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Metabolic Flux of Extracellular Heme Uptake in *Pseudomonas aeruginosa* Is Driven by the Iron-regulated Heme Oxygenase (HemO)*[S]

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Background: *Pseudomonas aeruginosa* utilizes extracellular heme as a source of iron.
Results: Deletion of *hemO* results in loss of ^{13}C-heme uptake and degradation to ^{13}C-BV IXδ and BV IXβ.
Conclusion: Extracellular heme uptake is dependent on the catalytic action of HemO.
Significance: Determining the role of metabolic flux in heme uptake and degradation is crucial in understanding the relationship between iron homeostasis and virulence.

Heme utilization by *Pseudomonas aeruginosa* involves several proteins required for internalization and degradation of heme. In the following report we provide the first direct *in vivo* evidence for the specific degradation of extracellular heme to biliverdin (BV) by the iron-regulated HemO. Moreover, through isotopic labeling (^{13}C-heme) and electrospray ionization-MS analysis we have confirmed the regioselectivity and ratio of ^{13}C-δ and β-BV IX (70:30) is identical *in vivo* to that previously observed for the purified protein. Furthermore, the ^{13}C-BV IXδ and BV IXβ products are effluxed from the cell by an as yet unidentified transporter. Conversion of extracellular heme to BV is dependent solely on the iron-regulated HemO as evidenced by the lack of BV production in the *P. aeruginosa hemO* deletion strain. Complementation of *P. aeruginosa* Δ*hemO* with a plasmid expressing either the wild type HemO or α-regioselective HemO mutant restored extracellular heme uptake and degradation. In contrast deletion of the gene encoding the cytoplasmic heme-binding protein, PhuS, homologs of which have been proposed to be heme oxygenases, did not eliminate ^{13}C-BV IXδ and IXβ production. In conclusion the metabolic flux of extracellular heme as a source of iron is driven by the catalytic action of HemO.

Iron is an essential micronutrient required by pathogenic bacteria for their survival, growth, and virulence. In addition to receptor-mediated iron-siderophore scavenging mechanisms, many bacteria utilize heme and hemeproteins as a source of iron (1, 2). The opportunistic Gram-negative pathogen *Pseudomonas aeruginosa* encodes two inter-dependent heme uptake systems, the *Pseudomonas* heme utilization (*phu*)[2] system and the heme assimilation system (*has*) (3). In addition to the outer-membrane receptor, PhuR, the operon encodes a periplasmic transport system comprising PhuT, a soluble receptor for the ATP-dependent permease (ABC transporter), PhuUV. The cytoplasmic heme-binding protein, PhuS, sequesters heme translocated to the cytoplasm by PhuUV. In contrast, the *has* system encodes a soluble hemophore (HasA), which is secreted to the extracellular media, extracts heme from hemoglobin and returns it to a TonB-dependent outer-membrane receptor (HasR). However, the *has* system lacks a periplasmic uptake system and is presumed to utilize the *phu*-encoded ABC transporter (4–6).

Heme uptake into the cytoplasm has been well characterized in Gram-negative organisms, however, the fate of heme once internalized has been the subject of some debate. Heme oxygenase enzymes have been identified and characterized in Gram-positive and Gram-negative pathogens including *Corynebacterium diphtheria* (7), *Neisseriae spp*., and *P. aeruginosa* (8). *P. aeruginosa* encodes a second heme oxygenase *bphO*, directly upstream of the phytochrome two-component sensor kinase, *bphP* (9). BphO, in contrast to the iron-regulated HemO produces BV IXα, which acts as a chromophore for the bacteriophytochrome kinase, BphP. Although the downstream two-component regulator of BphP has not been determined, the *bphOP* operon is not iron-regulated, and does not appear to be involved in extracellular heme uptake. The catalytic mechanism and structural fold of the bacterial heme oxygenases is strikingly similar to their more well characterized eukaryotic counterparts (10, 11). Moreover, in early studies a homolog of the cytoplasmic heme-binding protein PhuS, HemS from *Yersinia entercolitica*, had been proposed to be a heme oxygenase or hemin degrading factor, based solely on the observation of an inability to efficiently utilize heme on deletion of the *hemS* gene (12). More recently the *Escherichia coli* ChuS protein has been reported to be a heme oxygenase, although it has yet to be determined if the catalytic turnover is greater than one, or if the reaction product is BV (13). In contrast recent *in vitro* studies have suggested that PhuS acts as a heme chaperone in the transfer of heme to the iron-regulated HemO (14, 15). In an effort to further elucidate the role of HemO and PhuS in extracellular

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[2] The abbreviations used are: *phu*, *Pseudomonas* heme uptake; *has*, heme assimilation system; HemO, heme oxygenase; BV, biliverdin; TBS, Tris-buffered saline; ESI-MS, electrospray ionization mass spectrometry; ALA, δ-aminolevulinic acid.

TABLE 1
Bacterial strains and plasmids

Strain	Description	Source or Ref.
E. coli		
BL21(DE3)	F^-dcm *ompT hsdS* ($r_B^-m_B^-$) *gal*λ (DE3)	Stratagene
S17-1	*pro thi hsdR*_ Tpr Sm^r; chromosome::RP4–2 Tc::Mu-Km::Tn*7*/λpir	24
P. aeruginosa		
PAO1	Wild type	26
PAO1 *hemO*	PAO1 *hemO*::*aacC1* Gm	27
PAO1 *phuS*	PAO1 Δ*phus*	This study
Plasmids		
MRL2	Amp^R; pET-11a derivate harbouring the rat liver outer mitochondrial membrane cytochrome b_5 gene encoding a water-soluble domain of the cytochrome b_5	18
pFLP2	Amp^R; source of FLP recombinase	25
pEX18p-Δ*phuS*::*tet*	Amp^R; allelic replacement vector containing 1800-bp fragment of in-frame *phuS* deletion containing a tetracycline cassette	20
pBSPHemO	630-bp *hemO* gene cloned into a derivatve of pBBR1MCS3 under control of the *araC-*P_{BAD} cassette	This study

heme uptake we undertook a metabolic analysis of the *P. aeruginosa* wild type and *phuS* and *hemO* deletion strains.

