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Comparative analysis of mutants in the mycothiol biosynthesis pathway in *Mycobacterium smegmatis*

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Abstract

The role of mycothiol in mycobacteria was examined by comparative analysis of mutants disrupted in the four known genes encoding the protein machinery needed for mycothiol biosynthesis. These mutants were sensitive to acid stress, antibiotic stress, alkylating stress, and oxidative stress indicating that mycothiol and mycothiol-dependent enzymes protect the mycobacterial cell against attack from various different types of stresses and toxic agents.

Keywords: Mycothiol; Mycothiol deacetylase; Mycothiol ligase; Mycothiol synthase; Oxidative stress; Toxins; Xenobiotics

Mycobacteria, like most other Gram-positive bacteria do not make glutathione but produce another low molecular weight thiol, mycothiol (MSH) (Fig. 1), 1-D-myoinosityl-2-(*n*-acetyl-L-cysteinyl)-amido-2-deoxy-α-D-glucopyranoside. Since MSH is unique to actinomycetales [1], enzymes involved in MSH biosynthesis and metabolism are potential targets for drugs directed against pathogenic mycobacteria like *Mycobacterium tuberculosis*.

Mycothiol biosynthesis proceeds through a five-step pathway [2]. The initial substrates, 1L-inositol-1-phosphate and UDP-*N*-acetylglucosamine react to form *N*-acetylglucosaminylinositol phosphate, a reaction catalyzed by the *N*-acetylglucosamine transferase, MshA [3,4]. An unidentified phosphatase dephosphorylates this molecule to yield *N*-acetylglucosaminylinositol [4], which is deacetylated by an MshB deacetylase [5,6]. The resulting glucosaminylinositol (GI) is ligated with a cysteine in a reaction catalyzed by a ligase, MshC [7]. The cysteinylglucosaminylinositol (CGI) is then acetylated to form mycothiol in a reaction catalyzed by MshD acetyltransferase [8].

We have previously reported that *Mycobacterium smegmatis* mutants disrupted in the four known genes [3,9–11] involved in mycothiol biosynthesis are resistant to isoniazid, a front-line drug used in the treatment of tuberculosis. We have also reported that *M. smegmatis* mutants lacking mycothiol ligase activity and thus mycothiol are sensitive to a wide range of antibiotics, alkylating agents, and oxidative stress [11]. Lastly, we have demonstrated that a direct depletion of mycothiol pool occurs upon exposure to oxidative stress [12] and nitrosative stress [13]. This study further examines the role of mycothiol in mycobacteria by comparative analysis of mutants disrupted in four of the known genes involved in mycothiol biosynthesis.

Materials and methods

Growth and culture conditions. Mycobacterium smegmatis mc^2155 was the parent wild-type strain used for construction of the knockout mutants and transposon mutants and thus served as the control for the sensitivity studies. Initially, *M. smegmatis* and the mutants, A1 (Δ*mshA*), Myco504 (Δ*mshB*), I64 (Δ*mshC*), and *mshD*::Tn5 (Δ*mshD*) disrupted in genes involved in mycothiol biosynthesis were grown in Middlebrook 7H9 broth (DIFCO) with 0.05% Tween and OADC supplement. Media for mutants, Δ*mshA*, Δ*mshB*, and Δ*mshD*, were also supplemented with 25 μg ml^{-1} kanamycin. After the cultures were in log phase, wild-type and the

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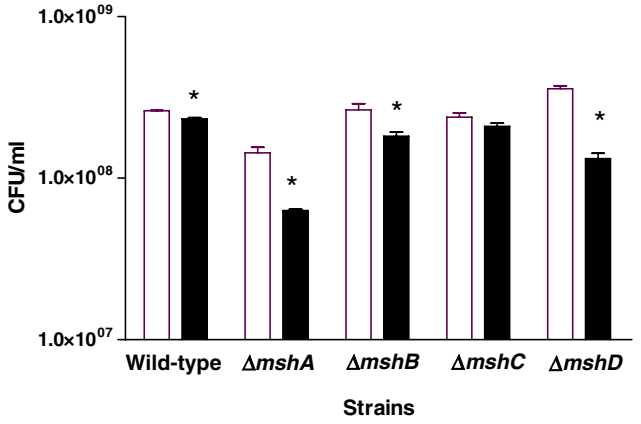

