Probing the Dynamic Nature of Mycobacterial Heme Homeostasis

A Thesis
Presented to
The Academic Faculty
By

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In Partial Fulfillment of the Requirements for the Degree
Bachelor of Science in Biology with the Research Option in the
School of Biological Sciences in the
College of Sciences

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Acknowledgements

I would like to thank my research mentor Dr. Rebecca Donegan for an outstanding education in laboratory research, Dr. Amit Reddi for providing me with and supporting me during this exceptional research opportunity, and the entire Reddi Lab for helpful conversations without which this work would not be possible.

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Abstract

Mycobacterium tuberculosis is responsible for more human deaths every year than any other bacterium. In order to establish an infection and cause disease, *M. tuberculosis* requires the nutrient heme. Heme is essential for numerous processes within the cell but is also cytotoxic, so its synthesis, uptake, and utilization must be tightly regulated by the bacterium. Despite the important role of this nutrient, relatively little is known about the regulation of its abundance and bioavailability. To better understand heme dynamics in mycobacteria, a total heme fluorescence assay and genetically encoded exchange labile heme sensors were used to observe changes in the abundance and bioavailability of heme in *Mycobacterium smegmatis* under various conditions. These experiments demonstrate that heme abundance and bioavailability in mycobacteria can change in response to physiologically relevant stresses and environmental cues.

Introduction

Mycobacterium tuberculosis (Mtb) is the etiological agent of tuberculosis and causes more deaths each year than any other pathogen. The World Health Organization estimates that one in four people are carriers of Mtb, with 10.0 million people developing tuberculosis and 1.4 million people dying from the disease in 2019 (World Health Organization, 2020). While most cases can be cured by antibiotics, drug resistant Mtb is a growing threat (World Health Organization, 2020). To facilitate the discovery of new treatments, a better understanding of the physiology of Mtb is required. This research project will investigate the dynamic nature of heme homeostasis in mycobacteria by observing changes in the concentration and bioavailability of heme in response to physiologically relevant conditions *in vitro*.

Tuberculosis is a respiratory disease which begins when a person inhales Mtb and the bacilli reach the alveoli. There, Mtb is engulfed by resident macrophages. If the macrophages are

successful in killing Mtb, the infection ends. If Mtb survives this initial encounter, it can invade the lung where the adaptive immune response will begin, a granuloma will form, and the bacteria will become dormant until the host's immune system is weakened (Pai et al., 2016). Within macrophage phagosomes, Mtb faces the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) as well as phagosomal maturation. In order to survive these threats, Mtb must produce virulence factors that inhibit these processes or neutralize them as they occur (Forrellad et al., 2012).

Mtb uses heme for a wide range of functions including energy production, gas sensing, and the neutralization of the ROS and RNS produced by macrophages (Forrellad et al., 2012). During infection, Mtb must acquire heme or synthesize it from nutrients it scavenges from the host; however, the host has evolved mechanisms to deny Mtb iron, heme, and other nutrients. Therefore, a successful infection requires Mtb to evade these defenses (Choby & Skaar, 2016).

While heme is an essential nutrient for Mtb, it can also be toxic. If let loose in the cell, heme localizes to membranes where it can damage lipids, proteins, and nucleic acids which can lead to death (Choby & Skaar, 2016). The paradoxical role of heme in the biology of Mtb is why the cell must precisely control the abundance of heme, store it safely, and be able to quickly mobilize it in response to potentially deadly changes in its environment. Mtb has many ways of regulating the amount of heme in the cell including making new heme, destroying existing heme, scavenging heme from the host, and pumping heme out of the cell (Dailey et al., 2015, Nambu et al., 2013, Mitra et al., 2017). The fact that so many cellular components are dedicated to controlling cellular heme levels suggests that it is an important part of the physiology of Mtb. Because the systems involved in regulating heme are so important, they may be potential targets for new antibiotics. The heme biosynthetic enzyme, coproheme decarboxylase (ChdC), has been proposed

as a drug target because it is only found in Firmicutes and Actinobacteria (Dailey et al., 2010). Targeting this enzyme could fatally dysregulate heme homeostasis in Mtb while leaving human cells unharmed, reducing side effects.

In order to better understand the role of heme in mycobacterial physiology it is necessary to directly observe heme within cells. Intracellular heme can either be inert, bound tightly to hemoproteins such as cytochromes, or exchange labile, bound loosely to proteins and easily transferred to other proteins to respond to the needs of the cell. To observe changes in the in these heme pools, two techniques have been developed which allow for the measurement of the total amount of intracellular heme by quantifying the fluorescence of the porphyrin component of each heme molecule and the exchange labile heme (ELH) within mycobacteria through the use of genetically encoded fluorescent heme sensors (Sassa, 1976, Hanna et al., 2016). Currently, the response of mycobacterial heme pools to challenges during infection has not been studied. To safely and efficiently gain insights into the biology of the pathogenic species Mtb, this work was performed on *M. smegmatis* (Msm), a nonpathogenic, rapid growing mycobacterium which is commonly used as a model organism for mycobacterial research. Using Msm allows for much faster experimentation without the risk of contracting tuberculosis. These experiments aim to observe the dynamic responses of the intracellular heme pools of Msm in response to some of the same stresses faced by Mtb in the host, which will shed light on the role of heme in mycobacterial pathogenesis.