In summary we have shown that the catalytic action of HemO drives the metabolic flux of extracellular heme uptake. Interestingly, under conditions whereby the extracellular heme uptake proteins are expressed the BphO protein did not appear to be able to compensate for the lack of HemO. Furthermore, lack of BV in the media on deletion of *hemO* is consistent with PhuS being a heme chaperone and not as previously suggested a heme-degrading enzyme.

EXPERIMENTAL PROCEDURES

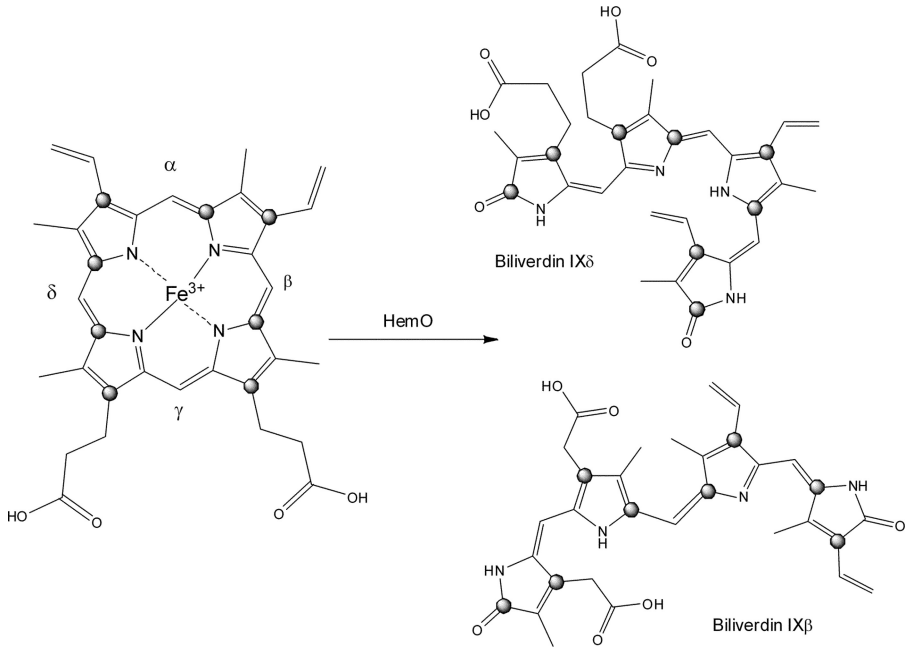
Bacterial Strains, Media, Growth Conditions, and Genetic Manipulation—All bacterial strains and plasmids used in this study are listed in Table 1. The *phuS* deletion was generated via diparental mating. The plasmid pEX18p-Δ*phuS*::tet was transferred to *P. aeruginosa* by conjugation with the *E. coli* S17-1 donor strain. Mutants were selected on tetracycline and double crossover mutants were obtained by sucrose challenge. To obtain a markerless mutant the tetracycline resistance cassette was removed using the flippase (FLP) recombinase encoded on the pFLP2 plasmid. Loss of the resistance cassette was confirmed by a lack of growth on tetracycline and the resulting *phuS* deletion mutant was verified by Southern blot analysis (data not shown). The pBSP*hemO* expression plasmid was constructed by cloning the 630-bp *hemO* or *hemO* N19K/K34A/F117Y/K132A mutant (hereon denoted as HemOα) gene into pBSP11 digested with NcoI and PstI.

Bacteria were routinely grown in Luria-Bertani (LB) broth at 37 °C at 200 rpm. Strains of *P. aeruginosa* and *E. coli* were maintained on *Pseudomonas* Isolation Agar (BD Biosciences) and LB plates, respectively. When necessary the following antibiotic concentrations were used: for *E. coli*, 100 μg/ml of ampicillin; for *P. aeruginosa*, 200 μg/ml of gentamicin.

For growth curves in nutrient defined medium, *P. aeruginosa* was grown overnight in LB media at 37 °C in a volume of 20 ml in 125-ml baffled Erlenmeyer flasks. The optical density of the overnight cultures was determined at 578 nm (A_{578}) and the cultures were then used to inoculate 100 ml of fresh M9 cultures (Na_2PO_4 6.78 g/liters; KH_2PO_4 3 g/liters; NaCl 0.5 g/liters; NH_4Cl 1 g/liters, 20 mM glucose; 2 mM $MgSO_4$; 0.1 mM $CaCl_2$) to a starting A_{578} of 0.05. Cells were grown at 37 °C in 250-ml Erlenmeyer baffled flasks at 200 rpm and cell density was monitored over a 24-h period. Heme was added to cultures to a final concentration of 5 or 0.5 μM as indicated. Heme stock solutions (500 μM) were prepared by dissolving biosynthetically prepared heme (see below) in 0.01 N NaOH and the concentration determined by the pyridine hemochrome assay (16). Heme solutions were prepared fresh for all supplementation experiments.

