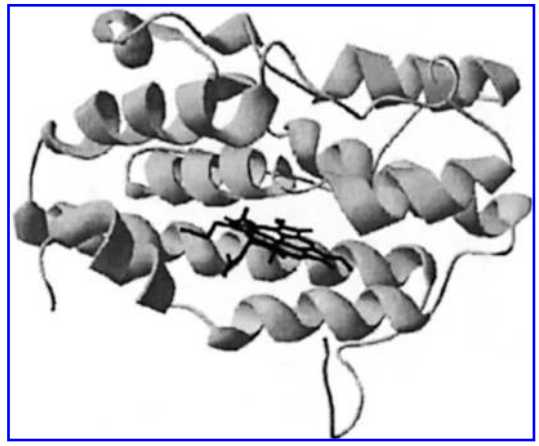

FIG. 5. Ribbon diagram of the crystal structure of *Neisseria meningitidis* HemO. Overall structure of *N. meningitidis* HemO shows the active site pocket and the bound heme molecule (41).

Crystal structures of bacterial HOs

Several crystal structures from mammalian and bacterial sources have been solved to date (9, 40, 41, 45). Strikingly, all structures have the same overall fold despite their low overall sequence similarity, a common phenomenon also present in other enzymes involved in heme biosynthesis/degradation (12). All HOs have a novel, mostly α-helical fold (Fig. 5). The heme is sandwiched between two α-helices with the propionate side chains being exposed at the molecular surface of the protein (41) (Fig. 5). One of these helices, the proximal helix, donates the histidine ligand that coordinates the heme through a water molecule. The recent elucidation of more refined crystal structures of bacterial HOs gives insights into the molecular understanding of proton transfer in these enzymes (20). (For more structural details, the reader is referred to the original literature and reference 49).

SUMMARY AND OUTLOOK

The availability of a large number of bacterial genomes during the last several years has largely contributed to the increasing number of known bacterial HOs. Especially among bacterial pathogens, HOs seem to play a so far underestimated role during iron acquisition from a host cell. The understanding of new HOs with varied regiospecificity for heme macrocycle cleavage is another emerging field that has to be elucidated especially if two HOs with different BV produced are present in one organism. Although the involvement of HOs in bacterial phytochrome chromophore biosynthesis appears to be understood, the function of this whole bacterial signal transduction system remains to be elucidated. In the future, a combination of biochemistry, microbiology, and structural biology will largely strengthen our knowledge of this emerging class of bacterial enzymes.

ACKNOWLEDGMENTS

Funding in the author's laboratory on the bacterial HOs from *Pseudomonas aeruginosa* is supported by the Emmy-Noether-Programm of the Deutsche Forschungsgemeinschaft and funds from the Fonds der chemischen Industrie. Thanks are due to Angela Wilks for helpful scientific discussions. I would especially like to thank J. Clark Lagarias for his mentorship and his continuing interest in my work, as well as Dieter Jahn for his continued support.

ABBREVIATIONS

BR, bilirubin; BV, biliverdin; BVR, biliverdin reductase; CO, carbon monoxide; DtxR, diphtheria toxin repressor; Fd, ferredoxin; Fur, ferric uptake repressor; HO, heme oxygenase; OM, outer membrane.

