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ANALYSIS OF ERGOTHIONEINE PRODUCTION IN
MYCOBACTERIUM TUBERCULOSIS

by

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A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
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ANALYSIS OF ERGOTHIONEINE PRODUCTION IN
MYCOBACTERIUM TUBERCULOSIS

LONI GUIDRY

MICROBIOLOGY

ABSTRACT

Mycobacterium tuberculosis (*Mtb*), the etiological agent of tuberculosis, currently infects more than two billion people worldwide. *Mtb* is a highly adaptable pathogen, capable of modifying its metabolism in response to its environment. We previously showed that *Mtb* WhiB3 is a redox-responsive regulator of core intermediary metabolism and lipid anabolism through which it maintains redox homeostasis. In this work, through the use of metabolomic analysis and a novel radioactive thin layer chromatography assay, we discovered that WhiB3 regulates the production of the intracellular thiol ergothioneine (ergo) when using immunomodulatory lipid precursors as the major carbon source. Our results suggest that this regulation is mediated by an altered redox environment due to loss of WhiB3. We also found that loss of the major redox buffer mycothiol or the thioredoxin regulator SigH results in increased ergo levels, suggesting that ergo functions similarly to these redox stress protection mechanisms. Correspondingly, ergo-deficient *Mtb* mutants are more sensitive than wild-type to cumene hydroperoxide, an oxidative stressor. The fact that ergo levels are affected by the loss of WhiB3 implicates WhiB3 not only in redox maintenance but also in redox stress protection. Altogether, our results indicate that ergo can protect *Mtb* from redox stress and is differentially produced depending on the redox environment, such that factors regulating redox homeostasis indirectly regulate ergo biosynthesis. Our data provide a link between carbon metabolism and ergo production and suggest that the *in vivo*

environment encountered by the bacilli determines ergo levels, thereby potentially predisposing *Mtb* to survive the many redox stresses it faces.

Key words: ergothioneine, WhiB3, redox homeostasis, mycothiol

DEDICATION

This dissertation is dedicated to my mother, Jacqueline Guidry, in appreciation of her unfailing support and encouragement.

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I would like to thank my mentor, the members of my committee and all past and present members of the Steyn lab for their advice and assistance throughout this journey. My sincere thanks also go to my family and friends for their love and support.

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LIST OF ABBREVIATIONS

AhpC	Alkyl hydroperoxide reductase subunit C
AhpD	Alkyl hydroperoxide reductase subunit D
AMAA	Acetonitrile:methanol:50 mM ammonium acetate (40:40:20)
CHP	Cumene hydroperoxide
Cys	Cysteine
DMD	Diamide
DTT	Dithiothreitol
Ergo	Ergothioneine
FA	Fatty acid
FAS	Fatty acid synthase
Fe-S	Iron-sulfur
GlcN-Ins	1- <i>O</i> -(2-amino-2-deoxy- α -D-glucopyranosyl)-D- <i>myo</i> -inositol
H ₂ O ₂	Hydrogen peroxide
His	Histidine
HPLC	High-performance liquid chromatography
Hyg	Hygromycin B
INH	Isoniazid
Kan	Kanamycin
LC/MS/MS	Liquid chromatography/mass spectrometry/multiple reaction monitoring

LMW	Low-molecular-weight
mBBr	Monobromobimane
MSA	Methanesulfonic acid
MSH	Mycothiol
<i>Msm</i>	<i>Mycobacterium smegmatis</i>
<i>Mtb</i>	<i>Mycobacterium tuberculosis</i>
Mtr	Mycothione reductase
NEM	<i>N</i> -ethylmaleimide
NO	Nitric oxide
PDIM	Phthiocerol dimycocerosate
PLP	Pyridoxal 5-phosphate
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SAM	<i>S</i> -adenosyl-methionine
SOD	Superoxide dismutase
TB	Tuberculosis
TCA	Tricarboxylic acid
TLC	Thin-layer chromatography
Tn	Transposon
TPx	Thiol peroxidase
TrxC	Thioredoxin C
TrxR	Thioredoxin reductase
Wbl	WhiB-like

INTRODUCTION

Tuberculosis (TB) is a potentially fatal, communicable disease caused by *Mycobacterium tuberculosis* (*Mtb*). In 2010, 8.8 million people developed TB and 1.4 million people died from the disease, including 350,000 who were human immunodeficiency virus (HIV)-positive. Not everyone infected with *Mtb* will develop TB, but co-infection with HIV increases the likelihood of TB development 21- to 34-fold (1). *Mtb* infection of immunocompetent individuals results in active disease in only 10% of cases (2), while the other 90% clear the infection completely or sequester the bacteria to minimize interaction between host and pathogen (3).

Humans are the only known reservoir for *Mtb*, and transmittance of the bacteria is from person to person through aerosolized respiratory droplets. Bacilli in droplets are first inhaled into the human lung and then engulfed by alveolar macrophages (3, 4). In activated macrophages, elimination of the bacteria occurs when *Mtb*-containing phagosomes fuse with lysosomes. However, in non-activated macrophages, *Mtb* can block phagolysosomal fusion and replicate in the phagosomes or may even escape to the nutrient-rich cytosol and multiply there (2, 3). Eventually, the bacteria are detected and surrounded by a collection of immune cells to form granulomas, which effectively walls off the infection and prevents spreading. Little is known of the biology of the bacilli residing in these granulomatous lesions. They are viable and resistant to killing by

existing antibiotics, but whether they actively replicate or persist in a dormant, metabolically inactive state is not yet clear (5).

Active TB can be successfully treated with a combination of antibiotics; however, in recent years multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB have emerged. Unfortunately, no new anti-TB drugs have been developed in the last 60 years (1). It is therefore critical to identify new targets and develop better drugs to eliminate the bacteria.

Maintenance of Redox Homeostasis in *Mtb*

Bacteria must maintain intracellular redox homeostasis in order to survive (6). Aerobic metabolism/respiration results in the formation of reactive oxygen species (ROS) which can cause endogenous damage (7). Further, *Mtb* residing in macrophages face additional stress from the oxidative burst of the phagolysosome (2). The ROS generated include superoxide, hydrogen peroxide (H₂O₂) and hydroxyl radical. Macrophages can also produce reactive nitrogen species (RNS), such as nitric oxide (NO) and peroxynitrite (8). The damage caused by redox stress is manifold and may include: (a) formation of disulfide bonds in proteins, which may inhibit their enzymatic activity; (b) destruction of iron-sulfur (Fe-S) clusters, which often act as sensors to dictate protein activity; and (c) harmful oxidation of various molecules, including lipids and DNA (7). However, *Mtb* contains both enzymes and low-molecular-weight (LMW) thiols to ensure that redox homeostasis is maintained, to detoxify reactive species and to repair the damage done by the reactive species. These mechanisms allow *Mtb* to survive the hostile environment of its host.

Antioxidant Enzymes

Mtb has numerous mechanisms at its disposal to detoxify oxidants and maintain the reducing environment of the cell, including a number of enzymes. Superoxide dismutase (SOD) converts superoxide into oxygen and H₂O₂ through a one-electron dismutation (9). Two SODs are produced by *Mtb*. SodA (or FeSOD, encoded by *sodA*) uses iron as cofactor and SodC (or CuZnSOD, encoded by *sodC*) is cofactored by copper and zinc. A null mutant of FeSOD has not been successfully created (10), which may indicate that *sodA* is essential. Using antisense RNA to decrease FeSOD levels, Edwards and coworkers showed that this enzyme protects cells from H₂O₂ exposure *in vitro* and contributes to the virulence of *Mtb* because FeSOD depleted cells were much less virulent than wild-type (11). A CuZnSOD deletion mutant of *Mtb* is more sensitive to superoxide and is killed more readily by activated macrophages compared to wild-type (12). Thus, both SODs of *Mtb* have important roles in protection from oxidative stress.

Another enzyme produced by *Mtb* to combat oxidative stress is KatG, a catalase peroxidase. It was one of the first virulence factors discovered in *Mtb* (13). Loss of functional KatG renders the bacilli resistant to isoniazid (INH), because INH is a prodrug that is activated by KatG, and decreases the virulence of *Mtb* (14-17). KatG can protect *Mtb* from ROS produced by NADPH oxidase; this is known because a *katG* deletion mutant that is attenuated in wild-type mice is as virulent as the parent *Mtb* strain when used to infect mice that do not produce the gp91 subunit of NADPH oxidase (18).

In *Mtb*, protection from alkyl hydroperoxides is provided by at least two peroxiredoxins: AhpC, (alkyl hydroperoxide reductase subunit C) and TPx (thiol peroxidase) (7). These enzymes reduce organic hydroperoxides to alcohols and, in the

process, form a disulfide bond between two intramolecular cysteines (Cys), which is in turn recycled by reducing systems. One system that can reduce AhpC includes NADPH, thioredoxin reductase (TrxR, also called TrxB2) and thioredoxin C (TrxC), one of the three *Mtb* thioredoxins (19). Another AhpC reducing system contains NADH, dihydrolipoamide dehydrogenase (Lpd), dihydrolipoamid succinlytransferase (SucB) and alkyl hydroperoxide reductase subunit D (AhpD) (20). TPx is reduced by NADPH, TrxR and thioredoxins B1 or C (19).

In addition to their roles as peroxiredoxins, AhpC and TPx can also reduce peroxynitrite; thus, these enzymes are involved in both ROS and RNS protection (19, 21). A TPx mutant is more sensitive to H₂O₂ and NO stress, and is less virulent than wild-type *Mtb* in mice (22). Deletion of *ahpC* in *Mtb* causes increased susceptibility to cumene hydroperoxide (23) and peroxynitrite, and the mutant grows less well in non-activated macrophages (24). Furthermore, AhpC has been implicated in resistance to INH (25).

ROS and RNS can oxidize methionine to methionine sulfoxide, which may disrupt protein function. Reduction of these oxidized residues is accomplished by methionine sulfoxide reductase in conjunction with NADPH, Trx and TrxR (26). *Mtb* has two methionine sulfoxide reductases, MsrA and MsrB, which reduce methionine-(*S*)-sulfoxide and methionine-(*R*)-sulfoxide, respectively. Disrupting only one of these reductases in *Mtb* does not affect susceptibility to ROS or RNS; however, loss of both renders *Mtb* more sensitive to nitrite and hypochlorite (27).

Low-Molecular-Weight Thiols

Mycothiol

In addition to the enzymes discussed above, *Mtb* contains mycothiol (MSH), a LMW thiol responsible for maintaining the reducing environment of the cytoplasm. Glutathione is the archetypal member of the LMW thiol family and is found in numerous eukaryotes and prokaryotes, but missing in actinomycetes, including mycobacteria (28). Instead, MSH is present in mycobacteria in millimolar concentrations. It is the major redox buffer in mycobacteria and also facilitates the removal of toxic compounds from the cell (29).

MSH biosynthesis proceeds through a five-step process (Fig. 1) and only one of the responsible enzymes remains to be identified. First, a glycosyltransferase encoded by *mshA* links 1L-*myo*-inositol-1-phosphate to UDP-*N*-acetylglucosamine (30). The MSH phosphatase MshA2 (whose gene has not yet been identified) then dephosphorylates the product (31). Deacetylation by MshB then produces glucosaminylinositol [1-*O*-(2-amino-1-deoxy- α -D-glucopyranosyl)-D-*myo*-inositol] (32). Next is the ATP-dependent ligation of Cys's carboxyl group to glucosaminylinositol catalyzed by MshC, the MSH ligase (33). Finally, the amino group of Cys is ligated to an acetyl group by MshD, an acetyltransferase (34). This acetylation results in mycothiol being more resistant to autoxidation than free Cys (35).

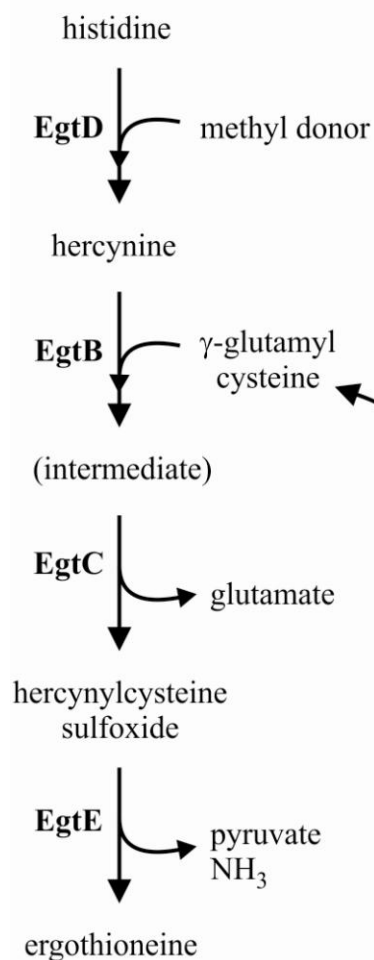
In the absence of active MshA, neither MSH nor its intermediates can be detected in *M. smegmatis* (*Msm*) or the *Mtb* strains H37Rv, Erdman and CDC1551 (30, 36, 37). *Msm* and *Mtb* mutants lacking MshB accumulate N-acetylglucosaminylinositol, the MshB substrate, but still produce low levels of MSH, probably due to an unidentified

deacetylase (38, 39). On the contrary, the loss of MshC activity in *Msm* results in undetectable levels of MSH but increased glucosaminylinositol levels (40). A viable *mshC* mutant of *Mtb* Erdman could not be isolated, prompting the authors to suggest that MSH is essential in *Mtb* (41). This is probably not the case, though, because viable *mshA* mutants of *Mtb* that do not produce MSH do exist (37). *Msm* and *Mtb mshD* mutants produce less MSH than wild-type, increased levels of the immediate MSH precursor and two novel thiols (42, 43).

