THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 1, pp. 56–66, January 2, 2009
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The Role of the Cytoplasmic Heme-binding Protein (PhuS) of *Pseudomonas aeruginosa* in Intracellular Heme Trafficking and Iron Homeostasis*[S]

Received for publication, August 6, 2008, and in revised form, November 5, 2008 Published, JBC Papers in Press, November 5, 2008, DOI 10.1074/jbc.M806068200

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The cytoplasmic heme-binding protein PhuS, encoded within the Fur-regulated *Pseudomonas* heme utilization (*phu*) operon, has previously been shown to traffic heme to the iron-regulated heme oxygenase (HO). We further investigate the role of PhuS in heme trafficking to HO on disruption of the *phuS* and *hemO* genes in a *Pseudomonas aeruginosa* siderophore-deficient and wild-type background. Previous studies have shown that deletion of *hemO* prevents the cells from utilizing heme as the sole source of iron. However, disruption of *phuS* alone resulted in a slow growth phenotype, consistent with its role as a heme-trafficking protein to HO. Furthermore, in contrast to the *hemO* and *hemO/phuS* deletion strains, the *phuS* knockout prematurely produced pyocyanin in the presence of heme. Western blot analysis of PhuS protein levels in the wild-type strain showed that Fur-regulation of the *phu* operon could be derepressed in the presence of heme. In addition the premature onset of pyocyanin production requires both heme and a functional HO. Suppression of the phenotype on increasing the external heme concentration suggested that the decreased heme-flux through HO results in premature production of pyocyanin. The premature production of pyocyanin was not due to lower intracellular iron levels as a result of decreased heme flux through HO. However, transcriptional analysis of the *phuS* mutants indicates that the cells are sensing iron deprivation. The present data suggest that PhuS has a dual function in trafficking heme to HO, and in directly or indirectly sensing and maintaining iron and heme homeostasis.

Iron is essential for the growth, survival, and virulence of most bacterial pathogens, with only a few exceptions (1–3). However, within the human body, bacteria encounter an extremely low iron milieu, because the majority of iron is sequestered in iron and heme proteins, such as transferrin and hemoglobin, respectively (4). Bacterial pathogens have therefore evolved multiple mechanisms to obtain iron from the iron- and heme-containing proteins of the host. The opportunistic pathogen *Pseudomonas aeruginosa*, which is responsible for severe nosocomial infections in immunocompromised patients (5, 6), secretes an array of high affinity iron binding siderophores (pyoverdin and pyochelin) and in addition can directly utilize the host iron- and heme-containing proteins (7, 8).

The *P. aeruginosa* genome encodes two heme acquisition systems: the *has* (heme assimilation system) and the *phu* (*Pseudomonas* heme utilization) operons. The *has* locus encodes the hemophore HasA, the hemophore receptor HasR, and a potential ABC transporter HasDEF for export of HasA (8, 9). The *phu* locus consists of six open reading frames encoding the outer membrane heme receptor PhuR, the periplasmic ABC (ATP binding cassette) transport system PhuTUV, PhuW whose function has not been determined, and the cytoplasmic heme-binding protein PhuS, which is essential for optimal heme utilization (8). The cytoplasmic protein PhuS has been shown *in vitro* to transfer heme to the iron-regulated heme oxygenase, HO[2] encoded by the *hemO* (also referred to as PigA in the literature) (10, 11). The expression of the *phu* and *has* operons is regulated by the ferric uptake regulator (Fur) protein, which has both negative and positive regulatory effects on the expression of iron-regulated genes (8, 12, 13). Under iron-replete conditions, Fe^{2+}-bound Fur represses gene expression by directly binding to the Fur box in the promoter regions of iron starvation-inducible genes (including the iron- and heme-uptake genes) and activating gene expression indirectly through a pair of small regulatory RNAs, PrrF1 and PrrF2 (*Pseudomonas* regulatory RNA involving iron (Fe)) (13–15). The regulation of iron and heme uptake by Fur is essential in maintaining the iron homeostasis, because free iron can catalyze formation of hydroxyl radicals via the Fenton reaction (1, 14). Additionally a recent proteomic analysis has shown that the ability to use hemoglobin as an iron source is quorum sensing (QS) regulated in *P. aeruginosa* (16).

QS is a cell density-dependent cell-to-cell communication mechanism, which allows bacteria to sense their environment and coordinate expression of various genes within a bacterial population (17–19). The QS network in *P. aeruginosa* is highly complex and consists of two interlinked *N*-acyl homoserine

* This work was supported, in whole or in part, by National Institutes of Health Grant AI-55912 (to A. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1, Figs. S1 and S2, and references.

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[2] The abbreviations used are: HO, heme oxygenase; QS, quinolone signal; PQS, *Pseudomonas* quinolone signal; Phu, *Pseudomonas* heme utilization; ABC, ATP binding cassette; Fur, ferric uptake regulator; PIA, *Pseudomonas* isolation agar; FDR, false discovery rate; RND, root nodule cell division.

TABLE 1
Strains and plasmids used in this study

Strain or plasmid	Description of strain or plasmid	Source
E. coli		
S17.1	*E. coli* shuttle vector. 294(*recA*, *pro res mod*$^+$) Tp^r, Sm^r, (pRP4-2-Tc::Mu-Km::Tn7)	(61)
P. aeruginosa		
MPAO1	Wild-type *P. aeruginosa PAO1*	Pseudomonas Genome Center, Seattle, WA
7520	*phuS* mutant containing IS*lacZ*/hah transposon insertion, derived from MAPO1	(as above)
CDC-5	*pvd-2* derivative of PAO1 strain lacking pyoverdin production	(35)
IA614	Pyochelin-deficient derivative of CDC-5 strain, obtained by ethylmethanesulfonate mutagenesis	(35)
IR1648	Chromosomal knockout of iron-regulated heme oxygenase gene, *hemO* derived from IA614	(46)
IA614-Δ*phuS*	Chromosomal knockout of *phuS* derived from IA614	This study
IR1648-Δ*phuS*	Chromosomal knockout of *phuS* derived from IR1648. This mutant lacks both *pigA* and *phuS* along with the siderophores pyochelin and pyoverdin	This study
Plasmids		
pEX18p	Amp^R; allelic replacement vector	(34)
pFlp2	Amp^R; source of Flp recombinase	(34)
pPS856	Source of Gent^R-GFP, Gent^R-conferring fragment flanked by *FRT* sites	(34)
pFTC1	Source of Tet^R-GFP, Tet^R-conferring fragment flanked by *FRT* sites	H. Schweizer
pEX18p-Δ*phuS*::*Gm*	Amp^R; allelic replacement vector containing 1.8-kb fragment of in-frame *phuS* deletion containing the Gent^R antibiotic marker	This study
pEX18p-Δ*phuS*::*tet*	Amp^R; allelic replacement vector containing 1.8-kb fragment of in-frame *phuS* deletion containing the Tet^R antibiotic marker	This study

lactone-dependent regulatory pathways, which are further modulated by the *Pseudomonas* quinolone signal (PQS) (20, 21). Furthermore, it has become increasingly evident that there exists a complex relationship between iron, QS, and virulence (22–26). A number of separate studies have shown that iron concentrations independent of cell density modulate expression of genes that are known to be QS-regulated (22). A recent study has shown the iron-regulated small regulatory RNAs PrrF1 and PrrF2 are linked to QS at the level of regulation of anthranilate (a biosynthetic precursor of PQS) metabolism providing further evidence of the physiological link between iron and QS (27). Furthermore it has been proposed that pyocyanin, the terminal QS signal, itself may play a role in iron uptake and in extracellular electron shuttling in biofilm formation (28–30).