Literature Review

The progression of each Mtb infection is governed by a series of complex interactions between the host and the invading microbe. The first obstacle which the tubercle bacillus must overcome is the innate immune system. Of particular interest in Mtb pathogenesis are the roles of

alveolar macrophages and nutritional immunity. Mtb infection begins when a bacillus is inhaled into an alveolus and subsequently phagocytosed by a resident macrophage (Pai et al., 2016). Within the macrophage, Mtb faces multiple threats including acidification, proteases, and the oxidative burst (Vandal et al., 2009). To protect itself from these attacks, Mtb produces proteins which inhibit the progression of these host defenses or neutralize them as they occur (Forrellad et al., 2012). In the latter case, KatG, the mycobacterial catalase-peroxidase, is required for survival and requires heme to function (Ng et al., 2004). While heme is understood to be an important component in mycobacterial physiology, the regulation of its synthesis, uptake, bioavailability, and utilization remains unknown. Through the use of genetically encoded ELH sensors and a total heme fluorescence assay, the extracellular conditions which alter heme homeostasis in mycobacteria may be better understood.

In addition to direct attacks against invading pathogens, the host immune system also restricts access to essential nutrients such as iron in a process termed nutritional immunity (Hood & Skaar, 2012). It is most commonly used to refer to the sequestration of iron but has recently been expanded to include other transition metals like copper and zinc. In the human body, the vast majority of iron is incorporated into heme bound to hemoglobin inside erythrocytes, while the rest of the body's iron is stored in ferritin, other iron storage proteins, or metalloenzymes (Hood & Skaar, 2012). The end result of this tightly controlled storage system is the withholding of iron and heme from Mtb. In order to establish a successful infection, Mtb must be able to take up enough iron and heme from the host to survive phagocytosis by alveolar macrophages (Skaar, 2010).

While iron and heme are absolutely essential for a wide range of biochemical processes performed by Mtb, they are also potentially toxic. Heme is lipophilic and, if free within a cell, can localize to membranes where it can damage membrane proteins and lipids, potentially resulting in

cell death (Choby & Skaar, 2016). Heme can also be broken down to release the iron atom at its center. Through Fenton chemistry and the Haber-Weiss reaction, free iron will react with hydrogen peroxide to produce free radicals which can damage DNA, lipids, and proteins (Choby & Skaar, 2016).

These conflicting functions of heme present some interesting questions about the regulation of this cofactor during infection. If heme is necessary but toxic, how does the cell ensure that it is tightly controlled but readily available to respond to stress? For which stresses does Mtb have to synthesize new heme or repurpose existing heme? How is heme synthesis and mobilization regulated? How much heme is required to successfully establish infection? While much work has been performed to elucidate the mechanisms of heme synthesis and uptake, the regulation of these processes is poorly understood. The most comprehensive study of the systems biology of heme came from a TnSeq experiment with Mtb grown on different iron sources, including heme (Zhang et al., 2020). Many genes required for heme uptake and utilization were uncovered, and the *zur* gene was identified as a possible heme uptake regulator. However, the specific mechanisms for the control of heme synthesis and utilization were not identified.

While the heme regulatory machinery is yet to be elucidated, advances in biochemical techniques for the precise measurement of heme within the cell have enabled researchers to observe dynamic changes in heme pools directly. The total quantity of heme within a cell can be split into two pools: exchange inert heme and ELH (Hanna et al., 2016). Exchange inert heme is tightly bound to proteins like cytochromes and is not readily transferred to other heme-binding proteins. ELH is more loosely bound to proteins and is readily exchanged between proteins (Hanna et al., 2016). This distinction is important when considering the dynamic mobilization of heme in

response to stress, in which Mtb must rapidly activate defense mechanisms which rely on heme, and therefore may scavenge ELH from its existing heme pool to produce those defenses.

To observe changes in ELH, Reddi and colleagues developed ratiometric ELH sensors (Hanna et al., 2016). This genetically encoded engineered protein contains three domains. The first is the heme-binding domain of cytochrome b_{562} (Cyt b_{562}), the second domain is enhanced green fluorescent protein (eGFP), and the third is Katushka 2 (mKATE2), a red fluorescent protein. When the Cyt b_{562} domain binds heme, eGFP fluorescence is quenched while mKATE2 fluorescence remains relatively unaffected. Thus, the ratio of eGFP fluorescence to mKATE2 fluorescence is inversely proportional to the concentration of ELH. The first-generation sensor, HS1, coordinates heme in the Cyt b_{562} domain between His and Met residues. These residues confer a sub-nanomolar K_d for ferrous heme, which is too tight to observe physiological changes in ELH. A second-generation sensor, HS1 M7A, was generated by replacing the heme-coordinating Met_7 residue with an Ala residue. This changed the ferrous heme K_d to 25 nM, which enables precise observations of changes in ELH. A third sensor, HS1 DM, was developed by mutating the heme-coordinating residues Met_7 and His_{102} to Ala to ablate heme binding and is used to control for the effects of sensor production on mycobacterial physiology (Hanna et al., 2016).

This project will leverage these ELH sensors and a total heme fluorescence assay to fill in gaps in the current understanding of mycobacterial heme homeostasis. Specifically, it will address the lack of specific knowledge of how heme is used by mycobacteria to respond to the fatal challenges posed by the innate immune system. This work will broadly enhance the understanding of the importance of mycobacterial pathogenesis and will provide a better physiological context for the effects of targeting heme synthesis and uptake with antimycobacterial compounds.