Various mutants in MSH biosynthesis display increased susceptibility to redox stress and are more resistant to antibiotics. *Msm* MSH mutants are more sensitive than wild-type to H₂O₂, the superoxide generator plumbagin and the reductant dithiothreitol (DTT) (36, 40, 44). The *Mtb mshB* mutant is more susceptible to cumene hydroperoxide, while the *mshD* mutant shows increased sensitivity to H₂O₂ (38, 42). Interestingly, all *Msm* MSH mutants (save *mshC*) have increased resistance to ethionamide (ETH) (39, 44), and the *Msm* mutants of *mshA* and *mshD* are more resistant to INH (30, 34). The *Mtb mshA* mutant is more resistant to both drugs (37), and the *mshB* mutant of *Mtb* also displays INH resistance (38). It is thought that MSH may be involved in the activation of ETH and INH, and this would explain the increased antibiotic resistance that occurs upon loss of MSH. Evidence for this includes the finding that EthA, the enzyme that activates ETH, is more active in the presence of MSH (37).

Little is known about the regulation of MSH biosynthesis. In *Mtb*, MSH levels are three-fold higher in stationary phase than in early exponential phase (42). It is not known why this is so; the expression levels of the MSH biosynthetic genes during these phases are not known (29), and they were not detected in a microarray experiment to identify

Ergo pathway



MSH pathway

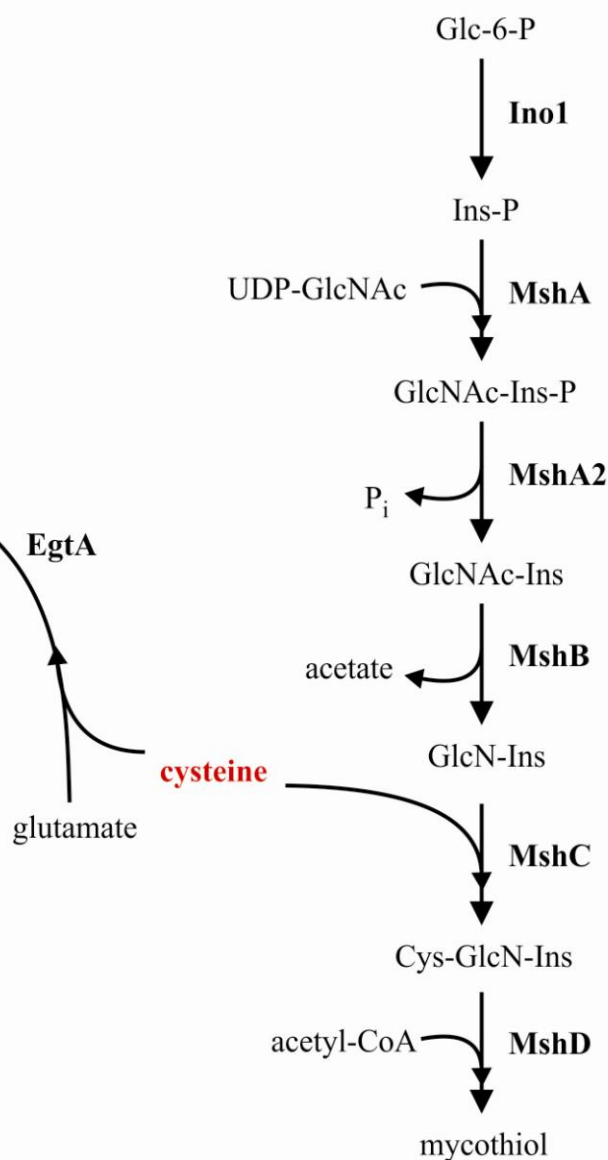


Figure 1. Ergothioneine and mycothiol biosynthetic pathways. The ergo pathway has not yet been verified *in vivo*. The first dedicated enzyme of the MSH pathway is MshA. Both pathways share a common intermediate, cysteine. Abbreviations: Glc-6-P, glucose-6-phosphate; Ino1, inositol phosphate synthase; Ins-P, 1L-*myo*-inositol-1-phosphate; UDP-GlcNAc, UDP-*N*-acetylglucosamine; GlcNAc-Ins-P, 1-*O*-(2-acetamido-2-deoxy- α -D-glucopyranosyl)-D-*myo*-inositol-3-phosphate; GlcN-Ins, 1-*O*-(2-amino-2-deoxy- α -D-glucopyranosyl)-D-*myo*-inositol; Cys-GlcN-Ins, 1-*O*-[2-[(2*R*)-2-amino-3-mercapto-1-oxopropyl]amino]-2-deoxy- α -D-glucopyranosyl]-D-*myo*-inositol.

genes with different expression levels in exponential versus stationary phases (45). In *Mtb*, there are transcriptional regulators upstream of *mshA* and *mshD*, but potential roles for these in MSH regulation have yet to be explored (29). It is possible that feedback inhibition regulates MSH biosynthesis because it was shown that MshB activity decreases in the presence of MSH (46).

MSH works with many enzymes in order to ensure mycobacterial survival. Mtr, the MSH disulfide reductase, reduces MSH that has become oxidized as a consequence of maintaining the reducing environment of the cell. The Mtr-catalyzed reaction requires FAD as a cofactor and consumes NADPH (47-49). MSH also functions to remove toxic compounds, such as the antibiotic cerulenin, with the aid of mycothiol S-conjugate amidase (Mca). After MSH conjugates the compound, Mca catalyzes the separation of the complex into GlcN-Ins, an MSH precursor, and a mercapturic acid, which is excreted from the cell (46, 50). To detoxify formaldehyde, formaldehyde dehydrogenase (MscR) oxidizes a MSH-formaldehyde conjugate in the presence of NAD^+ to produce formate, a less toxic metabolite (51). MscR also has NADH-dependent nitrosothiol reductase activity that breaks down MSNO (produced by the fusion of MSH and NO) into nitrate and MSH (51). Mycothiol has been shown to be important for the protection of mycobacteria from NO (52).

Ergothioneine (ergo)

Mycobacteria also produce ergothioneine, a LMW thiol with antioxidant capabilities whose *in vivo* function is still unknown. Ergo was initially discovered in 1909 in the fungus ergot (53). Prior to 1960, it was not known that bacteria are capable of

producing ergo; biosynthesis had only been shown in fungi (54, 55). The first bacterium shown to synthesize ergo was *Mtb* (56). Following this, various bacteria (including streptococci, *Salmonella*, *E. coli*, and *Corynebacterium*) were analyzed and found to be unable to produce ergo. In this same study, 101 strains of *Mycobacterium* comprising more than 15 different species were examined and the majority were capable of ergo biosynthesis (57). Other actinomycetes, including *Nocardia asteroides* and three *Streptomyces* strains, and cyanobacteria have also been shown to produce ergo (58, 59). Though ergo is present in millimolar quantities in the tissues of various plants and mammals, these organisms do not produce ergo themselves but instead acquire it from their surroundings (i.e., the soil) or through their diets (60).

Ergothioneine biosynthesis. The biosynthesis of ergo in fungi has been well studied, while the few early reports suggested that the mycobacterial biosynthetic pathway is similar to that in fungi (57, 61). Structurally, ergo is a histidine molecule that has a sulfur atom at position two of the imidazole ring and three methyl groups bound at the amine group of the alpha carbon (62). Using the fungus *Neurospora crassa*, which produces large quantities of ergo, Melville and coworkers showed that histidine is the starting precursor for ergo biosynthesis with subsequent incorporation of three methyl groups and a sulfur atom (63). Two possible intermediates are thiolhistidine, in which the sulfur group would be added prior to methylation, or hercynine, a methylated histidine with no sulfur. Using radioactive precursors, they determined that hercynine is the likely intermediate because addition of ^{14}C -thiolhistidine to the fungi did not result in the recovery of radioactive ergo (63). Further work established this scheme because supplementation with radio-labeled hercynine does lead to the production of labeled ergo

(64). The source of the methyl groups is methionine (63), and it was established that this assimilation is achieved through transmethylation (65). All three transmethylation reactions are catalyzed by a single unknown enzyme using *S*-adenosyl-methionine (SAM) as the methyl donor (66, 67). Cys is the preferred sulfur source (63), and the intermediate *S*-(β -amino- β -carboxyethyl) ergothioneine sulfoxide is formed from Cys and hercynine in a reaction requiring oxygen and Fe^{2+} . This intermediate is then converted to ergo through a pyridoxal 5-phosphate (PLP)-dependent reaction with the concomitant release of pyruvate (68).

Ergo biosynthesis in mycobacteria was investigated over 40 years ago using radioactive precursors. A 1964 study showed that the intact imidazole ring of histidine is incorporated into both hercynine and ergo, indicating that biosynthesis proceeds through the hercynine intermediate as in fungi. Supplementation with ^{35}S -sulfate results in labeling of ergo but not hercynine, as expected (57). Furthermore, ergo production decreases and hercynine accumulates in *Msm* cultures that are starved for sulfur. Addition of Cys or compounds easily converted to Cys rescues ergo production, while hercynine levels increase when L-histidine is added to the cultures (61).

Though the genes responsible for ergo biosynthesis in fungi have yet to be identified, a cluster of genes potentially involved in mycobacterial ergo biosynthesis was recently reported (*egtABCDE*; see Fig. 1 for proposed biosynthetic pathway) (69). Based on the studies done in fungi, the author searched for a methyltransferase in *M. avium* with a homologue in *N. crassa* but no homologues in *E. coli* or *Bacillus subtilis*, neither of which produce ergo. Ten genes were identified, and one was located next to an annotated PLP-dependent transferase in a cluster of five genes. Purification of the

methyltransferase's *Msm* homologue from recombinant *E. coli* and subsequent analysis revealed that the purified enzyme is indeed a histidine-specific methyltransferase. This enzyme was designated EgtD due to its position as the fourth gene in the five gene cluster (69).

Further analysis of the proteins in the cluster revealed that recombinant *Msm* EgtB catalyzes Fe-dependent oxidative sulfurization of hercynine and preferentially uses γ -glutamylcysteine as substrate over Cys, *N*-acetylcysteine and glutathione. The intermediate produced consists of γ -glutamylcysteine's oxidized sulfur group linked to position two of hercynine's imidazole ring. The addition of recombinant EgtC, annotated as a class-II glutamine amidotransamidase, to the reaction results in the release of glutamate from the intermediate to produce hercynylcysteine sulfoxide. This compound is converted to ergo presumably through the action of EgtE, a PLP-binding protein, though the author was unable to purify recombinant EgtE and instead used a β -lyase from *Erwinia tasmaniensis* to produce ergo (69). EgtA, a γ -glutamylcysteine ligase, was not tested and instead purified γ -glutamylcysteine was used in all reactions because a previous paper showed that the *Mtb* homologue of *Msm* EgtA could produce γ -glutamylcysteine (70).

Ergothioneine biochemistry. Ergo has distinctive chemical properties compared to other well known LMW thiols such as glutathione and mycothiol. While these compounds are generally found in the thiol form under physiological conditions, ergo favors the thione form (71). Unlike glutathione, which forms disulfides and peroxides in the presence of heavy metals, ergo is resistant to auto-oxidation mediated by heavy metals and instead can form complexes with divalent metals (72). In aerated aqueous

cultures, ergo remains in the reduced state while other LMW thiols tend to form disulfides (73). The E_o' value of ergo is -60 mV while glutathione has a value of -250 mV, meaning ergo remains reduced in environments that would lead to glutathione oxidation (74). The ergo disulfide is highly unstable, and if produced under physiological conditions would rapidly convert back to the reduced state. Oxidized ergo does not require a reductase to recycle to the thiol while many other LMW thiols have dedicated enzymes to maintain them in their reduced forms (75).

Functions of ergothioneine. Numerous studies have reported that ergo can function as an antioxidant *in vitro*. It scavenges ROS and RNS such as hydroxyl radicals, hypochlorous acid (HOCl) and peroxynitrite (76-80), thus shielding biomolecules from attack by these species. For example, addition of ergo inhibits the inactivation of α_1 -antiproteinase by HOCl and peroxynitrite (76, 80). Ergo also protects tyrosine from nitration by peroxynitrite better than either glutathione or Trolox C (80). The cytotoxic effect of peroxynitrite on rat adrenal pheochromocytoma (PC12) cells decreases in a dose-dependent manner with the addition of ergo (81). Lipid peroxidation by both the myoglobin/H₂O₂ and oxyhaemoglobin/H₂O₂ systems is inhibited by ergo (76). The ability of ergo to chelate metals contributes to its role as an antioxidant. It was shown that physiological concentrations of ergo reduces both the copper ion-induced oxidation of hemoglobin and the iron ion-induced degradation of deoxyribose (80).