Preparation of BV-IX Isomers—The BV IX isomers were synthesized via coupled oxidation of heme as described previously with some modification (17). Briefly, heme (25 mg) was added to a mixture of 100 ml of pyridine and 300 ml of deionized water. After 20 min of constant stirring and saturation with O_2, L-ascorbic acid (0.5 to 1 g) was added to the heme solution. The heme solution was stirred for a further 10 min under O_2 saturation until the reaction turned dark green. The products were extracted into 100 ml of chloroform ($CHCl_3$) and the resulting green organic phase was washed twice with 100 ml of 1 mM citric acid (pH 4–5) and once with 100 ml of water. Any remaining water was removed from the organic phase by addition of anhydrous Na_2SO_4 and filtered through Whatman filter paper. The resulting washed organic phase was dried to completion in a rotary evaporator (Buchi, New Castle, DE). The resulting dried solid was resuspended in 4 ml of $CHCl_3$, dried under nitrogen, re-dissolved in 2 ml of degassed methanol, and immediately bubbled with nitrogen. The BV isomers were prepared by the addition of 0.4 ml of degassed 2 M KOH in methanol under N_2 saturation for 2 min, following which acetic acid was added to a final concentration of 25% (v/v). The BV isomer solution was diluted 10-fold with 0.1% (v/v) trifluoroacetic acid (TFA) and loaded onto a C_{18} Sep-Pak column (Waters, Milford, MA) previously equilibrated as follows: 3 ml of acetonitrile, 3 ml of deionized water, 3 ml of 0.1% (v/v) TFA, and 3 ml of 10% methanol in 0.1% (v/v) TFA. The column was washed with 4 ml of 0.1% (v/v) TFA and 4 ml of a mixture of acetonitrile and 0.1% (v/v) TFA (20:80). The BV isomers were eluted in 2 ml of acetonitrile, dried down, and analyzed by HPLC as described below.

Production of ^{12}C- and ^{13}C-Labeled Heme—δ-Aminolevulinic acid (ALA) or [4-^{13}C]δ-ALA were used as a biosynthetic precursor to produce ^{12}C- or ^{13}C-labeled heme, respectively. Unlabeled δ-ALA was purchased from Sigma and [4-^{13}C]δ-ALA from Cambridge Isotope Laboratories (Andover, MA). ^{13}C-Heme was prepared by a slight modification of the method



SCHEME 1

previously described by Rivera and Walker (18). Expression of cytochrome b_5 in the presence of 1 mM δ-ALA induces heme biosynthesis and heme is captured by the overexpressed apocytochrome b_5. Briefly, following expression of cytochrome b_5 in *E. coli* BL21(DE3) and lysis of the cells, the supernatant was applied to a Q-Sepharose column (3 × 10 cm) equilibrated in 50 mM Tris-HCl (pH 7.4) containing 50 mM NaCl. The column was then washed (5–10 column volumes) with the same buffer. The protein was eluted with 50 mM Tris-HCl (pH 7.4) containing 350 mM NaCl. Heme extraction from purified cytochrome b_5 was carried out by the acid-butanone method as previously described (19). An aliquot of heme following extraction was used to calculate the final yield by pyridine hemochrome (16). The ^{13}C-heme labeling pattern obtained with [4-^{13}C]δ-ALA is shown in Scheme 1.

Extraction of BV-IX Isomers from P. aeruginosa Supernatant and Cell Lysates—For *in vivo* biliverdin analyses overnight cultures of the *P. aeruginosa* strains grown in LB medium were used to inoculate a fresh M9 culture (20 ml) at a final A_{578} of 0.1. The cultures were grown for 8 h and used to inoculate a fresh 50 ml of M9 culture to an A_{578} of 0.05. Heme was added to a final concentration of 5 or 0.5 μM and $FeCl_3$ to a final concentration of 200 μM as indicated. Cells were grown for 15 h at 37 °C in a 250-ml baffled flask at 200 rpm and pelleted by centrifugation (40 min, 6000 × *g*, 4 °C). The remaining media (from here on referred to as the supernatant) were retained for BV extraction. The supernatant fractions were spiked with 20 μM BV IXα as an internal standard and acidified to pH 3 by addition of 10% (v/v) TFA. As an exception, the HemOα supernatant was spiked with 20 μM BV IXδ. BV isomers were extracted and purified over a C_{18} Sep-Pak column (Waters) as follows. The column was washed with 2 ml of acetonitrile, 2 ml of methanol, 2 ml of water, and 2 ml of 10% methanol in 0.1% TFA (v/v). The acidified supernatant was loaded on the column, which was then washed with 4 ml of 0.1% (v/v) TFA, 4 ml of acetonitrile, 0.1% (v/v) TFA (20:80), and 450 μl of methanol. The BV isomers were eluted with 650 μl of methanol and dried down for further analysis by HPLC as described below. A set of control extractions without the BV IXα or BV IXδ internal standards were performed to ensure only that the expected BV isomers were detected as products of the reaction.

HPLC Analysis of BV—The BV isomers were analyzed by reverse-phase HPLC (Beckman System Gold 126 with a UV-visible 168 Detector) over a Phenomenex Ultarcarb 5 μM ODS (9) analytical column (4.6 × 250 mm). Samples were prepared by resuspending in 10 μl of dimethyl sulfoxide and further diluted with a 50-μl mobile phase. Particulate material was removed by centrifugation (1 min at 14,000 × *g*) and filtered through a 0.45 μm PTFE syringe filter. BV isomers were separated with a mobile phase of acetone, 20 mM formic acid (50:50 (v/v)) at a flow rate of 0.6 ml/min with 377 nm detection. The *in vivo* BV regioselectivity of HemO following growth in *P. aeruginosa* supplemented with 5 μM heme was assigned based on the retention times of the BV IX isomers prepared by coupled oxidation and as previously reported (Fig. 2*A*) (9). BV peaks were collected for further analysis by electrospray ionization (ESI)-MS.

ESI-MS Analysis—Mass spectra were obtained in the positive ion mode on an amaZon X ion trap mass spectrometer (Bruker Daltonics Inc., Fremont, CA). The isolated BV isomers following C_{18} Sep-Pak purification or the individual HPLC BV peaks were introduced into the electrospray source by injection (500 μl) at a flow rate of 5 μl/min. The capillary voltage was set at 4.5 kV, temperature to 180 °C, and the sample was measured with an Ultra Scan Mode at 32.500 (*m*/*z*)/s and a scan range from 250 to 650 *m*/*z*. Tandem MS/MS experiments were performed manually by entering the *m*/*z* of the precursor with a width of 4.0. Product ions were acquired over the same range.