Fig. 1. Viability of MSH mutants under acidic stress; blank bars: the number of colony forming units per ml (cfu/ml) for untreated samples, filled bars: the number of cfu/ml for samples incubated in acidified media for 2 h, $^{*}P \leqslant 0.005$ using Student's *t*-test. Strains were grown to mid-log phase in Middlebrook 7H9 liquid media supplemented with 0.05% Tween and 1% glucose at 37 °C. One set of cultures in quadruplicates was diluted to 0.5 OD_{600} in normal media and another set, also in quadruplicates, was diluted in media acidified to pH 4.0 by adding 20 mM HCl. After incubation for 2 h at 37 °C, cfus were determined on 7H10 1% glucose agar.

mutants were diluted and transferred to Middlebrook 7H9 broth with 0.05% Tween containing 1% glucose as the carbon source. When these diluted cultures reached mid-log phase, the cultures were diluted to 0.5 optical density (600) for sensitivity assays.

MSH determination. Derivitization of cell extracts with monobromobimane (mBBr) and HPLC analysis of the derivitized samples to determine the mycothiol content was performed essentially as described earlier [14]. Control samples, treated with *N*-ethylmalemide and then with mBBr, were also analyzed. Results are reported as μmol per gram of residual dry weight of the pellet obtained from the 50% acetonitrile–water extraction or as nmol per OD (600).

Sensitivity assays. Sensitivity was assessed by a number of different assays. First, *E*-test strips (AB Biodisk) were used according to the manufacturer's directions to determine the minimal inhibitory concentration (MIC) of the different mycobacterial strains for the antibiotics, rifampin, erythromycin, azithromycin, and vancomycin. Second, disk diffusion assays were performed for various antibiotics, oxidants, reductants, and toxins according to Rawat et al. [11]. Lastly, cell viability assays were performed by dividing cultures diluted to 0.5 OD_{600} into two sets; one set was left untreated and the second set was treated with various stresses for 2 h at 37 °C. Colony forming units (cfus) were determined on 7H10 1% glucose agar and MSH content was analyzed. These assays were performed with either five or four replicates.

Statistical analysis. Student's *t*-test was applied to statistically evaluate the results and *P* values were calculated to show significant differences.

Results and discussion

Mycothiol and mycothiol precursors levels in Mycobacterium smegmatis

In *M. smegmatis*, a nonpathogenic surrogate of *M. tuberculosis*, mutants disrupted in *mshA*, *mshB*, *mshC*, and *mshD*, have been isolated (Table 1); the gene(s) coding for the enzyme catalyzing the phosphatase activity, MshA2, has not been identified. The four biosynthetic pathway mutants differ in their mycothiol content ranging from <0.1% of the wild-type mycothiol level in Δ*mshA* strains [3,9], A1 and 49, to 5–10% of the wild-type mycothiol level in Δ*mshB* [10]. *M. tuberculosis* mutants disrupted in *mshB* [15] and *mshD* [16] have also been constructed and characterized. However, attempts to construct mutants disrupted in *mshA* [17] and *mshC* [18] by homologous recombination were unsuccessful until a second copy of the genes was inserted into the *M. tuberculosis* genome suggesting that these genes are essential in *M. tuberculosis,* although recently an *M. tuberculosis mshA* mutant was generated using allelic exchange (W.R. Jacobs, personal communication). Interestingly, the *M. smegmatis mshD* mutant produces two novel thiols, *N*-formyl CGI and *N*-succinyl-CGI [16,19].