In the present report we further address the role of PhuS in heme trafficking to HO. The *phuS* mutants in *P. aeruginosa* in the siderophore-deficient IA614 and wild-type MPAO1 strain were observed to prematurely produce pyocyanin in the presence of heme during transition to stationary phase. As PhuS plays a role in iron metabolism via heme trafficking, and pyocyanin has been suggested to play a direct role in iron acquisition (28, 31, 32), it was hypothesized that the premature pyocyanin phenotype is an indication of an imbalance in iron homeostasis on disruption of PhuS. To further investigate the cause of premature pyocyanin production in the *phuS* mutants and its relevance to iron homeostasis, we undertook a biochemical and global transcriptional profiling approach. The findings presented herein provide important insight into the critical role of heme in maintaining *P. aeruginosa* iron homeostasis.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Cultures—The *P. aeruginosa* strains used in this study are listed in Table 1. Luria-Bertani (LB) medium was routinely used for culture and maintenance of all strains. Tetracycline at 60 μg/ml concentration was used for maintenance and culture of the MPAO1 *phuS* transposon mutant strain. The location of the transposon insertion in the *phuS* mutant was confirmed by PCR with primers: PhuS1065R(5′-TCAGAGCGCCTTGAAGGAT-3′) and LacZ-148 (5′-GGGTAACGCCAGGGTTTTCC-3′). Analysis of *P. aeruginosa* growth in rich medium was carried out in LB medium, which has a heme content of ~5 μM based on the pyridine hemochrome assay (33). Overnight cultures (15 ml) of the *P. aeruginosa* strains grown in LB medium were spun down, and the bacterial pellets were resuspended in 5 ml of fresh LB medium followed by measurement of the optical density at 600 nm (A_{600}). The cultures were then used to inoculate LB medium (20 ml) in 250-ml baffled Erlenmeyer flasks to a final A_{600} of 0.05. The flasks were incubated at 37 °C with shaking at 220 rpm, and the A_{600} was measured every 30 min for a period of 6–8 h as indicated.

Succinate minimal medium supplemented with 5% glycerol (SM) was used to assess growth in nutrient-defined medium. The medium was made iron-replete with either 10 μM iron chloride ($FeCl_3$), 10 μM human hemoglobin, or both. Iron-restricted medium contained 2,2′-dipyridyl (500 μM) where indicated. Overnight cultures (15 ml) were supplemented with iron chloride and/or hemoglobin. The overnight cultures were then used to inoculate 250-ml baffled Erlenmeyer flasks containing the same medium to a final A_{600} of 0.1. The flasks were incubated at 37 °C with shaking at 220 rpm, and the A_{600} was measured every 1 h over a period of 7 h.

Construction of an In-frame Deletion of PhuS in P. aeruginosa IA614 and IR1648—Construction of the in-frame deletions utilized a four-primer PCR method. The EcoRI-KpnI and KpnI-PstI gene fragments were obtained from MPAO1 genomic DNA using the GeneAmp High Fidelity PCR kit. Primer 1 (5′-GATAGAATTCGAGGCTGCGGTCGGCGATC-3′) located upstream in the *phuT* gene and primer 2 (5′-GATAGGTAC-CAACCTGCACCTGAA-3′), located at the 3′-end of the *phuS* gene, generated a 900-bp fragment encoding EcoRI and KpnI

restriction sites. Similarly, primer 3 (5′-GATAGGTACCGTC-CTGCCAGGCGCGGT-3′), located at the start of the *phuS* gene and primer 4 (5′-GATACTGCAGGCTTGCCGAGCAG-GCTGT-3′), located within the *phuR* gene generated a 970-bp fragment introducing KpnI and PstI restriction sites at the 5′- and 3′-ends, respectively. The fragments were cloned in tandem into pEX18Ap insert was confirmed by restriction digest and DNA sequencing to ensure that no point mutations had occurred during PCR (34). The gentamycin antibiotic marker (Gm), flanked by FRT sites, was cloned into the KpnI site within the *phuS* deletion creating pEX18Ap-Δ*phuS*::Gm. The final construct was transformed into *Escherichia coli* S17.1 cells for mating (35).

Unmarked deletions of the *phuS* gene were constructed in the siderophore-deficient *P. aeruginosa* IA614 strain according to a modification of Hoang *et al.* (34). For the mating, *E. coli* S17.1 was grown with gentamycin in LB overnight at 37 °C, and the *P. aeruginosa* strains at 42 °C overnight. The single crossover was selected on *Pseudomonas* Isolation Agar (PIA) with 100 μg of gentamycin and 250 μg of carbenicillin. PCR confirmed the presence of both the wild-type and the deletion copy of *phuS*::*Gm*. Positive colonies were then plated on PIA containing 200 μg/ml gentamicin plus 5% sucrose to select for loss of the wild-type gene. Deletion of the integrated antibiotic marker with Flp recombinase was carried out by conjugally transferring pFlp2 in *E. coli* S17.1 into the IA614-Δ*phuS* deletion strain at 37 °C (34). Single colonies were screened for gentamycin sensitivity ($Gent^S$) on PIA plates containing 200 μg/ml gentamicin. Loss of plasmid was tested by screening for on PIA-sucrose and patching onto PIA carbenicillin (250 μg/ml) plates at 37 °C overnight. Colonies that were Suc^R-$Carb^S$ were streaked out on PIA alone and PIA-carbenicillin (200 μg/ml) and gentamycin (200 μg/ml) to confirm sensitivity to both antibiotics. Unmarked mutants were verified by PCR. The Δ*phuS*::*tet* pEX18Ap plasmid was constructed to create the Δ*phuS* knockout in strain IR1648 as described above.

Pyocyanin Purification and Quantification—Pyocyanin was purified and quantified as described previously (36). Briefly, supernatant from 1 ml of bacterial culture was extracted with 1 ml of chloroform. The chloroform phase containing pyocyanin was then extracted into 1 ml of 0.2 N HCl, yielding a pink solution indicating the presence of pyocyanin. The UV-visible spectrum was recorded in 0.2 N HCl. The pyocyanin concentration expressed as micrograms/ml of culture supernatant, was determined by multiplying the A_{520} by 17.072, as described previously (36). Reversed-phase high-performance liquid chromatography was performed on a 3.0- × 250-mm C18 column (Atlantis™ dC18 5 μm) with modification of a previously described procedure (37). Briefly the elution was carried out at 0.2 ml/min with the following stepwise gradient of acetonitrile/water/trifluoroacetic acid (10:90:0.01) for 0–5 min (10:90:0.1 to 70:30:0.1) for 5–40 min and (70:30:0.1 to 50:50:0.1) for 40–45 min. The elution of pyocyanin was monitored by UV-visible absorbance at 278 nm. The major peak was collected and analyzed on a Finnigan LCQ classic ion-trap Mass Spectrometer (Finnigan MAT, San Jose, CA) equipped with an electrospray ionization source for obtaining the *m/z*. The MS instrument was operated in a positive-ion mode with Finnigan Xcalibur software 1.1. The spectra were obtained in full scan mode (150–500 *m/z*).