Materials and Methods

Bacterial Strains & Growth Conditions

Mycobacterium smegmatis mc^2155 were grown from laboratory stocks. Cultures were grown in Middlebrook 7H9 media (Difco) which was supplemented with 0.05% tween 80 and 10% ADS. ADS consists of water supplemented with 0.85% sodium chloride, 2% glucose, and 5% bovine serum albumin (Fraction V Gemini Biosciences). All growth was overnight at 37°C shaking at 170-200 rpm. Growth was quantified by measuring the optical density at 600 nm (OD_{600}) of liquid cultures diluted 1:20 in Tween 80.

Exchange Labile Heme Assay

The OD_{600} of cultures grown as described above was obtained before pelleting and washing twice with water. Cells were then resuspended at an OD_{600} of 10 in phosphate buffered saline (PBS). 200 µL of this suspension was placed in a 96 well flat bottom Grenier Fluorotrac plate in technical duplicates. The fluorescence of WT Msm not expressing a sensor were measured and subtracted as background for the mKATE2 and eGFP channels. A Biotek Synergy MX plate reader was used to measure mKATE2 fluorescence with an excitation of 580 nm and an emission of 620 and eGFP fluorescence with an excitation of 480 nm and an emission of 510 nm. The mean of technical replicates was taken and used for the calculation of the eGFP/mKATE2 ratio for each sample. The means of the eGFP/mKATE2 ratios of the samples in each treatment group are reported in this study.

Total Heme Assay

The method for measuring total heme was described previously (Sassa, 1976). Samples were washed as described for ELH measurements above. 500 µL of each sample resuspended at an OD_{600} of 10 in PBS were pelleted, the supernatant was removed, and the pellets were frozen at -

80°C. They were resuspended in 500 µL of 20 mM oxalic acid and then incubated overnight at 4°C. 500 µL of 2M oxalic acid was added and mixed, and then each sample was divided into two 500 µL aliquots. One aliquot was incubated at room temperature protected from light and would serve as a blank. The other was boiled at 100°C for 30 minutes protected from light. Both the blank and boiled samples were centrifuged at 21,100 x g for 2 minutes. For each blank and boiled sample, two technical replicates of 200 µL of supernatant were plated in a 96 well black flat bottom Grenier Fluorotrac plate. A Tecan Infinite 200 Pro plate reader was used to measure fluorescence with an excitation wavelength of 400 nm and emission at 662 nm. When heme is boiled in acid, the iron separates from the porphyrin ring. Protoporphyrin IX is fluorescent, and by subtracting the fluorescence of an unboiled aliquot of a sample from the fluorescence of a boiled aliquot of the same sample, the total amount of heme can be determined.

Results

Cultures of Msm in 7H9 media were supplemented with 25, 50, and 100 µM heme dissolved in dimethyl sulfoxide. Compared to untreated cells, there was no change in the growth, but there was a significant decrease in the ELH heme sensor ratio at 50 and 100 µM heme (Fig. 1A & 1B). This indicates that there was an increase in ELH in response to greater amounts of environmental heme available to the cells. Another experiment showed that the total concentration of intracellular heme compared to untreated cultures increased when cells were supplemented with 100 µM heme (Fig. 1C). To see if Msm could grow using only exogenous heme, null mutants of the terminal heme biosynthetic enzyme coproheme decarboxylase were grown with and without a 25 µM heme supplement (Fig. 2). The heme starved mutant suffered an approximately five-fold decrease in growth compared to wild type. The supplemented mutant cultures were rescued to a level slightly below the wild type.

Cultures were treated with 5 µg/mL 5-aminolevulinic acid (ALA), a heme synthesis precursor common to humans and mycobacteria. ALA supplementation did not alter growth compared to untreated cells but did increase total heme and ELH (Fig. 3). After treatment in 7H9 with 150 µM iron, neither the growth, total heme, nor ELH was altered compared to untreated cultures (Fig. 4). Bathophenanthrolinedisolfonic acid (BPS) is an iron chelator. This means that it can bind iron in the culture media and has the potential to prevent cells from scavenging the element. When cultures were treated with 75 and 150 µM BPS dissolved in phosphate buffered saline, there was no significant change in growth or ELH compared to untreated cells (Fig. 5). However, there is a slight but not statistically significant trend towards increased ELH, and higher concentrations of BPS may induce significant increases in ELH. Interestingly, total heme increased in response to increasing amounts of BPS.

Exposing mycobacteria to acidic conditions is a key component of the immune response to infection (Ehrt and Schnappinger, 2009). *In vivo*, the pH of bacteria containing phagolysosomes can reach as low as 4.5, but previous studies suggest that when 7H9 media is acidified to that level, mycobacteria may encounter toxicity that is not physiologically relevant (Vandal et al., 2008). In order to observe changes in mycobacterial heme pools in response to acidification while avoiding this type of toxicity, the pH of the 7H9 media was lowered from the standard 6.75 to a slightly more acidic pH of 6.03. While this is a relatively small change in pH, it was enough to inhibit growth, increase intracellular heme abundance, and decrease ELH (Fig. 6).