Antibiotic sensitivity

In this study, we examined mutants disrupted in *mshA* (A1), *mshB* (myco504), and in *mshC* (I64), in *mshD* (*mshD*::Tn5) for sensitivity to a wide variety of drugs that are used in the treatment of tuberculosis as well as other drugs that are known to be ineffective against tuberculosis. We had already demonstrated that all *M. smegmatis* mutants disrupted in mycothiol are sensitive to streptomycin [20]. Also, in our previous work with the Δ*mshB*, we had reported that we did not see any difference in sensitivity to the antibiotics rifampin, vancomycin, and erythromycin based on disk assays [10]. In this report, we determined antibiotic sensitivity using *E* tests, which proved to be more sensitive determinant of susceptibility (Table 2). For the antibiotic rifampin, Δ*mshA* proved to be most sensitive, Δ*mshD* intermediate in its sensitivity, and Δ*mshB* and Δ*mshC* similar to wild-type. In contrast, Δ*mshA* is least sensitive and Δ*mshD* most sensitive to van-

Table 1
Definition, annotation and mycothiol levels in *M. smegmatis* mutants in the mycothiol biosynthetic pathway

Enzyme	Gene	ORF (*M. tuberculosis*)	ORF (*M. smegmatis*)	*M. smegmatis* mutant strain	Type of mutant	Relative mycothiol content (%)
Mycothiol glycosyl transferase	*mshA*	Rv0486	MSMEG0924	A1	Transposon	<0.1
				49	Chemically induced	<0.1
Phosphatase	*mshA2*	—	—	—	—	—
Mycothiol deacetylase	*mshB*	Rv1170	MSMEG5117	Myco504	Homologous recombination	5–10
Mycothiol ligase	*mshC*	Rv2130c	MSMEG4193	I64	Chemically induced	1–5
Mycothiol synthase	*mshD*	Rv0819	MSMEG5754	*mshD*::Tn5	Transposon	0.65–1.2

Table 2
Sensitivity of mycothiol deficient mutants to antibiotics as determined by *E*-test

Cell strain	Minimum inhibitory concentration (μg)			
	Rifampin	Erythromycin	Azithromycin	Vancomycin
mc^{2}155 (wild-type)	30–32	24–16	6–4	6–12
Δ*mshA*	3	0.5–1.5	0.5–1.0	6
Δ*mshB*	32	0.5–1.5	0.38–0.5	4
Δ*mshC*	32	0.75–2.0	0.75–1.0	1.9–6
Δ*mshD*	8	0.38–1.5	0.38–0.50	1

comycin. The *E*-test values for erythromycin and azithromycin demonstrate that all mutants are sensitive to these two antibiotics as compared to the wild-type strain (Table 2). The differences in sensitivity to rifampin, vancomycin, erythromycin, and azithromycin between different mutants suggest that different mechanisms involving different mycothiol-dependent enzymes may be at work. In *Amycolatopsis mediterraei* U32, a mutant disrupted in a gene with high sequence similarity to *mshD* was 2- to 3-fold more sensitive to apramycin and erythromycin, which is consistent with our results [21].

Previously, Δ*mshA* [3] and Δ*mshD* [8] were isolated by screening for mutants resistant to isoniazid, a pro-drug that is activated by KatG [22]. Another pro-drug that needs to be activated, albeit by a different enzyme, is ethioniamide [23]. We had previously reported that Δ*mshA* and Δ*mshC* are resistant to ethionamide while there is no difference in the sensitivity of Δ*mshB* as compared to wild-type [10]. We show that Δ*mshD* is also resistant to ethioniamide, showing no clearing on disks assays with 50 μg ethioniamide confirming that mycothiol plays a role in the activation of ethioniamide (Table 3). In line with earlier studies, wild-type *M. smegmatis* does not show any zone of clearings with fosfomycin (100 μg), pyrazinamide (100 μg), cycloserine (100 μg), penicillin (200 μg), and sulfonamide (25 μg), and thus is resistant to these agents as are mutants disrupted in mycothiol.

Sensitivity to alkylating agents

We have reported the construction and characterization of a mutant in the *mca* gene lacking mycothiol amidase hydrolase, a mycothiol-dependent detoxification enzyme [20]. Like GSH, MSH reacts with alkylating, electrophilic toxins and forms conjugates which are substrates for mycothiol amidase. Mycothiol amidase catalyzes the cleavage of an amide bond resulting in a mercapturic acid that is excreted [20,24,25]. Disk assays were performed in order to determine the sensitivity of mutant disrupted in genes involved in mycothiol biosynthesis to the alkylating agent, iodoacetamide, the GS-transferase substrate, chlorodinitrobenzene (CDNB), *N*-ethylmalemide, and the thiol derivitizing agent, mBBr. In Table 3, it can be seen that all mutants are more sensitive to iodoacetamide than wild-type with the exception of Δ*mshB,* which was also previously noted [10]. In addition, all mutants are also more sensitive to CDNB than wild-type. In contrast, although mBBr forms conjugates with MSH, which are substrates for mycothiol amidase [24,25], there was no difference in the sensitivity to mBBr. This lack of sensitivity to mBBr is also exhibited by Δ*mca* [20].

