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

Received for publication, March 6, 2012, and in revised form, March 27, 2012. Published, JBC Papers in Press, April 9, 2012, DOI 10.1074/jbc.M112.359265

Kylie D. Barker, Katalin Barkovits, and Angela Wilks

From the Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, Baltimore, Maryland 21201-1140

**Background:** *Pseudomonas aeruginosa* utilizes extracellular heme as a source of iron.

**Results:** Deletion of hemO results in loss of 13C-heme uptake and degradation to 13C-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 (13C-heme) and electrospray ionization-MS analysis we have confirmed the regioselectivity and ratio of 13C-δ and β-BV IX (70:30) is identical *in vivo* to that previously observed for the cellular degradation. Furthermore, the 13C-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* ΔhemO 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 13C-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 purification, growth, and virulence. In addition to receptor-mediated iron-siderophore scavenging mechanisms, many bacteria utilize heme and hemoproteins 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, sequencers 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 enterocolitica*, 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 or hemin degrading factor, based solely on the observation of an inability to efficiently utilize heme on deletion of the *hemS* gene (12).

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...
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-ΔphuS::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 Southern blot analysis (data not shown). The pBSPHemO expression plasmid was constructed by cloning the 630-bp phuS::::tet gene encoding a water-soluble domain of the cytochrome b3 gene into a derivative of pBBR1MCS3 under control of the araC-P~ara~ cassette. This study

---

**TABLE 1**

**Bacterial strains and plasmids**

| Strain            | Description                                   | Source or Ref. |
|-------------------|-----------------------------------------------|----------------|
| **E. coli**       |                                               |                |
| BL21(DE3)         | F’ dcm ompT lacz (rpsL mcrA) galA (DE3)        |                |
| S17-1             | pro thi isdR Tpr Sm r:: chromosome::RP4-2 Tc::Mu-Km::Tn7/Apir |                |
| **P. aeruginosa** |                                               |                |
| PAO1              | Wild type                                     | 26             |
| PAO1 hemO         | PAO1 hemO::aacC1 Gm                           | 27             |
| PAO1 phuS         | PAO1 ΔphuS                                    | This study     |
| **Plasmids**      |                                               |                |
| MRL2              | Amp4; pET-11a derivative harbouring the rat liver outer mitochondrial membrane cytochrome b3 | 18             |
|                   | gene encoding a water-soluble domain of the cytochrome b3 |                |
| pFLP2             | Amp4; source of FLP recombinase                | 25             |
| pEX18p-ΔphuS::tet | Amp4; 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 pBRR1MCS3 under control of the araC-P~ara~ cassette | This study     |

---

**Production of 12C- and 13C-Labeled Heme—δ-Aminolevulinic acid (ALA) or [4-13C]δ-ALA were used as a biosynthetic precursor to produce 12C- or 13C-labeled heme, respectively. Unlabeled δ-ALA was purchased from Sigma and [4-13C]δ-ALA from Cambridge Isotope Laboratories (Andover, MA). 13C-Heme was prepared by a slight modification of the method**
previously described by Rivera and Walker (18). Expression of cytochrome \( b_5 \) in the presence of 1 mM \( \Delta^5 \)-ALA induces heme biosynthesis and heme is captured by the overexpressed apo-
cytochrome \( b_5 \). 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]-\( \Delta^5 \)-ALA is
shown in Scheme 1.

Extraction of BV-IX Isomers from \( P. aeruginosa \) Supernatant
and Cell Lysates—For \( \textit{in vivo} \) biliverdin analyses overnight cul-
tures 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 \( \mu \)M and FeCl\(_3\) to a final concentration
of 200 \( \mu \)M 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 \( \times \) 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 \( \mu \)M BV IX as an
internal standard and acidified to pH 3 by addition of 10% (v/v)
TFA. As an exception, the HemO supernatant was spiked with
20 \( \mu \)M BV IX. 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 acid-
fied 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 \( \mu \)l of methanol. The BV isomers
were eluted with 650 \( \mu \)l of methanol and dried down for further
analysis by HPLC as described below. A set of control extrac-
tions without the BV IX or BV IX\(_8\) internal standards were
performed to ensure only that the expected BV isomers were
detected as products of the reaction.

