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NOTES

Mycobacterium tuberculosis Can Utilize Heme as an Iron Source[▽]

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Most iron in mammals is found within the heme prosthetic group. Consequently, many bacterial pathogens possess heme acquisition systems to utilize iron from the host. Here, we demonstrate that *Mycobacterium tuberculosis* can utilize heme as an iron source, suggesting that *M. tuberculosis* possesses a yet-unknown heme acquisition system.

Owing to its versatile redox potential under physiological conditions, iron is an essential nutrient for the vast majority of organisms (6, 31). Organisms tightly sequester and regulate their iron supplies to limit the toxicity of the ferrous ion and to deal with the insolubility of the ferric ion (2). In the mammalian host, these mechanisms create an iron-scarce environment for bacterial pathogens. As a result, all bacterial pathogens have evolved specialized iron acquisition systems to utilize host iron (30). Many bacteria produce siderophores, small high-affinity iron chelators, to acquire iron from their environments (13). However, 70% of total iron in an adult mammal is bound within the heme prosthetic group (1), and as a consequence many bacterial pathogens also use heme acquisition systems in addition to siderophores (29).

Mycobacterium tuberculosis, the causative agent of tuberculosis, is a global health problem. In 2008, *M. tuberculosis* caused more deaths than any other bacterial pathogen (32). A hallmark of *M. tuberculosis* virulence is its capability to proliferate within arrested phagosomes of macrophages. This growth is dependent on siderophore biosynthesis (11, 16). However, *M. tuberculosis* mutants deficient in siderophore transport are not completely attenuated following an aerosol infection in mice, suggesting that *M. tuberculosis* might possess alternative iron acquisition systems (25). Recently, a novel heme-degrading enzyme in *M. tuberculosis* (Rv3592, MhuD) was characterized (5). Furthermore, the slow-growing *Mycobacterium haemophilum* displays a strict heme requirement for growth *in vitro* (9, 28). These reports suggest that *M. tuberculosis* and other mycobacteria might be capable of utilizing heme as an iron source.

Construction of a siderophore-deficient strain of *M. tuberculosis*. The siderophores of *M. tuberculosis* are called mycobactins and chelate iron from a core unit assembled by nonribosomal peptide synthases and polyketide synthases, encoded by the genes *mbtA* to *mbtH* (7). In order to unambiguously determine if *M. tuberculosis* can utilize heme as an iron source, it is necessary to have a siderophore-deficient mutant. Deletion of the *mbtD* gene encoding the putative polyketide synthase completely eliminated mycobactin production in *Mycobacterium smegmatis* (27). Therefore, we deleted the *mbtD* gene by allelic exchange in the avirulent *M. tuberculosis* strain mc^{2}6230 (Δ*RD1* Δ*panCD*; referred to here as the wild type [wt]) (26) (Fig. 1A) using the plasmid pML1816 (Table 1), which contained 1,000-bp regions upstream and downstream of *mbtD*. The analysis of double-crossover candidate 1 by PCR of chromosomal DNA demonstrated the absence of the *mbtD* gene (Fig. 1B). This Δ*mbtD* mutant was named *M. tuberculosis* ML1600 and was used in further experiments. To test whether *M. tuberculosis* ML1600 displayed a low-iron growth defect, we prepared a low-iron minimal medium consisting of 500 μM $MgCl_2 \cdot 6H_2O$, 7 μM $CaCl_2 \cdot 2H_2O$, 1 μM $NaMoO_4 \cdot 2H_2O$, 2 μM $CoCl_2 \cdot 6H_2O$, 6 μM $MnCl_2 \cdot 4H_2O$, 7 μM $ZnSO_4 \cdot 7H_2O$, 1 μM $CuSO_4 \cdot 5H_2O$, 15 mM $(NH_4)_2SO_4$, 12 mM KH_2PO_4 (pH 6.8), and 1% (wt/vol) glucose, which was supplemented with 10% (vol/vol) oleic acid-albumin-dextrose-catalase (OADC; Middlebrook), and 0.2% (wt/vol) Casamino Acids. The iron content of this medium was determined to be approximately 500 nM by inductively coupled plasma-mass spectrometry (ICP-MS) (not shown). We inoculated this low-iron minimal medium and low-iron minimal medium supplemented with purified ferricarboxymycobactin from *Mycobacterium bovis* BCG (cMBT) with *M. tuberculosis* ML1600. Figure 1C shows that *M. tuberculosis* ML1600 does indeed display a growth defect in low-iron minimal medium and that this growth defect is rescued by the addition of purified cMBT. This biochemical complementation experiment provides evidence that the growth defect of *M. tuberculosis* ML1600 was caused by the elimination of siderophores and not by secondary mutations in siderophore acquisition or an incomplete medium.