Figures

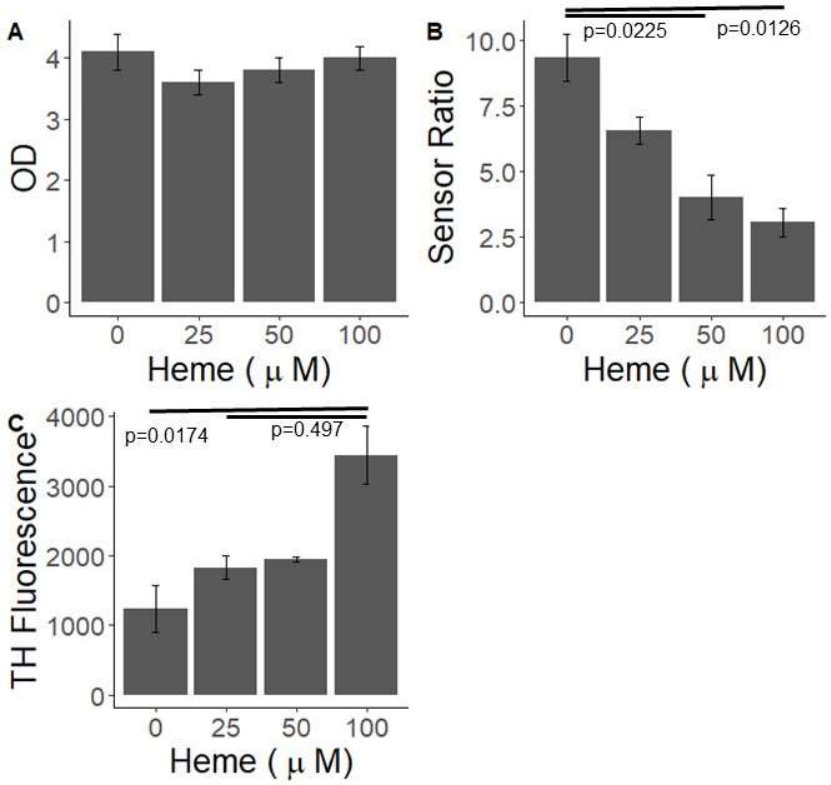

Figure 1. Environmental heme availability increases total and exchange labile heme. OD_{600} (**A**), M7A sensor ratio (**B**), and total heme fluorescence (**C**) of wild type Msm with 0, 25, 50, or 100 μM heme. Bars show the mean of two replicates. Error bars show one standard error from the mean. Data were analyzed with one-way ANOVA and Tukey-Kramer HSD post-hoc test. $p > 0.05$ for unlabeled comparisons.

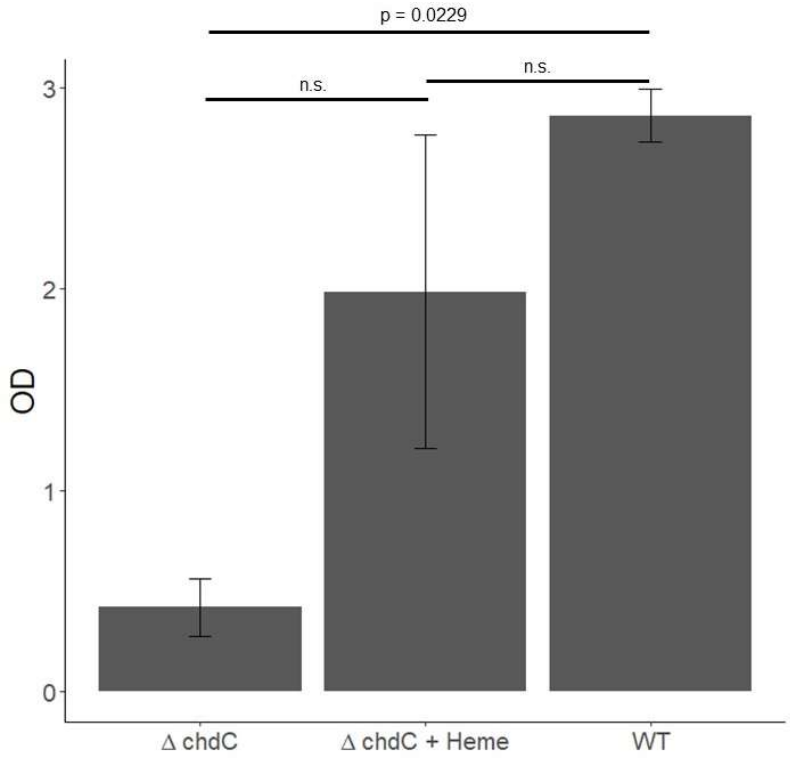

Figure 2. Msm can scavenge heme. Bars show the mean OD_{600} of three replicates of wild type (WT), ΔchdC, and ΔchdC with 25 μM heme. Error bars show one standard error from the mean. Data were analyzed with one-way ANOVA and Tukey-Kramer HSD post-hoc test. n.s. indicates $p > 0.05$.

