The Small Protein HemP Is a Transcriptional Activator for the Hemin Uptake Operon in *Burkholderia multivorans* ATCC 17616

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ABSTRACT Iron and heme play very important roles in various metabolic functions in bacteria, and their intracellular homeostasis is maintained because high concentrations of free forms of these molecules greatly facilitate the Fenton reaction-mediated production of large amounts of reactive oxygen species that severely damage various biomolecules. The ferric uptake regulator (Fur) from *Burkholderia multivorans* ATCC 17616 is an iron-responsive global transcriptional regulator, and its *fur* deletant exhibits pleiotropic phenotypes. In this study, we found that the phenotypes of the *fur* deletant were suppressed by an additional mutation in *hemP*. The transcription of *hemP* was negatively regulated by Fur under iron-replete conditions and was constitutive in the *fur* deletant. Growth of a *hemP* deletant was severely impaired in a medium containing hemin as the sole iron source, demonstrating the important role of HemP in hemin utilization. HemP was required as a transcriptional activator that specifically binds the promoter-containing region upstream of a Fur-repressive *hmuRSTUV* operon, which encodes the proteins for hemin uptake. A *hmuR* deletant was still able to grow using hemin as the sole iron source, albeit at a rate clearly lower than that of the wild-type strain. These results strongly suggested (i) the involvement of HmuR in hemin uptake and (ii) the presence in ATCC 17616 of at least part of other unknown hemin uptake systems whose expression depends on the HemP function. Our *in vitro* analysis also indicated high-affinity binding of HemP to hemin, and such a property might modulate transcriptional activation of the *hmu* operon.

IMPORTANCE Although the *hmuRSTUV* genes for the utilization of hemin as a sole iron source have been identified in a few *Burkholderia* strains, the regulatory expression of these genes has remained unknown. Our analysis in this study using *B. multivorans* ATCC 17616 showed that its HemP protein is required for expression of the *hmuRSTUV* operon, and the role of HemP in betaproteobacterial species was elucidated for the first time, to our knowledge, in this study. The HemP protein was also found to have two additional properties that have not been reported for functional homologues in other species; one is that HemP is able to bind to the promoter-containing region of the *hmu* operon to directly activate its transcription, and the other is that HemP is also required for the expression of an unknown hemin uptake system.

KEYWORDS *Burkholderia*, HemP, hemin, iron, regulator

Although iron is an essential nutrient that is used as a cofactor for many enzymes in almost all bacteria, available iron is very limited because of its low solubility in eukaryotic hosts and natural aerobic environments (1–3). Therefore, bacteria usually have multiple systems for the acquisition and metabolism of external iron. One

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representative and well-studied system is siderophore-mediated sequestration of external ferric ion and its subsequent transport into the cytoplasm and reduction to ferrous ion (4–6). However, high concentrations of intracellular ferrous ion under aerobic conditions are toxic and, for example, induce the Fenton reaction, by which metabolically produced hydrogen peroxide (a toxic reactive oxygen species [ROS]) is converted to hydroxyl radical, another ROS with a greater degree of toxicity ($\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{HO}^- + \text{OH}\cdot$) (7). Hydroxyl radical then reacts with a toxic reactive nitrogen species (RNS) (e.g., nitric oxide [NO], which is spontaneously generated in cells and natural environments) to generate more toxic RNS (8). ROS and RNS also react with various biomolecules to impair their functions severely, and they significantly facilitate the release of iron from the biomolecules (9). To avoid the extremely undesirable circumstance of high concentrations of free intracellular iron, bacteria have sophisticated systems to maintain iron homeostasis. One such system is mediated by Fur (ferric uptake regulator) or its functional homologue (7, 10, 11). Under iron-replete conditions, the Fur- Fe^{2+} complex interacts with a specific sequence (Fur box), usually leading to the transcriptional repression of genes that are located downstream of the Fur box. Fur is a global transcriptional regulator and regulates, either directly or indirectly, the transcription of a number of genes that are related to the acquisition and utilization of iron sources; Fur has also been indicated to regulate many genes that are not related to these functions (6, 12). A number of bacterial species possess, in addition to the siderophore-mediated iron uptake systems, other systems for the uptake of external heme as a sole iron source, and TonB-dependent ABC-type heme transport systems are usually involved in the uptake of heme into the cytoplasm, where ferrous ion is released from heme (13–19). Although heme is very important for various metabolic functions in bacteria (20), free intracellular heme is also very toxic, most likely due to the generation of hydroxyl radical by the heme-mediated Fenton reaction (21). Several regulatory mechanisms for the expression of genes for heme uptake have been clarified (16–19).

The species belonging to the genus *Burkholderia* in *Betaproteobacteria* are widely distributed in diverse natural environments (22). Some species are pathogenic to plants, animals, and humans, and other species are potentially useful for the biological suppression of plant diseases and the bioremediation of chemical pollutants. *Burkholderia multivorans* ATCC 17616 was isolated from a soil sample and is well known for its extraordinary metabolic versatility (12). We previously isolated a *fur* deletion mutant of ATCC 17616 (DF1) and showed that it exhibited pleiotropic phenotypes, i.e., constitutive secretion of siderophores, enhanced intracellular accumulation of free iron, increased sensitivity to ROS and RNS, drastically reduced assimilation of various carbon sources (including citrate and succinate), and reduced growth fitness in soil (12, 23). We also identified 13 ATCC 17616 loci to which the Fur protein can bind directly. Transcription of all of the loci was repressed under iron-replete conditions, through binding of the active Fur- Fe^{2+} complex to the Fur boxes that are located close to the promoter regions, and several such loci were suggested to be involved in production and/or secretion of siderophores, uptake of the siderophore- Fe^{3+} complex, and transcriptional regulation of the genes for these functions (12). To clarify the genetic mechanisms governing the pleiotropic phenotypes of DF1, we isolated spontaneous suppressor mutants of DF1 that restored tolerance to NO, and we found that they exhibited phenotypes very similar to those of the wild-type strain, except for constitutive siderophore secretion (24). The suppressor mutations were located within *oxyR*, whose product is a transcriptional regulator for H_2O_2 stress. A DF1 derivative with an *oxyR* deletion mutation constitutively overproduces enzymes (e.g., KatG, AhpC1, and AhpD) for the detoxification of ROS, thus reducing the amounts of ROS and RNS that are generated abundantly by DF1.