\textbf{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 Ultarbcarb 5 \( \mu \)M ODS
(9) analytical column (4.6 × 250 mm). Samples were prepared
by resuspending in 10 \( \mu \)l of dimethyl sulfoxide and further
diluted with a 50-\( \mu \)l mobile phase. Particulate material was
removed by centrifugation (1 min at 14,000 \( \times \) g) and filtered
through a 0.45 \( \mu \)m PTFE syringe filter. BV isomers were sepa-
rated 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 \( \textit{in}
\textit{vivo} \) BV regioselectivity of HemO following growth in \( P. aerugi-
nosa \) supplemented with 5 \( \mu \)M heme was assigned based on the
retention times of the BV IX isomers prepared by coupled oxi-
dation and as previously reported (Fig. 2A) (9). BV peaks were
collected for further analysis by electrospray ionization
(ESI)-MS.

\textbf{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 follow-
ing C\(_{18}\) Sep-Pak purification or the individual HPLC BV peaks
were introduced into the electrospray source by injection (500
\( \mu \)l) at a flow rate of 5 \( \mu \)l/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.

\textbf{SDS-PAGE and Western Blot Analysis—}Aliquots at various
time points during growth were analyzed by SDS-PAGE. All
samples were adjusted to an A₅₇₈ 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 membranes were 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. 1A, 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. 1B).

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. 2B). 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. 2C). 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 FeCl₃ 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. 2D). As expected in the presence of 0.5 μM heme and 200 μM FeCl₃ little to no BV IXδ or IXβ was detected due to the Fur-dependent repression of phuS and hemO (Fig. 1B). 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. 2D).

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 12C-heme or 13C-heme prepared biosynthetically as described. ESI-MS analysis of the biosynthetically generated heme gave an m/z of 616.1 for 12C-heme and 624.3 for 13C-heme (Fig. 3, B and C), confirming the incorporation of eight 13C atoms from [4-13C]δ-ALA (Fig. 3A).

The BV products extracted from cultures supplemented with 5 μM 13C-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. 4B). The 12C- and 13C-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. 4B).

In contrast, ESI-MS analysis of the excreted BV isomers following growth of P. aeruginosa in 13C-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. 4C). BV IXβ was further characterized by tandem ESI-MS/MS.
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. 4A). ESI-MS/MS analysis of BV IX 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. 4A). 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 $5 \, \mu M$ heme revealed only the internal standard BV IX$\alpha$ with no evidence of BV IX$\delta$ or BV IX$\beta$ (Fig. 5B). Extraction of BV isomers in the absence of the BV IX$\alpha$ internal standard showed no evidence of BV production (data not shown). The lack of HemO protein in the P. aeruginosa ΔhemO strain was confirmed by Western blot (Fig. 5A). On complementation of the hemO deletion strain with a plasmid expressing HemO, heme utilization was restored with BV IX$\delta$ and BV IX$\beta$ being detected at similar levels to the wild type strain (Fig. 5C). Western blot analysis confirmed that HemO protein levels were comparable with those of wild type P. aeruginosa (Fig. 5A). Interestingly ESI-MS analysis revealed that BV IX$\delta$ and BV IX$\beta$ were derived from both extracellular $^{13}$C-heme and intracellular $^{12}$C-heme (Fig. 5D). 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
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 \( \text{HemO} \) strain with a plasmid encoding \( \text{HemO} \), which yields BV IX \( \text{HemO} \) (Fig. 5). Expression levels of \( \text{HemO} \) were significantly higher than the wild type \( \text{HemO} \)-complemented \( \text{HemO} \) strain (Fig. 5A and supplemental Fig. S1). As a consequence of the elevated \( \text{HemO} \) at early time points the ratio of \( \text{BV IX} \) to \( \text{BV IX} \) was higher than that observed for the \( \text{HemO} \)-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 \( \text{HemO} \).

**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 \( \text{P. aeruginosa} \) wild type culture supplemented with \( \text{heme} \) (Fig. 5E). When PhuS was present, the BV IX \( \text{PhuS} \) strain (Fig. 5A and supplemental Fig. S1). As a consequence of the elevated PhuS at early time points the ratio of \( \text{BV IX} \) to \( \text{BV IX} \) was higher than that observed for the \( \text{PhuS} \)-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 \( \text{PhuS} \).

**DISCUSSION**

A combination of bacterial genetics and \( \text{C}-\)heme labeling techniques proved 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 \( \text{P. aeruginosa} \) wild type cultures supplemented with \( \text{heme} \) coincided with the excretion of \( \text{BV IX} \) and \( \text{BV IX} \) in the media. Furthermore, when supplemented with \( \text{C}-\)heme both \( \text{BV IX} \) and \( \text{BV IX} \) were exclusively \( \text{C}-\)labeled indicating that under iron limitation, exogenous heme is the major source of iron. Although \( \text{in vitro} \) production of both \( \text{BV IX} \) and \( \text{BV IX} \) by \( \text{HemO} \) is a result of rotation around the heme \( \alpha/\gamma \)-axis as a consequence of reconstitution with free heme, it was postulated that only one isomer would be produced \( \text{in vivo} \) due to the specific protein-mediated delivery of heme to \( \text{HemO} \) (8, 10, 19). The relevance if any of enzymatic cleavage to yield both \( \text{BV IX} \) and \( \text{BV IX} \) isomers is not known, but may reflect alternate heme delivery pathways to \( \text{HemO} \).