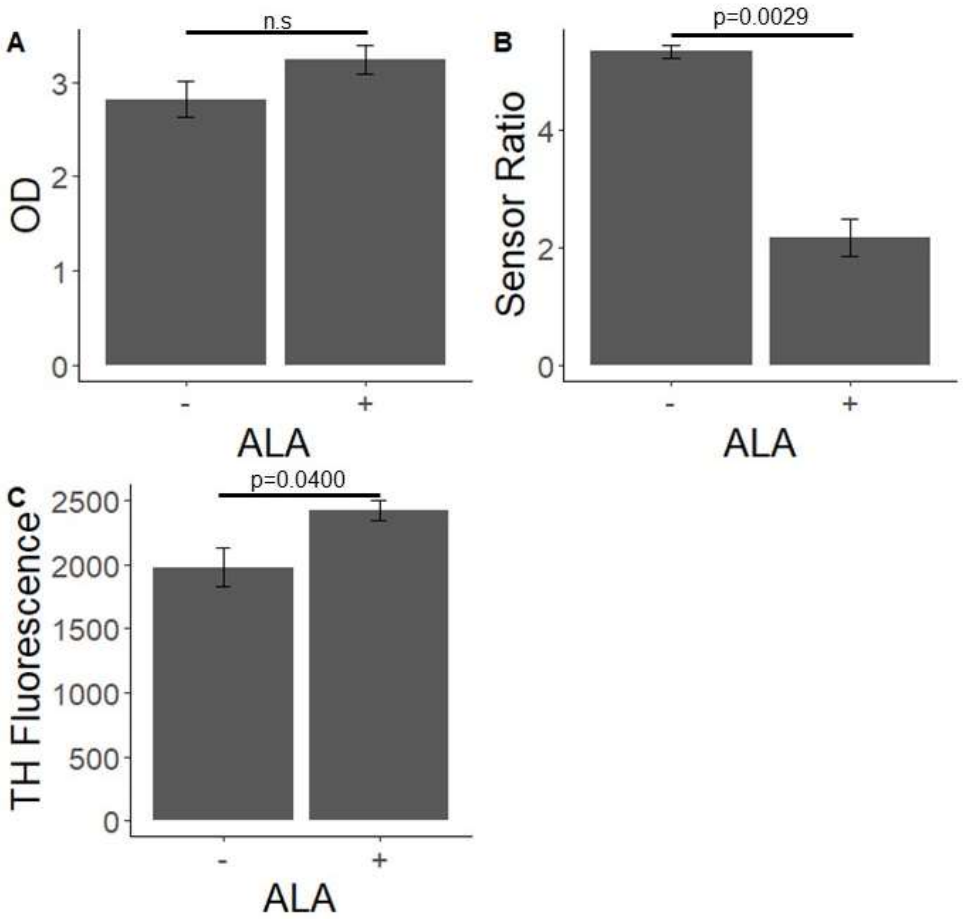

Figure 3. ALA supplementation increases heme abundance and bioavailability. OD_{600} (**A**), M7A sensor ratio (**B**), and total heme fluorescence (**C**) of wild type Msm with 0 or 5 μg/mL ALA. Bars represent the mean of three replicates. Error bars show one standard error from the mean. Data were analyzed with a one-tailed t-test. n.s. indicates $p > 0.05$.

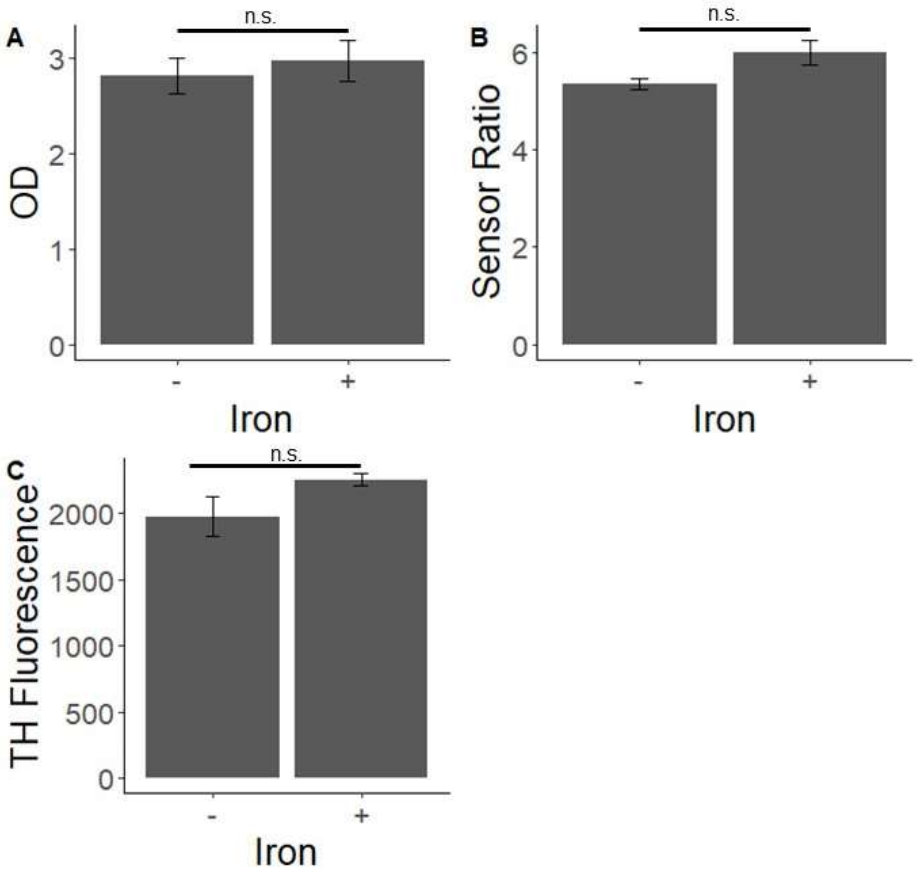

Figure 4. Heme abundance and bioavailability are unresponsive to iron supplementation in rich media. OD_{600} (**A**), M7A sensor ratio (**B**), and total heme fluorescence (**C**) of wild type Msm with 0 or 150 μM iron. Bars represent the mean of three replicates. Error bars show one standard error from the mean. Data were analyzed with a one-tailed t-test. n.s. indicates $p > 0.05$.