Attenuation of Pyocyanin Production—Overnight cultures of the *phuS* mutants were grown in LB medium from a single colony and used to inoculate 20 ml of LB medium in 250-ml baffled Erlenmeyer flasks to a final A_{600} of 0.05. The medium was supplemented with 0–100 μM human hemoglobin. The flasks were incubated at 37 °C with shaking at 220 rpm, and pyocyanin concentrations were determined following 24 h of growth and correction for optical density differences. Pyocyanin concentrations (micrograms/ml) were expressed as means ± S.E. and were compared by analysis of variance and *t*-tests for independent variables using SigmaPlot 10.0 (SPSS Inc., Chicago, IL). The pyocyanin increases were also monitored every 30 min over a period of 6 h for the *phuS* mutant cultured in LB medium ± 100 μM hemoglobin.

Spectroscopic Determination of Intracellular Iron Levels—The intracellular iron content of the *P. aeruginosa* strains was determined by modification of previously described procedure (38). Briefly, the bacterial cultures were grown with shaking at 37 °C in LB medium, starting from an A_{600} of 0.1. At an A_{600} of 1.5 and 3.0, 10-ml samples from each strain were harvested at 10,000 × *g* rpm for 15 min, and the supernatant was discarded. The pelleted cells were washed once with fresh LB medium and again pelleted. The pellets were then dried overnight at 80 °C in glass tubes after which they were dissolved in concentrated nitric acid, and the iron content was measured by atomic absorption spectroscopy. For each of the strains the measurements were carried out in triplicate. Control experiments to determine the background levels of iron in the nitric acid were also performed. All glassware and plastic ware were acid-washed before use. The iron concentrations (picograms of iron/μg of protein) were expressed as means ± S.E. and were compared by analysis of variance and *t*-tests for independent variables using SigmaPlot 10.0.

Western Blot Analysis—The determination of PhuS and/or HO expression levels in nutrient-defined succinate medium was determined by Western blot analysis. Cultures were grown overnight in the following nutrient-defined conditions: succinate (iron-restricted), containing either 100 μM $FeCl_3$, or 25 μM hemoglobin, or both. The overnight cultures were used to inoculate 20 ml of the same nutrient-defined medium to an A_{600} of 0.1. The medium was supplemented with higher concentrations of $FeCl_3$ and hemoglobin than in the previous growth studies to ensure optimal growth at 6 h. Aliquots of the wild-type MPAO1 strain (1 ml) were pelleted every hour over a 7-h growth period, lysed in 100 μl of Bugbuster® (Novagen, EMD Biosciences, Inc.) and pelleted at 10,000 × *g* at 4 °C to obtain the soluble protein fraction. The total protein content was measured by Bradford assay (39) using Bio-Rad reagent (Bio-Rad Laboratories Inc.), and 20 μg of total protein was loaded and separated on 4–15% Tris-HCl SDS-PAGE gels (Bio-Rad). Proteins were electrophoretically transferred to Immobilon-P transfer membrane (Millipore Corp., Bedford, MA) as described previously (40). Membranes were blocked with Tris-buffered saline containing 5% skim milk and probed with a 1:500 dilution of primary anti-PhuS polyclonal antibody in Tris-buffered saline containing 0.1% Tween 20. The membrane

was then probed with the secondary antibody goat-anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (KPL, Inc., Gaithersburg, MD) at a dilution of 1:10,000 in Tris-buffered saline-containing 0.1% Tween 20, and the protein was visualized by enhanced chemiluminescence using the Super-Signal chemiluminescence kit (Pierce).

RNA Extraction, Labeling, and Hybridization—Overnight cultures of the *P. aeruginosa* strains were grown from a single colony in 15 ml of RNase-free LB medium at 37 °C for 16–18 h. Aliquots from the samples were spun down, the bacterial pellets were resuspended in 5 ml of fresh RNase-free LB medium, and the A_{600} was measured. The resuspended cells were then used to inoculate triplicate 20-ml cultures in RNase-free LB medium in 250-ml baffled Erlenmeyer flasks to a final A_{600} of 0.1.

Aliquots (1 ml) of the bacterial cultures were removed at an A_{600} of 1.5 and 3.0 for the 7520 *phuS*::*Tn* mutant and MPAO1 strains. For the IA614, IR1648, IA614-Δ*phuS*, and IR1648-Δ*phuS* strains, samples were removed at an A_{600} of 1.5. RNAprotect Bacteria Reagent (Qiagen) was added to the samples to stabilize the RNA prior to extraction. Total RNA was then extracted from all strains using the RNeasy Mini kit (Qiagen). Contaminating DNA was removed with an on-column RNase-free DNase I treatment (Qiagen) followed by an off-column DNase I treatment (0.1 unit/μg of RNA) according to the manufacturer's recommendations (Qiagen). The RNA was concentrated to a final volume of 15 μl using the RNeasy MinElute kit (Qiagen), and the RNA was quantified by measurement of the absorbance at 260 nm. The quality of the purified RNA was determined on a 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA) at the University of Maryland, Baltimore, School of Medicine, Biopolymer/Genomics Core Facility.

Microarray Analysis—Synthesis of cDNA, target hybridization, staining, scanning, and extraction of the hybridization intensity data were performed at the University of Maryland, Baltimore, School of Medicine, Biopolymer/Genomics Core Facility using an Affymetrix GeneChip® system (GeneChip® hybridization oven 640, fluidics workstation 450, Scanner, and Operating Software, GCOS version 1.4).

The determination of normalized gene expression signals by statistical analysis was performed at the Microarray Core Facility, Johns Hopkins University, School of Medicine. The quality of the microarray experiment was assessed with affyPLM and Affy, two Bioconductor packages for statistical analysis of microarray data. To estimate the normalized gene expression signals, data analysis was conducted on the probe signal values in the Chips' CEL file at the Affymetrix probe pair (perfect match probe and mismatch probe) level, using the statistical algorithm RMA (Robust Multi-array expression measure) with Affy (41). This probe level data processing includes a quantile normalization method (42) to reduce the obscuring variation between microarrays, which might be introduced during the processes of sample preparation, manufacture, fluorescence labeling, hybridization, and/or scanning. With the signal estimates, principal component analysis was performed in R to assess sample variability. With the signal data in a log-transformed format, differential gene expression between controls and mutant strains was assessed by statistical linear model analysis using the bioconductor package limma, in which an empirical Bayes method is used to moderate the standard errors of the estimated log-fold changes of gene expression, and results in more stable inference and improved power, especially for experiments with small numbers of microarrays (43). The moderated *t*-statistic *p* values derived from the linear model analysis above were further adjusted by Benjamini and Hochbergs' method to estimate false discovery rate (FDR). The FDR was used to obtain the list of differentially expressed genes. All Bioconductor packages are available at www.bioconductor.org, and all computation was performed under the R environment (www.r-project.org) (44), which is a highly extensible language and environment for statistical computing and graphics and provides a wide variety of statistical (linear and nonlinear modeling, classic statistical tests, time-series analysis, classification, clustering, etc.) and graphical techniques. The annotations were obtained from the Pseudomonas Genome project at www.pseudomonas.com (45). Confirmation of the transcriptional analysis was carried out by reverse transcription-PCR analysis of a select set of genes (supplemental Table S1). Changes in transcript levels by reverse transcription-PCR compared well with those obtained by microarray analysis (supplemental Table S2).

RESULTS

PhuS Is Required for Efficient Heme Utilization in P. aeruginosa—It has previously been shown *in vitro* that PhuS functions to traffic heme to HO. The *in vivo* role of PhuS in trafficking heme to HO was examined in a series of bacterial knockout strains in which the *phuS* and *hemO* genes were deleted individually, and as a double *hemO*/*phuS* knockout. The knockouts were constructed in the IA614 siderophore-deficient strain to study the effects on heme utilization in the absence of high affinity iron-uptake systems that are able to scavenge trace amounts of iron.