REFERENCES

1. Abraham NG, Drummond GS, Lutton JD, and Kappas A. The physiological significance of heme oxygenase. *Cell Physiol Biochem* 6: 129–168, 1996.
2. Andrews SC, Robinson AK, and Rodriguez-Quinones F. Bacterial iron homeostasis. *FEMS Microbiol Rev* 27: 215–237, 2003.
3. Bhoo S-H, Davis SJ, Walker J, Karniol B, and Viestra RD. Bacteriophytochromes are photochromic histidine kinases using a biliverdin chromophore. *Nature* 414: 776–779, 2001.
4. Bibby TS, Nield J, and Barber J. Iron deficiency induces the formation of an antenna ring around trimeric photosystem I in cyanobacteria. *Nature* 412: 743–745, 2001.
5. Blattner FR, Plunkett G 3rd, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA, Goeden MA, Rose DJ, Mau B, and Shao Y. The complete genome sequence of *Escherichia coli* K-12. *Science* 277:1453–1474, 1997.
6. Caignan GA, Deshmukh R, Wilks A, Zeng Y, Huang HW, Moenne-Loccoz P, Bunce RA, Eastman MA, and Rivera M. Oxidation of heme to beta- and delta-biliverdin by *Pseudomonas aeruginosa* heme oxygenase as a consequence of an unusual seating of the heme. *J Am Chem Soc* 124: 14879–14892, 2002.
7. Cannon JB. Pharmaceutics and drug delivery aspects of heme and porphyrin therapy. *J Pharm Sci* 82: 435–446, 1993.
8. Chu GC, Katakura K, Zhang X, Yoshida T, and Ikeda-Saito M. Heme degradation as catalyzed by a recombinant bacterial heme oxygenase (HmuO) from *Corynebacterium diphtheriae. J Biol Chem* 274: 21319–21325, 1999.
9. Chu GC, Park SY, Shiro Y, Yoshida T, and Ikeda-Saito M. Crystallization and preliminary x-ray diffraction analysis of a recombinant bacterial heme oxygenase (HmuO) from *Corynebacterium diphtheriae. J Struct Biol* 126: 171–174, 1999.
10. Clarke TE, Tari LW, and Vogel HJ. Structural biology of bacterial iron uptake systems. *Curr Top Med Chem* 1: 7–30, 2001.
11. Cornejo J, Willows RD, and Beale SI. Phytobilin biosynthesis: cloning and expression of a gene encoding soluble ferredoxin-dependent heme oxygenase from *Synechocystis* sp. PCC 6803. *Plant J* 15: 99–107, 1998.
12. Dailey HA, Dailey TA, Wu CK, Medlock AE, Wang KF, Rose JP, and Wang BC. Ferrochelatase at the millennium: structures, mechanisms and [2Fe-2S] clusters. *Cell Mol Life Sci* 57: 1909–1926, 2000.
13. Davis SJ, Kurepa J, and Vierstra R. The *Arabidopsis thaliana HY1* locus, required for phytochrome-chromophore biosynthesis, encodes a protein related to heme oxygenases. *Proc Natl Acad Sci U S A* 96: 6541–6546, 1999.
14. Drazek ES, Hammack CA, and Schmitt MP. *Corynebacterium diphtheriae* genes required for acquisition of iron from haemin and haemoglobin are homologous to ABC haemin transporters. *Mol Microbiol* 36: 68–84, 2000.
15. Engel RR, Matsen JM, Chapman SS, and Schwartz S. Carbon monoxide production from heme compounds by bacteria. *J Bacteriol* 112: 1310–1315, 1972.
16. Frankenberg N, and Largarias JC. Biosynthesis and biological functions of bilins. In: *The Porphyrin Handbook, Vol. 13,* edited by Kadish KM, Smith KM, and Guilard R. San Diego, CA: Academic Press, pp. 211–235.
17. Frankenberg N, Mukougawa K, Kohchi T, and Lagarias JC. Functional genomic analysis of the HY2 family of ferredoxin-dependent bilin reductases from oxygenic photosynthetic organisms. *Plant Cell* 13: 965–978, 2001.
18. Giraud E, Fardoux J, Fourrier N, Hannibal L, Genty B, Bouyer P, Dreyfus B, and Vermeglio A. Bacteriophytochrome controls photosystem synthesis in anoxygenic bacteria. *Nature* 417: 202–205, 2002.
19. Hess WR, Rocap G, Ting CS, Larimer F, Stilwagen S, Lamerdin J, and Chisholm SW. The photosynthetic apparatus of *Prochlorococcus:* insights through comparative genomics. *Photosynth Res* 70: 53–71, 2001.
20. Hirotsu S, Chu GC, Unno M, Lee D-S, Yoshida T, Park S-Y, Shiro Y, and Ikeda-Saito M. The crystal structures of the ferric and ferrous forms of the heme complex of HmuO, a heme oxygenase of *Corynebacterium diphtheriae. J Biol Chem* 279: 11937–11947, 2004.
21. Kakuta Y, Horio T, Takahashi Y, and Fukuyama K. Crystal structure of *Escherichia coli* Fdx, an adrenodoxin-type ferredoxin involved in the assembly of iron-sulfur clusters. *Biochemistry* 40: 11007–11012, 2001.
22. Kaneko T, Sato S, Kotani H, Tanaka A, Asamizu E, Nakamura Y, Miyajima N, Hirosawa M, Sugiura M, Sasamoto S, Kimura T, Hosouchi T, Matsuno A, Muraki A, Nakazaki N, Naruo K, Okumura S, Shimpo S, Takeuchi C, Wada T, Watanabe A, Yamada M, Yasuda M, and Tabata S. Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions (supplement). *DNA Res* 3: 185–209, 1996.
23. Karniol B and Vierstra RD. The pair of bacteriophytochromes from *Agrobacterium tumefaciens* are histidine kinases with opposing photobiological properties. *Proc Natl Acad Sci U S A* 100: 2807–2812, 2003.
24. Lamparter T, Michael N, Mittmann F, and Esteban B. Phytochrome from *Agrobacterium tumefaciens* has unusual spectral properties and reveals an N-terminal chromophore