As described above, our previous study indicated the advantages of a genetic approach using suppressor mutations (e.g., those in *oxyR*) to elucidate the pleiotropic phenotypes of DF1. Accordingly, in this study we isolated suppressor mutants of DF1 that were able to efficiently use succinate as a sole source of carbon. The relevant

FIG 1 Phenotypes of ATCC 17616 and its derivatives. (A) Carbon assimilation assay. Aliquots of cell suspensions (5 μ l) containing 10³ to 10⁷ CFU/ml were spotted on a 1/3LB agar plate (left) and M9 minimal agar plates supplemented with 0.2% citrate (middle) or succinate (right) as the sole carbon source. These plates were incubated at 30°C for 4 days. (B) Secretion of siderophores. The cell suspension used for carbon assimilation was adjusted to a concentration of 10⁷ CFU/ml, and 5 μ l of the suspension was spotted on a CAS agar plate containing 30 μ M FeCl₃. The plate was incubated at 30°C for 4 days. The change of color from blue to orange around the colony indicates the secretion of siderophores. (C) Comparison of intracellular free iron levels with the streptonigrin sensitivity assay. This assay was used to estimate intracellular free iron levels (9). A cell suspension was diluted 100-fold in fresh 1/3LB medium supplemented with 1 mg/ml streptonigrin. The OD₆₆₀ value of this culture was measured after incubation at 30°C for 16 h, and the sensitivity to streptonigrin was expressed as a percentage of the value for the control culture without the addition of streptonigrin. Mean $\pm$ standard deviation (SD) values from three independent experiments are shown. (D) Cellular sensitivity to acidified nitrite. A cell suspension was diluted 100-fold in fresh 1/3LB medium supplemented with 1 mM acidified nitrite (a NO producer). The OD₆₆₀ value of this culture was measured after incubation at 30°C for 16 h, and the sensitivity to acidified nitrite was expressed as a percentage of the value for the control culture without the addition of acidified nitrite. Mean $\pm$ SD values from three independent experiments are shown. (E) Cellular sensitivity to H₂O₂. A cell suspension was diluted with 1/3LB to a concentration of 10⁷ CFU/ml, and 100 μ l of the suspension was spread on a 1/3LB agar plate. A sterilized filter paper disc was placed on the plate, and 10 μ l of 30% H₂O₂ was spotted on each disc. After incubation of the plate at 30°C for 2 days, the diameter of the growth inhibition zone around the disc was measured. Mean $\pm$ SD values for the diameters of the inhibition zones from three independent experiments are shown. (F) SDS-PAGE analysis of crude cell extracts. Twenty milligrams of protein from crude extracts of ATCC 17616, DF1, SUC5, and DFP1 cells at mid-exponential phase was loaded in each lane of a 15% SDS-PAGE gel. Bands A, B, and C correspond to KatG, AhpC1, and AhpD, respectively. WT, wild type.

mutation in one such mutant was mapped within *hemP*, whose product was found to be a transcriptional regulator for the genes involved in the uptake system of hemin (i.e., b-type heme with a chlorinated ligand). We also show two unique properties of the ATCC 17616 *hemP* gene product that are not associated with the functional homologues in other heme-utilizing genera.

RESULTS

Identification of spontaneous suppressor mutations of DF1 that restored the ability to use carbon sources efficiently. The colony-forming ability of DF1 on M9 citrate or succinate minimal agar plates is drastically reduced, by more than 10⁴-fold, compared with the wild-type strain (12). DF1 spontaneously formed large-colony derivatives on such plates, and our investigation of those derivatives revealed that the DF1-associated pleiotropic phenotypes were restored to those of the wild-type strain, except for siderophore secretion (Fig. 1A to E). Subsequent determination of the draft genome sequences of several such derivatives and their comparison with the DF1 sequence indicated that the derivatives had point mutations in *oxyR* or a 1-base insertion mutation at the 56th codon within a 243-bp gene, *bmuj02186*. Since *bmuj02186* is annotated to encode the probable hemin transporter HemP in the NCBI database, this gene was designated *hemP* (see Fig. S1 in the supplemental material). To clarify whether the *hemP* mutation in one DF1 derivative (designated SUC5) was indeed responsible for the suppression of the DF1-associated phenotypes, a *hemP* deletion mutation was introduced into DF1. The resulting strain, DFP1, exhibited phenotypes very similar to those of SUC5, and a DFP1 derivative carrying a *hemP*-containing

Genetic analysis of ATCC 17616 *hemP* and *hmuR* genes for hemin utilization.

The *hemP* gene of *B. multivorans* ATCC 17616 was located on its main chromosome (chromosome 1), and the gene is conserved in many other *Burkholderia* species (Fig. 2A). Some strains in these species have been shown to utilize extracellular heme as a sole source of iron (15). Kvitko et al. (25) experimentally identified the *Burkholderia pseudomallei* *hmuRSTUV* cluster that encodes a TonB-dependent ABC transporter system for the uptake and utilization of heme, and in the present study we found a similar gene cluster on chromosome 2 in ATCC 17616 (Fig. 2A). Reverse transcription (RT)-PCR analysis using the primer sets for detection of transcriptional read-through between two neighboring genes within the ATCC 17616 *hmuRSTUV* cluster supported the structure of the *hmu* operon, whose transcriptional start site is located upstream of *hmuR* (data not shown). Our previous ATCC 17616 study (12) showed that (i) the promoter-containing fragments of both the *hemP* gene (which was designated a gene for a conserved hypothetical protein at the time of publication) and the *hmuR* gene carry functional Fur boxes (Fig. 2) and (ii) the two genes are transcriptionally repressed and derepressed under iron-replete and iron-poor conditions, respectively. Such transcriptional regulation of two genes was confirmed in this study with the use of M9

FIG 3 Growth of ATCC 17616 and its derivatives in liquid media with different iron sources. The strains inoculated were ATCC 17616 (black), DP1 (red), DP1(pCP1) (pink), DR1 (blue), DR1(pCR1) (purple), and DPR1 (green). Growth media were M9 glucose minimal liquid medium supplemented with Dpr and hemin in panels A, D, and G, with FeCl₃ in panels B, E, and H, and with Dpr in panels C, F, and I. See Materials and Methods for details. Each OD₆₆₀ value with a SD (vertical bar) was obtained in at least three independent experiments.

minimal liquid medium supplemented with ferric ion or its chelator, 2,2-dipyridyl (Dpr) (Fig. S2). To clarify the involvement of the ATCC 17616 HemP product in the utilization of hemin, the *hemP* deletant DP1 was constructed. The growth of DP1 was impaired severely in M9 minimal liquid medium supplemented with hemin as the sole iron source but was normal in medium supplemented with FeCl₃ (Fig. 3A to C), indicating a crucial role of HemP in hemin utilization.

The HmuP proteins from *Bradyrhizobium japonicum* and *Sinorhizobium meliloti* are transcriptional activators for the genes encoding outer membrane heme receptors in the heme uptake system (26, 27). Although the overall amino acid sequence similarity of the two proteins with the ATCC 17616 HemP protein is approximately 30% (Fig. 2A), much greater similarity is conserved among the C-terminal portions of the three proteins (Fig. S1B). Taking these facts into consideration, the regulatory role of the

FIG 4 Effects of HemP on the expression of *hmuR* and *bfd* genes. (A and B) qRT-PCR analysis of the ATCC 17616 *hmuR* (A) and *bfd* (B) genes, which are both transcriptionally repressed by the Fur-Fe²⁺ complex (12). The cells were cultivated in 1/3LB, washed with M9 minimal medium, and suspended in the same medium. The suspension was exposed to Dpr at 30°C for 30 min and was used to prepare RNA for qRT-PCR analysis. Transcriptional levels are expressed as relative values, compared with the wild-type strain. (C) qRT-PCR analysis of *hmuR* under conditions of constitutive expression of the *hemP* gene. A cell suspension, prepared as described above, was exposed to FeCl₃ alone or FeCl₃ and 0.2 mM arabinose and was used for qRT-PCR analysis. Each value with a SD (vertical bar) was obtained in at least three independent experiments. WT, wild type.