The siderophore-deficient IA614 strain when supplemented with 10 μM $FeCl_3$ is capable of growth to an optical density of 1.4 (Fig. 1*A*). All of the mutants grew at the same rate as the parent IA614 strain to optical densities of 1.2–1.4 (data not shown). As expected the parent IA614 strain when supplemented with heme as the sole iron source grew to a similar optical density as the iron-supplemented strains (Fig. 1*A*). However, in contrast the *phuS* mutant had a slower growth profile and reached a lower optical density. As previously shown the *hemO* mutant was unable to grow when given heme as the sole source of iron (46). Similarly, in the absence of a functional heme oxygenase to release iron from the porphyrin macrocycle, the *hemO/phuS* mutant is also unable to grow (Fig. 1*A*). These data taken together are consistent with our previous *in vitro* studies, which have characterized PhuS as a heme-trafficking protein to the iron-regulated HO for degradation and release of iron (10, 11, 47). Interestingly, the *phuS* mutant in contrast to the parent strain or the mutants carrying the *hemO* gene deletion began to produce a blue-green pigment (supplemental Fig. S1*A*).

The growth profile and phenotype of the *phuS* transposon mutant and the wild-type MPAO1 strains were also analyzed to

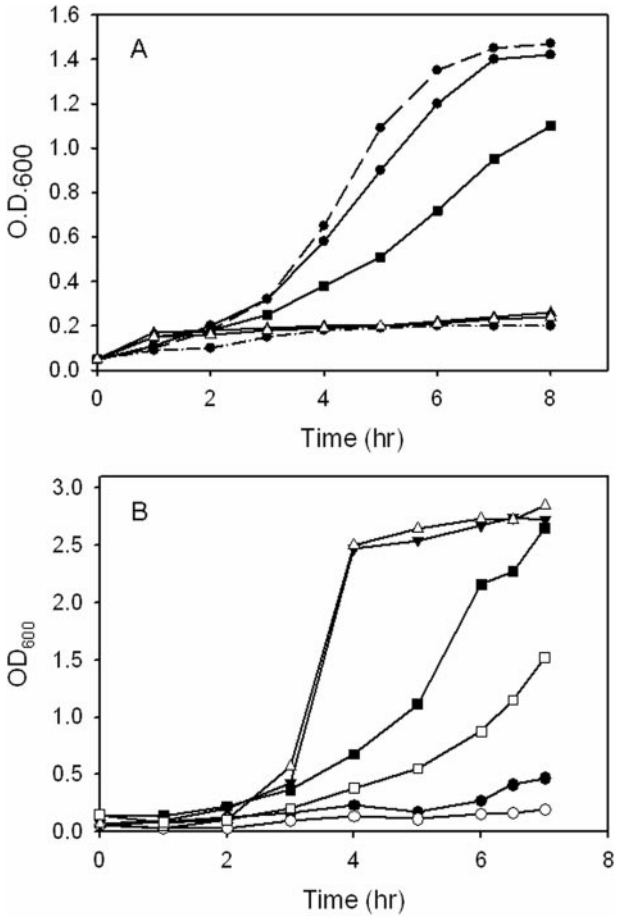

FIGURE 1. **Growth phenotypes of the *P. aeruginosa* heme utilization mutant strains in the wild-type MPAO1 and siderophore-deficient strains.** *A*, *P. aeruginosa* IA614 siderophore-deficient mutants. Isogenic IA614 siderophore-deficient strain (●) in iron-restricted medium (500 μM dipyridyl) (*dashed-dotted line*); supplemented with 10 μM $FeCl_3$ (*dashed line*); supplemented with 10 μM hemoglobin (*solid line*); IA614-Δ*phuS* deletion strain (■) supplemented with 10 μM hemoglobin; IR1648 (*hemO* mutant) (△) supplemented with 10 μM hemoglobin; IR1648-Δ*phuS* (*hemO*/Δ*phuS* double mutant) (▲) supplemented with hemoglobin. The *phuS* and *hemO* mutants supplemented with $FeCl_3$ all had similar growth curves as the isogenic IA614 strain and for clarity are not shown. *B*, *P. aeruginosa* MPAO1 and 7520 *phuS*::*Tn* in nutrient-defined succinate media. MPAO1 (●) and 7520 *phuS*::*Tn* (*phuS* transposon mutant) (○) in iron-restricted medium (500 μM dipyridyl); MPAO1 (▼) and 7520 *phuS*::*Tn* (△) supplemented with 10 μM $FeCl_3$; MPAO1 (■) and 7520 *phuS*::*Tn* (□) supplemented with 10 μM hemoglobin.

determine if the pigment production is related to the compromised ability of the IA614 strain to acquire iron via siderophore mechanisms. As observed for the IA614 strain the growth rate of the *phuS* transposon mutant was similar to that of the parent MPAO1 strain in nutrient-rich LB medium (data not shown). However, in iron-restricted conditions or when hemoglobin is the sole iron source, the growth of the *phuS* mutant was much slower when compared with the parent MPAO1 strain (Fig. 1*B*). Furthermore, as for the *phuS* mutant in the siderophore-deficient strain, the *phuS* transposon mutant also began to secrete the blue-green pigment on entering stationary phase (supplemental Fig. S1*A*).

The phuS Mutants Exhibit Premature Pyocyanin Production—The *phuS* mutant in either the wild-type or siderophore-deficient strain upon reaching the transition to stationary phase (A_{600} of ~3.0) secretes a blue-green pigment (supplemental Fig. S1*A*). Interestingly, the pigment was not observed when the *hemO* (IR1648) or both the *phuS* and *hemO* genes (IR1648-Δ*phuS*) were deleted (supplemental Fig. S1*A*). The excreted pigment was further identified as pyocyanin by a combination of UV-visible spectroscopy, high-performance liquid chromatography, and electrospray ionization-mass spectrometry (supplemental Fig. S1*B*).

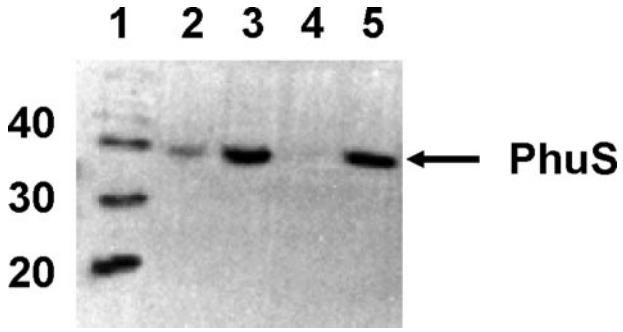

FIGURE 2. **Western blot analysis of the wild-type MPAO1 strain.** Cells were grown in succinate minimal medium with the indicated iron and heme supplements. *Lane 1*, molecular mass markers in kilodaltons; *lane 2*, 100 μM $FeCl_3$ and 25 μM hemoglobin; *lane 3*, 25 μM hemoglobin; *lane 4*, 100 μM $FeCl_3$; *lane 5*, 500 μM 2,2′-dipyridyl. Cells were harvested 6 h post-inoculation, and 20 μg of total protein was loaded in each well and separated by SDS-PAGE.