Table 3
Sensitivity of mutants lacking mycothiol to toxins, antibiotics, oxidants and reductants as determined by disk assays

	Zone of clearing (mm) of various strains				
	Wild-type	Δ*mshA*	Δ*mshB*	Δ*mshC*	Δ*mshD*
Toxins (μmol)					
Iodoacetamide (0.05)	11.6 ± 0.4	20.2 ± 0.4**	12.4 ± 0.2	16.6 ± 0.7**	14.2 ± 0.4**
NEM (0.05)	12.6 ± 0.5	17.2 ± 0.4**	20.8 ± 0.5**	19.4 ± 0.6**	19.8 ± 2.1**
MBBR (0.5)	35.4 ± 1.2	34.8 ± 1.8	37.8 ± 0.8	38.0 ± 1.7	36.8 ± 2.9
CDNB (0.2)	15.0 ± 0.4	32.0 ± 0.8**	34.0 ± 0.8**	19.0 ± 0.7**	28.0 ± 0.8**
Methylglyoxal (1.0)	18.8 ± 0.2	21.6 ± 2.1	18.8 ± 0.4	19.8 ± 0.5	25.6 ± 1.2**
Antibiotics (μg)					
Isoniazid (10)	28.4 ± 0.7	Resistant	24.2 ± 1.1*	20.2 ± 1.5**	Resistant
Ethioniamide (50)	34.5 ± 0.0	Resistant	Resistant	33.5 ±1.0	Resistant
Oxidants (μmol)					
H_2O_2 (1.0)	21.8 ± 0.5	26.6 ± 0.5**	26.6 ± 0.7**	23.8 ± 0.5*	37.8 ± 0.6**
Menadione (0.3)	11.4 ± 0.5	23.8 ± 0.8**	14.2 ± 0.5**	40.0 ± 1.8**	23.0 ± 0.9**
Plumbagin (0.02)	22.4 ± 0.8	43.6 ± 1.6**	34.4 ± 1.2**	42.0 ± 1.1**	28.8 ± 0.5**
Cumene hydroperoxide (0.1)	12.0 ± 0.4	10.8 ± 0.2	12.2 ± 0.2	11.6 ± 0.2	12.0 ± 0
t-Butyl hydrogen peroxide (5)	21.5 ± 1.0	39.5 ± 2.1**	34.5 ± 1.0**	24.2 ± 0.9*	46.8 ± 1.5**
Diamide (15)	20.5 ± 0.5	22.0 ± 0.8	22.00 ± 0.8	20.0 ± 0.0	22.8 ± 1.1
Reductant (μmol)					
DTT (10)	11.7 ± 0.5	23.5 ± 0.5**	23.0 ± 1.3**	18.7 ± 1.3**	33.0 ± 1.0**

* $P \leqslant 0.05$.
** $P \leqslant 0.005$ using Student's *t*-test.

Another toxin, methylglyoxal, which is produced during the normal course of metabolism, must be detoxified by the cell since small amounts are toxic. In GSH containing organisms, methylglyoxal reacts with GSH to form hemithioacetal, which is converted to *S*-D-lactoylglutathione by the action of glyoxalase I; the glutathione is recycled by glyoxalase II, which catalyzes the production of D-lactate and glutathione [26]. Unlike glutathione deficient mutants in *Rhizobium tropici* [27], mutants lacking mycothiol are insensitive to methylglyoxal with the exception of Δ*mshD* (Table 3).