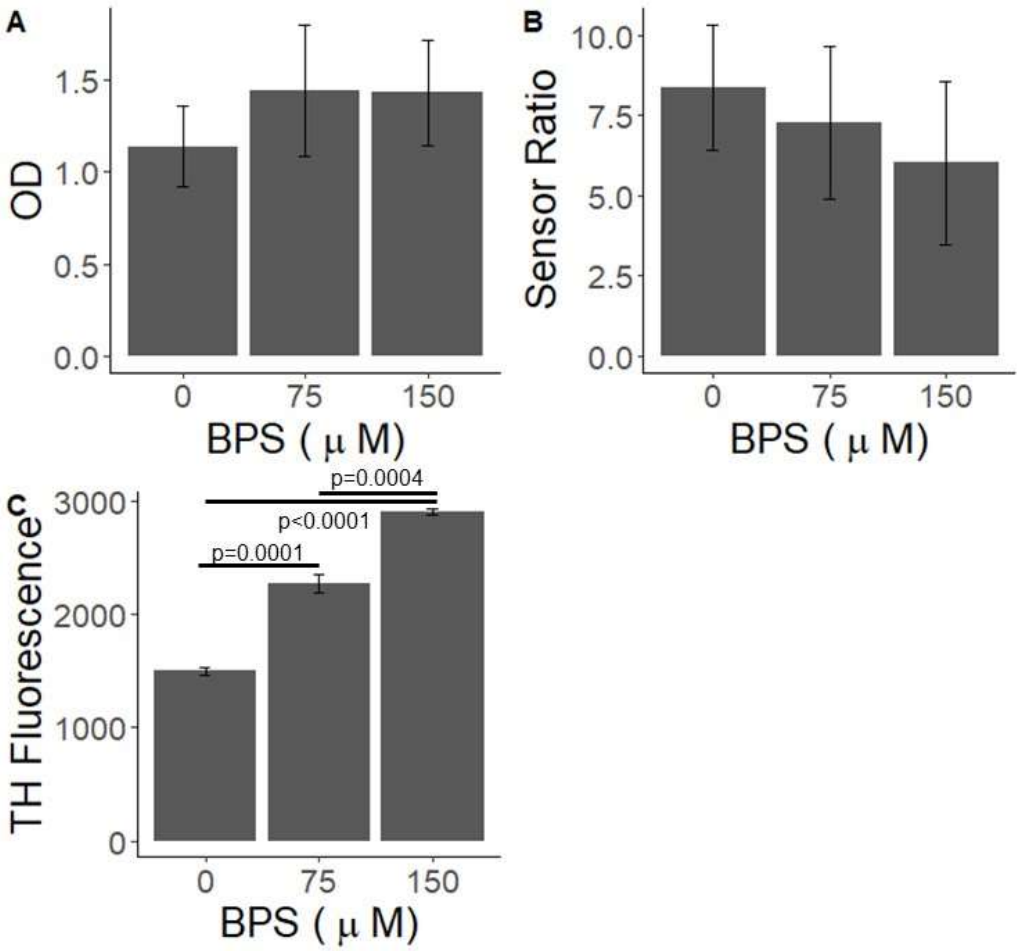

Figure 5. Iron depletion increases intracellular heme. OD_{600} (**A**), M7A sensor ratio (**B**), and total heme fluorescence (**C**) of wild type Msm with 0, 75, or 150 μM of the iron chelator BPS. Bars show the mean of three replicates. Error bars show one standard error from the mean. Data were analyzed with one-way ANOVA and Tukey-Kramer HSD post-hoc test. $p > 0.05$ for unlabeled comparisons.