attachment site. *Proc Natl Acad Sci U S A* 99: 11628–11633, 2002.
25. Lill R and Kispal G. Maturation of cellular Fe-S proteins: an essential function of mitochondria. *Trends Biochem Sci* 25: 352–356, 2000.
26. Liu Y and Ortiz de Montellano PR. Reaction intermediates and single turnover rate constants for the oxidation of heme by human heme oxygenase-1. *J Biol Chem* 275: 5297–5307, 2000.
27. Loeb MR. Ferrochelatase activity and protoporphyrin IX utilization in *Haemophilus influenzae. J Bacteriol* 177: 3613–3615, 1995.
28. Maines MD and Trakshel GM. Purification and characterization of human biliverdin reductase. *Arch Biochem Biophys* 300: 320–326, 1993.
29. Mazmanian SK, Skaar EP, Gaspar AH, Humayun M, Gornicki P, Jelenska J, Joachmiak A, Missiakas DM, and Schneewind O. Passage of heme-iron across the envelope of *Staphylococcus aureus. Science* 299: 906–909, 2003.
30. Migita CT, Zhang X, and Yoshida T. Expression and characterization of cyanobacterium heme oxygenase, a key enzyme in the phycobilin synthesis. Properties of the heme complex of recombinant active enzyme. *Eur J Biochem* 270: 687–698, 2003.
31. Muramoto T, Kohchi T, Yokota A, Hwang I, and Goodman HM. The *Arabidopsis* photomorphogenic mutant *hy1* is deficient in phytochrome chromophore biosynthesis as a result of a mutation in a plastid heme oxygenase. *Plant Cell* 11: 335–347, 1999.
32. Muramoto T, Tsurui N, Terry MJ, Yokota A, and Kohchi T. Expression and biochemical properties of a ferredoxin-dependent heme oxygenase required for phytochrome chromophore synthesis. *Plant Physiol* 130: 1958–1966, 2002.
33. Ochsner UA and Vasil ML. Gene repression by the ferric uptake regulator in *Pseudomonas aeruginosa:* cycle selection of iron-regulated genes. *Proc Natl Acad Sci U S A* 93: 4409–4414, 1996.
34. Ortiz de Montellano PR. The mechanism of heme oxygenase. *Curr Opin Chem Biol* 4: 221–227, 2000.
35. Otto BR, Sparrius M, Verweij-van Vught AM, and MacLaren DM. Iron-regulated outer membrane protein of *Bacteroides fragilis* involved in heme uptake. *Infect Immun* 58: 3954–3958, 1990.
36. Ratliff M, Zhu W, Deshmukh R, Wilks A, and Stojiljkovic I. Homologues of neisserial heme oxygenase in Gram-negative bacteria: degradation of heme by the product of the pigA gene of *Pseudomonas aeruginosa. J Bacteriol* 183: 6394–6403, 2001.
37. Richaud C and Zabulon G. The heme oxygenase gene (pbsA) in the red alga *Rhodella violacea* is discontinuous and transcriptionally activated during iron limitation. *Proc Natl Acad Sci U S A* 94: 11736–11741, 1997.
38. Schmitt M. Transcription of the *Corynebacterium diphtheriae hmuO* gene is regulated by iron and heme. *Infect Immun* 65: 4634–4641, 1997.
39. Schmitt M. Utilization of host iron sources by *Corynebacterium diphtheriae:* identification of a gene whose product is homologous to eukaryotic heme oxygenases and is required for acquisition of iron from heme and hemoglobin. *J Bacteriol* 179: 838–845, 1997.