HemP protein in the expression of the ATCC 17616 *hmu* operon was investigated by quantitative RT-PCR (qRT-PCR) analysis. DP1 cells that were exposed to the Dpr-supplemented M9 minimal medium exhibited no detectable transcription of *hmuR* but showed normal transcription of *bfd* (12), a Fur-regulated gene for bacterioferritin-associated ferredoxin (Fig. 4A and B; also see Fig. S1B). DR1 (ATCC 17616 $\Delta hmuR$) was still able to grow using hemin as the sole iron source, albeit at a rate clearly lower than that of the wild-type strain (Fig. 3A and D to F), whereas the growth of DPR1 (ATCC 17616 $\Delta hemP \Delta hmuR$) in the same medium was severely impaired, similar to that of DP1 (Fig. 3G to I). These results suggested (i) the involvement of HmuR in hemin utilization and (ii) the presence in ATCC 17616 of at least a part of one or more unknown efficient hemin utilization systems whose expression depends on the HemP function.

DNA-binding ability of HemP. The possibility that HemP binds to the promoter-containing region of *hmuR* was investigated with an electrophoretic mobility shift assay (EMSA) (Fig. 5A). The 81-amino-acid HemP protein (8.5 kDa in size) was tagged with six histidine residues at the C terminus (HemP_{6×His}) and purified (Fig. S3); it was found to be specifically bound to a 221-bp promoter-containing region (Fig. 5B). Subsequent DNase I footprinting analysis of this region using HemP_{6×His} (Fig. 5C) revealed its protection of a 12-bp tract within an inverted repeat sequence whose center was located 22 bp upstream of the −35 box for *hmuR*.

The *in vitro* experimental results presented above strongly suggested that the HemP-binding site in the *hmuR*-upstream region is followed, in order, by the −35 box, the Fur box, and the −10 box (Fig. 5B). It was of interest to determine whether the binding of HemP at its cognate site overcomes the Fur-mediated transcriptional repression of the *hmuR* gene under iron-replete conditions. To investigate this plausible event, we constructed a plasmid, pBAD-*hemP*, whose *hemP* transcription is designed to be induced, resulting in protein overproduction, in the presence of arabinose in the medium. Transcriptional levels of *hmuR* in ATCC 17616 and ATCC 17616(pBAD-*hemP*) under iron-replete conditions were measured in the presence of arabinose. As shown in Fig. 4C, the *hmuR* transcriptional levels were similar in the two strains, and there was no apparent increase in *hmuR* transcription in the latter strain. This result strongly suggested that the Fur-Fe²⁺ complex that binds to the Fur box in the *hmuR* promoter region does not allow HemP-mediated transcriptional activation of the *hmuR* operon.

Hemin-binding ability of HemP. The C-terminal histidine and tyrosine residues of the heme-binding protein HasA from *Pseudomonas aeruginosa* have been shown to participate in the binding to heme (28, 29), and the two residues are conserved among

FIG 5 Binding of HemP_{6xHis} to the fragment upstream of *hmuR*. (A) EMSA for HemP_{6xHis} binding to the FAM-labeled 221-bp fragment. The mobility shift was not affected by the addition of a 100-fold excess of salmon sperm DNA and was almost completely abolished by the addition of a 100-fold excess of the nonlabeled fragment (data not shown). (B) Sequence of the 221-bp fragment used for this analysis. The protected region (HemP binding) is shown by red shading and an inverted repeat by arrows. (C) DNase I footprinting analysis. The FAM-labeled 221-bp fragment was digested by DNase I in a reaction mixture supplemented with (red) or without (blue) the purified HemP_{6xHis} protein. The data obtained were analyzed with TraceViewer software. See Materials and Methods for details.

the HemP superfamily proteins (Fig. S1B). Our three-dimensional structure analysis of another heme-binding protein, i.e., YdiE from *Escherichia coli* (30), which exhibits 43% amino acid sequence similarity with ATCC 17616 HemP, showed that the two residues are located in close proximity to each other. Homology modeling using SWISS-MODEL (<https://swissmodel.expasy.org>) was applied to HemP and YdiE. The results predicted close localization of the two residues in the three-dimensional structure of HemP, suggesting the possible binding of HemP to heme. This possibility was investigated *in vitro* using a quantitative horseradish peroxidase (HRP)-based heme assay system in which a protein possessing heme-binding ability generates chemiluminescence (see Materials and Methods for details) (31). After the incubation of HemP_{6xHis} with hemin and subsequent removal of free hemin, the amount of hemin bound to HemP_{6xHis} was measured. The results indicated that much stronger chemiluminescence was detected with the HemP_{6xHis} protein than with the AhpD_{6xHis} protein (an AhpD derivative with six histidine residues at the C terminus), which is not expected to bind hemin (Fig. 6A). Based on a calibration curve of chemiluminescence intensities determined using various known concentrations of hemin, 1 μ M HemP_{6xHis} was calculated to bind 460 pM hemin (Fig. 6B). Taking into account the hemin extraction efficiency in this study, the calculated value suggested stoichiometric binding of HemP_{6xHis} to hemin.

DISCUSSION

The transcriptional regulation of gene clusters for heme uptake systems in various Gram-negative bacterial species has been investigated, and the transcription of such genes in most species (except for alphaproteobacterial species) was found to be commonly repressed under iron-replete conditions, through the involvement of the active form of Fur. The transcription of heme uptake gene clusters is also positively regulated by various mechanisms in the presence of heme. Such mechanisms are exemplified by (i) the system consisting of an inner membrane-spanning heme-sensor protein with anti-sigma-factor activity and an extracytoplasmic sigma factor (ECF) protein in *P. aeruginosa* and *Serratia marcescens* (19), (ii) the two-component heme-responsive sensor-regulator system in *Neisseria meningitidis* (17), (iii) the LysR-type activator system in *Vibrio* spp. (18), and (iv) the HmuP activator (belonging to the HemP superfamily) system (Fig. S1) in *B. japonicum* and *S. meliloti*, which belong to the alphaproteobacterial genera (16). The genes encoding such regulatory systems have

FIG 6 Binding of HemP_{6xHis} to hemin. The HemP_{6xHis} protein incubated with hemin was repurified, and bound hemin was extracted with acidified acetone. Apo-HRP and HRP substrates were added to the extract, and chemiluminescence was measured. See Materials and Methods for details. (A) Well images of reactions with the extract using 1 μ M HemP_{6xHis} (left) or AhpD_{6xHis} (right). (B) Calibration curve prepared by using different concentrations of hemin (0, 100, 200, 400, 600, and 800 pM). Each value with a SD (vertical bar) was obtained from at least three independent experiments. The intensity value measured with 1 μ M HemP_{6xHis} in panel A is depicted as a horizontal dashed line.

been shown to be almost always located adjacent to the structural gene clusters for heme uptake.

Although analysis of the genomes of various *Burkholderia* species has suggested their use of hemin as a sole source of iron, only a few *Burkholderia cenocepacia* and *B. pseudomallei* strains have been experimentally confirmed to utilize external hemin (6, 25, 32). However, the mechanisms for transcriptional regulation of the genes for heme uptake systems in these strains have remained unknown. In our previous study, *B. multivorans* ATCC 17616 was suggested to use hemin as an iron source, and the putative gene cluster for hemin uptake (*hmu* operon) and the *hemP* gene (which was uncharacterized at that time) were both shown to be transcriptionally repressed by the Fur-Fe²⁺ complex under iron-replete conditions. Our investigation of ATCC 17616 in this study clearly showed (i) its ability to use hemin as a sole source of iron and (ii) the requirement for HemP for transcription of the *hmu* operon. The ATCC 17616 *hemP* gene was found to be located far from its *hmu* operon. This distant localization of the regulator gene from the structural *hmu* gene cluster is apparently conserved in various other *Burkholderia* species, which is not generally the case in other bacterial genera that encode HemP superfamily proteins (Fig. 2A).