Steffek et al. [25], demonstrated that the removal of the methyl residue of the acetyl group of the mBBr conjugate, MS-monobimane to produce *N*-formyl-CGI-monobimane led to a 20-fold loss of activity in amidase activity whereas complete removal of the acetyl group resulting in CGI-mB led to a 1000-fold loss of activity. If mycothiol amidase is still able to react with *N*-formyl CGI conjugates such as those of iodoacetamide and monobromobimane albeit at a lesser level than with MSH conjugates, the Δ*mshD* should be less sensitive to these toxins than the other mutants lacking mycothiol. Since Δ*mshD* is just as sensitive as the other mutants, it appears that *N*-formyl CGI and CGI are not able to compensate for the loss of MSH.

Sensitivity to oxidants and reductants

In higher organisms and Gram-negative bacteria, glutathione protects the cells against the effects of oxidants by serving as a cofactor for GSH-dependent peroxidases [28] and by serving as a reducing agent for glutaredoxins which act as oxidoreductases to maintain sulfhydryl groups of proteins in the reduced state [29]. Presumably, mycothiol-dependent peroxidases and "mycoredoxins" with similar function also exist [30]. In Ung and Av-Gay [12], mycothiol levels decreased by 75% in *M. bovis* BCG grown in Middlebrook media supplemented with ADS and 5 mm diamide, a thiol oxidizing agent that preferentially oxidizes low molecular weight thiols. However, by 4 h, the mycothiol levels had recovered to normal amounts. In contrast, there was no change in mycothiol levels when *M. bovis* BCG was treated with 10 mM hydrogen peroxide. We also checked *M. smegmatis* mycothiol levels after treatment with sublethal amounts of various oxidants. When wild-type *M. smegmatis* was treated with hydrogen peroxide (10 mM), *t*-BOH (0.1 mM), and CHP (0.1 mM) for 2 h, there was no significant change in mycothiol levels. In contrast, treatment with 10 mM diamide resulted in a decrease of 30–60%. There was also a decrease of 75% and a decrease of 60% when 0.005 mM plumbagin and 0.05 mM menadione, respectively, were added to the culture, indicating a direct interaction between mycothiol and these oxidants.

Previously, we have shown that the chemical mutant I64 (Δ*mshC*) is sensitive to oxidative stress [11]. As seen in Table 3, all mutants disrupted in genes coding for mycothiol biosynthesis are sensitive to hydrogen peroxide and *t*-butyl hydroperoxide, an organic peroxide. However, these mutants are insensitive to cumene hydroperoxide, an aromatic peroxide; indeed, there was no clearing in disk assays even at such high amounts as 10 μmol (data not shown) indicating that MSH does not play a role in the detoxification of this peroxide. Furthermore, none of the mutants are sensitive to diamide, which is not surprising since addition of diamide to mycobacterial cultures only causes a transient decrease in mycothiol levels [12]. In Table 3, it can be observed that mutants disrupted in genes coding for mycothiol biosynthetic enzymes are sensitive to the redox cycling agent, plumbagin and menadione.

Recently Newton et al. [19], reported a lack of difference in the minimum inhibitory concentration for hydrogen peroxide, cumene hydroperoxide and *t*-butyl hydroperoxide, between the *M. smegmatis* Δ*mshD* mutant and wild-type strain as measured by the broth dilution assay. The discrepancy in the results may be due to the two methods used to determine sensitivity, disk sensitivity assays versus broth dilution assay. It should be noted that both *M. tuberculosis* Δ*mshD* [16] and *Amycolaptosis mediterranei* Δ*mshD* [21] strains are sensitive to hydrogen peroxide.

In actinomycetes, MscR codes for an enzyme with dual MSH-dependent formaldehyde dehydrogenase activity and nitrosothiol reductase activity requires mycothiol for activity [31]. When mutants disrupted in mycothiol biosynthesis were checked for sensitivity to nitrosative stress, there was no difference in sensitivity to sodium nitrite as determined by broth dilution assays (data not shown). We also did not see a difference in viability of *M. smegmatis* and mutants lacking mycothiol treated with 2 mM sodium nitroprusside, a NO donor (data not shown). In contrast, when the wild-type and Δ*mshA* were treated with 200 ppm, the mutant was more sensitive than Δ*mshA* [13]. This difference in sensitivity may be a result of the greater permeability of gaseous NO.