SDS-PAGE and Western Blot Analysis—Aliquots at various time points during growth were analyzed by SDS-PAGE. All

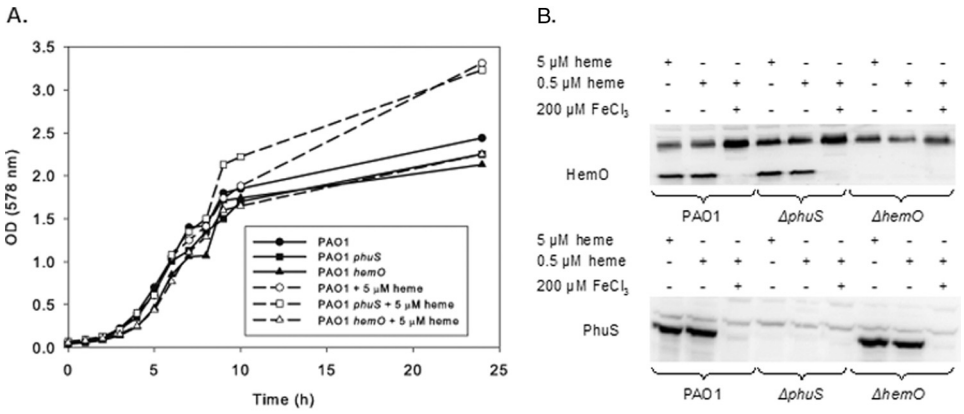


FIGURE 1. *A*, growth curves of the *P. aeruginosa* wild type (●), *phuS* (■), and *hemO* (▲) strains of *P. aeruginosa* in M9 media. *Solid lines* indicate cells grown without heme supplementation. *Dashed lines* indicate cells supplemented with 5 μM heme. *B*, Western blot analysis of the *P. aeruginosa* wild type, *phuS* and *hemO* deletion strains. Cells were harvested 15 h post-inoculation and 20 μg of total protein was separated by SDS-PAGE.

samples were adjusted to an A_{578} of 0.5/ml, centrifuged (1 min at 14,000 × *g*), and the pellets were resuspended in 60 μl of SDS sample buffer. Samples were heated at 100 °C for 10 min, centrifuged for 5 min at 14,000 × *g*, loaded (10 μl), and separated on 12.5% (w/v) SDS-PAGE. Following SDS-PAGE, proteins were electrophoretically transferred to PVDF membranes (Millipore, Billerica, MA) as previously described (21). The membranes were blocked with 5% blocking buffer (5% (w/v) skim milk in Tris-buffered saline (TBS) containing 0.2% (v/v) Tween 20) and probed with a 1:500 dilution of primary anti-HemO or anti-PhuS polyclonal antibody in 5% (w/v) blocking buffer. The membrane was then probed with goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (KPL, Inc., Gaithersburg, MD) at a dilution of 1:10,000 in TBS containing 0.2% (v/v) Tween 20. Proteins were visualized by enhanced chemiluminescence using the Super-Signal Chemiluminescence kit (Pierce).

RESULTS

HemO and PhuS Are Constitutively Expressed in Defined Minimal Medium—The growth of *P. aeruginosa* in M9 media was followed for 24 h. As shown in Fig. 1*A*, *P. aeruginosa* wild type, *phuS* and *hemO* deletion strains grew at similar rates in M9 media despite limited iron availability. When supplemented with 5 μM heme as an iron source the wild type and *phuS* deletion strains grew to a higher optical density. As expected, addition of 5 μM heme to the *hemO* deletion mutant did not result in a significant increase in growth due to the inability to utilize heme. Western blot analysis confirmed the loss of PhuS or HemO in the respective deletion strains (Fig. 1*B*).

The BV IXδ and BV IXβ Products of HemO-catalyzed Heme Degradation Are Excreted into Media—Whereas all previously characterized heme oxygenases oxidize heme at the α-meso carbon to yield BV IXα, *P. aeruginosa* HemO-dependent oxidative cleavage yields both BV IXδ and BV IXβ (Scheme 1) (8). It was unclear at the time if the production of both BV isomers was the result of *in vitro* heme reconstitution, where rotation of the heme around the α/γ-axis can place either the β- or δ-meso carbon in a position for cleavage. However, HPLC analysis of the BV isomers excreted into the media revealed both BV IXδ and BV IXβ (Fig. 2*B*). Extracts from 5 μM heme-supplemented cultures have an additional peak at 44 min corresponding to the remaining heme in the medium.

Extraction of the BV isomers from cultures supplemented with 0.5 μM heme was less efficient based on the recovery of the BV IXα internal standard (Fig. 2*C*). The less efficient BV extraction is most likely due to increased siderophore production as a consequence of the iron-restricted conditions. This was confirmed by supplementing the media with 0.5 μM heme and 200 μM $FeCl_3$ to suppress siderophore production. Extraction of the internal standard (BV IXα) from the siderophore-deficient cultures was similar to that in the 5 μM culture (Fig. 2*D*). As expected in the presence of 0.5 μM heme and 200 μM $FeCl_3$ little to no BV IXδ or IXβ was detected due to the Fur-dependent repression of *phuS* and *hemO* (Fig. 1*B*). An additional background peak is observed at 34 min in all extracts. This peak is unrelated to heme utilization as it is observed when the heme acquisition system is Fur-repressed (Fig. 2*D*).

BV IXδ and BV IXβ Products Are Derived from Oxidative Cleavage of Extracellular Heme—To determine the origin of the excreted BV IXδ and IXβ isomers, isotopic-labeling experiments were performed. *P. aeruginosa* cultures were supplemented with ^{12}C-heme or ^{13}C-heme prepared biosynthetically as described. ESI-MS analysis of the biosynthetically generated heme gave an *m*/*z* of 616.1 for ^{12}C-heme and 624.3 for ^{13}C-heme (Fig. 3, *B* and *C*), confirming the incorporation of eight ^{13}C atoms from [4-^{13}C]δ-ALA (Fig. 3*A*).