The HemP superfamily proteins show very high levels of similarity in their C-terminal 20 amino acid residues, with the conserved KLILXK motif at their C termini (Fig. S1B) (26, 27). Despite of the high level of conservation of these proteins among various proteobacteria, their experimental characterization has been limited to the HmuP proteins from *S. meliloti* and *B. japonicum*, and both proteins are required for heme uptake (26, 27). Each HmuP protein is a transcriptional activator for the gene encoding the outer membrane receptor protein for heme uptake but not for the *hmuP*-containing operon that encodes the remaining proteins for the heme uptake system (Fig. 2A). The KLILXK motif in the former HmuP protein has been shown to be important for transcriptional activity (26). Expression of the two *hmu* transcriptional units in each species is also

transcriptionally regulated by the concentrations of external iron; expression is repressed in the former species by RirA, a pleiotropic regulator that is active under iron-replete conditions, and is activated in the latter species by Irr (iron response regulator), a pleiotropic regulator that is produced abundantly under iron-depleted conditions. It should be noted that the two species do not encode Fur for transcriptional regulation of the iron regulon (16). The mechanism for transcriptional regulation of genes for heme uptake in *B. multivorans* ATCC 17616 resembles that in these two species, in that (i) each HemP homologue is a transcriptional activator for the heme receptor-encoding gene, and (ii) transcription of each *hemP* homologue is regulated by a pleiotropic regulator (Fur, RirA, or Irr) whose activity or abundance depends on the external iron concentration. However, the three pleiotropic regulators differ significantly in their amino acid sequences. One feature associated only with ATCC 17616 is that its HemP protein activates the transcription of a whole set of structural genes required for hemin uptake, which reflects the operon structure of the *hmuRSTUV* cluster. Considering our findings that the *hemP* and *hmuR* deletants of ATCC 17616 exhibited nearly complete and partial deficiencies, respectively, of growth in the hemin-containing M9 minimal medium (Fig. 3), the HemP protein most likely regulates at least the expression of other, currently unidentified genes (other than the *hmu* operon) for alternative hemin uptake systems. The presence of such predicted alternative systems is unlikely in the two alphaproteobacterial species and *B. pseudomallei*, because their derivatives lacking the identified heme-receptor-encoding genes demonstrated the loss of heme-utilizing ability (25–27). To our knowledge, the predicted multiple regulatory roles of HemP in ATCC 17616 represent a novel property that is not observed for the other HemP superfamily proteins.

There have been no experimental results clearly showing the direct binding of HmuP to its target DNA sequences in the two alphaproteobacterial species (16). However, our EMSA and DNase I footprinting analysis in this study revealed the specific binding of the ATCC 17616 HemP_{Hisx6} protein to the sequence upstream of the Fur-box-containing promoter region of the *hmu* operon (Fig. 5). Since transcriptional activation of the ATCC 17616 *hmu* operon was similarly observed when the gene encoding HemP_{6xHis} was supplied instead of the wild type (data not shown), it is quite unlikely that the addition of six histidine residues to the C terminus of HemP drastically affects the binding affinity of HemP_{6xHis} for the target sequence. The relative positions of the HemP- and Fur-Fe²⁺-binding sites in the *hmuR*-upstream region are suitable to explain our observation that the Fur-mediated repression of the *hmu* operon is epistatic to the HemP-mediated activation (Fig. 4C); binding of the Fur-Fe²⁺ complex to the Fur box within the *hmu* promoter region hinders RNA polymerase from binding to the promoter even in the situation in which HemP is allowed to bind to its binding site for activation of transcription of the *hmu* operon. Our *in vitro* analysis showed strong binding affinity of HemP for hemin (Fig. 6). If this is also the case *in vivo*, then the HemP-heme complex might contribute to modulating its interaction with the *hmuR*-upstream region so as to more effectively regulate transcription of the *hmu* operon. In spite of the ability of HemP to bind to its target DNA sequence, no canonical DNA-binding helix-turn-helix motifs were predicted in HemP, and this was also the case with other HemP superfamily proteins, including the HmuP proteins from the two alphaproteobacterial species (26, 27). Three-dimensional structure modeling of the *S. melliloti* HmuP protein has predicted that it consists of three β -sheets and one small α -helix, probably with a dimer form, and that the KLILXK motif is located within the C-terminal β -sheet (26). It is presently not known which amino acid residues in the ATCC 17616 HemP protein and which nucleotide residues in the HemP-binding site are critical for binding. Our future detailed mutational analysis of HemP and its binding site will help to clarify the mechanisms for HemP-mediated transcriptional activation.

The mechanism by which almost all of the pleiotropic phenotypes observed in the ATCC 17616 Δfur mutant (DF1) are suppressed by the additional introduction of the *hemP* mutation remains unclear. However, this mechanism is quite likely to differ from the mechanism by which the *oxyR* mutation suppresses the pleiotropic phenotypes of

the Δfur mutant, since the constitutive overproduction of KatG and AhpC1D proteins in the $\Delta fur \Delta oxyR$ mutant (21) was not detected in the $\Delta fur \Delta hemP$ mutant (DFP1) (Fig. 1F). Our present study showed a constitutive and high level of *hemP* transcription in DF1. When we further consider that HemP is suggested to positively regulate at least the expression of unidentified heme-utilizing genes (Fig. 3 and 4), it is plausible that HemP also regulates the expression of other genes. As a transcriptional regulator, HemP might function either directly or indirectly (i) to positively regulate the expression of unknown genes for the enhanced generation of ROS or (ii) to negatively regulate the expression of unknown genes for the detoxification of ROS. Such a mechanism might account for the phenotypes of DFP1 in which ROS are not overproduced. Constitutive and high-level production of HemP in DF1 would be expected to lead to overproduction of HmuS. Some HmuS homologues from other genera have been reported to function as heme oxygenases that degrade heme to release free iron (15, 17). If the ATCC 17616 HmuS protein has such an activity, then the overproduced HmuS protein in DF1 would result in drastic increases in the levels of free iron produced from intracellular heme, and the increased levels of free iron would in turn generate large amounts of ROS. Introduction of the *hemP* mutation into DF1 might be able to block the release of iron from intracellular heme, thereby reducing the generation of ROS. This alternative possibility will be clarified by investigating the heme oxygenase activity of HmuS and the amounts of free heme in DF1 and DFP1. For investigation of the latter point, it is necessary to establish an assay system to measure the amounts of free intracellular heme in bacteria. Use of such an experimental system in our future work will greatly contribute to our understanding of the detailed role of HemP in heme uptake.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Table 1 lists the bacterial strains and plasmids used in this study. *B. multivorans* and *E. coli* cells were cultivated at 30°C and 37°C, respectively. The liquid media used were LB broth, 1/3LB broth, and M9 minimal medium supplemented with an appropriate carbon source (glucose, citrate, or succinate) at a concentration of 0.2% (24). Solid media were prepared by the addition of 1.5% agar. Chrome azurol S (CAS) agar plates (33) were used to monitor the secretion of siderophores. When required, FeCl₃, hemin (Sigma-Aldrich Japan, Tokyo, Japan), and Dpr (Sigma-Aldrich) were added to the media at final concentrations of 30, 30, and 200 μ M, respectively. The antibiotics added to media were as follows: ampicillin (Ap) at 100 μ g/ml, kanamycin (Km) at 50 μ g/ml, and tetracycline (Tc) at 10 μ g/ml for *E. coli* and chloramphenicol (Cm) at 100 μ g/ml, Km at 100 μ g/ml, and Tc at 50 μ g/ml for *B. multivorans*.