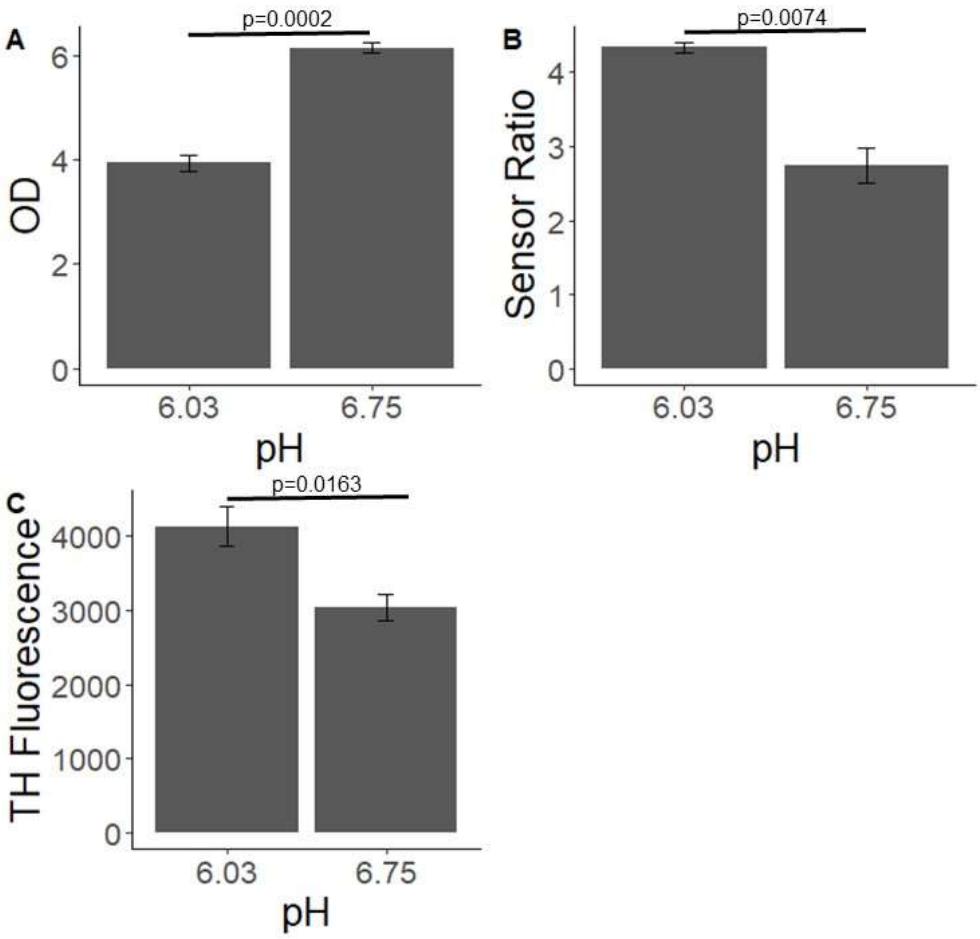

Figure 6. Acidification inhibits growth, increases total heme, and decreases heme bioavailability. OD_{600} (**A**), M7A sensor ratio (**B**), and total heme fluorescence (**C**) of wild type Msm cultured in untreated (pH = 6.75) and acidified (pH = 6.03). Bars represent the mean of three replicates. Error bars show one standard error from the mean. Data were analyzed with a one-tailed t-test.

Discussion

Heme is required for mycobacterial growth and pathogenesis (Forrellad et al., 2012). It serves as a cofactor for numerous essential enzymes and is an important source of nutritional iron (Jones & Niederweis, 2011). Heme's importance is further evidenced by the fact that mycobacteria evolved at least two heme uptake mechanisms and a *de novo* heme synthesis pathway (Mitra et al., 2019 & Dailey et al., 2015). Despite the essential role of heme and the wide range of processes it is involved in, whether the abundance and bioavailability of mycobacterial heme are responsive to environmental conditions is unknown. To better understand the dynamics of heme pools in mycobacteria, ELH sensors and a total heme fluorescence assay were used to observe changes in heme quantities in response to pathologically relevant environmental conditions. Treatment with heme, ALA, and BPS, as well as acidification increased the total quantity of heme in Msm. Heme and ALA treatment increased ELH while acidification decreased it. Iron treatment had no effect on heme pools. Of all the treatments, only acidification altered growth, decreasing it by about one third compared to control. This work demonstrates that heme pools within Msm are dynamic and are altered in response to pathologically relevant environmental conditions modeled *in vitro*.

The metallobiology of bacteria is a wide-ranging field with implications for microbial cell biology, ecology, and pathogenesis. Building an understanding of bacterial metallostasis is an important endeavor that is not limited to any one microbe. It must be noted that while the aim of these experiments was to gain insights into mycobacterial biology, one must be cautious when generalizing findings from a model organism. Msm and Mtb are both mycobacteria, but they are quite different. Msm is a soil microbe that rarely causes infections in humans, and the vast majority of those infections are skin and soft tissue infections that result from the contamination of wounds (Pierre-Audigier et al., 1997). In contrast, Mtb is an obligate human pathogen which commonly

causes severe disease (Pai et al., 2016). The different lifestyles of these microbes expose them to very different evolutionary pressures.

As mentioned earlier, one of the greatest challenges in becoming a successful pathogen is evolving mechanisms to overcome nutritional immunity (Skaar, 2010). Because the vast majority of iron in the host is bound in heme, one way to scavenge a sufficient amount of iron would be to develop a heme uptake pathway. Recent work has uncovered two independent heme uptake systems in Mtb (Mitra et al., 2019). One involves outer membrane associated PPE proteins that likely pass heme to DppA, which in turn transfers heme to the DppBCD transporter to bring it through the inner membrane. The factors involved in the second pathway are unknown, but it is dependent on heme bound to the host protein albumin.

While Msm and Mtb use the same heme synthesis pathway, the characterized heme uptake pathway of Mtb is not found in Msm. According to the comprehensive mycobacterial genome browser MycoBrowser, no orthologues of the seven genes coding for the known components of the Mtb heme uptake system can be found in the Msm genome (Kapopoulou et al., 2011). However, the uncharacterized Mtb heme uptake pathway allows for the use of albumin bound heme, which is in 7H9 media and may be the pathway used by Msm. The finding that the total heme and ELH increase after heme treatment and that heme auxotrophs can be rescued by heme supplementation is consistent with the idea that exogenous heme is not simply a nutritional source of iron but can supplement endogenous heme pools. Heme that enters the cell may be directly transferred to a hemoprotein to perform heme dependent processes such as the neutralization of hydrogen peroxide by KatG or it can be broken down into mycobilin and iron, which can be used for iron dependent activities or recycled in *de novo* heme synthesis. When Msm synthesizes new

heme, at least some of it enters the ELH pool, as evidenced by the fact that ALA treatment increases total and ELH.