Heme Positively Regulates the Expression of PhuS in P. aeruginosa—The premature pyocyanin phenotype on disruption of *phuS* in iron-replete LB medium suggests that in the wild-type strain the protein must be expressed. Therefore, the *phu* operon must be positively regulated in a manner distinct form that of the negative repression by Fur. We hypothesized that heme if present may act as a positive regulator of the *phu* operon. Because LB medium contains ~6 μM heme in yeast extract (as calculated by pyridine hemochrome), we postulated that the presence of heme alleviates the iron-regulated Fur suppression of the heme utilization genes. In keeping with this hypothesis we observed a constitutive expression of PhuS in the wild-type PAO1 strain grown in iron-replete LB medium (data not shown). To directly test this hypothesis, the expression of PhuS in the wild-type PAO1 was analyzed in nutrient-defined succinate medium supplemented with either $FeCl_3$ or hemoglobin. As shown in Fig. 2 in iron-restricted succinate medium the PhuS protein is expressed at high levels. On supplementation with 100 μM $FeCl_3$ the expression of PhuS is repressed. In contrast, addition of hemoglobin to the medium induces the expression of PhuS. Furthermore, in the presence of both $FeCl_3$ and hemoglobin the PhuS protein levels are detectable in contrast to the culture with $FeCl_3$ alone. Taken together the data indicate that, even in the presence of iron, heme positively regulates the expression of the heme uptake genes.

Premature Production of Pyocyanin Is Not Due to Iron Starvation—If the disruption of the *phuS* gene results in inefficient heme utilization, it might be expected that the intracellular iron levels in the *phuS* mutants would be lower than the wild-type strains. Interestingly in the MPAO1 strain the intracellular iron levels between the wild-type and *phuS* mutant were not significantly different (Fig. 3). However, in the siderophore-deficient background the *phuS* mutant has significantly lower levels of intracellular iron, whereas both the *hemO*

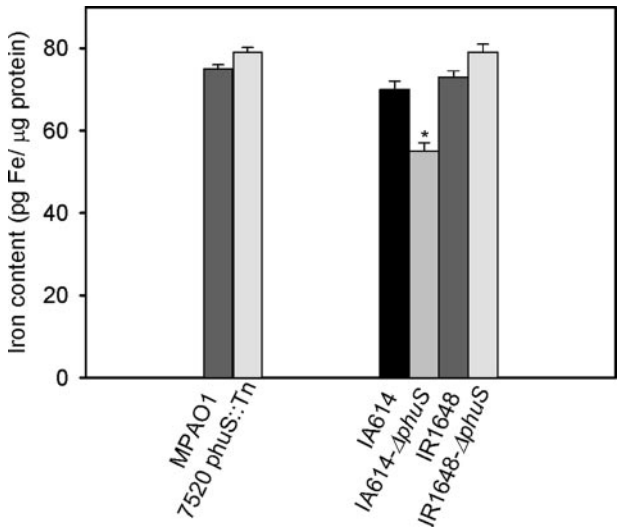

FIGURE 3. **Iron content in the MPAO1 and IA614 mutant strains.** Iron content as measured by atomic absorption spectroscopy. Cells were analyzed for iron content as described under "Experimental Procedures." Data are means ± S.E. of three experiments. *, $p < 0.02$ (*versus* control strain IA614).

and *hemO*/*phuS* knockouts were similar to that of the siderophore-deficient IA614 strain (Fig. 3). On loss of the PhuS protein in the MPAO1 strain it appears that the cells' iron requirements are met by alternate iron-uptake mechanisms. However, the siderophore-deficient strain on loss of PhuS is unable to adequately maintain its iron balance when combined with the inability to efficiently utilize heme. The relationship between heme utilization and iron balance will be discussed in greater detail in the following section.

The Premature Production of Pyocyanin Is Directly Linked to the Cells Ability to Utilize Heme as an Iron Source—The premature production of pyocyanin on disruption of *phuS* in the presence of a functional HO, suggests the pyocyanin phenotype is directly related to inefficient utilization of heme. Therefore to test this hypothesis we attempted to override the inefficiency by increasing the concentration of heme in the extracellular environment. Pyocyanin production was monitored over a period of 24 h following the addition of 100 μM exogenous hemoglobin. As shown in Fig. 4*A* over a 24-h period the level of pyocyanin was significantly decreased. The decrease in pyocyanin on addition of hemoglobin to the medium was further examined as a function of hemoglobin concentration. The *phuS* transposon mutant in the wild-type background showed a concentration-dependent suppression of pyocyanin (Fig. 4*B*). However, the *phuS* mutant in siderophore-deficient background did not show a concentration-dependent suppression of pyocyanin (Fig. 4*B*). Although it is clear from the manifestation of the phenotype on mutation of *phuS* in the wild-type MPAO1 background that iron alone is not responsible for the premature production of pyocyanin, the siderophore-deficient strain produced higher levels of pyocyanin, most likely as a result of the additive effect of the lower intracellular iron levels. These data suggest that, in addition to the previously documented relationship between iron availability and pyocyanin biosynthesis (27, 48), heme utilization also independently influences pyocyanin biosynthesis.

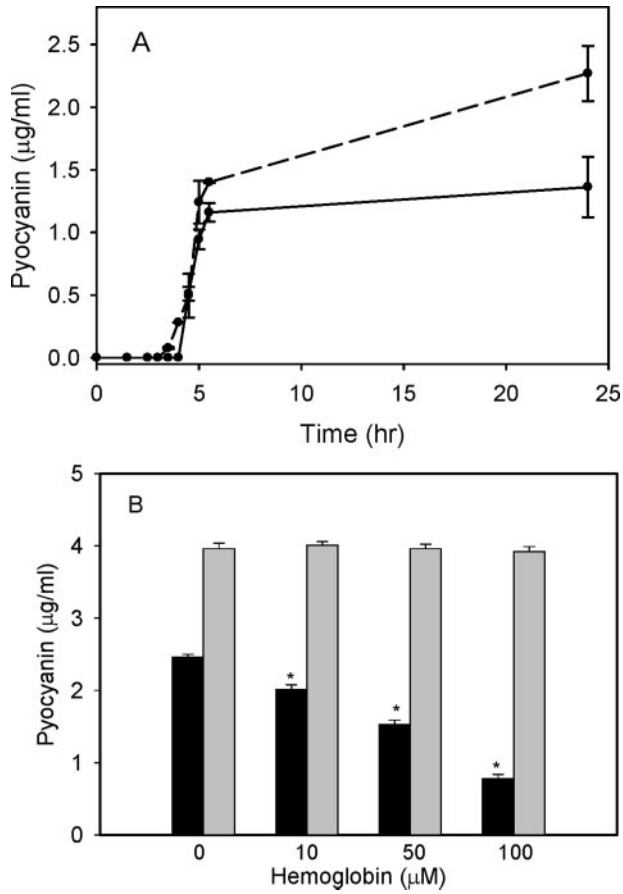

FIGURE 4. **Attenuation of pyocyanin production in the *P. aeruginosa* MPAO1 and IA614 *phuS* mutants on supplementing the medium with hemoglobin.** *A*, pyocyanin production over a 24-h growth period in the absence (*dashed line*) or presence (*solid line*) of 100 μM hemoglobin. All pyocyanin extractions were carried out on cultures of the wild-type MPAO1 and 7520 *phuS*::*Tn* mutant strains at equivalent cell densities. Data are means ± S.E. of three separate experiments. *B*, pyocyanin production in the 7520 *phuS*::*Tn* (*black bars*) and IA614-Δ*phuS* (*gray bars*) mutants as a function of hemoglobin concentration. Extraction and quantification of pyocyanin were carried out as described under "Experimental Procedures." Data are means ± S.E. of three separate experiments. *, $p < 0.001$ (*versus* control value no exogenous hemoglobin).