Bacterial growth in liquid medium was monitored using the Infinite M200 microplate reader system (Tecan Japan, Kawasaki, Japan). Bacterial cells cultivated overnight in 1/3LB broth were washed three times with M9 minimal medium and suspended in the same medium so that the optical density at 600 nm (OD₆₆₀) value was adjusted to 1.0. A 0.1% aliquot of such suspension was inoculated in 200 μ l of appropriate liquid medium in each well of a 96-well microtiter plate and used for measurements of OD₆₆₀ values at different time points during the incubation.

Basic DNA and RNA manipulations. Established procedures were employed for the preparation of genomic and plasmid DNA, DNA digestion with restriction enzymes, ligation, agarose gel electrophoresis, and transformation of *E. coli* and *B. multivorans* (12, 24). PCR amplification of DNA fragments was carried out using Ex Taq DNA polymerase (TaKaRa, Otsu, Japan) or KOD-plus DNA polymerase (Toyobo, Osaka, Japan), and the primers used in this study are listed in Table 2. PCR products were cloned into multiple cloning sites (MCS) on vector plasmids using restriction enzymes followed by ligation mix (TaKaRa) or a Gibson assembly cloning kit (New England BioLabs, Beverly, MA).

Sequence determination was performed using an ABI Prism model 3130xl sequencer and an ABI Prism BigDye termination kit (Thermo Fisher Scientific, Waltham, MA). Draft genome sequencing was carried out using the HiSeq 1000 sequencing system (Illumina, San Diego, CA) and the 454 GS FLX titanium system (Roche, Mannheim, Germany), and mutation sites were identified by using ShortRead-Manager software (34). An RNeasy minikit (Qiagen, Hilden, Germany) was used to prepare total cellular RNA from *B. multivorans* cells cultivated in liquid medium. RT-PCR was carried out with Superscript II reverse transcriptase (Thermo Fisher Scientific) or a ReverTra Ace kit (Toyobo), and the resulting cDNA samples were employed for qRT-PCR analysis using the Opticon 2 system (Bio-Rad, Hercules, CA) and a SYBR Premix Ex Taq kit (TaKaRa). The mRNA levels were normalized to those of a housekeeping gene, *dnaA*.

Construction of plasmids and strains. *E. coli* strain DH5 α or JM109 was used for construction of plasmids. To delete the *hemP* gene from the ATCC 17616 genome, ~800-bp fragments upstream and downstream of *hemP* were PCR amplified using the ATCC 17616 genome as a template and the primer pairs of *hemP*_UP_FW/*hemP*_UP_RV and *hemP*_DOWN_FW/*hemP*_DOWN_RV, respectively. A Gibson assembly cloning kit was used to insert the two amplicons into the EcoRI and HindIII sites on pEX18Tc so that they were arranged in the same order as in the genome. The resulting plasmid was introduced

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Reference or source
<i>E. coli</i>		
DH5 α	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 $\Delta(lac)U169$ (ϕ80dlacZΔM15)</i>	39
JM109	<i>recA1 endA1 gyrA96 thi-1 hsdR17(r_K⁻ m_K⁺) supE44 relA1 $\Delta(lac-pro)$ [F' <i>traD36 proAB</i>⁺ <i>lacI^q</i>ΔM15]</i>	40
BL21Star(DE3)	<i>ompT hsd_B (r_B⁻ m_B⁻) gal dcm λ (DE3)</i>	Thermo Fisher Scientific
<i>B. multivorans</i>		
ATCC 17616	Soil isolate; type strain	ATCC
DF1	Cm ^r ; ATCC 17616 <i>fur</i> deletion mutant	12
SOF8	Spontaneous suppressor mutant of DF1 that restored tolerance to NO	24
SUC5	Spontaneous suppressor mutant of DF1 that restored ability to use succinate as carbon source	This study
DP1	ATCC 17616 <i>hemP</i> deletion mutant	This study
DFP1	Cm ^r ; ATCC 17616 <i>fur</i> and <i>hemP</i> deletion mutant	This study
DR1	ATCC 17616 <i>hmuR</i> deletion mutant	This study
DPR1	ATCC 17616 <i>hemP</i> and <i>hmuR</i> deletion mutant	This study
Plasmids		
pEX18Tc	Tc ^r ; <i>sacB</i> <i>oriT</i> ; suicide vector for gene replacement, carrying pUC18-derived MCS	41
pBBR1MCS-3	Tc ^r ; shuttle vector able to replicate in <i>E. coli</i> and <i>B. multivorans</i>	42
pCF1	Km ^r ; pME6041 (shuttle vector able to replicate in <i>E. coli</i> and <i>B. multivorans</i>) derivative carrying <i>fur</i> gene	12
pCP1	Tc ^r ; pBBR1MCS-3 carrying <i>hemP</i> gene with its authentic promoter	This study
pCR1	Tc ^r ; pBBR1MCS-3 carrying <i>hmuR</i> gene with its authentic promoter	This study
pNIT6012	Tc ^r ; shuttle vector able to replicate in <i>E. coli</i> and <i>B. multivorans</i>	43
pNIT6012dS	Tc ^r ; SmaI site-added derivative of pNIT6012	36
pKD46	Ap ^r ; <i>oriR101(repA101^{ts})</i> ; λ Red recombinase expression plasmid	35
pNITara	pNIT6012dS carrying <i>araC</i> gene and pBAD promoter from pKD46	This study
pBAD-hemP	Tc ^r ; pNITara carrying <i>hemP</i> gene without its authentic promoter; arabinose-inducible expression of <i>hemP</i>	This study
pET22b(+)	Ap ^r ; C-terminal 6 $\times$ His tag	Novagen
pEThemP	Ap ^r ; pET22b(+) derivative for overexpression of C-terminal His-tagged HemP protein	This study
pETAhpD	Ap ^r ; pET22b(+) derivative for overexpression of C-terminal His-tagged AhpD protein	This study

^ar, resistant.

into ATCC 17616 or its derivatives by transformation to select Tc-resistant clones, in which the plasmid was integrated into the genome by single crossover-mediated homologous recombination between the fragment shared by the genome and the introduced plasmid. Subsequent exposure of the transformants to 10% sucrose led to the formation of Tc-sensitive segregants, and some of them were confirmed by PCR to lack the *hemP* gene, with concomitant loss of the pEX18Tc portion through a second single crossover-mediated homologous recombination event. The *hemP* deletants of ATCC 17616 and DF1 were designated DP1 and DFP1, respectively. A similar procedure was employed to delete the *hmuR* gene from the ATCC 17616 genome. In this case, the primer pairs *hmuR*_UP_FW/*hmuR*_UP_RV and *hmuR*_DOWN_FW/*hmuR*_DOWN_RV were used to PCR amplify the DNA fragments upstream and downstream of *hmuR*, respectively. The *hmuR* deletants of ATCC 17616 and DP1 were designated DR1 and DPR1, respectively.