The BV products extracted from cultures supplemented with 5 μM ^{12}C-heme were analyzed by HPLC and ESI-MS and a peak at *m*/*z* 583.1 consistent with the $[M + H]^+$ of BV was observed for both BV IXβ and BV IXδ isomers (Fig. 4*B*). The ^{12}C-BV IXδ and BV IXβ isomers were further analyzed by tandem ESI-MS/MS where fragmentation yields dominant product ions at *m*/*z* 402.1 and 343.1 for BV IXβ and BV IXδ, respectively (Fig. 4*B*).

In contrast, ESI-MS analysis of the excreted BV isomers following growth of *P. aeruginosa* in ^{13}C-heme yielded an ion species at *m*/*z* 591.3, confirming that the excreted BV products arise from uptake and utilization of extracellular heme (Fig. 4*C*). BV IXβ was further characterized by tandem ESI-MS/MS

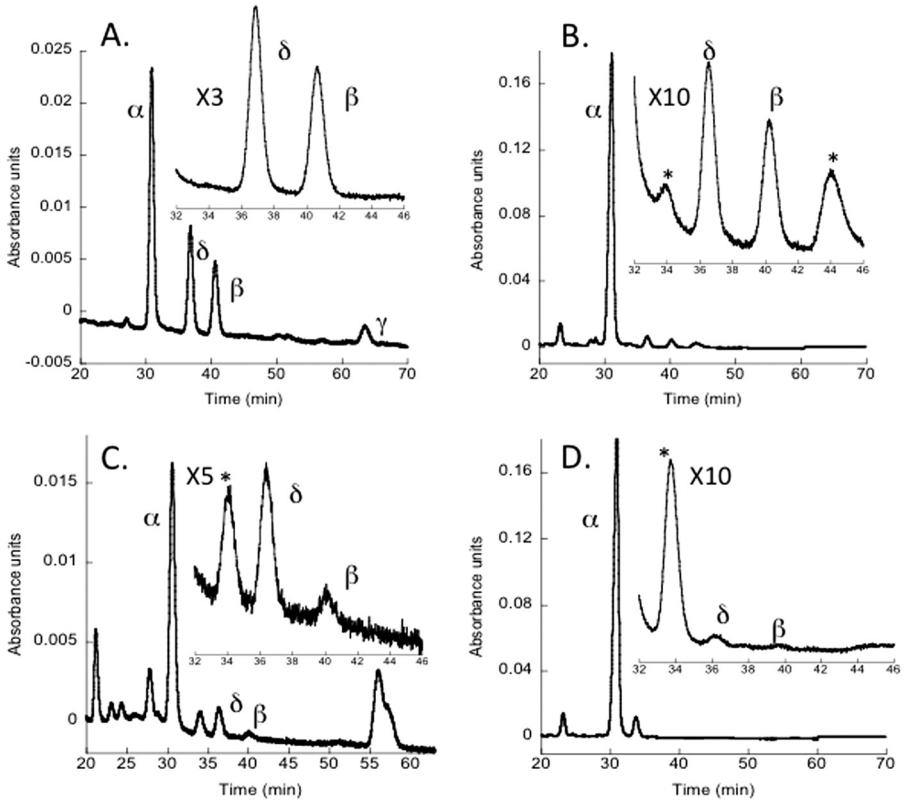


FIGURE 2. **HPLC trace of the supernatant extracts of *P. aeruginosa* wild type cells grown with heme.** *A*, 20 μM biliverdin standards generated by coupled oxidation of heme; *B*, extracts from cells grown with 5 μM heme; *C*, cells grown with 0.5 μM heme; *D*, cells grown with 0.5 μM heme and 200 μM $FeCl_3$. Extracts were spiked with BV IXα (20 μM) to assess extraction efficiency. *Inset*, magnification of BV IXδ and BV IXβ elution profiles.

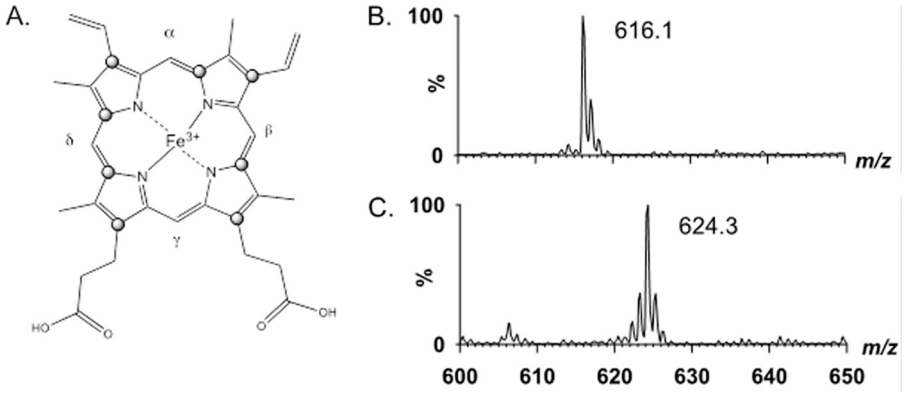


FIGURE 3. **ESI-MS of biosynthetically prepared ^{12}C-heme and ^{13}C-heme.** *A*, ^{13}C-labeling pattern of heme generated from [4-^{13}C]δ-ALA; *B*, ESI-MS of ^{12}C-labeled heme with *m*/*z* of 616.1; *C*, ESI-MS of ^{13}C-labeled heme with *m*/*z* of 624.3.

where a major fragment ion at *m*/*z* 408.1 corresponds to an increased mass of six from cleavage between the C and D pyrroles (Fig. 4*A*). ESI-MS/MS analysis of BV IXδ gave a fragment at *m*/*z* 347.1 corresponding to an increased mass of four as a result of cleavage between the D and A pyrroles (Fig. 4*A*). The present results confirm that the excreted BV isomers arise solely from utilization and degradation of extracellular heme.