Even though mammals do not produce ergo, they accumulate high levels in various cells and tissues that are exposed to oxidative stress, including red blood cells, kidney, liver, and eye lens (82-84). Ergo cannot permeate plasma membranes and a specific transporter, OCTN1, is responsible for this cell/tissue-specific accumulation of

ergo (85). Knocking out OCTN1 in mice leads to nearly complete loss of ergo from tissues. Thus, this transporter is the major ergo uptake mechanism (86). Depletion of OCTN1 through RNA silencing in HeLa cells leads to increased susceptibility to H₂O₂ stress. Cells lacking OCTN1 also have higher levels of protein carbonylation, lipid peroxidation, and mitochondrial DNA damage following treatment with H₂O₂ (87).

Studies done *in vivo* have also shown a role for ergo in protection from oxidative stress. When wild-type and OCTN1 knockout (KO) mice were subjected to intestinal ischemia and reperfusion injury, the KO mice had increased loss of intestinal structures and decreased survival following the injury (86). The damage caused by ischemia and reperfusion injury has been shown to involve ROS (88); thus, these results suggest that ergo can protect the small intestine from oxidative stress. In another study, mice that were fed ergo during treatment with cisplatin had significant restoration of the neuronal deficits associated with this treatment compared to mice that were not fed ergo. Additionally, the ergo supplementation prevented lipid peroxidation and restored the glutathione/glutathione disulfide ratio in the brain (89).

WhiB Proteins in *Mtb*

The Wbl (WhiB-like) protein family is widespread in actinomycetes (90). Members of this protein family are involved in numerous and diverse cellular processes, which may be explained by the heterogeneity in domain architecture among the proteins (91). The first Wbl protein described, the eponymous WhiB, was discovered by Chater in 1972 as a protein necessary for sporulation in *Streptomyces coelicolor* (92). Sporulation mutants remain white while wild-type colonies turn gray, and this characteristic is

responsible for the name (Whi) of the family. Further analysis of WhiB indicated that it is not necessary for survival, and a single amino acid change in a putative helix-loop-helix domain was enough to abolish sporulation (93). It has been speculated that the *Mtb* WhiBs may be involved in dormancy due to their homology to this sporulation factor (94, 95).

Nearly all Wbl proteins contain four conserved Cys residues, which suggests that these proteins coordinate a metal cofactor (90). This is indeed the case, and *S. coelicolor* WhiD was the first shown to bind an Fe-S cluster (96). Like WhiB, WhiD is also involved in sporulation; the *whiD* mutant produces heat-sensitive spores of irregular size that lyse easily (97, 98). Purified WhiD contains a [2Fe-2S] cluster than can be anaerobically reconstituted to a [4Fe-4S] cluster. Exposure to oxygen eventually causes complete degradation of the cluster. All four conserved Cys residues are individually required for *in vivo* function of the protein, though the authors did not determine how loss of each individual residue affects coordination of the Fe-S cluster (96). *Mtb* contains seven proteins that belong to the Wbl family (90), and all of these have been shown to coordinate redox-sensitive Fe-S clusters (99-101).

Mtb WhiB1

A 2010 study established that *Mtb* WhiB1 is essential for survival and is a transcription factor that can bind to the promoter region of *whiB1* and inhibit its transcription (101). Wbl proteins had long been suspected to function as transcription factors, but before this publication no direct evidence had been presented to support this notion. DNA binding by WhiB1 can only occur in the absence of its [4Fe-4S]²⁺ cluster.

The authors show that the cluster is not degraded upon exposure to air and can react with NO (101). The expression of *whiB1* is also regulated by Rv3676, a cAMP receptor protein (CRP) family member, in response to cAMP levels (102, 103). Regulation by this CRP protein is different from the well-established CRP regulation in *E. coli*, possibly to accommodate the much higher levels of cAMP in *Mtb* compared to *E. coli* (104).

Two of the four conserved Cys residues in the majority of Wbl proteins are present in a C-X-X-C arrangement, and in thiol-disulfide oxidoreductases this motif acts as the active site. Analysis of WhiB1 showed that it has general protein disulfide reductase activity in its cluster-free (apo) form (105). This is significant because it suggests a role for this protein in the redox biology of *Mtb*. Further support for this activity was presented by the same researchers when they showed that WhiB1 can bind GlgB and reduce its intramolecular disulfide bond. This reduction was shown to be specific because two *Mtb* thioredoxins were not able to reduce the GlgB disulfide bond (106). Another group was unable to detect general disulfide reductase activity for WhiB1 (101), so whether the protein has this ability remains controversial.

Mtb *WhiB2*

WhiB2 is an Fe-S cluster containing, essential protein in *Mtb* and is involved in cellular division (100, 107). Strains producing low levels of WhiB2 produce smaller colonies than wild-type, have elongated cells and decreased viability. Overexpression of *whiB2* leads to changes in colony phenotype that indicate alteration of the cell envelope (107). Sherrid and coworkers identified a group of genes termed the Reaeration Response that is upregulated in *Mtb* when the bacilli are transferred from hypoxic growth

conditions to normal aerated growth. One of these genes is *whiB2*, which is fitting based on its role in cell division; higher levels of WhiB2 may facilitate the recovery of cells from a dormant state induced by oxygen depletion (108). Treatment with capreomycin, an antibiotic that is active against non-replicating *Mtb*, upregulates the expression of *whiB2* (109). This finding is consistent with the above observations; by increasing the levels of WhiB2, capreomycin may promote emergence of the bacilli from their dormant state, at which point they can be killed by capreomycin through inhibition of protein translation.

Mtb *WhiB3*

WhiB3 was identified as a virulence factor of *Mtb* in 2002 when it was shown that a *whiB3* deletion mutant grew as well as wild-type in mice but caused less severe lung pathology. Additionally, mice infected with the mutant strain survived nearly two times longer than their counterparts infected with wild-type *Mtb* (110). Furthermore, WhiB3 was shown to interact with wild-type RpoV (SigA), but not with RpoV containing an Arg-515→His mutation that was previously shown to cause the attenuation of *Mycobacterium bovis* (110, 111). It has also been shown that *whiB3* is induced in *Mtb* present in the lungs of mice and that there is an inverse relationship between induction and bacterial number, suggesting that *whiB3* expression may be regulated through quorum sensing (112).

WhiB3 contains a $[4\text{Fe-4S}]^{2+}$ cluster, coordinated by the four conserved Cys residues, that is degraded by oxygen and can bind NO (99). WhiB3 regulates intermediary metabolism, which was discovered based on the growth phenotypes of wild-

type *Mtb* and *MtbΔwhiB3*. The mutant displayed a growth defect when grown on media containing glucose, succinate, fumarate or pyruvate as the sole carbon source.

Complementation with wild-type WhiB3, but not WhiB3 having mutated Cys residues, can rescue the growth defect on glucose and succinate, signifying that redox responsiveness is crucial for metabolic regulation by WhiB3 (99). This study pointed to a role for WhiB3 in the switch over from carbohydrates to fatty acids as the major carbon source *in vivo* because the *whiB3* mutant grew better than wild-type on media containing acetate as the sole carbon source (99).

Utilization of long chain fatty acids as carbon source *in vivo* leads to the accumulation of reducing equivalents in *Mtb* due to the multiple rounds of β -oxidation necessary to completely break down FAs to acetate and propionate. It has been demonstrated that redox balance can be maintained through *Mtb* lipid anabolism, which consumes reducing equivalents, acetate and propionate (113). WhiB3 regulates the expression and levels of methyl-branched immunomodulatory lipids and the storage lipid triacylglycerol (TAG), indicating that WhiB3 plays a role in redox homeostasis. Indeed, a *whiB3* mutant that has an altered lipid profile accumulates high levels of reducing equivalents when grown in macrophages (113). WhiB3 also functions in the detoxification of propionate, which is a precursor of methyl-branched lipids, since it was shown that *MtbΔwhiB3*, but not wild-type, can grow at high concentrations of propionate (113). Thus, by regulating the production of propionate-derived lipids, WhiB3 contributes to both redox homeostasis and propionate detoxification.

WhiB3 was the first of the Wbl proteins shown to bind DNA, and it does so in a redox-responsive manner (113). The promoter regions of both *pks2* and *pks3*, polyketide

lipid biosynthetic genes, are bound by WhiB3. The redox state of WhiB3's Fe-S cluster does not affect DNA binding, but when the cluster is lost from the protein, oxidation of the Cys residues enhances DNA binding. Expression analysis of various polyketide lipid biosynthetic genes indicates that WhiB3 can positively regulate the transcription of a subset of these, explaining the altered lipid profile seen in *MtbΔwhiB3* (113).

WhiB3 regulation of polyketides involved in the inflammatory response of the host was proposed in 2002 to explain the attenuation of the *whiB3* mutant (110). This hypothesis was supported by the altered spot colony phenotype of *MtbΔwhiB3*, wherein it displays less cording than wild-type, suggesting differences in cell wall lipids (99). Finally, a 2009 study corroborated the hypothesis when it was shown that macrophages infected with *MtbΔwhiB3* secreted higher levels of pro- and anti-inflammatory cytokines than did macrophages infected with wild-type *Mtb* (113).

Mtb *WhiB4*

Purified WhiB4 contains a [2Fe-2S] cluster than can be chemically reconstituted *in vitro* to presumably a [4Fe-4S] cluster (114). This cluster is completely degraded upon exposure to oxygen. A protein having all four conserved Cys residues mutated to serines is unable to coordinate a cluster and, of the four Cys residues, the two present in the C-X-X-C motif are more important for cluster coordination. When the Fe-S cluster is completely lost from WhiB4, two intramolecular disulfide bonds can be formed, one being between the C-X-X-C Cys residues. As shown for WhiB1, this same group also demonstrated that apo-WhiB4 can act as a protein disulfide reductase. Interestingly, they

suggest that the protein has two active sites, one at the C-X-X-C motif and the other composed of the remaining Cys residues (114).

Mtb *WhiB5* and *WhiB6*

As is the case with the other *WhiB* proteins, *WhiB5* and *WhiB6* coordinate oxygen labile Fe-S clusters that are in the [2Fe-2S] form when the proteins are aerobically purified. Enzymatic reconstitution results in the formation of [4F-4S] clusters. The *WhiB6* Fe-S cluster is more resistant to oxygen degradation than all others except *WhiB7*. *WhiB5* is the only *Mtb* *WhiB* protein that does not have the C-X-X-C motif; instead, it has a C-X-X-X-C arrangement. Nevertheless, the apo form still displays protein disulfide reductase activity, as does the apo form of *WhiB6*. *WhiB5* and *WhiB6* are structurally distinct from the other *Mtb* *WhiB* proteins, as evinced by minimal amino acid sequence homology with other *WhiBs* (100). Furthermore, *WhiB5* and *WhiB6* are present only in mycobacteria, and *WhiB5* is found only in pathogenic strains (91).

Mtb *WhiB7*

A screen to discover a regulator that mediates multidrug resistance in *Streptomyces lividans* identified *whiB7* as being involved in this process. Subsequently, a *whiB7* mutant of *Mtb* was shown to be more sensitive than wild-type to a range of antibiotics. Exposure to certain antibiotics and fatty acids leads to induction of *whiB7* in *Mtb*, and a *whiB7* regulon was identified that includes genes involved in antibiotic resistance, including an efflux pump (115). The induction of *whiB7* by the presence of fatty acids is interesting since *Mtb* is thought to subsist mainly on fatty acids *in vivo*; thus,

genes that confer antibiotic resistance would be expressed *in vivo* even before any antibiotics are present. Recently, *whiB7* expression was found to be induced by reducing conditions (DTT), and the authors suggested that upregulation of *whiB7* in response to antibiotics and fatty acids is due to a more reducing environment present in the bacilli under these conditions (116). This theory is interesting because it suggests that WhiB7 may induce antibiotic resistance mechanisms in response to the intracellular redox environment.

Present Study: Investigation of Mycobacterial Ergothioneine

The presence of Fe-S clusters in the *Mtb* WhiB proteins suggests that they may function as sensors of the intracellular redox environment or external host signals. This has indeed been shown to be the case for WhiB3 and WhiB7, wherein WhiB3 regulates lipid biosynthesis to balance the redox environment of *Mtb* (113). The goal of the current project was to investigate whether additional WhiB3-dependent mechanisms exist for maintaining redox homeostasis. We identified the antioxidant ergothioneine through metabolomic analysis of wild-type *Mtb* and a *whiB3* mutant and went on to determine other factors regulating ergo production using a radioactive TLC assay we developed. We found that conditions that cause a more reducing intracellular environment led to decreased production of ergo. Additionally, loss of other redox stress protection mechanisms increased ergo production. Using ergo-deficient *Mtb* mutants, we also show that ergo can protect *Mtb* from oxidative stress.