40. Schuller DJ, Wilks A, Ortiz de Montellano PR, and Poulos TL. Crystal structure of human heme oxygenase-1. *Nat Struct Biol* 6: 860–867, 1999.
41. Schuller DJ, Zhu WM, Stojiljkovic I, Wilks A, and Poulos TL. Crystal structure of heme oxygenase from the Gram-negative pathogen *Neisseria meningitidis and a comparison with mammalian heme oxygenase-1. Biochemistry* 40: 11552–11558, 2001.
42. Singh AK, McIntyre LM, and Sherman LA. Microarray analysis of the genome-wide response to iron deficiency and iron reconstruction in the cyanobacterium Synechocystis sp. PCC 6803. *Plant Physiol* 132: 1825–1839, 2003.
43. Skaar EP, Gaspar AH, and Schneewind O. IsdG and IsdI, heme-degrading enzymes in the cytoplasm of *Staphylococcus aureus. J Biol Chem* 279: 436–443, 2004.
44. Smith H. Phytochromes and light signal perception by plants—an emerging synthesis. *Nature* 407: 585–591, 2000.
45. Sugishima M, Omata Y, Kakuta Y, Sakamoto H, Noguchi M, and Fukuyama K. Crystal structure of rat heme oxygenase-1 in complex with heme. *FEBS Lett* 471: 61–66, 2000.
46. Tai SS, Wang TR, and Lee CJ. Characterization of hemin binding activity of *Streptococcus pneumoniae. Infect Immun* 65: 1083–1087, 1997.
47. Takahashi S, Wang J, Rousseau DL, Ishikawa K, Yoshida T, Host JR, and Ikeda-Saito M. Heme–heme oxygenase complex. Structure of the catalytic site and its implication for oxygen activation. *J Biol Chem* 269: 1010–1014, 1994.
48. Vasil ML and Ochsner UA. The response of *Pseudomonas aeruginosa* to iron: genetics, biochemistry and virulence. *Mol Microbiol* 34: 399–413, 1999.
49. Wilks A. Heme oxygenase: evolution, structure and mechanism. *Antioxid Redox Signal* 4: 603–614, 2002.
50. Wilks A and Schmitt MP. Expression and characterization of a heme oxygenase (HmuO) from *Corynebacterium diphtheriae.* Iron acquisition requires oxidative cleavage of the heme macrocycle. *J Biol Chem* 273: 837–841, 1998.
51. Willows RD, Mayer SM, Foulk MS, DeLong A, Hanson K, Chory J, and Beale SI. Phytobilin biosynthesis: the *Synechocystis* sp. PCC 6803 heme oxygenase-encoding *ho1* gene complements a phytochrome-deficient *Arabidopsis thalianna hy1* mutant. *Plant Mol Biol* 43: 113–120, 2000.
52. Zhu W, Hunt DJ, Richardson AR, and Stojiljkovic I. Use of heme compounds as iron sources by pathogenic neisseriae requires the product of the *hemO* gene. *J Bacteriol* 182: 439–447, 2000.
53. Zhu W, Wilks A, and Stojiljkovic I. Degradation of heme in Gram-negative bacteria: the product of the *hemO* gene of Neisseriae is a heme oxygenase. *J Bacteriol* 182: 6783–6790, 2000.

Address reprint requests to:
Nicole Frankenberg-Dinkel
Institute for Microbiology
Technical University Braunschweig
Spielmannstr. 7
38106 Braunschweig, Germany

E-mail: n.frankenberg@tu-bs.de

Received for publication May 20, 2004; accepted June 13, 2004.

This article has been cited by:

1. Prof. Mahin D. Maines . 2005. The Heme Oxygenase System: Update 2005. *Antioxidants & Redox Signaling* **7**:11-12, 1761-1766. [Citation] [Full Text PDF] [Full Text PDF with Links]