The primer pairs pBBR_hemP_FW/pBBR_hemP_RV and pBBR_hmuR_FW/pBBR_hmuR_RV were used to PCR amplify the ATCC 17616 *hemP* and *hmuR* genes, respectively, with their authentic promoter-containing fragments. A Gibson assembly cloning kit was used to insert the two amplicons into the XbaI and XhoI sites on pBBR1MCS-3 to obtain pCP1 and pCR1, respectively. To construct pNITara, a DNA fragment containing the *araC* gene and pBAD promoter on pKD46 (35) was amplified by PCR using the primer set of XhoI_paraBC and paraBC_NheI, and the resulting amplicon was inserted into the MCS on pNIT6012dS, after treatment with XhoI and NheI (36). The ATCC 17616 *hemP* gene without its promoter-containing fragment was PCR amplified using the primer set of pNITara_hemP_EcoRI and pNITara_hemP_HindIII, and the EcoRI- and HindIII-treated amplicon was inserted into the corresponding sites of pNIT6012dS so that the *hemP* gene was just downstream of the pBAD promoter. The resulting plasmid was designated pBAD-hemP. The primer pairs NdeI_hemP/XhoI_hemP and NdeI_ahpC/XhoI_ahpC were used to PCR amplify the ATCC 17616 *hemP* and *ahpD* genes, respectively. After treatment with NdeI and XhoI, the amplicons were inserted into the corresponding sites on pET22b(+) to obtain pEThemP and pETAhpD, respectively.

Phenotype analysis. ATCC 17616 and its derivatives were analyzed for their carbon assimilation ability, secretion of siderophores, levels of free intracellular iron, and sensitivity to RNS and ROS, as in our previous studies (12, 24). Cells grown to late-exponential phase in 1/3LB broth were used for the phenotype analysis. Cells were washed three times with M9 minimal medium and suspended in the same medium to yield suspensions with OD₆₆₀ values of 1.0, which corresponded to CFU values of approximately 10⁹ CFU/ml. These suspensions were serially diluted 100-fold to obtain suspensions with CFU

TABLE 2 Primers used in this study

Purpose and primer name	Sequence (5' to 3') ^a	Use
Allelic exchange mutagenesis		
hemP_UP_FW	CGACTCTAGAGGATCTACCGAAGAAGAGATTCGG	Upstream region of <i>hemP</i>
hemP_UP_RV	GCCCAACACCTCTCAGTCGATAGCTTGAAGCAAAC	
hemP_DOWN_FW	TGAGAGGTGTTGGGCATTA	Downstream region of <i>hemP</i>
hemP_DOWN_RV	CGGTACCCGGGGATCGCAGATCAAGCAGTGGCT	
hmuR_UP_FW	TAAAACGACGGCCAGTGCCATTCAACGAACGCGGCGTGACC	Upstream region of <i>hmuR</i>
hmuR_UP_RV	CTTGAGCTTCAGGGAACGGGCTGCGCCC	
hmuR_DOWN_FW	CCCGTTCCCTGAAGCTCAAGCGCGAGCG	Downstream region of <i>hmuR</i>
hmuR_DOWN_RV	CAGCTATGACCATGATTACGCCGGATCGAGCACGTTGATC	
Complementation analysis		
pBBR_hemP_FW	CCACCGCGGTGGCGGCCGCTTACGGTGCGCCTGATTTCGC	Construction of pCP1
pBBR_hemP_RV	GCTGGGTACCGGGCCCCCGCGAGCGAATGGAGGCGC	Construction of pCR1
pBBR_hmuR_FW	CCACCGCGGTGGCGGCCGCTCCGGGCGCAGCCCGTTCC	
pBBR_hmuR_RV	GCTGGGTACCGGGCCCCCGACGAACGCGTCGCGCAGC	
XhoI_paraBC	CCCCTCGAGCCAAAAAACGGGTATGGAG	Construction of pNITara
paraBC_NheI	CCCGCTAGCTTTATTATGACAACCTTGACG	Construction of pBAD-hemP
pNITara_hemP_EcoRI	GGGGAATTCATTATCCATCGTATCGACAGT	
pNITara_hemP_HindIII	GGGAAGCTTCGAATGGAGGCGCACAAATAA	
qRT-PCR		
dnaA_F	GGCGTTCGACGATTTCAAGC	Transcription of <i>dnaA</i>
dnaA_R	CGAACGCGTAGAAGAATTCC	Transcription of <i>hemP</i>
hemP_F	CAAGGTCACAGCCATATCAG	
hemP_R	CTTCGTCAGGATCAGCTTGC	
hmuR_F	GTATCTGCAGTACGCACATG	Transcription of <i>hmuR</i>
hmuR_R	GATTGCCGATCGAGGTATAG	Transcription of <i>bfd</i>
bfd_F	GTTTCCGATCGGAAGATTTCG	
bfd_R	GTACCGACTCCTCGCATTTG	
Protein purification		
NdeI_hemP	GGGCATATGACCGACACCATGCGCCC	Construction of pEThemP
XhoI_hemP	GGGCTCGAGCTTCGTCAGGATCAGC	Construction of pETahpD
NdeI_ahpD	GGGCATATGGAATTCATCGATTTCGATTAAGG	
XhoI_ahpD	GGGCTCGAGTTCGCCTTCGGCGG	
EMSA		
pNIT_hmuR_pro_NheI	GGGGCTAGCGTCTCCGAATTGATCGCGCG	Upstream region of <i>hmuR</i>
pNIT_hmuR_pro_HindIII	GGGAAGCTTAGGTGGTCTCCCATGGTTGA	Construction of probe
pNIT_5041	CTGACACCCTCATCAGTGCCAA	
pNIT_5548	GTGAGAAATCACCATGAGTGACG	

^aRestriction sites are underlined.

values of 10^3 CFU/ml to 10^7 CFU/ml. A 5- μ l portion of each cell suspension was spotted on an M9 minimal agar plate supplemented with 0.2% citrate or succinate as the sole carbon source for the carbon assimilation assay or on a CAS agar plate containing 30 μ M FeCl₃ for assessment of siderophore secretion. Cells cultivated in 1/3LB broth (see above) were washed three times with 1/3LB broth, and cell suspensions in fresh 1/3LB broth were used for determination of cellular free iron levels and sensitivities to RNS and ROS.

Preparation of crude cell extracts. Cells grown to mid-exponential phase in 1/3LB broth were disrupted by sonication. The lysates were recovered by centrifugation at $13,000 \times g$ for 15 min, and proteins were separated by using SDS-PAGE. The protein bands were stained using Coomassie brilliant blue.

Purification of His-tagged derivatives of HemP and AhpD and their biochemical analysis. When LB cultures of *E. coli* BL21Star(DE3) cells carrying pEThemP or pETahpD reached mid-log phase, isopropyl- β -D-thiogalactopyranoside (IPTG) was added at a final concentration of 0.5 mM. After additional incubation for 8 h at 25°C, the cells were collected and disrupted using CellLytic B reagent (Sigma-Aldrich). The IPTG-induced HemP or AhpD derivative tagged with six histidine residues at the C terminus was purified using Talon metal affinity resins (TaKaRa), according to the manufacturer's protocol.