Trotter and Grant [32] have demonstrated that mutants lacking thioredoxin are not only sensitive to oxidative stress but also sensitive to reducing stress in the form of DTT, whereas mutants lacking glutathione are insensitive. We checked the sensitivity of the wild-type strain and mycothiol mutants to DTT with disk assays. As described in Table 3, we did see a difference in the sensitivity to this reducing agent between wild-type and mutants lacking mycothiol. We also checked the sensitivity to glutathione with disk assays and broth dilution assays as it has been reported that *M. tuberculosis* is sensitive to glutathione [33]; there was no difference in sensitivity between wild-type and mutants (data not shown).

Acid stress

Acidic conditions occur in soil and aquatic habitats where saprophytic mycobacteria reside and in granulamatous lesions in the lungs and the phagosomes (pH 6.1–6.5) where the pathogenic *M. tuberculosis* thrive. Although microarray analysis of *M. tuberculosis* transcripts induced by acidic conditions did not identify any of the genes

involved in mycothiol biosynthesis [34], *M. tuberculosis* Δ*mshD* showed restricted growth in acidic medium (pH 5.5) [17]. We tested the viability of wild-type and all four MSH deficient mutants in acidified media (pH 4.0) to which 20 mM HCl had been added. There was a decrease in viability for wild-type and mutant strains with the greatest decrease in viability for Δ*mshA* and Δ*mshD* mutants. Δ*mshB* was also affected but not as severely and the decrease in viability for Δ*mshC* was not significant using Student's *t*-test (Fig. 1). As with *M. tuberculosis*, MSH levels after acid treatment were lower in the wild-type (5.08 ± 0.488–3.56 ± 0.260, $P = 0.03$). Treatment with 20 mM NaOH to mimic basic stress did not result in a significant difference (5.96 ± 0.472, $P = 0.243$) in MSH levels. The reason for this decrease in mycothiol after acid treatment is not clear in mycobacteria although in *Escherichia coli*, glutathione and glutathione adducts control the potassium efflux activity of KefB/KefC channels [35]. An increase in glutathione adducts such as GS-*N*-ethylmalemide causes the opening of these channels, resulting in a potassium efflux with concurrent proton influx, followed by cytoplasmic acidification, which protects against further electrophilic attack.

Osmotic stress

Glutathione is known to play a role in protection against osmotic stress in many organisms. For example, a *R. tropicii* mutant lacking glutathione is sensitive to osmotic stress [27]. In the case of mycothiol, treatment with 0.2 M KCl results in the increase in transcription of the genes involved in the mycothiol biosynthesis and the amount mycothiol increased by 30% within an hour of osmotic challenge with 0.2 M KCl in *Streptomyces coelicolor* [36]. In *M. smegmatis,* addition of either 0.2 M NaCl or KCl to log-phase wild-type cells followed by incubation for two hours did not affect mycothiol levels. The mycothiol levels in the untreated cells are 5.08 ± 0.49 nmol/OD while the levels in 0.2 M NaCl treated cells and 0.2 M KCl treated cells are 5.59 ± 0.38 nmol/OD and 5.18 ± 0.22 nmol/OD, respectively. Moreover, there is no difference in the growth of the wild-type strain and the *M. smegmatis* mutants in solid media amended with 0.4 M NaCl or 0.4 M KCl. Since mycothiol levels in *M. smegmatis* are substantially higher as compared to *S. coelicolor*, the amount of mycothiol present in *M. smegmatis* may be sufficient to counteract the effects of osmotic stress [1].

Conclusions

In this report, we have established that it is MSH and in extension mycothiol-dependent enzymes play a role in detoxification of electrophiles, alkylating agents, oxidants, and antibiotics. MSH and MSH-dependent enzymes also play a role in protection against acid stress but not osmotic stress. We postulate that MSH-dependent enzymes are highly specific for mycothiol since a closely related species such as *N*-formyl CGI that is present in Δ*mshD* cannot substitute for mycothiol to provide the same levels of resistance to xenobiotics. The results presented herein broaden the scope of mycothiol action and establish the role of mycothiol in providing mycobacteria with innate resistance to xenobiotics.

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