Extracellular Heme Uptake and Metabolism Is Driven by Catalytic Activity of HemO—HPLC analysis of the *hemO* deletion strain supplemented with 5 μM ^{13}C-heme revealed only the internal standard BV IXα with no evidence of BV IXδ or BV IXβ (Fig. 5*B*). Extraction of BV isomers in the absence of the BV IXα internal standard showed no evidence of BV production (data not shown). The lack of HemO protein in the *P. aeruginosa* Δ*hemO* strain was confirmed by Western blot (Fig. 5*A*). On complementation of the *hemO* deletion strain with a plasmid expressing HemO, heme utilization was restored with BV IXδ and BV IXβ being detected at similar levels to the wild type strain (Fig. 5*C*). Western blot analysis confirmed that HemO protein levels were comparable with those of wild type *P. aeruginosa* (Fig. 5*A*). Interestingly ESI-MS analysis revealed that BV IXδ and BV IXβ were derived from both extracellular ^{13}C-heme and intracellular ^{12}C-heme (Fig. 5*D*). The turnover of intracellular heme is a result of elevated HemO levels at early time points in the complemented strains compared with the wild type strain (supplemental Fig. S1). Hence the presence of

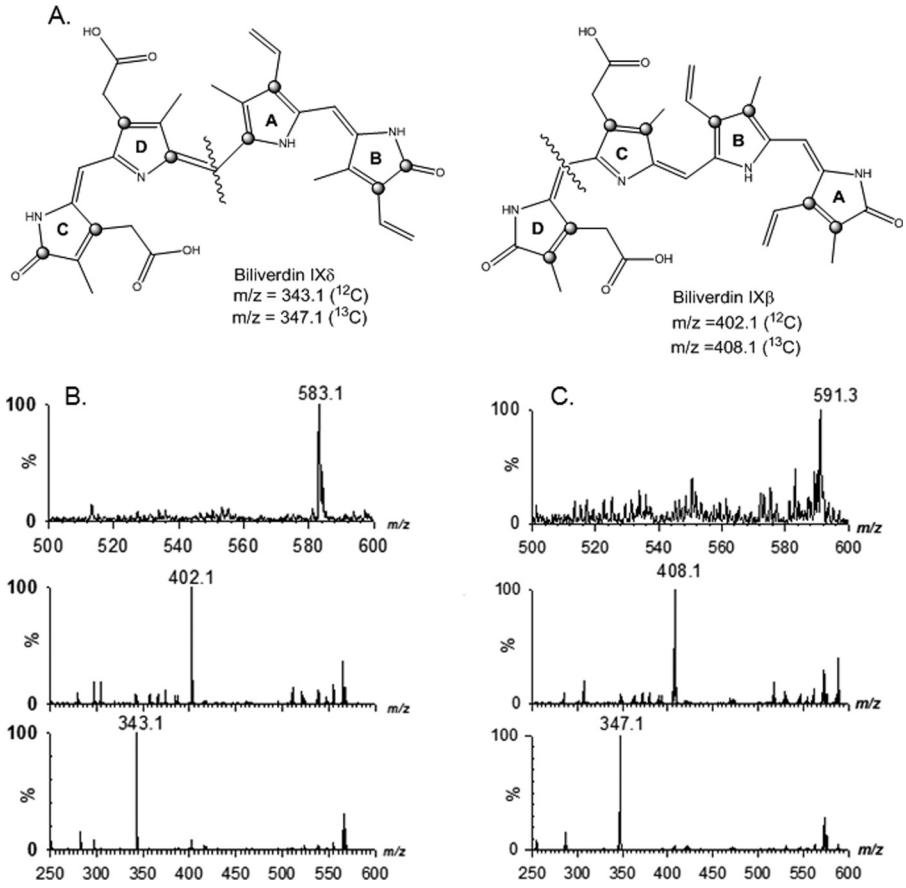


FIGURE 4. **ESI-MS and ESI-MS/MS analyses of the BV IXδ and BV IXβ peaks from the supernatant of the *P. aeruginosa* wild type strain supplemented with 5 μM ^{12}C-heme or ^{13}C-heme.** *A*, fragmentation pattern and expected *m/z* for ^{12}C- and ^{13}C-labeled BV IXδ and BV IXβ; *B*, ESI-MS of ^{12}C-BV IXδ (BV IXβ not shown as both isomers have an *m/z* of 583.1) (*upper panel*) fragmentation pattern of ^{12}C-BV IXβ showing an *m/z* peak at 402.1 (*middle panel*), and fragmentation of ^{12}C-BV IXδ showing an *m/z* at 343.1 (*lower panel*). *C*, ESI-MS of ^{13}C-BV IXδ (*upper panel*), fragmentation pattern of ^{13}C-BV IXβ showing an *m/z* peak at 408.1 (*middle panel*), and fragmentation of ^{13}C-BV IXδ showing an *m/z* at 347.1 (*lower panel*).

HemO in the complemented strains prior to up-regulation of heme uptake proteins in iron-restricted conditions shunts heme from the intracellular pool.

This was further confirmed on complementation of the Δ*hemO* strain with a plasmid encoding HemOα, which yields BV IXα (Fig. 5*E*). Expression levels of HemOα were significantly higher than the wild type HemO-complemented Δ*hemO* strain (Fig. 5*A* and supplemental Fig. S1). As a consequence of the elevated HemOα at early time points the ratio of ^{12}C-BV IXα to ^{13}C-BV IXα was higher than that observed for the HemO-complemented strain. Taken together the present data confirms that the metabolic flux of extracellular heme through the uptake system is controlled by the catalytic action of HemO.

Cytoplasmic Heme-binding Protein PhuS Is Not a Heme Degrading Factor—The function of the intracellular cytoplasmic heme-binding protein PhuS was determined by analysis of the BV metabolite profile in the *P. aeruginosa* Δ*phuS* strain. HPLC analysis of the BV products from the supernatant of *P. aeruginosa* Δ*phuS* supplemented with 5 μM heme revealed a similar yield of BV IXβ and BV IXδ to that of the wild type strain (Fig. 6*A*). Analysis by ESI-MS revealed peaks for BV isomers at *m/z* 591.3 indicating conversion of extracellular heme (^{13}C-heme) to BV IXδ and BV IXβ (Fig. 6*B*). The lack of BV in the absence of HemO but not PhuS confirms that the cytoplasmic heme-binding proteins are not heme oxygenases, as has been previously reported.