For EMSA of the HemP_{6 $\times$ His}-binding DNA sequence, a 221-bp DNA fragment upstream of *hmuR* was amplified by PCR using the primers pNIT_hmuR_pro_NheI and pNIT_hmuR_pro_HindIII, and the amplicon was digested with NheI and HindIII and then inserted into the MCS on pNIT6012. The *hmuR*-upstream region of the resulting plasmid, pNITupR, was amplified by PCR using the primers pNIT_5041 and pNIT_5548, each 5' end of which was labeled with 6-carboxyfluorescein (FAM) (Eurofins Genomics, Tokyo, Japan). A reaction mixture containing 2 μ g/ μ l of the PCR-amplified and FAM-labeled DNA fragment and the HemP_{6 $\times$ His} protein at final concentrations ranging from 0 to 120 nM, in 20 μ l of binding

buffer (10% glycerol in phosphate-buffered saline [PBS]), was incubated for 3 min at 30°C. The reaction mixture was loaded onto a 12.5% (wt/vol) nondenaturing polyacrylamide gel in PBS. After electrophoresis for 1 h at 80 mA, the gel was exposed to an imaging plate, and the gel image was analyzed using the BAS-1000 system (Fujifilm, Tokyo, Japan).

For DNase I footprinting analysis, pNITupR was used as a template to PCR amplify the 221-bp upstream fragment of *hmuR* using the primer set of pNIT_5548 and 5'-FAM-labeled pNIT_5041. A 20- μ l aliquot of a reaction mixture containing 200 nM HemP_{6 $\times$ His}, 400 ng of labeled DNA, 10% glycerol in PBS, and 0.0014 unit of DNase I (TaKaRa) was kept at 30°C for 3 min and then at 95°C for 10 min. The DNase I-digested fragments were purified by phenol-chloroform/isoamyl alcohol extraction and ethanol precipitation. The purified fragments and GeneScan-500 LIZ size standard (Thermo Fisher Scientific) were dissolved in HiDi formamide. A Maxam-Gilbert G+A sequencing ladder was prepared as described previously (37), using the same DNA fragment as used for detection of the HemP_{Hisx6}-binding site. The samples were analyzed using a 3130xl genetic analyzer, a G5 dye set, the POP-7 polymer, and 50-cm capillaries. The data were analyzed by using TraceViewer software (<http://www.ige.tohoku.ac.jp/joho/gmProject/gmdownload.html>) (38), and the LIZ bands were used for calibration.

In vitro hemin-binding assay. The HemP_{6 $\times$ His} or AhpD_{6 $\times$ His} (negative control) protein was mixed with 100 μ M hemin in 100 mM Tris buffer (pH 7.0) for 30 min at 25°C; the protein was subsequently affinity purified as described above and resolved in 100 mM Tris buffer (pH 7.0). Four hundred microliters of acidified acetone was added to 100 μ l of solution with 5 μ M protein, and the mixture was incubated overnight at 4°C. The solution was then centrifuged to precipitate the protein, 1 μ l of the supernatant was added to a well of a 96-well microtiter plate containing 100 μ l of 100 mM Tris buffer (pH 7.0) and 2.5 nM apoHRP (Funakoshi, Tokyo, Japan), and the mixture was incubated for 30 min at 25°C. One hundred microliters of an HRP substrate, the Immobilon Western chemiluminescent HRP substrate (Merck Millipore, Darmstadt, Germany), was then added to the well, and the chemiluminescence intensity was measured using the ChemoDoc XRS system (Bio-Rad).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.00479-17>.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.

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REFERENCES

- Omidvari M, Sharifi RA, Ahmadzadeh M, Dahaji PA. 2010. Role of fluorescent pseudomonads siderophore to increase bean growth factors. *J Agric Sci* 2:242–247.
- Oppenheimer SJ. 2001. Iron and its relation to immunity and infectious disease. *J Nutr* 131:616S–635S.
- Becker KW, Skaar EP. 2014. Metal limitation and toxicity at the interface between host and pathogen. *FEMS Microbiol Rev* 38:1235–1249. <https://doi.org/10.1111/1574-6976.12087>.
- Li K, Chen WH, Bruner SD. 2016. Microbial siderophore-based iron assimilation and therapeutic applications. *Biometals* 29:377–388. <https://doi.org/10.1007/s10534-016-9935-3>.
- Schalk IJ, Hannauer M, Braud A. 2011. New roles for bacterial siderophores in metal transport and tolerance. *Environ Microbiol* 13:2844–2854. <https://doi.org/10.1111/j.1462-2920.2011.02556.x>.
- Thomas MS. 2007. Iron acquisition mechanisms of the *Burkholderia cepacia* complex. *Biometals* 20:431–452. <https://doi.org/10.1007/s10534-006-9065-4>.
- Cornelis P, Wei Q, Andrews SC, Vincx T. 2011. Iron homeostasis and management of oxidative stress response in bacteria. *Metallomics* 3:540–549. <https://doi.org/10.1039/c1mt00022e>.
- Reiter TA, Pang B, Dedon P, Demple B. 2006. Resistance to nitric oxide-induced necrosis in heme oxygenase-1 overexpressing pulmonary epithelial cells associated with decreased lipid peroxidation. *J Biol Chem* 281:36603–36612. <https://doi.org/10.1074/jbc.M602634200>.
- Justino MC, Almeida CC, Teixeira M, Saraiva LM. 2007. *Escherichia coli* di-iron YtfE protein is necessary for the repair of stress-damaged iron-sulfur clusters. *J Biol Chem* 282:10352–10359. <https://doi.org/10.1074/jbc.M610656200>.
- Troxell B, Hassan HM. 2013. Transcriptional regulation by ferric uptake regulator (Fur) in pathogenic bacteria. *Front Cell Infect Microbiol* 3:59. <https://doi.org/10.3389/fcimb.2013.00059>.
- Yu C, Genco CA. 2012. Fur-mediated activation of gene transcription in the human pathogen *Neisseria gonorrhoeae*. *J Bacteriol* 194:1730–1742. <https://doi.org/10.1128/JB.06176-11>.
- Yuhara S, Komatsu H, Goto H, Ohtsubo Y, Nagata Y, Tsuda M. 2008. Pleiotropic roles of iron-responsive transcriptional regulator Fur in *Burkholderia multivorans*. *Microbiology* 154:1763–1774. <https://doi.org/10.1099/mic.0.2007/015537-0>.
- Stojiljkovic I, Hantke K. 1992. Hemin uptake system of *Yersinia enterocolitica*: similarities with other TonB-dependent systems in Gram-negative bacteria. *EMBO J* 11:4359–4367.
- Tong Y, Guo M. 2009. Bacterial heme-transport proteins and their heme-coordination modes. *Arch Biochem Biophys* 481:1–15. <https://doi.org/10.1016/j.abb.2008.10.013>.
- Runyen-Janecky LJ. 2013. Role and regulation of heme iron acquisition in Gram-negative pathogens. *Front Cell Infect Microbiol* 3:55. <https://doi.org/10.3389/fcimb.2013.00055>.
- O'Brian MR. 2015. Perception and homeostatic control of iron in the rhizobia and related bacteria. *Annu Rev Microbiol* 69:229–245. <https://doi.org/10.1146/annurev-micro-091014-104432>.
- Choby JE, Skaar EP. 2016. Heme synthesis and acquisition in bacterial pathogens. *J Mol Biol* 428:3408–3428. <https://doi.org/10.1016/j.jmb.2016.03.018>.
- Payne SM, Mey AR, Wyckoff EE. 2016. *Vibrio* iron transport: evolutionary adaptation to life in multiple environments. *Microbiol Mol Biol Rev* 80:69–90. <https://doi.org/10.1128/MMBR.00046-15>.
- Sheldon JR, Laakso HA, Heinrichs DE. 2016. Iron acquisition strategies of bacterial pathogens. *Microbiol Spectr* 4:VMBF-0010-2015.
- Brewitz HH, Hagelueken G, Imhof D. 2017. Structural and functional diversity of transient heme binding to bacterial proteins. *Biochim Biophys Acta* 1861:683–697. <https://doi.org/10.1016/j.bbagen.2016.12.021>.
- Anzaldi LL, Skaar EP. 2010. Overcoming the heme paradox: heme toxicity