Utilization of heme by the *M. tuberculosis* Δ*mbtD* mutant. To test whether *M. tuberculosis* can utilize heme as an iron source, we measured growth of wt *M. tuberculosis* and the siderophore-deficient *M. tuberculosis* mutant ML1600 in low-iron medium

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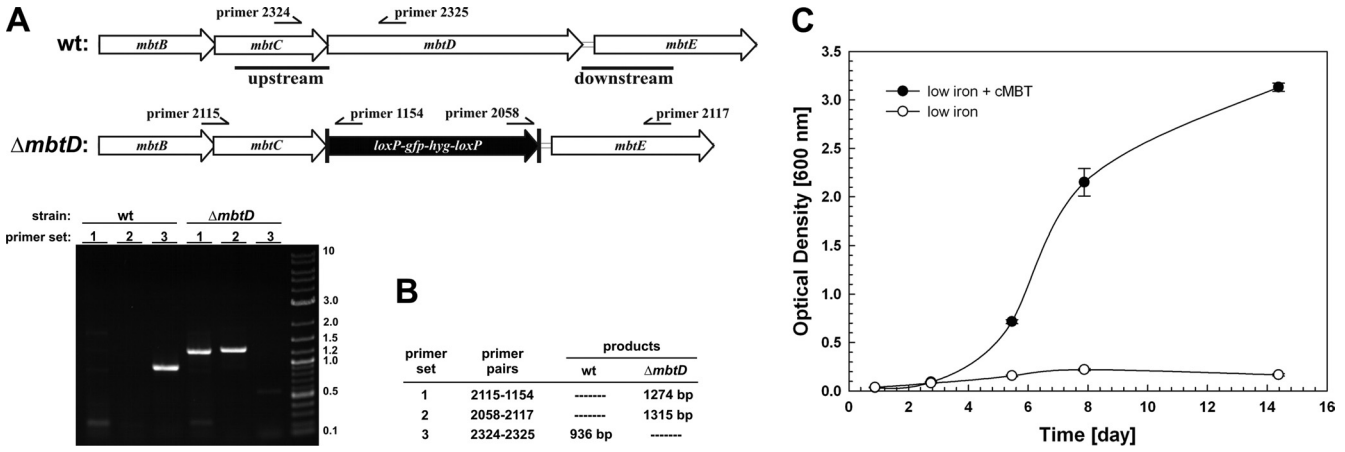

FIG. 1. Construction of a mycobactin-deficient *M. tuberculosis* strain. (A) Scheme of the mycobactin biosynthesis locus of *M. tuberculosis* encompassing *mbtD*. Genomic deletion of the *mbtD* gene was obtained by allelic exchange as described in the text. (B) Verification of the Δ*mbtD* mutant ML1600 by colony PCR. Bands in the DNA marker are denoted with their lengths in kilobase pairs. (C) Rescue of the ML1600 by purified ferri-carboxymycobactin (cMBT) from *M. bovis* BCG. Error bars represent standard deviations from the mean of results from biological duplicates.