In contrast, the \( \text{hemO} \) deletion strain under iron-restricted conditions produced no detectable levels of BV confirming that \( \text{HemO} \) is absolutely required for driving the metabolic flux of heme-binding proteins are not heme oxygenases, as has been previously reported.
heme through the heme uptake system. The role of HemO in driving heme utilization is independent of regioselectivity as observed by complementation of the ΔhemO 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 ΔhemO 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

**FIGURE 5.** HPLC and ESI-MS analyses of BV products from *P. aeruginosa* ΔhemO supplemented with 5 μM heme. A, Western blot analysis of HemO in *P. aeruginosa* cultures at 15 h; B, HPLC of *P. aeruginosa* ΔhemO; C, HPLC of *P. aeruginosa* ΔhemO complemented with pBSPHemO; D, ESI-MS analysis of the BV IXδ and BV IXβ products from *P. aeruginosa* ΔhemO complemented with pBSPHemO; E, HPLC of *P. aeruginosa* ΔhemO complemented with pBSPHemOα; F, ESI-MS of the BV IXδ and BV IXβ products from *P. aeruginosa* ΔhemO 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 13C-heme (5 μM).
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 bacteriochlorophyll a kinase, BphP, and that BV IXδ and BV IXβ are not substrates for BphP (9). In vitro experiments had shown holophoS 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 13C-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 ΔphuS 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 ΔphuS 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 13C labeling of the BV IXδ and BV IXβ products. Based on the previous P. aeruginosa ΔphuS 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 13C-BV IXδ and IXβ were detected in the ΔphuS mutant grown in the presence of extracellular 13C-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 13C- and 12C-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 2A). On loss of PhuS an uncoupling of extracellular heme delivery to HemO under heme limiting conditions results in inefficient heme utilization (Scheme 2B). 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 2C). 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.

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.

Acknowledgment—We thank Professor Mario Rivera for the kind gift of the cytochrome b₅ expression plasmid.

REFERENCES

1. Wilks, A., and Burkhard, K. A. (2007) Heme and virulence. How bacterial pathogens regulate, transport and utilize heme. Nat. Prod. Rep. 24, 511–522
2. Wilks, A., and Barker, K. D. (2011) in Handbook of Porphyrin Science (Kadish, K. M., Smith, K. M., and Guillard, R., eds) 1st Ed., pp. 357–398, World Scientific, Singapore
3. Ochsnert, U. A., Johnson, Z., and Vasil, M. L. (2000) Genetics and regulation of two distinct haem-uptake systems, phu and has; in Pseudomonas aeruginosa. Microbiology 146, 185–198
4. Krieg, S., Huché, F., Diederichs, K., Izadi-Pruneyre, N., Lecroisey, A., Wandersman, C., Delepeleire, P., and Welte, W. (2009) Heme uptake across the outer membrane as revealed by crystal structures of the receptor-hemophore complex. Proc. Natl. Acad. Sci. U.S.A. 106, 1045–1050
5. Cescau, S., Cwerman, H., Létóffé, S., Delepeleire, P., Wandersman, C., and Biville, F. (2007) Heme acquisition by hemophores. Biometals 20, 603–613
6. Létóffé, S., Deniau, C., Wolff, N., Dassa, E., Delepeleire, P., Lecroisey, A., and Wandersman, C. (2001) Haemophore-mediated bacterial heme transport. Evidence for a common or overlapping site for heme-free and heme-loaded haemophore on its specific outer membrane receptor. Mol. Microbiol. 41, 439–450
7. Wilks, A., and Schmitt, M. P. (1998) 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
8. Ratliff, M., Zhu, W., Deshmukh, R., Wilks, A., and Stojilkovic, I. (2001) Homologues of Neisseria heme oxygenase in Gram-negative bacteria. Degradation of heme by the product of the pigA gene of Pseudomonas aeruginosa. J. Bacteriol. 183, 6394–6403
9. Wegele, R., Tasler, R., Zeng, Y., Rivera, M., and Frankenberg-Dinkel, N. (2004) The heme oxygenase(s)-phytochrome system of Pseudomonas aeruginosa. J. Biol. Chem. 279, 45791–45802
10. Friedman, J., Lad, L., Li, H., Wilks, A., and Poulos, T. L. (2004) Structural basis for novel δ-regioselective heme oxygenation in the opportunistic pathogen Pseudomonas aeruginosa. Biochemistry 43, 5239–5245
11. Schuller, D. J., Zhu, W., Stojiljkovic, I., Wilks, A., and Poulos, T. L. (2001) Crystal structure of heme oxygenase from the Gram-negative pathogen Neisseria meningitidis and a comparison with mammalian heme oxygenase-1. *Biochemistry* **40**, 11552–11558