- and tolerance in bacterial pathogens. *Infect Immun* 78:4977–4989. <https://doi.org/10.1128/IAI.00613-10>.
22. Chiarini L, Bevivino A, Dalmastri C, Tabacchioni S, Visca P. 2006. *Burkholderia cepacia* complex species: health hazards and biotechnological potential. *Trends Microbiol* 14:277–286. <https://doi.org/10.1016/j.tim.2006.04.006>.
 23. Nagata Y, Senbongi J, Ishibashi Y, Sudo R, Miyakoshi M, Ohtsubo Y, Tsuda M. 2014. Identification of *Burkholderia multivorans* ATCC 17616 genetic determinants for fitness in soil by using signature-tagged mutagenesis. *Microbiology* 160:883–891. <https://doi.org/10.1099/mic.0.077057-0>.
 24. Kimura A, Yuhara S, Ohtsubo Y, Nagata Y, Tsuda M. 2012. Suppression of pleiotropic phenotypes of a *Burkholderia multivorans* *fur* mutant by *oxyR* mutation. *Microbiology* 158:1284–1293. <https://doi.org/10.1099/mic.0.057372-0>.
 25. Kvitko BH, Goodyear A, Propst KL, Dow SW, Schweizer HP. 2012. *Burkholderia pseudomallei* known siderophores and heme uptake are dispensable for lethal murine melioidosis. *PLoS Negl Trop Dis* 6:e1715. <https://doi.org/10.1371/journal.pntd.0001715>.
 26. Amarelle V, Koziol U, Rosconi F, Noya F, O'Brian MR, Fabiano E. 2010. A new small regulatory protein, HmuP, modulates haemin acquisition in *Sinorhizobium meliloti*. *Microbiology* 156:1873–1882. <https://doi.org/10.1099/mic.0.037713-0>.
 27. Escamilla-Hernandez R, O'Brian MR. 2012. HmuP is a coactivator of *Irr*-dependent expression of heme utilization genes in *Bradyrhizobium japonicum*. *J Bacteriol* 194:3137–3143. <https://doi.org/10.1128/JB.00071-12>.
 28. Smith AD, Wilks A. 2015. Differential contributions of the outer membrane receptors PhuR and HasR to heme acquisition in *Pseudomonas aeruginosa*. *J Biol Chem* 290:7756–7766. <https://doi.org/10.1074/jbc.M114.633495>.
 29. Shirataki C, Shoji O, Terada M, Ozaki SI, Sugimoto H, Shiro Y, Watanabe Y. 2014. Inhibition of heme uptake in *Pseudomonas aeruginosa* by its hemophore (HasAp) bound to synthetic metal complexes. *Angew Chem Int Ed Engl* 53:2862–2866. <https://doi.org/10.1002/anie.201307889>.
 30. Nishimura K, Addy C, Shrestha R, Voet ARD, Zhang KYJ, Ito Y, Tame JRH. 2015. The crystal and solution structure of YdiE from *Escherichia coli*. *Acta Crystallogr F Struct Biol Commun* 71:919–924. <https://doi.org/10.1107/S2053230X15009140>.
 31. Espinas NA, Kobayashi K, Takahashi S, Mochizuki N, Masuda T. 2012. Evaluation of unbound free heme in plant cells by differential acetone extraction. *Plant Cell Physiol* 53:1344–1354. <https://doi.org/10.1093/pcp/pcs067>.
 32. Tyrrell J, Whelan N, Wright C, Sá-Correia I, McClean S, Thomas M, Callaghan M. 2015. Investigation of the multifaceted iron acquisition strategies of *Burkholderia cenocepacia*. *Biometals* 28:367–380. <https://doi.org/10.1007/s10534-015-9840-1>.
 33. Schwyn B, Neilands J. 1987. Universal chemical assay for the detection and determination of siderophores. *Anal Biochem* 160:47–56. [https://doi.org/10.1016/0003-2697\(87\)90612-9](https://doi.org/10.1016/0003-2697(87)90612-9).
 34. Ohtsubo Y, Maruyama F, Mitsui H, Nagata Y, Tsuda M. 2012. Complete genome sequence of *Acidovorax* sp. strain KKS102, a polychlorinated-biphenyl degrader. *J Bacteriol* 194:6970–6971. <https://doi.org/10.1128/JB.01848-12>.
 35. Datsenko K, Wanner BL. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* 97:6640–6645. <https://doi.org/10.1073/pnas.120163297>.
 36. Nagayama H, Sugawara T, Endo R, Ono A, Kato H, Ohtsubo Y, Nagata Y, Tsuda M. 2015. Isolation of oxygenase genes for indigo-forming activity from an artificially polluted soil metagenome by functional screening using *Pseudomonas putida* strains as hosts. *Appl Microbiol Biotechnol* 99:4453–4470. <https://doi.org/10.1007/s00253-014-6322-2>.
 37. Kishida K, Inoue K, Ohtsubo Y, Nagata Y, Tsuda M. 2017. Host range of the conjugative transfer system of IncP-9 naphthalene-catabolic plasmid NAH7 and characterization of its *oriT* region and relaxase. *Appl Environ Microbiol* 83:e02359-16.
 38. Ohtsubo Y, Nagata Y, Tsuda M. 2017. Efficient N-tailing of blunt DNA ends by Moloney murine leukemia virus reverse transcriptase. *Sci Rep* 7:41769. <https://doi.org/10.1038/srep41769>.
 39. Green MR, Sambrook J. 2012. *Molecular cloning: a laboratory manual*, 4th ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
 40. Yanisch-Perron C, Vieira J, Messing J. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* 33:103–119. [https://doi.org/10.1016/0378-1119\(85\)90120-9](https://doi.org/10.1016/0378-1119(85)90120-9).
 41. Hoang TT, Karkhoff-Schweizer RR, Kutchma AJ, Schweizer HP. 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. [https://doi.org/10.1016/S0378-1119\(98\)00130-9](https://doi.org/10.1016/S0378-1119(98)00130-9).
 42. Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM, Peterson KM. 1995. Four new derivatives of the broad host range cloning vector pBBR1MCS, carrying different antibiotic resistance cassettes. *Gene* 166:175–176. [https://doi.org/10.1016/0378-1119\(95\)00584-1](https://doi.org/10.1016/0378-1119(95)00584-1).
 43. Heeb S, Itoh Y, Nishijyo T, Schnider U, Keel C, Wade J, Walsh U, O'Gara F, Haas D. 2000. Small, stable shuttle vectors based on the minimal pVS1 replicon for use in Gram-negative, plant-associated bacteria. *Mol Plant Microbe Interact* 13:232–237. <https://doi.org/10.1094/MPMI.2000.13.2.232>.