and low-iron medium supplemented with 10 μM ferric citrate (prepared in a 1:200 iron-to-citrate ratio), 10 μM holo-transferrin (Sigma), and 10 μM hemin (Sigma). *M. tuberculosis* ML1600 displayed a severe growth defect in the low-iron medium which cannot be restored with 10 μM ferric citrate, indicating that in the absence of siderophores, *M. tuberculosis* cannot utilize ferric citrate under these conditions (Fig. 2B). Similar results were obtained with ferric chloride (not shown). Additionally, the low-iron growth defect of ML1600 was not rescued by 10 μM holo-transferrin (Fig. 2B), suggesting that *M. tuberculosis* does not possess a mechanism to liberate iron from transferrin in the absence of siderophores in contrast to *Haemophilus influenzae* and *Neisseria gonorrhoeae* (3). However, the growth defect of ML1600 under these conditions was rescued by hemin at a concentration of either 1 μM (not shown) or 10 μM (Fig. 2B), demonstrating that *M. tuberculosis* ML1600 can utilize hemin as an iron source. The growth rate of the Δ*mbtD* mutant grown with 1 μM hemin (doubling time ± standard deviation, 31.3 ± 0.1 h) was significantly lower than the growth rate of the mutant grown with 1 μM cMBT (doubling time of 27.1 ± 0.5 h; $P = 0.01$, two-tailed Student's *t* test) (not shown). In addition, stationary-phase optical densities were lower in the presence of hemin than in the presence of cMBT. In contrast, the growth rates of wt *M. tuberculosis* were similar in the presence of hemin or cMBT (not shown). Taken together, these data indicate that heme utilization is not as efficient as siderophore utilization under the conditions tested. It was reported that *M. haemophilum* requires heme concentrations greater than 7 μM in order to grow to visible colonies on solid medium, whereas heme concentrations of 2 μM did not yield any colonies (28). We observed that *M. tuberculosis* grows well with 1 μM hemin, suggesting that heme utilization in *M. tuberculosis* is more efficient than in *M. haemophilum*.

TABLE 1. Plasmids used in this work

Plasmid	Parent vector, relevant genotype, and properties[a]	Source or reference
pML523	pUC origin, PAL5000 origin; *sacB xylE loxP-gfp*$^{2+}$$_{m}$-Hyg^{R}-*loxP*; 9,845 bp	This study
pML1815	pML523; *mbtD*$_{up}$ *loxP-gfp*$^{2+}$$_{m}$-*hyg-loxP*; 10,766 bp	This study
pML1816	pML1815; *loxP-gfp*$^{2+}$$_{m}$-*hyg-loxP mbtD*$_{down}$; 11,825 bp	This study

[a] Up- and downstream homologous sequences of genes are noted with a subscript "up" and "down," respectively. Origin, origin of replication. The annotation Hyg^{R} indicates that the plasmid confers resistance to hygromycin.

Heme utilization often involves lysins and proteases to make host heme available (12). Contact-dependent hemolytic activity has been reported in *Mycobacterium avium* (24) and *M. tuberculosis* (10). However, the molecular determinants of the *M. tuberculosis* hemolytic activity are not entirely clear. A putative hemolysin in *M. tuberculosis* was first identified based on sequence homology to the hemolysin TlyA from the porcine pathogen *Serpula hyodysenteriae* (21). Expression of the *M. tuberculosis tlyA* homolog in *Escherichia coli* JM109 and *M. smegmatis* conferred contact-dependent hemolytic activity to these otherwise nonhemolytic strains (33). However, the *tlyA* gene product was later shown to possess 2′-*O*-methyltransferase activity that methylates the 16S and 23S rRNA subunits of both *M. tuberculosis* and *M. smegmatis* (15). The 2′-*O*-methyltransferase activity of TlyA renders these strains sensitive to the antibiotic capreomycin (20). Most recently, both *in vitro* hemolytic activity and *in vitro* methyltransferase activity were demonstrated for recombinant TlyA (23). Those authors argue that TlyA has evolved functions as a hemolysin and an rRNA 2′-*O*-methyltransferase. However, this notion is challenged by the fact that mutations in *M. smegmatis tlyA* result in capreomycin resistance, while no contact-dependent hemolytic activity has been observed in this organism. Additionally, TlyA-dependent hemolytic activity has not been reported in *M. tuberculosis*, only in *E. coli* and *M. smegmatis* overexpressing *M. tuberculosis tlyA*. Further research is necessary to determine if TlyA is indeed a bona fide hemolysin of *M. tuberculosis*.