Iron Homeostasis Is Perturbed in the phuS Mutants—To further investigate the effect of disruption of heme utilization on the premature pyocyanin phenotype a global microarray approach was undertaken. The global transcriptional changes were performed on the wild-type and siderophore-deficient strains at an A_{600} 1.5, prior to the production of pyocyanin. In addition the expression profile of the wild-type and *phuS* transposon mutant strain were analyzed at an A_{600} of 3.0, the time point at which the mutant strains begin to produce pyocyanin. The wild-type strain was performed at the later time point to assess the expression profiles of the heme and iron-uptake systems in a non-compromised iron-uptake background.

Consistent with the premature pyocyanin production the genes involved in pyocyanin biosynthesis, including *phzABCDEFG* operons 1 and 2, *phzS* and *phzM* (49) were significantly up-regulated at A_{600} of 3.0 in the *phuS* transposon mutant (Table 2). Similarly, genes previously reported to be specifically regulated by pyocyanin namely, the root nodule cell division (RND) efflux pump MexGHI-OmpD (29), were also up-regu-

TABLE 2

Differential transcription of the PQS and pyocyanin biosynthesis and efflux genes

O.D., optical density at 600 nm.

Open reading frame	Gene name	Product name	IA614[a] *versus*			MPAO1[b] *versus*	
			IA614-Δ*phuS*	IR1648 (*hemO*)	IR1648-(*hemO*/Δ*phuS*)	7520 *phuS::Tn*	7520 *phuS::Tn*
				O.D. 1.5		*O.D. 1.5*	*O.D. 3.0*
PQS biosynthesis genes							
PA0996	*pqsA*	Probable coenzyme A ligase	4.2	NS[c]	NS	6.4	10.2
PA0997	*pqsB*	Homologous to β-keto-acyl-acyl carrier protein synthase	7.8	NS	1.2	12.1	5.3
PA0998	*pqsC*	Homologous to β-keto-acyl-acyl carrier protein synthase	10.0	NS	NS	16.2	5.3
PA0999	*pqsD*	3-Oxoacyl-[acyl-carrier-protein] synthase III	6.5	NS	NS	10.4	5.2
PA1000	*pqsE*	Quinolone signal response protein	3.0	NS	NS	6.0	7.0
PA1001	*phnA*	Anthranilate synthase component I	3.4	NS	NS	5.8	10.0
PA1002	*phnB*	Anthranilate synthase component II	1.7	NS	NS	3.0	5.4
PA1003	*pqsR*	Transcriptional regulator MvfR	1.2	NS	NS	NS	2.0
Phenazine biosynthesis genes							
PA1898	*qscR; phzR*	Quorum-sensing control repressor	NS	NS	NS	NS	2.7
PA1901	*phzC2*	Phenazine biosynthesis protein PhzC	NS	NS	1.2	1.20	15.4
PA1902	*phzD2*	Phenazine biosynthesis protein PhzD	NS	−1.20	NS	1.30	13.9
PA1903	*phzE2*	Phenazine biosynthesis protein PhzE	NS	NS	NS	1.32	18.2
PA1904	*phzF2*	Probable phenazine biosynthesis protein	NS	NS	NS	NS	17.9
PA1905	*phzG2*	Probable pyridoxamine 5′-phosphate oxidase	1.1	NS	NS	1.5	23.0
PA4209	*phzM*	Probable phenazine-specific methyltransferase	NS	NS	NS	1.3	7.8
PA4210	*phzA1*	Probable phenazine biosynthesis protein	NS	NS	NS	NS	40.32
PA4211	*phzB1*	Probable phenazine biosynthesis protein	1.3	NS	NS	1.8	34.0
PA4217	*phzS*	Flavin-containing monooxygenase	NS	−1.2	−1.1	1.5	12.7
Pyocyanin regulated efflux pumps							
PA4205	*mexG*	Hypothetical protein	2.6	NS	NS	NS	81.9
PA4206	*mexH*	Probable RND efflux membrane fusion	2.5	NS	NS	NS	26.6
PA4207	*mexI*	Probable RND efflux transporter	1.8	NS	NS	NS	12.1
PA4208	*opmD*	Probable outer membrane protein precursor	1.7	NS	NS	NS	5.8

[a] Ratio of expression of the IA614-Δ*phuS*, IR1648, and IR1648-Δ*phuS* deletion strains compared to IA614 from three separate microarray experiments. Changes were considered significant at or below a 0.05 FDR as described under "Experimental Procedures."

[b] Ratio of expression of the 7520 *phuS::Tn* strain compared to MPA01 from three separate microarray experiments. Changes were considered significant at or below a 0.05 FDR as described under "Experimental Procedures."

[c] NS, not significant.

lated (Table 2). Additionally at the earlier time point genes involved in PQS biosynthesis (a positive signal for pyocyanin biosynthesis), the *phnAB* and *pqsABCDE* operons were up-regulated in the *phuS* mutants in both the wild-type and siderophore-deficient strains (Table 2). The transcriptional data agree with the increased excretion of PQS and other HAQs compared with the wild-type strain as determined by high-performance liquid chromatography with a known PQS standard (supplemental Fig. S2) and confirmed by electrospray ionization-mass spectrometry analysis. In contrast the HO-deficient (IR1648 or IR1648-Δ*phuS*) strains did not show significant up-regulation of the QS networks, consistent with the hypothesis that both heme and a functional HO are required for premature pyocyanin production (Table 2).

Not surprisingly the lower intracellular levels of iron on the loss of PhuS in the siderophore-deficient background correlated with the up-regulation of genes involved in iron uptake and storage (Tables 3 and 4). However, interestingly, in the wild-type strain a similar up-regulation of the heme and iron-uptake systems is observed, including genes involved in siderophore-mediated iron acquisition, the extra cytoplasmic function σ-factor *pvdS*, the PvdS-regulated pyoverdin biosynthesis genes (*pvd*), and the ferripyoverdin receptor *fpvA* (8, 50) (Table 3). Furthermore the accessory iron-uptake gene *tonB*, the ferrous iron-uptake receptor (*feoA*), and the heme-uptake system (*phuR* and *phuTUV*) were also up-regulated (Table 4). In addition the up-regulation of genes such as *fumC*, *sodA*, ferredoxin *bfd* and *fpr*, which encode proteins that do not require iron for their function and can substitute for essential iron-containing proteins under iron-starvation conditions further confirm an iron starvation response (15). Therefore, despite the fact that the intracellular iron levels in the transposon mutant are not different from the wild-type strain, the *phuS* transposon mutant is sensing iron deprivation (Tables 3 and 4). At the earlier time point in both the *phuS* transposon mutant and the siderophore-deficient *phuS* strain, genes involved in heme uptake (*phuRTV*) are also up-regulated (Table 4).

Perhaps more interestingly in the *hemO*/*phuS* mutant the expression profile for the iron-uptake systems is very similar to that of the *phuS* mutant alone, despite the fact that it does not display a pyocyanin phenotype (Table 4). Therefore the microarray analysis indicates that deletion of the *phuS* gene despite the intracellular iron levels disrupts the ability to sense the iron homeostasis and is distinct from the premature pyocyanin phenotype. The current data suggest that the PhuS protein may have a dual function in intracellular heme trafficking to HO, and in sensing and maintaining the balance between heme and iron uptake.