METABOLOMIC DISCOVERY OF ERGOTHIONEINE REGULATION
BY REDOX RESPONSIVE WHIB3

by

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Abstract

The role of redox homeostasis in the persistence of *Mycobacterium tuberculosis* (*Mtb*) is poorly understood. The intracellular redox sensor WhiB3 mediates redox homeostasis in *Mtb* by regulating lipid anabolism. Here we used metabolomic analysis to identify other WhiB3-regulated *Mtb* redox maintenance mechanisms. We show that production of the thiol ergothioneine (ergo) is under the control of WhiB3 in a carbon source-dependent manner. We developed a novel radioactive TLC assay and demonstrated that growth on immunomodulatory lipid precursors results in altered ergo production in an *Mtb whiB3* mutant. Additionally, we show that a reducing environment causes decreased levels of ergo regardless of the presence of WhiB3. Notably, we discovered that ergo protects *Mtb* from oxidative stress. Altogether, our data suggest that ergo contributes to *Mtb* redox maintenance. Identification of mechanisms that maintain redox balance in *Mtb* is important due to the link between *Mtb* central metabolism and persistence.

Introduction

As the etiological agent of tuberculosis (TB), *Mycobacterium tuberculosis* (*Mtb*) latently infects more than two billion people worldwide and resulted in approximately 1.4 million deaths in 2010 (1). After HIV, TB is the second most common cause of death from an infectious agent largely due to the ability of *Mtb* to remain in a dormant, drug-unresponsive state for decades in humans. In 10% of infected hosts, *Mtb* can emerge from dormancy to cause active disease (1). Throughout the establishment and progression of TB, *Mtb* encounters diverse environments and must adapt its metabolism to ensure that redox equilibrium is preserved (2). This metabolic modification can directly affect the pathogenesis of *Mtb*; e.g., through increased production of immunomodulatory lipids to rid the cells of excess reducing equivalents produced as a consequence of using fatty acids as the primary carbon source. This detoxification through lipid anabolism is facilitated by WhiB3, an iron-sulfur (Fe-S) cluster protein that regulates the synthesis of virulence and storage lipids in a redox-dependent manner (3). Thus, WhiB3 integrates the intracellular redox state of *Mtb* with the production of virulence determinants to maintain redox balance.

WhiB3 is one of seven WhiB-like (Wbl) proteins present in *Mtb*. Members of this family are found only in actinomycetes and are involved in diverse cellular processes including cell division, antibiotic resistance and metabolism (4). It has been shown that all seven of the *Mtb* Wbl proteins (WhiB1-WhiB7) coordinate Fe-S clusters through which they can monitor the redox state of the cell (5). WhiB3 is the best studied *Mtb* WhiB protein, and its $[4\text{Fe-4S}]^{2+}$ cluster is responsive to both oxygen and nitric oxide (NO) (6). WhiB3 has been shown to facilitate the switch-over from carbohydrate

metabolism to that of its preferred *in vivo* carbon source (fatty acids) under reducing conditions (3).

Redox buffers are key contributors to redox homeostasis in living organisms and maintain the reducing environment of the cytosol. In *Mtb*, mycothiol is the major redox buffer which, once oxidized, is reduced by mycothione reductase (Mtr), an enzyme that uses NADPH as a cofactor (7, 8). Mycobacterial cells lacking mycothiol have decreased resistance to various redox stressors (9-13). Additional *Mtb* proteins that help maintain redox balance include three thioredoxins (Trx), which reduce disulfide bonds in collaboration with Trx reductase and NADPH, and are involved in protection of *Mtb* from reactive oxygen species (ROS) and reactive nitrogen species (RNS) (14). Enzymes that contribute to redox homeostasis include: KatG, the only *Mtb* catalase-peroxidase; AhpC, an alkylhydroperoxidase; superoxide dismutase (SOD); methionine sulfoxide reductases (MSRs); and others (see (2) for review).

In addition to mycothiol, *Mtb* produces a second thiol buffer, ergothioneine (ergo), which is a sulfur-containing derivative of histidine that has garnered much interest due to its antioxidant properties (15, 16). Though ergo was first discovered over 100 years ago in fungi and was shown to be produced by *Mtb* in 1960, the genetics of ergo biosynthesis and its physiological role are still unknown (15, 17). While ergo can be detected in mammalian cells and tissues, it must be acquired through the diet as there is no evidence that mammals can produce ergo (16). Ergo is accumulated in certain human cells by a specific transporter, OCTN1, and blocking this transporter in HeLa cells results in increased susceptibility to hydrogen peroxide and superoxide (18, 19). However, little

is known about ergo's direct role in *Mtb* as a potential antioxidant, or its function in maintaining redox homeostasis.

WhiB3 is an intracellular redox sensor that regulates core intermediary metabolism in response to the prevailing redox environment to maintain redox homeostasis (3, 6). In this study, we hypothesized that WhiB3 regulates other factors involved in the maintenance of redox homeostasis. We used a comprehensive metabolomics approach to identify metabolites whose levels are affected by the loss of WhiB3 and discovered that WhiB3 regulates ergo production in *Mtb* in a carbon source-dependent manner. The link between central metabolism and ergo production was further investigated using a newly developed radioactive thin layer chromatography (TLC)-based assay to quantify ergo levels after growth on various metabolic intermediates. Moreover, we examined ergo production under oxidizing and reducing conditions and in response to the loss of key redox stress defense mechanisms. We also used ergo-deficient mutants to assess the contribution of ergo to oxidative stress protection in *Mtb*. Persistence in *Mtb* requires the continued functioning of certain metabolic pathways (20) and, since successful metabolism depends on a properly balanced redox environment, the identification of mechanisms used by *Mtb* to maintain redox homeostasis will allow a better understanding of persistent *Mtb* infections.

Materials and Methods

Bacterial Strains and Culture Conditions

Mtb strains were grown at 37°C in 7H9 (broth) or 7H10/7H11 (agar) media with 1x AS enrichment (albumin- NaCl), 0.02% Tyloxapol, and various concentrations of

glucose, sodium acetate, succinate or sodium propionate. When necessary, 5 mM diamide (DMD) or 5 mM dithiothreitol (DTT) was added to the 7H10 medium. Hygromycin B (Hyg; 50 µg/ml) was added to CDC1551 Δ *mshA*, H37Rv Δ *whiB3*, and H37Rv Δ *whiB3 tetRO:whiB3* cultures. The latter also contained 25 µg/ml Kanamycin (Kan), as did the CDC1551 *egt* transposon mutants. Complemented *egt* mutant cultures received Kan and 50 µg/ml Hyg. *M. smegmatis* (*Msm*) and its mutants were grown essentially the same as the *Mtb* strains, with the addition of 25 µg/ml Kan to the mutants' media. *Escherichia coli* strains were grown in LB medium with 150 µg/ml Hyg.

Metabolic Labeling and Extraction of Ergo from Mycobacterial Strains

Filter-culturing: Mycobacteria grown to early- to mid-log phase in 7H9 were set to an OD₆₀₀ of 0.8-1.0 in 400 µl and inoculated onto mixed cellulose ester membranes, which were then transferred to 7H10 plates containing the same carbon source as the broth. For radioactive labeling, 2-3 µCi/ml of ³⁵S-cysteine (American Radiolabeled Chemicals) or 5-10 µCi of [2-¹⁴C]-acetate was spread on the plates prior to placement of the membranes. The cells were grown on plates until a confluent film was achieved, and growth times varied for different carbon sources. For metabolome analysis, bacteria were metabolically quenched by immersion into acetonitrile:methanol:50 mM ammonium acetate (40:40:20) [AMAA] pre-cooled to -40°C and metabolites were extracted by mechanical lysis followed by clarification and filtration across a 0.22 µm filter.

Radioactively labeled bacteria were quenched and extracted in AMAA or water by heating at 90°C for 20 min prior to clarification and filtration. When necessary, bacterial biomass of individual samples was determined by measuring residual protein content.

Broth culturing: Mycobacteria grown to mid- to late-log phase in the presence of 2-3 $\mu\text{Ci/ml}$ ^{35}S -cysteine were pelleted, washed two times with medium and, after the addition of AMAA or water, extracts were prepared by heating as above.

Liquid Chromatography/Mass Spectrometry (LC/MS) for Metabolome Analysis

Metabolites were separated on a Cogent Diamond Hydride Type C column as detailed by de Carvalho *et al* (21). The mass spectrometer used was an Agilent Accurate Mass 6220 TOF coupled to an Agilent 1200 LC system. Dynamic mass axis calibration was achieved by continuous infusion of a reference mass solution using an isocratic pump with a 100:1 splitter. This configuration achieved mass errors of approximately 5 parts-per-million (ppm), mass resolution ranging from 10,000-25,000 (over m/z 121-955 amu), and 5 \log_{10} dynamic range. Detected ions were deemed metabolites on the basis of unique accurate mass-retention time identifiers for masses exhibiting the expected distribution of accompanying isotopomers. Ergothioneine was confirmed by demonstrating co-elution with chemical standard.

TLC Analysis of Ergo

Radioactive extracts were concentrated to dryness and resuspended in 30-50 μl AMAA. At least 30,000 cpm in no more than 15 μl was loaded on silica gel TLC plates and developed in a 3:1 methanol:water solvent system. Radiolabeled ergo (identified by its R_f value; see below) was visualized by exposing the TLC plate to a phosphor storage screen followed by phosphorimaging on a Storm 820 phosphorimager (GE Healthcare). ImageQuant software (GE Healthcare) was used for quantitative analysis. The R_f value of

ergo in this solvent system was determined by TLC analysis of pure ergo followed by application of 0.2% 2,6-dichloroquinonechloroimide (Gibb's reagent) in ethanol, which makes ergo appear brick-red. The R_f value of pure histidine was also determined after visualization with 0.25% ninhydrin in acetone, with which histidine appears violet.

Liquid Chromatography/Mass Spectrometry/Multiple Reaction Monitoring (MRM)

Analysis of TLC Ergo Spot

Concentrated, unlabeled *Msm* extract was loaded on a silica gel TLC plate and developed as above. The suspected ergo spot was visualized under UV light, scraped from the plate, and extracted four times with 50 μ l AMAA. It was then concentrated to dryness and resuspended in 20 μ l AMAA. For LC/MS/MRM analysis, samples and calibration standards were diluted into AMAA and injected onto a Waters T3 (C18) column (2 x 150 mm). Samples were eluted with 10 mM NH_4OAc (buffer A) and acetonitrile + 10 mM NH_4OAc (buffer B) with the following gradient: at 0 min, 0% buffer B; at 5 min, 95% buffer B; at 6 min, 0% buffer B; at 10min, stop. The flow rate was 0.2 ml/min. Mass spectrometry was carried out on an AB Sciex API-3200 Triple Quadrupole coupled to a Waters Acquity LC system. Data was collected in positive electrospray mode.

Cloning and Complementation

To complement the *egt* transposon (Tn) mutants, *egt* genes were PCR amplified and cloned into the *Nco*I/*Hind*III sites of pMV762. The *egtA* mutant was complemented with the entire *egt* gene cluster (forward primer 5'-TGACTCATGACGCTTGCCGCCA

TGACC-3', reverse primer 5'-TATGAAGCTTAAGGCCGGCTGAGGCTAAC-3') and the *egtD* mutant with *egtD* (forward primer 5'-CAGTCATGAGAGTGTCTGGTTGCCA ACCATC-3', reverse primer 5'-TATGAAGCTTGCCGGCGCGGCTCACTTG-3'). The PCR products were digested with BspHI and HindIII, ligated with pMV762 and transformed into DH10B *E. coli* cells. Sequenced clones were transformed into the *egt* mutants and ergo-producing strains were identified with the radioactive TLC assay. Not all transformants carrying the complementation vectors produced ergo, a previously reported phenomenon that can occur when the *hsp60* promoter is used for gene expression (22). We are in the process of confirming that loss of these genes is responsible for loss of ergo by curing the vector from the complemented strains and verifying their reversion to ergo-deficient phenotypes.

Microplate alamarBlue Analysis of Cumene Hydroperoxide (CHP)-treated Cells

Cultures were grown to 0.7-0.9 OD₆₀₀ in 7H9 media with 50 mM glucose, set to an OD₆₀₀ of 0.6-0.7 in medium of the same composition, and treated with 250 μ M CHP (prepared in 100% ethanol) at 37°C with shaking. Control cultures were treated with equivalent concentrations of ethanol. After 24 hours, cells were washed twice and 1x10⁶ cells were put in a 96-well plate in triplicate wells and incubated at 37°C with shaking for one day. alamarBlue (AbD Serotec), mixed 1:1 with 0.04% tyloxapol, was added to achieve a final concentration of ~9%. After ~40 hours of incubation at 37°C with shaking, fluorescence (530nm/590nm) was read with a sensitivity value of 70 in bottom-read mode on a BioTek Synergy H1 microplate reader running Gen5 software.

Thiol/Disulfide Extraction and High-Performance Liquid Chromatography (HPLC)

A 20 ml sample of culture grown to OD₆₀₀ of 1.2-1.4 was centrifuged at 2500 x g and the cell pellet was washed twice with 1X phosphate buffered saline (PBS). For mycothiol analysis, cells were extracted at 60°C for 15 min in 600 µl of warm (60°C) 50% aqueous acetonitrile containing 20 mM HEPES (pH 8.0) and 2 mM monobromobimane (mBBBr). For control samples, 5 mM *N*-ethylmaleimide (NEM) replaced the mBBBr. The samples were clarified by centrifugation at 15,000 x g and those with mBBBr were acidified with 20 mM of methanesulfonic acid (MSA). NEM control samples were treated with 2 mM mBBBr at 60°C for 15 min before acidification with 50 mM MSA.