It is unknown how heme is taken up by *M. tuberculosis*. In Gram-negative bacteria, heme uptake is mediated by TonB-dependent high-affinity receptors in the outer membrane, periplasmic binding proteins, and inner membrane ABC trans-

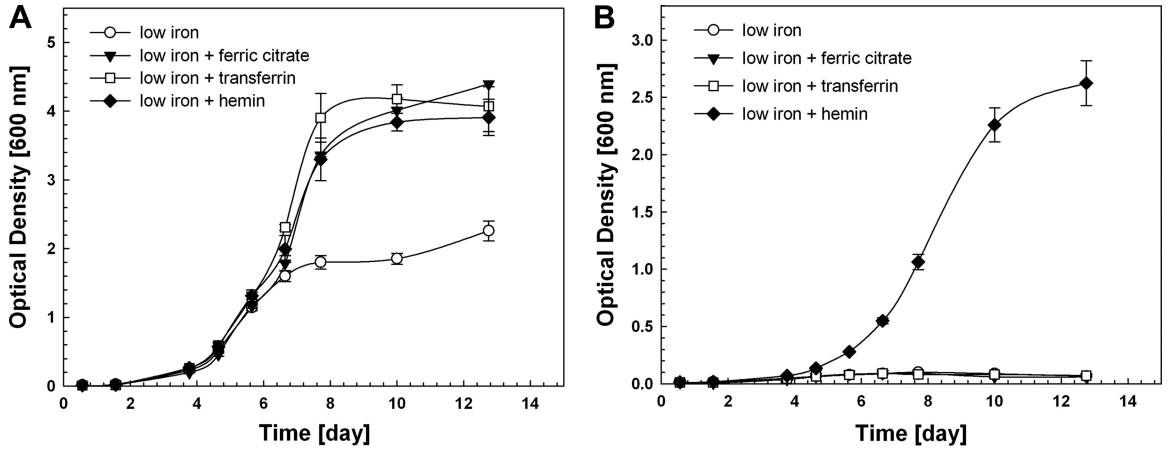

FIG. 2. *M. tuberculosis* can utilize heme as an iron source. (A) Growth of wt *M. tuberculosis* in low-iron medium or low-iron medium supplemented with 10 μM ferric citrate, 10 μM holo-transferrin, or 10 μM hemin. (B) Growth of *M. tuberculosis* ML1600 in low-iron medium or low-iron medium supplemented with 10 μM ferric citrate, 10 μM holo-transferrin, or 10 μM hemin. The media for both these growth experiments were identical. Error bars represent standard deviations from the mean of results from biological triplicates.

porters to facilitate passage into the cytosol (17). In *M. tuberculosis*, heme uptake would also require transport across the outer and inner membranes (14, 22). However, bioinformatic analysis of the *M. tuberculosis* genome did not reveal homologs to TonB or TonB-dependent receptors in Gram-negative bacteria. Homology searches for heme acquisition systems using the heme uptake operon *phuRSTUVW* and hemophore export system *hasRADEF* from *Pseudomonas aeruginosa* as models revealed only Rv1747 of *M. tuberculosis* (49% similar to the heme permease PhuV) and Rv0194 of *M. tuberculosis* (54% similar to the hemophore transporter HasD). However, Rv0194 was recently shown to be a multidrug efflux protein of *M. tuberculosis* (8), making it unlikely that it is part of a hemophore export system. Furthermore, a sequence similarity of 43% between *M. tuberculosis* FecB and the *Staphylococcus aureus* heme binding protein HtsA (43%) was observed but not any other proteins. A possible explanation for the apparent absence of sequence similarity of *M. tuberculosis* proteins to known heme uptake systems is that heme uptake in *M. tuberculosis* occurs nonspecifically, such as that seen in *Escherichia coli* K-12, where heme uptake across the inner membrane is dependent on the dipeptide transporter DppBCDF (18).

The molecular weight of heme exceeds the diffusion limit of the outer membrane in Gram-negative bacteria (4). Thus, acquisition is mediated by dedicated uptake machinery. However, heme has been shown to diffuse through liposome membranes (19), suggesting that it could passively diffuse through the mycobacterial outer membrane. We find this unlikely due to the fact that free heme is toxic and is always bound by proteins (12). Any bacterial uptake mechanism would then have to compete for heme binding, whether it is a secreted hemophore or a cell surface receptor. Therefore, *M. tuberculosis* likely has evolved a novel mechanism for the utilization of heme.

Conclusions. Heme uptake by bacterial pathogens is a common mechanism for iron acquisition in the host. Here, we demonstrate that *M. tuberculosis* can utilize heme as an iron source, suggesting that it has evolved a heme acquisition system like many other bacterial pathogens to exploit the most abundant iron source inside the human body.

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