DISCUSSION

A combination of bacterial genetics and ^{13}C-heme labeling techniques provided a platform to determine the metabolic profile and functional relationship between the cytoplasmic heme-binding protein PhuS and iron-regulated HemO. The increased growth rate of *P. aeruginosa* wild type cultures supplemented with heme coincided with the excretion of BV IXδ and BV IXβ in the media. Furthermore, when supplemented with ^{13}C-heme both BV IXδ and BV IXβ were exclusively ^{13}C-labeled indicating that under iron limitation, exogenous heme is the major source of iron. Although *in vitro* production of both BV IXδ and IXβ by HemO is a result of rotation around the heme α/γ-axis as a consequence of reconstitution with free heme, it was postulated that only one isomer would be produced *in vivo* due to the specific protein-mediated delivery of heme to HemO (8, 10, 19). The relevance if any of enzymatic cleavage to yield both BV IXδ and BV IXβ isomers is not known, but may reflect alternate heme delivery pathways to HemO.

In contrast, the *hemO* deletion strain under iron-restricted conditions produced no detectable levels of BV confirming that HemO is absolutely required for driving the metabolic flux of

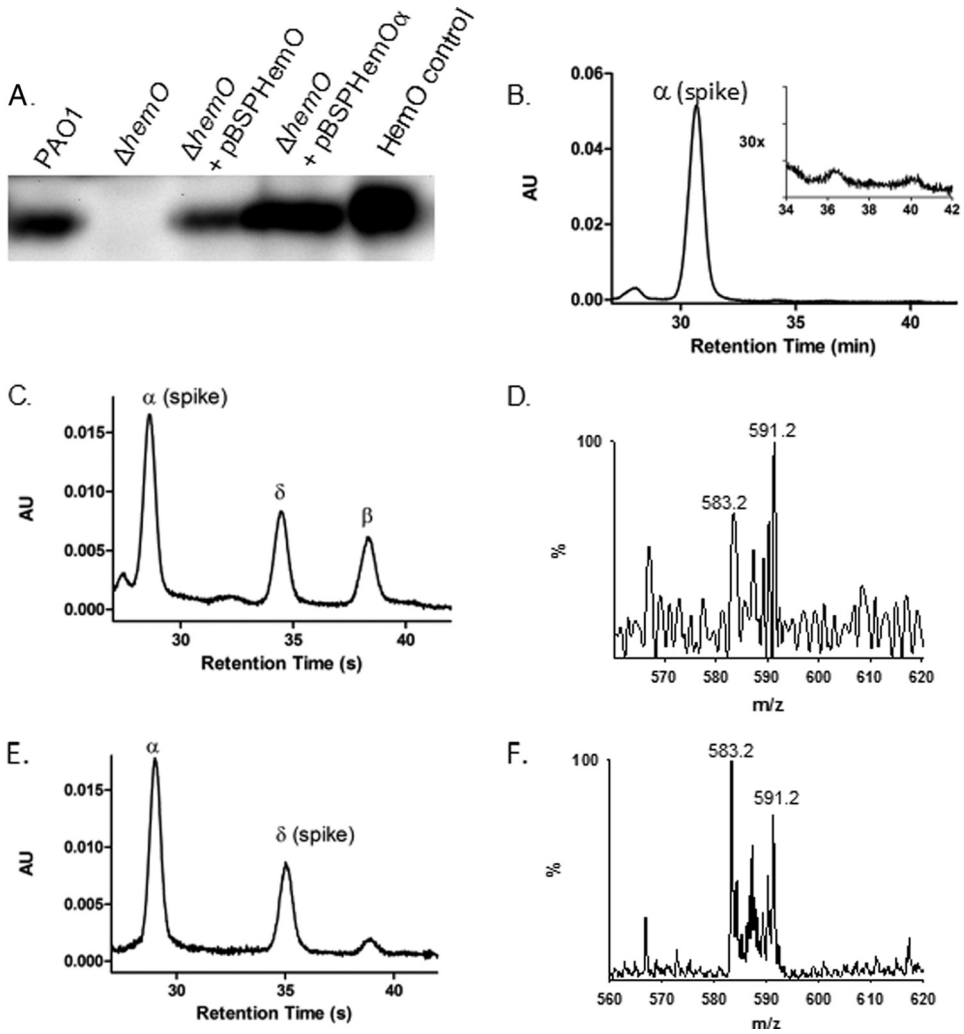


FIGURE 5. **HPLC and ESI-MS analyses of BV products from *P. aeruginosa* Δ*hemO* supplemented with 5 μM heme.** *A,* Western blot analysis of HemO in *P. aeruginosa* cultures at 15 h; *B,* HPLC of *P. aeruginosa* Δ*hemO*; *C,* HPLC of *P. aeruginosa* Δ*hemO* complemented with pBSPHemO; *D*, ESI-MS analysis of the BV IXδ and BV IXβ products from *P. aeruginosa* Δ*hemO* complemented with pBSPHemO; *E,* HPLC *P. aeruginosa* Δ*hemO* complemented with pBSPHemOα; *F,* ESI-MS of the BV IXδ and BV IXβ products from *P. aeruginosa* Δ*hemO* complemented with pBSPHemOα. Experiments were performed as described under "Experimental Procedures."