For disulfide analysis, extraction of 20 ml cells was at 60°C for 15 min in 600 µl warm 50% aqueous acetonitrile containing 20 mM HEPES (pH 8.0) and 5 mM NEM. Clarified samples were treated with 5 mM β-mercaptoethanol at room temperature (RT) for 10 min before the addition of 2 mM DTT and incubation at RT for 10 min. Disulfide samples were then treated with 9 mM mBBBr at 60°C for 15 min. Control samples were incubated with 5 mM NEM at 60°C for 10 min before treatment with 9 mM mBBBr at 60°C for 15 min. Recovered supernatants from all samples were kept on ice for 5 min, then acidified with 20 mM MSA. To determine the ratio of MSH to MSM, two 20 ml aliquots were taken from one culture, with one aliquot used for thiol analysis and the other aliquot used for disulfide analysis. Thus, MSH/MSM values were determined for three individual cultures and the results averaged to produce the final result.

HPLC (Waters 1525 Binary HPLC) analysis of the thiols was carried out by injecting 15-25 µL of a 1:4 - 1:10 dilution of the samples onto an Inertsil ODS-4 column (5 µm; 250 by 4.6 mm; GL Sciences Inc, Japan). Elution was accomplished with 0.25%

glacial acetic acid (pH 3.6 with NaOH; buffer A) and 95% methanol (buffer B) by using the following gradient: at 0 min, 10% buffer B; at 5 min, 18% buffer B; at 30 min, 27% buffer B; at 32 min, 100% buffer B; at 34 min, 10% buffer B; and at 45 min, 10% buffer B (reinjection). The flow rate was 1 ml/min, and fluorescence detection was carried out on a 2001A Fluorotec (370 nm excitation filter and a 418–700 emission filter). The software used was Waters Breeze GPC.

Disk Diffusion Assays

7H11 plates supplemented with 1X ADS (albumin- dextrose- NaCl) and 0.02% tyloxapol were covered with 0.5 OD₆₀₀ mid-log phase cells and allowed to dry. Disks (6 mm) were placed on the plates and 10 µl of stressors was added to the disks to get the following concentrations: 10% and 20% hydrogen peroxide; 1 mM and 10 mM menadione; and 500 µM, 1 mM, 5 mM and 10 mM pyrogallol. After incubation at 37°C, zones of inhibition were measured.

Results

Metabolomic Analysis of Mtb WhiB3

To identify metabolites regulated by WhiB3 in *Mtb*, we analyzed the metabolomes of H37Rv and H37RvΔ*whiB3*. Metabolomics allows the unbiased comparison of small molecular weight compounds between different strains or one strain cultured under different conditions. This method is advantageous because direct examination of the levels of metabolites gives the truest picture of a cell's phenotype (23). We used carefully controlled conditions for optimal retention of metabolites when

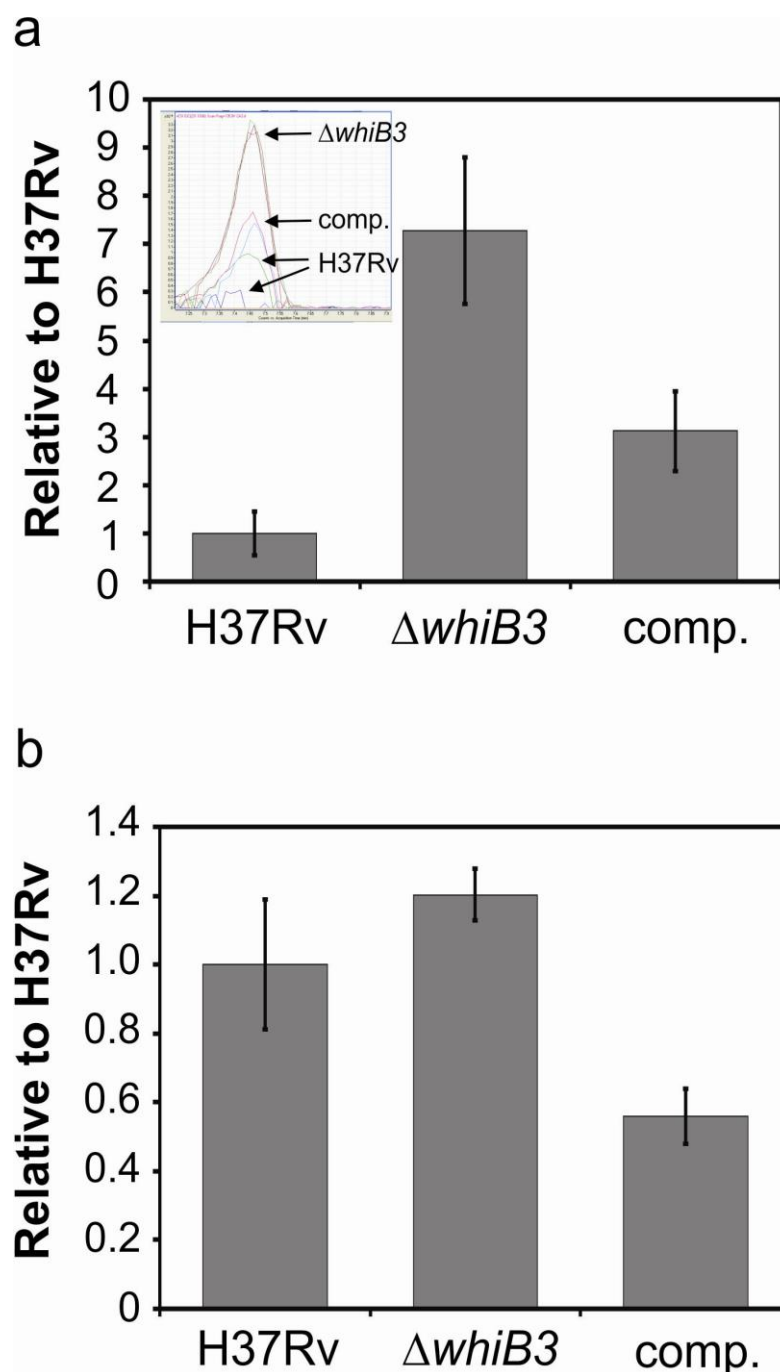


Figure 1. Ergo levels based on metabolomic analysis of *Mtb* WhiB3. Abundance of ergo based on LC/MS analysis after growth on solid medium containing 5 mM acetate (**a**) or 50 mM glucose (**b**). Data are expressed as ergo ion counts in each strain relative to H37Rv, and have been normalized to total cellular protein content. Error bars represent standard error of the mean of triplicate samples (except H37Rv on acetate, which was done in duplicate). Inset: Ergo LC/MS data for acetate samples (ion counts vs. acquisition time).

processing the samples. The bacteria were cultured on solid media to minimize the release of metabolites and were promptly transferred from plates to below freezing solvent to limit changes to the metabolomes due to handling (23). The cells were cultured on medium containing either glucose or acetate as the major carbon source before extraction and LC/MS analysis.

Among the differences that were detected between the two strains was the increased production of ergo in H37Rv Δ *whiB3* cultured on acetate (Fig. 1a). We observed a 7.3-fold increase in ergo in the WhiB3 mutant strain when grown on acetate (Fig. 1a), and the *p* value based on the Student's t-test was 0.0508. In contrast, ergo levels after growth on glucose were similar between the strains (Fig. 1b). Complementation of the knockout strain with *whiB3* decreased ergo content to near wild-type levels (Fig. 1a). These data suggest that the redox-responsive regulator WhiB3 influences the levels of the antioxidant ergo in *Mtb*.

Radioactive TLC Assay Development

We next developed a radioactive labeling assay based on thin-layer chromatography (TLC) to qualitatively and quantitatively measure mycobacterial ergo production to confirm our LC/MS findings. First, we showed that pure ergo has an R_f value of approximately 0.46 (Fig. 2a) when analyzed by TLC using a 3:1 methanol:water solvent system. Concentrated *Msm* and *Mtb* extracts examined with this method each showed a band consistent with the pure ergo band (Fig. 2b). The identity of the band in the mycobacterial extracts was confirmed to be ergo by LC/MS/MRM analysis of the *Msm* band (Fig. 2c-e).

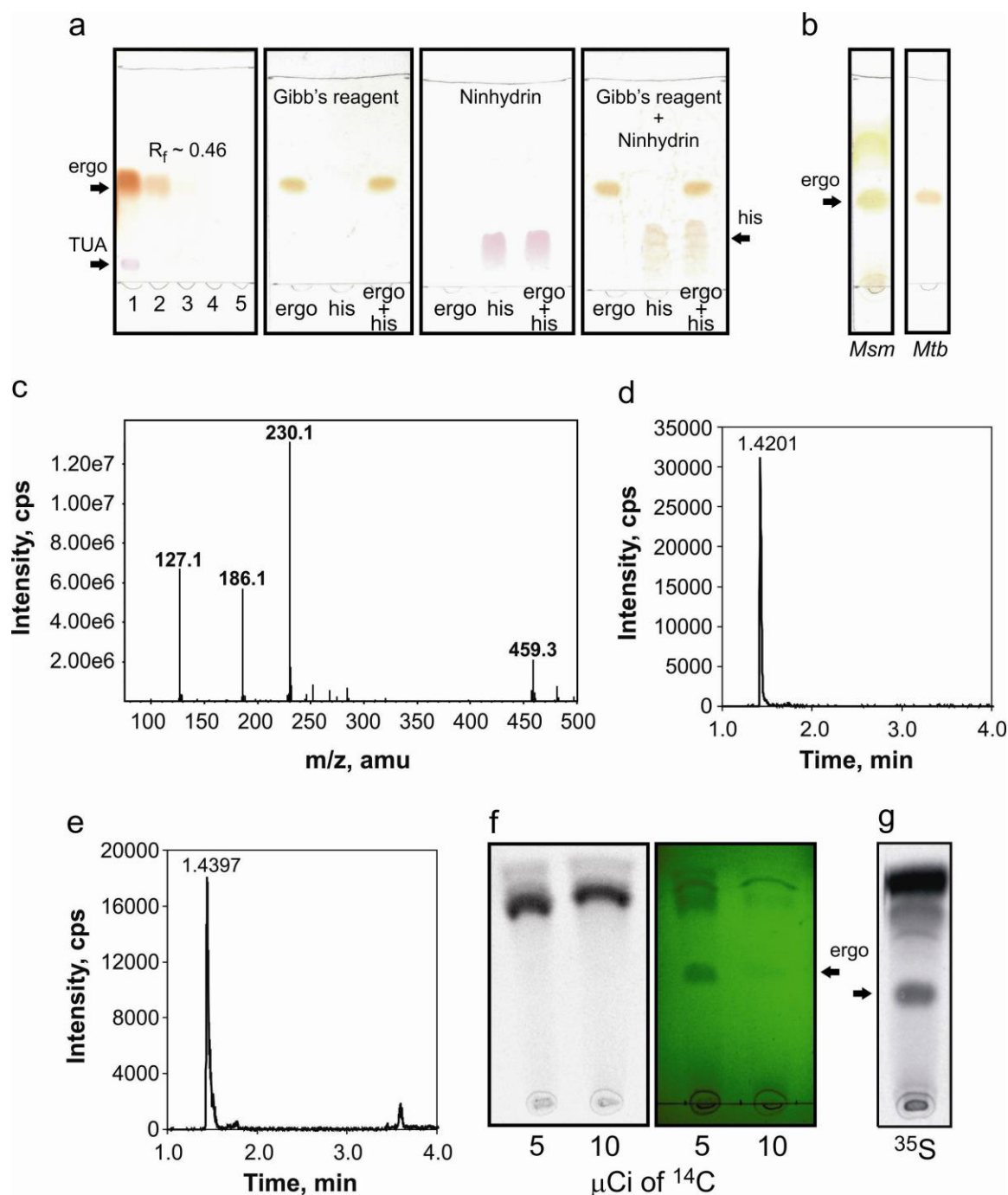


Figure 2. Detection of ergo by TLC analysis and LC/MS/MS. (a) Various concentrations of pure ergo (10 μl) dissolved in water were spotted on a TLC plate and run in 3:1 methanol:water followed by application of Gibb's reagent. Ergo (brick-red spot) runs with an R_f value of ~ 0.46 . Thiolutrocanic acid (TUA) is a breakdown product of ergo. (1) 10 mM, (2) 1 mM, (3) 100 μM , (4) 10 μM , (5) 1 μM . Ergo and histidine (10 μl of 1 mM solutions) were visualized with Gibb's reagent and 0.25% ninhydrin in acetone, respectively (middle plates). Histidine (his) turns violet in the presence of

ninhydrin. Applying both reagents (right) alters the color of his, but it is clear that the compounds have distinct R_f values. **(b)** TLC analysis of *Msm* and *Mtb* extracts followed by application of 0.2% ethanolic Gibb's reagent. Ergo appears as a brick-red spot at R_f values near 0.45. **(c)** The most abundant fragment ions of ergo were identified for use in LC/MS/MS. **(d)** Pure ergo was subjected to LC/MS/MS to determine the elution time of specific ergo fragments. **(e)** The suspected ergo band in the *Msm* TLC was extracted in AMAA and analyzed by LC/MS/MS. Comparison with the pure ergo standard indicates that ergo is present in the *Msm* band. **(f)** *Msm* was grown on plates in the presence of 5 or 10 μCi of $[2-^{14}\text{C}]$ -acetate followed by extraction in water and TLC analysis using 50,000 counts. The TLC plate was exposed to a phosphor storage screen and visualized by phosphorimaging (left) or viewed with a UV lamp at 256 nm (right). **(g)** *Msm* was labeled with ^{35}S -cysteine, extracted in water, and analyzed via TLC followed by phosphorimaging of the TLC plate.