DISCUSSION

The slow growth phenotype observed for the *phuS* mutants in the presence of heme as the sole source of iron

TABLE 3

Differential transcription of the pyoverdin and pyochelin biosynthesis and uptake genes

O.D., optical density at 600 nm.

Open reading frame	Gene name	Product name	IA614[a] *versus*			MPAO1[b] *versus*	
			IA614-Δ*phuS*	IR1648 (*hemO*)	IR1648-Δ*phuS*	7520 *phuS*::*Tn*	7520 *phuS*::*Tn*
				O.D. 1.5		*O.D. 1.5*	*O.D. 3.0*
Pyoverdin biosynthesis and uptake system							
PA2383		Probable transcriptional regulator	−1.2	NS[c]	−1.2	NS	2.3
PA2384		Hypothetical protein	1.2	NS	NS	−1.3	11.2
PA2385	*pvdQ*	PvdQ	NS	NS	NS	NS	2.4
PA2386	*pvdA*	L-ornithine N5-oxygenase	NS	NS	1.30	−1.4	NS
PA2392	*pvdP*	PvdP	NS	NS	NS	NS	2.3
PA2393		Probable dipeptidase precursor	NS	NS	NS	NS	4.17
PA2394	*pvdN*	PvdN	NS	−1.1	1.3	NS	2.9
PA2396	*pvdF*	Pyoverdine synthetase F	NS	NS	NS	NS	3.2
PA2397	*pvdE*	Pyoverdine biosynthesis protein PvdE	NS	NS	NS	NS	2.8
PA2398	*fpvA*	Ferripyoverdine receptor	−1.2	NS	NS	−2.9	2.4
PA2399	*pvdD*	Pyoverdine synthetase D	NS	NS	1.4	NS	2.4
PA2413	*pvdH*	L-2,4-diaminobutyrate:2-ketoglutarate 4-aminotransferase	NS	NS	1.3	NS	3.8
PA2425	*pvdG*	PvdG	NS	NS	NS	NS	2.6
PA2426	*pvdS*	Sigma factor PvdS	1.1	NS	NS	−1.3	13.5
Pyochelin biosynthesis and uptake genes							
PA4218	*fptX*	Probable transporter	NS	NS	1.4	7.4	1.4
PA4219	*fptC; yfpB*	Hypothetical protein	NS	NS	1.3	3.3	NS
PA4220	*fptB*	Hypothetical protein	NS	NS	1.5	13.4	2.0
PA4221	*fptA*	Fe(III)-pyochelin outer membrane receptor precursor	NS	NS	1.3	4.7	NS
PA4222	*pchI*	Probable ATP-binding component of ABC transporter	NS	NS	1.2	4.1	NS
PA4223	*pchH*	Probable ATP-binding component of ABC transporter	NS	NS	1.2	6.2	NS
PA4224	*pchG*	Pyochelin biosynthetic protein PchG	NS	NS	NS	5.5	NS
PA4225	*pchF*	Pyochelin synthetase	NS	NS	1.2	5.1	NS
PA4226	*pchE*	Dihydroaeruginoic acid synthetase	NS	NS	NS	4.7	NS
PA4227	*pchR*	Transcriptional regulator PchR	−1.1	NS	NS	NS	4.0
PA4228	*pchD*	Pyochelin biosynthesis protein PchD	NS	−1.2	1.2	2.6	NS
PA4229	*pchC*	Pyochelin biosynthetic protein PchC	NS	NS	1.4	2.7	NS
PA4230	*pchB*	Salicylate biosynthesis protein PchB	NS	NS	1.2	6.5	NS
PA4231	*pchA*	Salicylate biosynthesis isochorismate synthase	NS	NS	1.3	4.5	NS

[a] Ratio of expression of the IA614-Δ*phuS*, IR1648, and IR1648-Δ*phuS* deletion strains compared to IA614 from three separate microarray experiments. Changes were considered significant at or below a 0.05 FDR as described under "Experimental Procedures."

[b] Ratio of expression of the 7520 *phuS*::*Tn* strain compared to MPA01 from three separate microarray experiments. Changes were considered significant at or below a 0.05 FDR as described under "Experimental Procedures."

[c] NS, not significant.

confirmed that the cytoplasmic heme-binding protein, PhuS, is required for efficient heme utilization. This is consistent with previous *in vitro* reports that PhuS functions to traffic heme to the iron regulated HO (10, 11, 47). The observation of the premature production of pyocyanin in the *phuS* mutants and the confirmation that heme can override suppression of the *phu* operon by Fur, indicates that there is a separate and distinct heme-dependent regulation of the *phu* operon. Therefore, whereas Fur acts as a global negative regulator of the iron and heme-uptake systems, heme can independently and selectively up-regulate the heme uptake system via an as yet unidentified mechanism. Regulation of heme utilization by heme itself has previously been reported in a number of bacteria, including *Bordetella* sp. and *Corynebacterium diphtheriae* (51–53). The ability of the organism to respond to the environmental iron source and differentially express the iron and heme uptake systems has relevance not only for survival (54) but also for the ability to establish infection. It has previously been shown in *Staphylococcus aureus* that, during the early stages of infection, the cell preferentially utilizes heme as an iron source and switches to iron-containing proteins such as transferrin much later in the disease progression (55).

The premature pyocyanin phenotype observed with the *phuS* mutants but not evident in either the *hemO*-deficient (IR1648) or the *hemO/phuS* double mutant (IR1648-Δ*phuS*) strains confirmed that, in addition to heme in the external environment, a functional HO is also required. This led us to hypothesize that the pyocyanin phenotype in the absence of PhuS is a result of decreased flow of heme to HO. This is further supported with the concentration-dependent suppression of the phenotype by hemoglobin in the *phuS* transposon mutant. Therefore, an inability to efficiently utilize heme signals the cell to acquire iron via alternative mechanisms. Addition of exogenous hemoglobin did not suppress the premature production of pyocyanin in the siderophore-deficient strain, presumably as a result of the increased iron restriction and lower intracellular iron levels. It has previously been reported that pyocyanin production is influenced not only by cell density and QS but also by several environmental factors, including iron (48, 56–58). Therefore, in the siderophore-deficient strain the cumulative effect of both inefficient heme and iron utilization appear to have an additive effect. Furthermore, it has been previously reported that pyocyanin itself can participate in iron uptake due to its ability to reduce Fe^{3+}-transferrin and ferric iron oxides to

TABLE 4

Differential transcription of iron-regulated genes involved in iron acquisition and metabolism

O.D., optical density at 600 nm.