Comparative experiments have demonstrated that there is much variation in mycobacterial nutrient uptake systems. It is believed that as Mtb became a more specialized pathogen, its genome was simplified. Mtb has approximately 2.5 million fewer base pairs in its genome than Msm (Salah et al., 2009). One analysis showed that Msm can express twenty-eight putative carbohydrate transporters whereas Mtb only has five (Titgemeyer et al., 2007). Msm also produces a class of siderophores that are not produced by Mtb. Siderophores are small molecules that tightly bind ferric iron and are used to scavenge the nutrient from the environment. Msm and Mtb make carboxymycobactins and mycobactins and Msm can also make exochelins (Sritharan, 2016). Carboxymycobactins are water soluble siderophores which are secreted to bind iron that is in close proximity to the bacterium. In contrast, mycobactins are water insoluble and localize to the waxy outer membrane of the cell. It is hypothesized that the soluble carboxymycobactins transfer iron to mycobactins in order to bring the nutrient into the cell. Exochelins are mycobacterial siderophores which are only produced by the nonpathogenic members of the genus such as Msm and are thought to perform similarly to carboxymycobactins. Like heme, iron is potentially cytotoxic, and its cellular abundance and bioavailability are also tightly regulated. In Mtb, it has been shown that siderophore biosynthesis is downregulated by iron availability and upregulated during iron depletion (Pandey, et al., 2014).

One of the most interesting results from these experiments was that iron supplementation in rich media does not affect growth, ELH, or total heme. This is in contrast to supplementation with heme, so it may be that heme uptake regulation is less sensitive to iron availability than the factors that regulate iron uptake. Unlike mycobacterial siderophores which bind ferric, BPS binds

ferrous iron. This means that in the BPS experiment, the siderophores and BPS were not directly competing for iron. However, by decreasing the total amount of free iron, BPS did likely decrease the ferric iron available to siderophores. Despite decreasing the availability of iron, which is required for heme synthesis, BPS at and above 75 μM increased the total intracellular concentration of heme. Oddly, BPS treatment was the only treatment that increased the total abundance of heme without changing the amount of ELH. One explanation for this could be that when iron and heme are abundant, more heme is transiently bound to hemoproteins and is not being used, but when iron is limited, the cell must partition its heme to only the most essential heme dependent processes, preventing the ELH pool from growing. Many bacteria use iron depletion as a signal that they have entered the host, so the increase in total heme may be part of the cell's preparation to survive challenges from the immune system (Skaar, 2010).

One of the main differences between Msm and Mtb is the ability to survive engulfment by phagocytes. Despite some similarities, Msm is much worse at persisting in phagocytes (Sharbati-Tehrani et al., 2005). When mycobacteria are phagocytosed by eukaryotes, they are engulfed in a compartment called a phagosome where they can usually survive. However, after phagosome maturation, a process in which the phagosome fuses with the lysosome, the compartmental environment becomes much more hostile. Specifically in human macrophages, mycobacteria face oxidative and nitrosative stress, nutrient limitation, hydrolases, and acidification (Forrellad et al., 2013). Some of the factors used to defend against these threats are common to both species like KatG and an iron containing superoxide dismutase SodA (Kapopoulou et al., 2011). The pH of the phagosome is approximately 6.5 but after lysosome fusion, the pH drops to 4.5 (Forrellad et al., 2013).

When the pH of 7H9 media was reduced slightly from 6.75 to 6.05, total heme in Msm increased while the ELH pool became smaller (Fig. 7). A crucial component of mycobacterial acid persistence is the integrity of the cell envelope. In screens of genes important for acid resistance, many factors involved in cell envelope maintenance have been identified (Vandal et al., 2009). Additionally, the increased permeability of the Msm cell envelope was identified as a limiting factor in its ability to tolerate phagocytosis (Sharbati-Tehrani et al., 2005). Heme is a lipophilic compound and if free will localize to the cell envelope where it is capable of damaging the cell membrane and membrane proteins (Choby & Skaar, 2016). The decrease in the ELH pool that occurs during growth in acidic conditions may be due to cells buffering heme with more tightly binding hemoproteins in order to avoid membrane damage that may reduce acid tolerance.

Further research is needed to understand the full range of conditions for which heme and its dynamic mobilization occur in mycobacteria and the extent to which heme homeostasis is involved in mycobacterial growth and pathogenesis. It is also important to gain an understanding of the factors involved in regulating heme homeostasis in mycobacteria. Additionally, future work should address the partitioning of heme within the cell. Acidification and iron deprivation increased total heme without growing the ELH pool, indicating that under certain conditions, specific heme dependent processes are prioritized over other potential destinations for newly synthesized or scavenged heme. With a more complete understanding of mycobacterial heme homeostasis, new drugs disrupting heme synthesis, uptake, and utilization could be used to prevent the growing threat of drug resistant Mtb (McLean & Munro, 2017).

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