To maximize the sensitivity of the TLC assay, we chose to radioactively label ergo by growing mycobacteria in the presence of labeled ergo precursors. An earlier study on ergo production by the fungus *Claviceps purpurea* showed that $[2-^{14}\text{C}]$ -acetate could be used to label ergo (24). Because it was thought that fungi and mycobacteria have similar ergo biosynthetic pathways, this radioactive precursor was used. However, ^{14}C -labeled ergo was not detected after TLC analysis of extracts from *Msm* cultured in the presence of $[2-^{14}\text{C}]$ -acetate. Inspection of the plate under UV exposure revealed a band at the same position as pure ergo, indicating that ergo is present in the sample but was not radioactively labeled (Fig. 2f). This indicates that there are differences in the biosynthetic pathways of either ergo or ergo precursors between fungi and mycobacteria.

To radioactively label ergo, we next used $[^{35}\text{S}]$ -cysteine as the labeled precursor in the mycobacterial cultures. This isotope was chosen because a 1968 study by Genghoff and Van Damme showed that addition of cysteine to cells grown under low-sulfur conditions results in the production of ergo (25). Accordingly, *Msm* was grown in the presence of $[^{35}\text{S}]$ -cysteine and the extracts were analyzed via TLC. A labeled spot was detected that had the same R_f value as the mycobacterial ergo identified by LC/MS/MS.

analysis (Fig. 2g). Subsequent studies to determine conditions affecting ergo production in mycobacteria were performed using this sensitive TLC assay.

Conditions Affecting Ergo Production in Mycobacteria

WhiB3 regulates core intermediary metabolism in *Mtb*, specifically the switchover to utilization of fatty acids, which led us to ask whether WhiB3 regulation of ergo production is influenced by the type of available carbon source (6). Using the radioactive TLC method, we verified the LC/MS results for growth on the carbohydrate glucose and the fatty acid precursor acetate. We also analyzed ergo levels after growth on the tricarboxylic acid (TCA) cycle intermediate succinate, on which H37Rv Δ *whiB3* has a growth defect, and the immunomodulatory lipid precursor propionate, the detoxification of which involves WhiB3 (3, 6). As expected, ergo levels were not greatly altered in the WhiB3 mutant compared to wild-type following growth on 50 mM, 100 mM, or 200 mM glucose (Fig. 3a). Surprisingly, no difference in ergo levels was observed between the strains on 5 mM acetate using radioactive TLC analysis, contrary to the LC/MS results (Fig. 3b). However, when the acetate concentration was increased to 50 mM or 100 mM, ergo levels in H37Rv Δ *whiB3* increased ~2 to 5 fold compared to H37Rv (Fig. 3b). While increased acetate concentrations led to decreased ergo concentrations in both H37Rv and H37Rv Δ *whiB3*, the decrease was less pronounced in H37Rv Δ *whiB3*. These results demonstrate that increasing the acetate concentration decreases ergo levels, and that WhiB3 influences ergo production when *Mtb* grows on acetate as carbon source.

Growth on increasing concentrations of succinate (5 mM, 50 mM and 100 mM) did not lead to altered levels of ergo in H37Rv or H37Rv Δ *whiB3* (Fig. 3c). In contrast,

growth on increasing levels of propionate resulted in decreasing concentrations of ergo in both H37Rv and H37Rv Δ *whiB3* (Fig. 3d). At 5 mM propionate, H37Rv Δ *whiB3* produced ~30% less ergo than did H37Rv. At 50 mM and 100 mM propionate, ergo levels of wild-type and mutant *Mtb* were decreased to the same degree. Thus, a *whiB3*-specific effect was present at 5 mM propionate (which is the concentration having the greatest production of ergo), but was lost as propionate concentration increased.

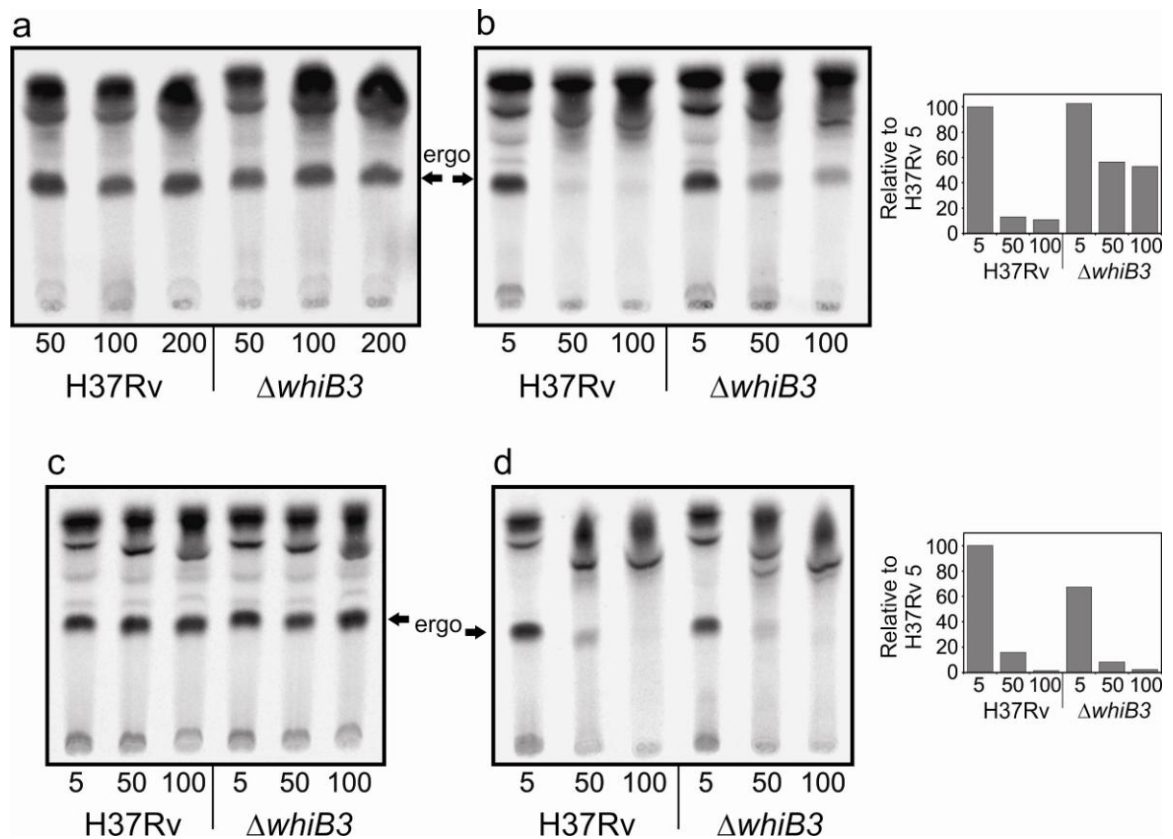


Figure 3. TLC analysis of ^{35}S -labeled H37Rv and H37Rv Δ *whiB3* on different carbon sources. Cells were grown on solid medium containing: (a) 50 mM, 100 mM or 200 mM glucose; (b) 5 mM, 50 mM or 100 mM acetate; (c) 5 mM, 50 mM or 100 mM succinate; and (d) 5 mM, 50 mM or 100 mM propionate. Quantitation (graphs to the right of b and d) was performed with ImageQuant software. For each individual culturing condition, the strains were grown for the same number of days before extraction in AMAA. Experiments were performed two independent times with similar results.

As already demonstrated, WhiB3 senses the redox state of *Mtb* and regulates lipid anabolism accordingly to maintain redox homeostasis (3). Therefore, we sought to determine if the cytoplasmic redox state of *Mtb* affects ergo levels in a WhiB3-dependent manner by measuring ergo levels in H37Rv and H37Rv Δ *whiB3* during oxidative or reductive stress. Addition of 5 mM DMD, a thiol-specific oxidant, to glucose-containing medium did not change ergo levels (Fig. 4). However, this was not the case when 5 mM DTT was used. After growth of H37Rv and H37Rv Δ *whiB3* on medium containing 50 mM glucose and 5 mM DTT, we observed a 4-fold decrease in ergo levels compared to untreated samples. This effect was not dependent on WhiB3, as ergo levels in both strains decreased to the same extent upon DTT exposure (Fig. 4).

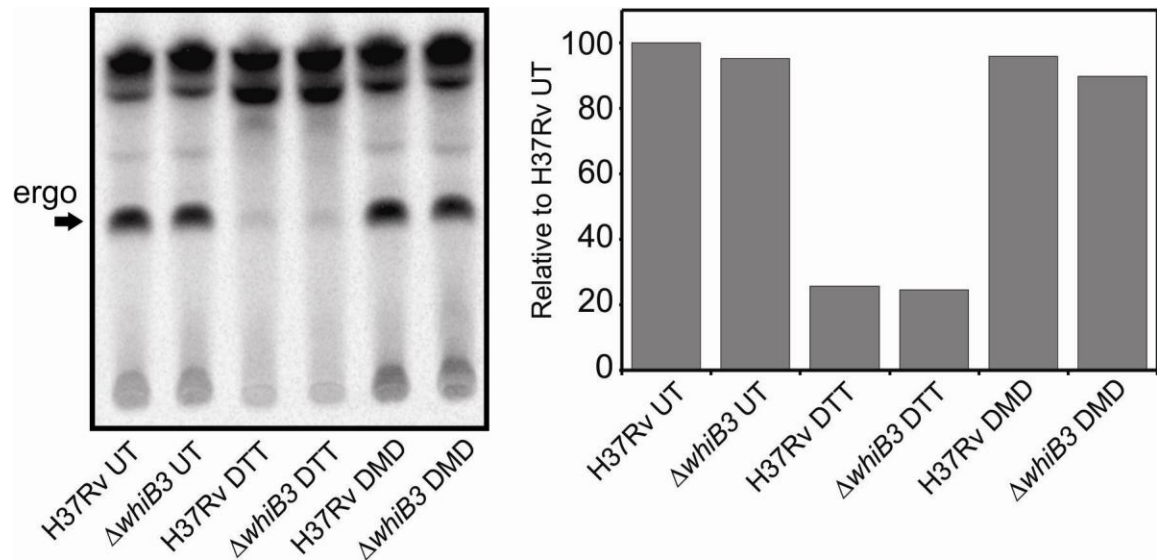


Figure 4. ^{35}S TLC analysis of strains grown in the presence of redox agents. H37Rv and H37Rv Δ *whiB3* were grown on 50 mM glucose-containing solid medium with no additives (untreated, UT) or containing either 5 mM DTT or 5 mM DMD in the presence of ^{35}S -cysteine before extraction in AMAA and TLC analysis. Quantitation of the results is presented as percent of untreated H37Rv. Experiments were performed two independent times with similar results.

Genetic Characterization of Ergo Biosynthesis

In vitro biochemical studies suggest that the following enzymes are involved in the production of ergo: EgtA, a γ -glutamyl cysteine synthetase thought to produce the ergo sulfur donor; EgtB, thought to catalyze oxidative sulfurization of hercynine; EgtC, a glutamine amidotransferase; EgtD, a methyltransferase thought to methylate histidine to produce hercynine; and EgtE, a PLP-binding protein homolog (26). However, genetic evidence is lacking. To test the hypothesis that EgtA and EgtD are involved in ergo biosynthesis, we used two CDC1551 *Mtb* strains with transposons that disrupt either *egtA* or *egtD* (hereafter referred to as *egtA*::Tn and *egtD*::Tn). Ergo was not detected in either of these strains using the radioactive TLC method (Fig. 5a). We successfully complemented the production of ergo in *egtA*::Tn by expressing the whole *egt* gene cluster and in *egtD*::Tn by expressing *egtD* (Fig. 5b).

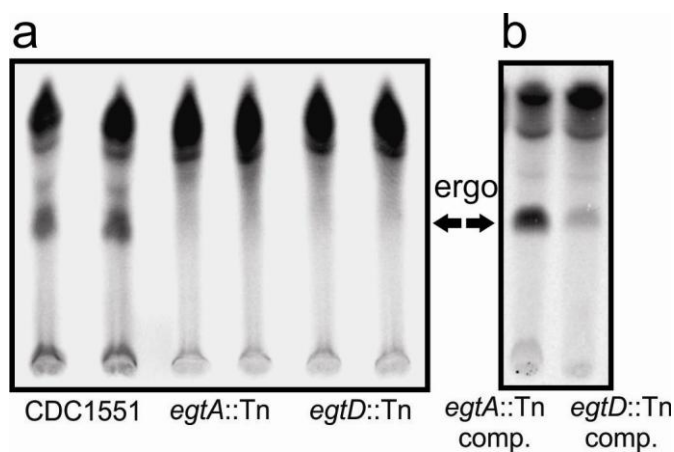


Figure 5. ³⁵S TLC analysis of ergo levels in *egt* mutants and complemented strains. (a) TLC analysis of wild-type CDC1551, *egtA*::Tn and *egtD*::Tn (duplicate samples) indicates that these Tn mutants do not produce ergo. (b) Ergo production in two complemented Tn mutants. *egtA*::Tn was transformed with pMV762-*egtABCDE* and *egtD*::Tn was transformed with pMV762-*egtD*.