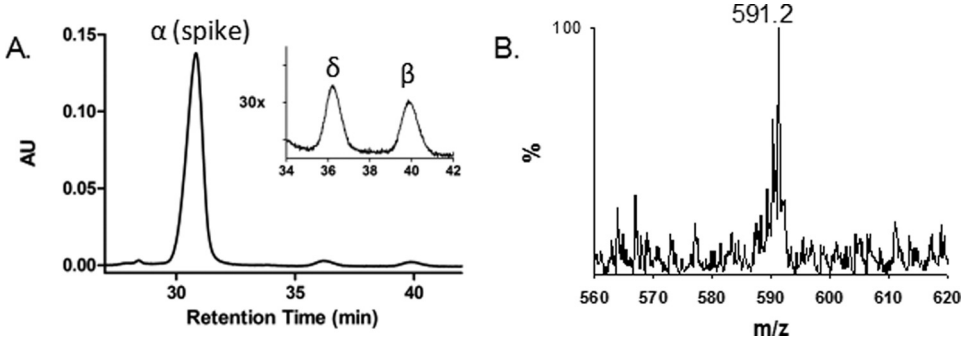


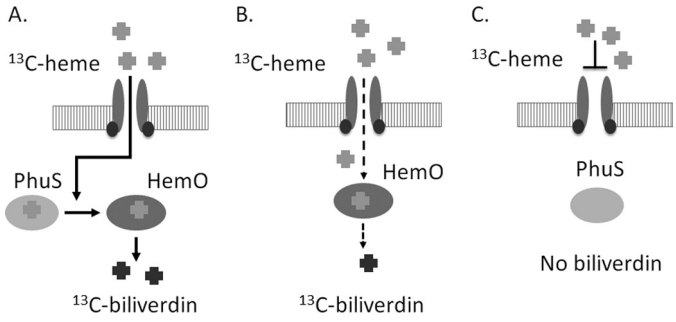
FIGURE 6. **HPLC (*A*) and ESI-MS (*B*) of the supernatant from the *P. aeruginosa phuS* strain supplemented with ^{13}C-heme (5 μM).**

heme through the heme uptake system. The role of HemO in driving heme utilization is independent of regioselectivity as observed by complementation of the Δ*hemO* strain with a plasmid expressing either wild type HemO or the BV IXα producing HemOα. Interestingly, uncoupling HemO expression from transcriptional regulation in the complemented Δ*hemO* strain results in degradation of intracellular heme as up-regulation of heme uptake proteins lags behind HemO levels. Therefore, synchronization of HemO levels with the heme uptake proteins is essential for maintaining the metabolic flux of extracellular heme and hence intracellular heme homeostasis. Although the *phu* and *hemO* operons are iron-regulated the *hemO* operon also encodes a putative extra-cytoplasmic function σ factor-dependent signaling system (22). At the present time it is not

known if HemO is regulated by heme or BV IXδ and/or BV IXβ through an extra-cytoplasmic function σ factor-dependent signaling pathway, similar to that described for siderophore uptake (22).

The lack of BV on deletion of the *hemO* gene definitively shows there is no redundancy between the HemO and BphO enzymes as BphO cannot substitute for HemO. It has previously been shown that BphO-dependent cleavage of heme to BV IXα is required for the signaling activity of the bacteriophytochrome kinase, BphP, and that BV IXδ and BV IXβ are not substrates for BphP (9). *In vitro* experiments had shown holo-PhuS interacts with HemO but not BphO suggesting the pathways are independent (15). Furthermore, previous reports have suggested homologs of the cytoplasmic heme-binding protein PhuS are heme oxygenases (13). In the current studies the lack of ^{13}C-BV IXδ and IXβ in the media on deletion of *hemO*, but not *phuS*, definitively shows that the cytoplasmic heme-binding proteins are not heme oxygenases. Indeed the current studies are more consistent with previous reports of inefficient heme utilization on deletion of *hemS* and *shuS* in *Y. enterocolitica* and *Shigella dysenteriae*, respectively (12, 23). In an earlier study the *P. aeruginosa* Δ*phuS* mutant phenotype of inefficient heme utilization was accompanied by the early production of pyocyanin, which could be suppressed by increasing heme concentrations (20). In addition transcriptional analysis of the *P. aeruginosa* Δ*phuS* mutant at low heme concentrations indicated the cells were manifesting an iron starvation response, despite intracellular iron levels similar to those of the wild type strain. However, it is clear that under the present conditions PhuS is not essential for heme uptake as revealed by exclusive ^{13}C labeling of the BV IXδ and BV IXβ products. Based on the previous *P. aeruginosa* Δ*phuS* phenotype and transcriptional analysis we might have expected that the loss of PhuS would disrupt extracellular heme uptake with the resulting turnover of intracellular heme. Although no ^{12}C-BV IXδ and IXβ were detected in the Δ*phuS* mutant grown in the presence of extracellular ^{13}C-heme we propose this is a result of the higher than physiologically relevant levels of heme (0.5 μM). At such heme levels, the lack of PhuS is not manifested in the metabolite profile, whereas at lower levels of extracellular heme, we would expect to detect ^{13}C- and ^{12}C-BV IXδ/β. However, in the current studies we were not able to reliably detect the BV products at heme concentrations below 0.5 μM.

Based on the current findings we propose a model for heme uptake and utilization in *P. aeruginosa* where the concerted action of PhuS and HemO drive the metabolic flux of heme into the cell (Scheme 2*A*). On loss of PhuS an uncoupling of extracellular heme delivery to HemO under heme limiting conditions results in inefficient heme utilization (Scheme 2*B*). In contrast, deletion of *hemO* shuts down the metabolic flux of extracellular heme through the system, despite the presence of heme in the extracellular environment, and the bacteria switch to utilizing alternative iron-acquisition systems (Scheme 2*C*). Thus, the effective coupling of heme catabolism to BV IXδ and/or IXβ to heme availability provides a potential mechanism by which the organism can rapidly respond at the transcriptional level to the extracellular environment.



SCHEME 2

In summary, the present studies have identified a central role for HemO in driving heme uptake via the catalytic conversion of extracellular heme to BV. Furthermore, the present *in vivo* studies definitively show that the cytoplasmic heme-binding proteins are not heme oxygenases. Metabolic studies such as those described herein are critical in combination with genetic complementation studies to confirm biological function. We suggest that future studies aimed at disrupting heme uptake and utilization may lead to novel therapeutic strategies targeting global iron-homeostasis and virulence.

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