12. Stojiljkovic, I., and Hantke, K. (1994) Hemin uptake system of Yersinia enterocolitica. Similarities with other TonB-dependent systems in Gram-negative bacteria. *Mol. Microbiol.* **13**, 719–732

13. Suits, M. D., Pal, G. P., Nakatsu, K., Matte, A., Cygler, M., and Jia, Z. (2005) Identification of an *Escherichia coli* O157:H7 heme oxygenase with tandem functional repeats. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 16955–16960

14. Block, D. R., Lukat-Rodgers, G. S., Rodgers, K. R., Wilks, A., Bhakta, M. N., and Lansky, I. B. (2007) Identification of two heme-binding sites in the cytoplasmic heme-trafficking protein PhuS from *Pseudomonas aeruginosa* and their relevance to function. *Biochemistry* **46**, 14391–14402

15. Lansky, I. B., Lukat-Rodgers, G. S., Block, D., Rodgers, K. R., Ratliff, M., and Wilks, A. (2006) The cytoplasmic heme-binding protein (PhuS) from the heme uptake system of *Pseudomonas aeruginosa* is an intracellular heme-trafficking protein to the β-regioselective heme oxygenase. *J. Biol. Chem.* **281**, 13652–13662

16. Fuhrop, J. H., and Smith, K. M. (eds) (1975) *Porphyrins and Metalloporphyrins*, Elsevier, Amsterdam

17. Bonnett, R., and McDonagh, A. F. (1973) The meso-reactivity of porphyrins and related compounds. VI. Oxidative cleavage of the haem system. The four isomeric biliverdins of the IX series. *J. Chem. Soc. Perkin Trans. 1* **9**, 881–888

18. Rivera, M., and Walker, F. A. (1995) Biosynthetic preparation of isotopically labeled heme. *Anal. Biochem.* **230**, 295–302

19. Caignan, G. A., Deshmukh, R., Wilks, A., Zeng, Y., Huang, H. W., Moëne-Loccoz, P., Bunce, R. A., Eastman, M. A., and Rivera, M. (2002) Oxidation of heme to β- and δ-biliverdin by *Pseudomonas aeruginosa* heme oxygenase as a consequence of an unusual seating of the heme. *J. Am. Chem. Soc.* **124**, 14879–14892

20. Kaur, A. P., Lansky, I. B., and Wilks, A. (2009) The role of the cytoplasmic heme-binding protein (PhuS) of *Pseudomonas aeruginosa* in intracellular heme trafficking and iron homeostasis. *J. Biol. Chem.* **284**, 56–66

21. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

22. Beare, P. A., For, R. J., Martin, L. W., and Lamont, I. L. (2003) Siderophore-mediated cell signaling in *Pseudomonas aeruginosa*. Divergent pathways regulate virulence factor production and siderophore receptor synthesis. *Mol. Microbiol.* **47**, 195–207

23. Wyckoff, E. E., Lopreato, G. F., Tipton, K. A., and Payne, S. M. (2005) *Shigella dysenteriae* ShuS promotes utilization of heme as an iron source and protects against heme toxicity. *J. Bacteriol.* **187**, 5658–5664

24. de Lorenzo, V., and Timmis, K. N. (1994) Analysis and construction of stable phenotypes in Gram-negative bacteria with Tn5- and Tn10-derived minitransposons. *Methods Enzymol.* **235**, 386–405

25. Hoang, T. T., Karkhoff-Schweizer, R. R., Kutchma, A. J., and Schweizer, H. P. (1998) A broad host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences. Application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* **212**, 77–86

26. Holloway, B. W. (1955) Genetic recombination in *Pseudomonas aeruginosa*. *J. Gen. Microbiol.* **13**, 572–581

27. Oglesby-Sherrouse, A. G., and Vasil, M. L. (2010) Characterization of a heme-regulated noncoding RNA encoded by the prrF locus of *Pseudomonas aeruginosa*. *PLoS One* **5**, e9930