Role of Ergo in Protection from Oxidative Stress

A physiological role for ergo in mycobacteria has not yet been established. Based on its antioxidant properties, we investigated the contribution of ergo to the survival of *Mtb* after exposure to oxidative stress. When *egtA::Tn* and *egtD::Tn* *Mtb* strains are exposed to hydrogen peroxide, menadione, or pyrogallol, little difference in zone sizes was noted between wild-type CDC1551 and the *egt* mutants using disk diffusion assays (data not shown). However, both *egtA::Tn* and *egtD::Tn* are more sensitive to cumene hydroperoxide (CHP) than is wild-type CDC1551 (Fig 6). Complementation of ergo production in *egtD::Tn* rescues the growth of the mutant, indicating that ergo can protect *Mtb* from the oxidative stress caused by CHP.

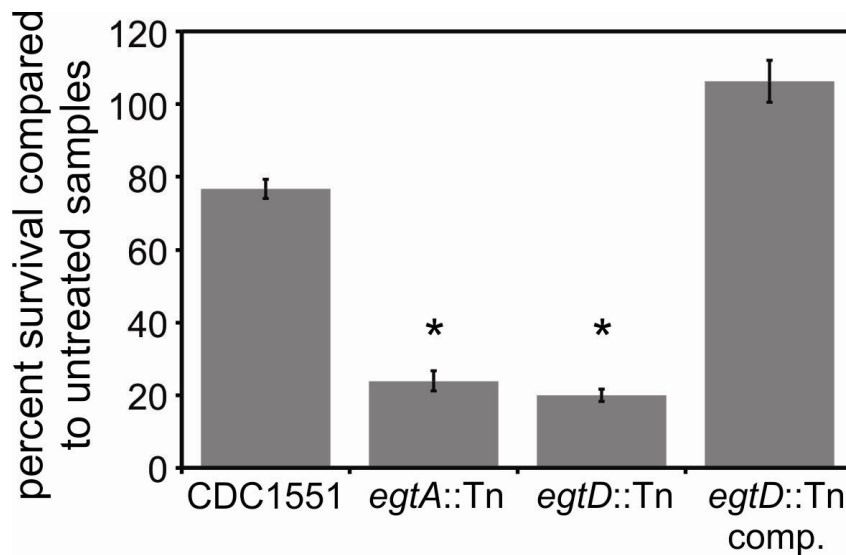


Figure 6. Role of ergo in protection of *Mtb* from CHP stress. alamarBlue analysis of cell viability after treatment with 250 μ M CHP for 24 hours. Shown are the percentage differences between CHP-treated and untreated cells for each strain (mean \pm standard deviation, n=3). Asterisks denote *p* values of <0.001 by Student's *t*-test compared to CDC1551.

Effect of Loss of Redox Stress Protection Mechanisms on Ergo Production

Our results indicate that ergo protects *Mtb* from oxidative stress, and we hypothesized that loss of other redox buffer systems may affect ergo levels. In mycobacteria, mycothiol is the major redox buffer responsible for maintaining the reducing environment of the cell, and SigH is an alternative sigma factor that regulates oxidative stress responses (27-29). Using the ^{35}S TLC assay, slightly lower ergo levels were found in an *Msm* *mshD* mycothiol mutant than in wild-type *Msm* (Fig. 7a).

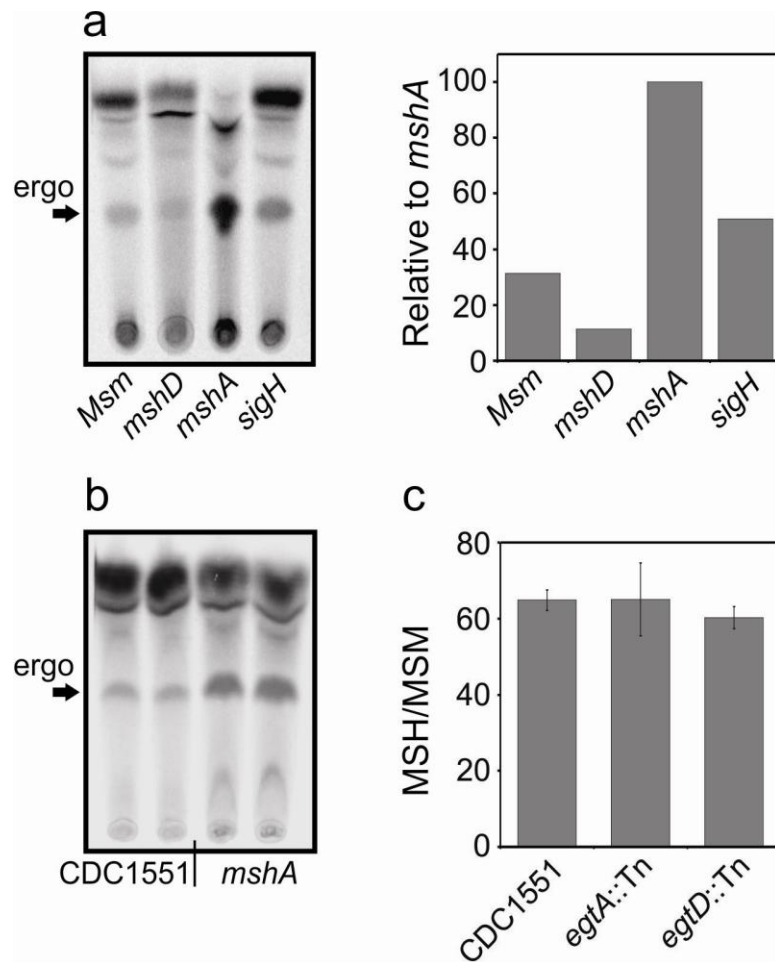


Figure 7. Analysis of thiol levels. (a) ^{35}S TLC analysis of *Msm* strains reveals increased ergo production by the *mshA* and *sigH* mutants. Quantitation (right) was done relative to the ergo levels in the *mshA* mutant. (b) ^{35}S TLC analysis of CDC1551 and its *mshA* mutant strain (duplicate samples) indicates that *Mtb*, like *Msm*, has increased ergo

production in the absence of mycothiol. (c) Ratios of reduced mycothiol to oxidized mycothiol in CDC1551 and the *egt* Tn mutants. Presented are the means \pm standard deviation of three independent cultures. All experiments were performed at least two times with similar results.

However, we saw increased ergo levels compared to wild-type strains in *mshA* mycothiol-deficient mutants of both *Msm* and *Mtb* (Fig. 7a, b). Similarly, a *sigH* mutant of *Msm* had slightly higher levels of ergo than did wild-type *Msm* (Fig. 7a). Thus, complete loss of mycothiol and SigH is associated with increased production of ergo in mycobacteria.

We next determined whether the loss of ergo has any effect on mycothiol levels. HPLC analysis of reduced mycothiol (MSH) and mycothiol disulfide (MSM) levels in CDC1551, *egtA::Tn* and *egtD::Tn* indicated that there were no significant differences in MSH or MSM levels between the strains. Accordingly, the MSH/MSM ratio did not change significantly due to loss of ergo (Fig. 7c).

Discussion

Based on its role as an intracellular redox sensor that regulates lipid metabolism to ensure redox balance (3), we hypothesized that WhiB3 may regulate other factors that are involved in maintaining the redox homeostasis of *Mtb*. Using metabolomic analysis, we have shown production of the antioxidant ergothioneine to be under the control of WhiB3. We developed a novel radioactive TLC assay to assess ergo levels in mycobacteria and showed that the WhiB3-dependent regulation of ergo production occurs when *Mtb* is grown in the presence of immunomodulatory lipid precursors. We also demonstrated that ergo levels decrease under reducing conditions. Additionally, loss

of either the major redox buffer mycothiol or the oxidative stress response regulator SigH led to increased levels of ergo. Importantly, we showed that ergo protects *Mtb* from oxidative stress caused by CHP. Notably, there is evidence that ergo is clinically relevant to TB disease. A 1951 study reported that decreased ergo levels were associated with the severity of TB in Indian/Eskimo patients (30). More recently, TB susceptibility was shown to be associated with single-nucleotide polymorphisms (SNPs) in *SLC22A4*, which encodes an ergo transporter in humans (31). These studies indicate a potential role for ergo in the pathogenesis of *Mtb*.

Metabolomics is a powerful tool to assess how cells react to specific physiological conditions. An interesting finding from the metabolomic analysis of H37Rv and H37Rv Δ *whiB3* was the carbon source-dependent increased production of ergothioneine by the mutant. Ergo is a low-molecular-weight (LMW) thiol with antioxidant properties that has been shown to protect eukaryotic cells and tissues from oxidative stress (19, 32, 33). It has been known since the 1960s that many actinomycetes, including mycobacteria, produce ergo, but very few studies have been done in bacteria and its function in mycobacteria is still unknown (15, 34). The link between WhiB3 and ergo is noteworthy considering the importance of WhiB3 in maintaining redox homeostasis in *Mtb*, and regulation of ergo may be another means through which WhiB3 ensures redox balance.

The carbons of fungal ergo are derived from two sources: histidine, which is assimilated into ergo with no rearrangements, and *S*-adenosyl methionine (SAM), which contributes the methyl groups (35). Carbons from both these sources are radiolabeled in ergo when the fungus *C. purpurea* is grown in the presence of [2-¹⁴C]-acetate (24). However, this is not the case in mycobacteria; we observed no radiolabeled ergo after

culturing *Msm* in the presence of [2-¹⁴C]-acetate. One possible explanation for this difference is that SAM is not the source of the ergo methyl groups in mycobacteria. It has been shown that SAM can be used as a methyl group donor by EgtD to produce ergo *in vitro*, but no other substrates were tested and it may be that some other donor may be preferred over SAM *in vivo* (26). Another possibility is that differences in the synthesis of histidine and methionine may exist between mycobacteria and fungi such that the 2-carbon of acetate is not incorporated into these amino acids in mycobacteria as it is in fungi (24).

Under physiological conditions, ergo is predominantly a thione, as opposed to thiol, and is more difficult to oxidize than typical LMW thiols (36-38). These properties may make ergo more useful under conditions of oxidative vs. reductive stress, which would explain the loss of ergo production in the presence of the reducing agent DTT, but the continued production of ergo when the oxidant DMD is present. Furthermore, the altered ergo production by H37RvΔ*whiB3* may be explained by the changes in its redox environment due to the loss of *whiB3*. H37RvΔ*whiB3* grows better than wild-type on acetate media, reflected by an increased NAD⁺/NADH ratio in the mutant (3, 6). The increased ratio indicates that there is a more oxidizing environment in H37RvΔ*whiB3*, which is in accordance with the increased production of ergo by this strain on higher concentrations of acetate. In contrast, when the strains are grown on propionate media, we see the opposite result wherein H37RvΔ*whiB3* produces less ergo than wild-type. This finding can be explained by the fact that the *whiB3* mutant assimilates propionate into methyl-branched lipids differently than does wild-type, which results in a more reducing environment in the mutant in the presence of excess propionate (3).

Mycothiol and SigH are major components of mycobacterial stress protection (4, 28, 29); therefore, we determined the levels of ergo in *mshA*, *mshD*, and *sigH* mutants of *Msm* and an *mshA* mutant of *Mtb*. We found that ergo levels are increased in strains that completely lack mycothiol (*mshA* mutants of *Msm* and *Mtb*) but not in a strain that produces some mycothiol and compensatory thiols (*mshD* mutant of *Msm*) (39-41). These findings indicate that ergo production is linked to the amount of mycothiol present, and are in agreement with a previous study (42). The *Msm* strain deficient for SigH also produced noticeably more ergo than wild-type, suggesting that ergo production increases to compensate for the loss of other redox stress defense systems.

A significant finding is the ability of ergo to protect cells from oxidative stress caused by CHP. This result affirms what has been proposed, that ergo is used as a means to resist redox stress (43). To our knowledge, this is the first report using an ergo-deficient mutant to assess whether ergo can protect against oxidative stress. Previously, Ta *et al* showed that protection from CHP in *Msm* is mediated by Ohr (organic hydroperoxide resistance protein) (42). *Mtb* does not produce Ohr, and our data indicate that, in this slow-growing strain, ergo is involved in protection from CHP stress.