Open reading frame	Gene name	Product name	IA614[a] *versus*			MPAO1[b] *versus*	
			IA614-Δ*phuS*	IR1648 (*hemO*)	IR1648-Δ*phuS*	7520 *phuS*::*Tn*	7520 *phuS*::*Tn*
				O.D. 1.5		*O.D. 1.5*	*O.D. 3.0*
PA0470	*fiuA*	Ferrichrome receptor FiuA	−1.2	−1.2	NS[c]	NS	NS
PA0471	*fiuR*	Probable transmembrane sensor	1.2	NS	1.2	−1.8	1.7
PA0472	*fiuI*	Probable sigma-70 factor, ECF subfamily	1.2	NS	1.3	−1.9	2.5
PA0672	*hemO*	Heme oxygenase	NS	NS	1.2	−1.6	2.4
PA0693	*exbB2*	Transport protein ExbB2	4.8	NS	NS	NS	1.8
PA0694	*exbD2*	Transport protein ExbD	2.59	NS	NS	NS	NS
PA0929	*pirR*	Two-component response regulator	1.2	−1.2	NS	−2.6	2.1
PA1302	*hxuC*	Probable heme utilization protein precursor	NS	NS	1.3	NS	NS
PA1365		Probable siderophore receptor	NS	NS	NS	NS	−1.3
PA2686	*pfeR*	Two-component response regulator PfeR	NS	NS	NS	NS	1.3
PA3397	*fpr*	Ferredoxin–NADP+ reductase	NS	NS	−1.1	NS	2.8
PA3410		Probable sigma-70 factor, ECF subfamily	NS	NS	1.2	−1.3	2.7
PA3530	*bfd*	Conserved hypothetical protein	1.1	NS	1.2	−1.3	23.5
PA3531	*bfrB*	Bacterioferritin	−1.3	1.6	−1.1	1.5	−6.5
PA3812	*iscA*	Probable iron-binding protein IscA	NS	NS	NS	NS	1.6
PA3814	*iscS*	L-cysteine desulfurase	NS	NS	NS	−1.3	3.0
PA3899		Probable sigma-70 factor, ECF subfamily	NS	NS	1.2	−1.3	1.6
PA3901	*fecA*	Fe(III) dicitrate transport protein FecA	−1.5	NS	−1.2	−1.5	−2.5
PA4359	*feoA*	Conserved hypothetical protein	1.9	NS	−1.2	−2.2	8.7
PA4513	*piuB*	Probable oxidoreductase	NS	NS	1.13	−1.1	NS
PA4514	*piuA*	Probable outer membrane iron-receptor	−1.4	NS	1.4	−2.0	NS
PA4515	*piuC*	Conserved hypothetical protein	1.2	−1.3	1.2	−3.4	4.4
PA4675	*optH; iutA*	Probable TonB-dependent receptor	−2.0	NS	NS	−2.2	−2.4
PA4687	*hitA*	Ferric iron-binding periplasmic protein HitA	NS	−1.4	−1.3	−2.4	NS
PA4688	*hitB*	Iron (III)-transport system permease HitB	−1.2	NS	−1.1	−1.8	NS
PA4706	*phuV*	ABC transporter (ATPase)	1.2	NS	1.5	8.6	3.4
PA4708	*phuT*	Heme-transport protein, PhuT	1.8	NS	1.9	13.2	10.8
PA4709	*phuS*	Probable hemin degrading factor	NS	NS	NS	NS	1.8
PA4710	*phuR*	Heme/hemoglobin uptake outer membrane receptor PhuR precursor	2.4	NS	2.5	1.7	2.3
PA4896		Probable sigma-70 factor, ECF subfamily	NS	−1.2	NS	−1.4	2.1
PA5217		Probable component of ABC iron transporter	1.2	NS	1.2	−3.4	NS
PA5531	*tonB*	TonB protein	1.3	−1.3	1.2	−3.4	4.2

[a] Ratio of expression of the IA614-Δ*phuS*, IR1648, and IR1648-Δ*phuS* deletion strains compared to IA614 from three separate microarray experiments. Changes were considered significant at or below a 0.05 FDR as described under "Experimental Procedures."

[b] Ratio of expression of the 7520 *phuS*::*Tn* strain compared to MPA01 from three separate microarray experiments. Changes were considered significant at or below a 0.05 FDR as described under "Experimental Procedures."

[c] NS, not significant.

release the Fe^{2+} ion (28, 30). Interestingly the premature production of pyocyanin in the *phuS* mutants indicates that heme utilization may be coupled to iron acquisition via transferrin, as suggested by the up-regulation of the ferrous iron uptake protein (*feoA*) required for active transport of the Fe^{2+} ion. This also may be physiologically relevant during infection where both heme and transferrin are likely sources of iron.

It is intriguing to speculate that the distinct regulation of heme utilization *versus* that of iron is linked to the flux of heme through HO. In such a model the by-product of heme degradation by the iron-regulated HO, δ-biliverdin, acts as a feedback regulator in maintaining the up-regulation of the heme uptake system. The premature production of pyocyanin may be a result of decreased production of δ-biliverdin, which maintains the up-regulation of the pathway but provides lower levels of available iron. In contrast deletion of HO shuts down the heme flux such that the cell switches to alternate non-heme iron sources. In effect HO acts as the valve for controlling heme utilization via a metabolic feedback loop. We are currently testing this hypothesis by complementation of the *phuS* mutant in the siderophore-deficient strain with either the wild-type HO, which oxidatively cleaves heme at the δ-meso-carbon to yield δ-biliverdin, or a mutant HO that yields α-biliverdin. If our hypothesis is correct, the complementation with the wild-type HO will induce the premature pyocyanin phenotype in the absence of PhuS. In contrast complementation with the α-biliverdin-producing HO should have no effect on pyocyanin production. It has previously been reported that the *P. aeruginosa* genome encodes a second heme oxygenase BphO, in an operon containing a bacterial phytochrome, BphP (59). The BpHO is an α-selective heme oxygenase that does not accept heme from PhuS (11). Furthermore, it has been shown that δ-biliverdin, in contrast to α-biliverdin, the product of BphO oxidative cleavage, does not bind to the downstream acceptor phytochrome, BphP (60). These data further suggest that the unique regioselectivity of the differentially expressed heme oxygenases in *P. aeruginosa* is required for their distinct functions in phytochrome signaling and iron metabolism, respectively.

Interestingly, despite the fact that the premature production of pyocyanin is not observed in either the *hemO* or *hemO/phuS* mutant strains, it is evident from the microarray analysis that disruption of *phuS* with or without a functional HO causes a disruption in iron homeostasis. As can be seen in the transcriptional changes of the *phuS* mutants several iron-uptake systems are up-regulated despite the iron-replete conditions, suggesting that PhuS in addition to facilitating heme utilization also acts to directly or indirectly sense the intracellular heme/iron levels. In a separate study we have recently shown that the PhuS homolog ShuS of *S. dysenteriae* binds DNA in a heme-depend-

ent manner (60). Recent studies with the apo- and holo-PhuS have shown similar results whereby the apo-PhuS binds to DNA, and the holo-PhuS has no DNA binding ability.[3] We are actively pursuing the physiological relevance of this observation in light of the current studies.

Therefore, we hypothesize that the disruption of iron homeostasis is a function of the cells' inability to sense the intracellular heme levels, whereas, the premature production of pyocyanin is solely related to the flux of heme through heme oxygenase. This is further supported by the disruption of iron homeostasis in the *hemO/phuS* mutant (IR1648-Δ*phuS*), which does not display the pyocyanin phenotype as the heme flux or valve is shut off. However, because PhuS also acts as an intracellular sensor of heme and/or iron, the up-regulation of the iron- and heme-uptake systems is similar to that of the *phuS* mutants. In contrast as would be expected there is no increase in the transcriptional levels of the heme- and iron-uptake systems in the *hemO* mutant strain (IR1648), because the presence of PhuS allows the cell to sense the intracellular heme/iron levels and the absence of HO shuts down heme flux maintaining iron homeostasis.

In summary the PhuS protein plays a pivotal role in maintaining the iron homeostasis of the cell and facilitating heme utilization. Furthermore this central role in cellular metabolism suggests that disruption of PhuS may be an effective mechanism in reducing virulence.

Acknowledgments—We thank Dr. Susanne Häussler, Helmholtz Center for Infection Research, Inhoffenstrasse, Braunschweig, Germany, for providing the PQS standard and Dr. Valeria Cullotta, The Johns Hopkins School of Public Health, Baltimore, MD, for use of her atomic absorption spectrometer.

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