In summary, we have shown that WhiB3 regulates ergo production during growth on immunomodulatory lipid precursors, likely due to the effect of the loss of WhiB3 on the intracellular *Mtb* redox environment under these conditions. Our data indicate that a more reducing environment is associated with lower levels of ergo, and that loss of redox stress defense mechanisms (mycothiol or SigH) increases ergo levels. Additionally, viability assays reveal that ergo protects *Mtb* from CHP oxidative stress. Overall, our results indicate that ergo production is influenced by the redox environment, and that

ergo is likely produced when it can most effectively contribute to defense against oxidative stress. Redox homeostasis likely plays an important role in *Mtb* persistence due to the link between cellular metabolism and persistent infections; thus, understanding how *Mtb* maintains redox balance may have implications for the development of chemotherapies that target latent infection.

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DISCUSSION AND CONCLUSIONS

Despite the fact that ergothioneine was discovered over 50 years ago in *Mtb*, very little is currently known about what role this LMW thiol plays in mycobacteria (56). Early studies looked at the levels of ergo produced by various actinomycetes, and explored the biosynthetic pathway in mycobacteria using radioactive precursors (57, 58). Our metabolomic discovery that WhiB3 regulates ergo levels was the impetus for us to investigate conditions that affect ergo production in mycobacteria as a step toward understanding the physiological function of this molecule. Importantly, the use of ergo-deficient mutants of *Mtb* allowed us to assess the contribution of ergo to oxidative stress protection.

Because of its primary host niche, *Mtb* requires protection from redox stress to survive and establish infection. It is therefore important to understand the mechanisms utilized by the bacterium to subsist in the harsh conditions in which it resides. Though *Mtb* encounters numerous environments over the course of infection, its initial and main residence is the alveolar macrophage, which phagocytizes *Mtb* once it enters the lung. It has been shown that *Mtb* can prevent fusion of phagosomes and lysosomes, allowing it to replicate in the relatively permissive environment of the phagosome (117), or even escape to the cytosol (118, 119). However, *Mtb* cannot stop phagolysosomal fusion in activated macrophages, and in these cells it encounters numerous stresses, including oxidative, nitrosative, acid and nutritional stress (117).

Among the methods *Mtb* employs to protect itself from redox stress is the use of the LMW thiol mycothiol as an antioxidant (7). Our results suggest that ergo performs a similar function. *In vitro* and *in vivo* studies on ergo activity, which show that it can scavenge certain free radicals of oxygen and nitrogen, indicate that ergo has antioxidant capabilities (76-81). Additionally, our study suggests that ergo production is influenced by the redox environment; more specifically, that it is produced when the environment is more oxidizing. Thus, ergo may be produced when the redox balance is shifted toward an oxidized state, as part of the *Mtb* oxidative stress response system. Furthermore, we show that ergo levels are increased in the absence of mycothiol and SigH, which are both used by mycobacteria to resist redox stress (7, 120, 121). Finally, ergo-deficient mutants are more susceptible to the oxidant CHP. This study supports a role for ergo in the defense against oxidative stress and, therefore, in the maintenance of redox homeostasis.

Ergo Levels are Affected by the Carbon Source and Presence of WhiB3

The carbon source utilized by *Mtb* affects the intracellular redox environment of the bacterium because complete oxidation of the carbon source is accomplished through a set of reactions which, depending on the type of carbon substrate and the number of subunits it comprises, result in the production of varying amounts of reducing equivalents. For example, one molecule of glucose is catabolized to two molecules of pyruvate via glycolysis with the production of two molecules of NADH. Pyruvate can then be decarboxylated to acetyl-CoA (producing one NADH), which can enter the TCA cycle for further oxidation to two molecules of carbon dioxide with the production of three molecules of NADH and one molecule of FADH₂. Fatty acids are metabolized to

acetate and/or propionate via β -oxidation. The more carbons that make up the fatty acid, the more rounds of β -oxidation are needed for complete breakdown. Each round of β -oxidation produces one NADH and one FADH₂; thus, the longer the fatty acid chain, the more reducing equivalents are generated (122).

Mtb is capable of metabolizing various types of carbon sources to produce energy and anabolic substrates, which is a reflection of the diverse environments it is exposed to over the course of infection. It is prototrophic, meaning that once *Mtb* has oxidized the available carbon source, it can use the resulting catabolites to synthesize all compounds required for growth, including amino acids, vitamins, nucleic acids, etc. (123). As is to be expected based on its diverse metabolic capacity, *Mtb* contains all the major carbon utilization pathways, with the exception of the Entner-Doudoroff pathway (123, 124). Research indicates that *Mtb* metabolizes carbohydrates during the early stages of infection (125), and at later stages uses fatty acids as the major carbon source. This is known because *Mtb* recovered from mice after approximately 3 weeks of infection can metabolize fatty acids, but not carbohydrates (126). Furthermore, *Mtb* Icl1 and Icl2, which are required for assimilation of acetate and propionate (the two possible products of β -oxidation) are necessary for *in vivo* survival and virulence (127, 128). Mirroring the situation *in vivo*, *Mtb* likely also uses FAs as a carbon source during growth in macrophages, suggested by the upregulation of genes associated with FA metabolism in macrophage-cultured *Mtb* (129).

Because *Mtb* subsists on FAs *in vivo*, it must have mechanisms available to deal with the influx of reducing equivalents due to β -oxidation. Lipid anabolism is one such mechanism employed by the bacilli (113). *Mtb* contains both the FAS (fatty acid

synthase) I and FAS II systems of FA biosynthesis, and both use NADH and NADPH as cofactors. One cycle of each pathway adds a two-carbon unit with the consumption of one NADH and one NADPH. Thus, the synthesis of long chain fatty acids expends many reducing equivalents. *Mtb* FAs may contain as many as 90 carbons, making lipid anabolism an efficient redox sink for ridding the cell of excess reducing equivalents (130). An important precursor to lipid anabolism in *Mtb* is propionate, which is incorporated into methyl-branched lipids such as the virulence factor phthiocerol dimycocerosate (PDIM) (131).

Lipid anabolism in *Mtb* is regulated in part by WhiB3, which makes WhiB3 a significant contributor to the maintenance of redox homeostasis in *Mtb*. It has been shown that *Mtb* cultured in macrophages produces more methyl-branched lipids than *Mtb* cultured *in vitro* (113). This is probably due to the presence of excess propionate from the β -oxidation of host FAs, since it was previously established that the addition of propionate to *Mtb* cultures results in the increased production of the methyl-branched lipids sulfolipid-1 (SL-1) and PDIM (131). The increased production of lipids by *Mtb* cultured in macrophages correlates to a higher NADP⁺/NADPH ratio of macrophage-grown *Mtb* than *in vitro*-grown *Mtb*. The *Mtb whiB3* mutant exhibited altered production of methyl-branched lipids when cultured in macrophages, which correlated to a lower NADP⁺/NADPH ratio than wild-type (113). Thus, the differential assimilation of propionate into methyl-branched lipids by H37Rv and H37Rv $\Delta whiB3$ was associated with more efficient recycling of NADPH by H37Rv.

In addition to regulating lipid anabolism, WhiB3 has also been implicated as a regulator of core intermediary metabolism because an *Mtb whiB3* mutant has different

growth profiles compared to wild-type on various carbon sources (99). It was previously shown that H37Rv Δ *whiB3* has a slightly higher NAD⁺/NADH ratio than wild-type after growth *in vitro* in 5 mM acetate media (113). Also, the mutant is better able to metabolize acetate as evinced by better growth on acetate media compared to wild-type (99). These results suggest that there is a positive correlation between acetate metabolism and NADH recycling, and that the redox environment of the mutant will be more oxidized than that of the wild-type during growth on acetate.

Ergo production decreases when the cells experience a more reducing environment, as indicated by the decrease in ergo levels due to the addition of DTT in both H37Rv and H37Rv Δ *whiB3*. We therefore expect that the altered cytoplasmic redox environment of the *whiB3* mutant, which has a higher or lower NAD(P)⁺/NAD(P)H ratio than wild-type depending on the available carbon source, will lead to different ergo levels in the mutant than in wild-type. This is indeed what we observed; the strain with the more reducing environment based on the carbon source had lower ergo levels. The mutant produced less ergo on 5 mM propionate, while wild-type produced less ergo on higher concentrations of acetate. We have thus identified WhiB3 as a regulator of ergo production, likely due to WhiB3's role in the maintenance of redox homeostasis.

Ergo Levels are Affected by the Loss of Certain Redox Stress Defense Mechanisms

As the major redox buffer in mycobacteria, mycothiol has an important role in maintaining a reducing cytoplasmic environment and protecting the bacilli from redox stress, alkylating agents and antibiotics. For example, strains with mutations in various enzymes of the MSH biosynthetic pathway are more susceptible to oxidative, reductive

and nitrosative stress (29, 44). SigH is a regulator of heat and oxidative stress response systems in *Mtb*, including TrxB2 (TrxR) and TrxC (121). A *sigH* mutant is more susceptible to diamide and plumbagin, a superoxide generator (121). Our results showing that ergo levels are increased in mycothiol- and SigH-deficient *Msm* strains are not unexpected given the antioxidant functions of ergo. In the absence of these protective systems, ergo may play a compensatory role.

Besides being increased to make up for the loss of mycothiol, an alternate explanation for the greater production of ergo in the *mshA* mutants may be the altered redox environment of these strains. It has previously been shown that various H37Rv *mshA* missense, nonsense or frameshift mutants had higher NAD⁺/NADH ratios than wild-type (37). If this holds true for the *Msm* and CDC1551 *mshA* mutants, the situation may be as discussed above wherein the more oxidizing environments of the *mshA* mutants correlate to higher ergo levels in these strains than in wild-type.

Ergo Levels may be Affected by Anion Imbalance

An altered redox environment due to the loss of *whiB3* is one factor that affected ergo levels in our study. Another condition we observed that caused changes in the ergo levels of our strains was growth on high levels of fatty acid precursors. As we increased the concentration of acetate or propionate, ergo levels decreased in both strains. However, H37Rv Δ *whiB3* produced more ergo than wild-type even at the highest level of acetate tested.

The decreased ergo production due to increasing concentrations of FA precursors may not have been related to the redox environment, but rather to anion imbalance

caused by an influx of weak acids and the subsequent depletion of ergo precursors to correct the imbalance. Addition of weak acids such as acetate or propionate to *E. coli* results in an increase in the amount of anions in the cell, and this leads to the loss of other anions to achieve equilibrium. In *E. coli*, the pool of negatively charged amino acids, especially glutamate, is decreased when the cells are grown in the presence of acetate (132). However, this does not completely compensate for the increase in acetate anions and the amounts of other, unidentified anions must also be lowered. It has also been shown that the addition of acetate or propionate to *E. coli* results in depleted methionine levels, and the authors hypothesized that this could be due to a decrease in some anion(s) involved in methionine biosynthesis (133).

If growth of *Mtb* in acetate or propionate leads to decreased glutamate levels to achieve ionic balance, the production of ergo would be affected because this metabolite is likely involved in ergo biosynthesis. Glutamate is a component of γ -glutamyl cysteine, which is the product of the reaction catalyzed by EgtA and is the probable source of sulfur for ergo production (69). Loss of methionine may also contribute to decreased ergo levels because this amino acid is necessary for the production of *S*-adenosyl methionine (SAM), which is a potential methyl donor for EgtD (69). Ergo levels are higher in H37Rv Δ *whiB3* than in wild-type at increasing concentrations of acetate, and this may be due to the fact that the mutant metabolizes acetate better than wild-type. That is, increased metabolism of acetate correlates to fewer acetate anions present in the cell, so less of the other anions would be depleted to compensate for acetate. Thus, H37Rv Δ *whiB3* would have higher levels of ergo precursors, such as glutamate and methionine, allowing it to produce more ergo.

There is a precedent for decreasing levels of a low-molecular-weight thiol upon addition of acetate to media. Treatment of *E. coli* with 50 mM acetate reduces glutathione levels two-fold (134). Glutamate is a precursor of glutathione, so this could be due to the decreased levels of glutamate in acetate-exposed *E. coli* as discussed above. Another possible explanation is that since glutathione is in the anion form at physiological pH, its levels may be decreased to compensate for the influx of acetate anions (134). Alternatively, acetate treatment can reduce the pH of the cytoplasm and this may inhibit the activities of γ -glutamyl cysteine synthetase and GSH-synthetase, the two enzymes required for glutathione biosynthesis (135). This could also explain the decreased levels of ergo we see at increasing concentrations of acetate since γ -glutamyl cysteine synthetase (EgtA) is likely required for ergo biosynthesis (69).

Summary

We discovered that ergothioneine is a WhiB3-regulated component of the oxidative stress response system in *Mtb*. It has been shown that WhiB3 is involved in maintaining the redox homeostasis of *Mtb*, and the data presented here indicate that WhiB3 also has a role in protection from redox stress through regulation of ergo levels in response to carbon source availability. Based on this work, it is clear that the regulation of ergo production in mycobacteria is complex and influenced by multiple factors. The production of ergo by *Mtb* is in itself very interesting; eukaryotic and microbial systems typically only produce one major redox buffer. Perhaps ergo is an auxiliary mechanism that has evolved in *Mtb* due to the unusual life cycle of this bacterium, wherein it persists in human tissues for decades, under attack by both endogenous and exogenous reactive

species, while retaining the ability to cause disease. Future work should focus on verification of the *egt* genes in ergo biosynthesis and further characterization of the role of ergo in *Mtb*, including determination of the virulence of an ergo-deficient mutant.

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