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Characterization of *Mycobacterium smegmatis* Mutants Defective in 1-D-*myo*-Inosityl-2-amino-2-deoxy-α-D-glucopyranoside and Mycothiol Biosynthesis

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Mycothiol (MSH) is the major low molecular weight thiol in mycobacteria. Two chemical mutants with low MSH and one with no MSH (strain 49) were produced in *Mycobacterium smegmatis* mc^{2}155 to assess the role of MSH in mycobacteria. Strain 49 was shown to not produce 1-D-*myo*-inosityl-2-amino-2-deoxy-α-D-glucopyranoside (GlcN-Ins), an intermediate in MSH biosynthesis. Relative to the parent strain, mutant 49 formed colonies more slowly on solid media and was more sensitive to H_2O_2 and rifampin, but less sensitive to isoniazid. Complementation of mutant 49 with DNA from *M. tuberculosis* H37Rv partially restored production of GlcN-Ins and MSH, and resistance to H_2O_2, but largely restored colony growth rate and sensitivity to rifampin and isoniazid. The results indicate that MSH and GlcN-Ins are not essential for *in vitro* survival of mycobacteria but may play significant roles in determining the sensitivity of mycobacteria to environmental toxins.

Mycothiol, 1-d-*myo*-inosityl-2-(*N*-acetyl-l-cysteinyl) amido-2-deoxy-α-d-glucopyranoside (MSH, AcCys-GlcN-Ins), is a novel conjugate of *N*-acetylcysteine, glucosamine and *myo*- inositol (Fig. 1) that is produced by most actinomycetes but apparently not by other bacteria or eukaryotes (1). These bacteria, which include the medically important mycobacteria, do not produce GSH, an important coenzyme in reactions which protect against oxygen toxicity and electrophilic toxins (2,3). Since MSH is more resistant than GSH to autoxidation (4) and, like GSH, occurs at millimolar levels, it may have functions in actinomycetes analogous to those of GSH in GSH-producing organisms. A recent example is the demonstration for the actinomycete *Amycolatopsis methanolytica* that MSH is the factor for NAD/factor-dependent formaldehyde dehydrogenase, playing an analogous role to that of GSH in the well-characterized NAD/GSH-dependent formaldehyde dehydrogenase (5,6). If MSH is a key protectant in these bacteria, then the biochemical steps involved in its production and utilization may represent important new targets for drug development (1,4,7,8). The last steps of MSH biosynthesis are thought to involve coupling of Cys to GlcN-Ins followed by acetylation (Fig. 1) based upon studies by Bornemann *et al.* (8) with partially purified cell extracts from *Mycobacterium smegmatis* and by Anderberg *et al.* on cellular levels of possible biosynthetic intermediates in *M. smegmatis* (9).

We wanted to answer two questions in the present studies. First, is MSH essential for survival of mycobacteria, thereby making mutants in its production inaccessible? Second, does MSH play an important role in the sensitivity of mycobacteria to peroxides and drugs? To answer these questions we sought chemical mutants blocked in MSH production. This was facilitated by development of an immunoassay for MSH (10) and of sensitive high performance liquid chromatography (HPLC) assays for key intermediates in MSH biosynthesis (9). We describe here the production and characterization of three mutants of *Mycobacterium smegmatis* mc^{2}155 having significantly reduced MSH levels.

MATERIALS AND METHODS

The organisms used were *M. smegmatis* mc^{2}155, kindly provided by W. R. Jacobs, and *M. tuberculosis* H37Rv strain 102 (ATCC 27294). *N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) was from

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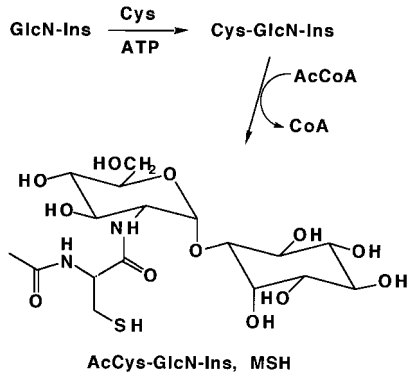

FIG. 1. Structure and biosynthesis of mycothiol in *M. smegmatis.*

Aldrich; rifampin (RIF) and isoniazid (INH) were from Sigma; hygromycin B and diamide were from Calbiochem. Growth media were described previously (9). All other chemicals were of reagent grade or higher purity from Fisher.

Mutants of *M. smegmatis* mc^{2}155 were produced from mid-log phase cultures on Middlebrook 7H9 liquid medium supplemented with 0.05% Tween 80 and 0.4% glucose. Cells were harvested by centrifugation and the pellets resuspended in the original medium at ~2.5 x 10^9 colony forming units per ml (CFU ml^{-1}), using the factor 2.5 x 10^8 CFU ml^{-1} for A_{600} = 1.0 (11). A 1 ml aliquot in a 15-ml centrifuge tube was vortexed after addition of 4 μl of a freshly-made solution of 100 μg/μl MNNG in dimethylsulfoxide (DMSO). A control was similarly prepared using pure DMSO and both samples were incubated 30 min at 37°C with shaking (240 rpm). The cell suspensions were diluted with 9 ml of fresh medium, vortexed, and centrifuged 10 min at 1000 x *g;* 9 ml of the supernatant was discarded. This washing protocol was repeated twice after which the cells were resuspended in 10 ml fresh medium, transferred in 5 ml aliquots to sterile culture tubes, and incubated 8.5 h at 37°C with shaking (240 rpm). The cells were diluted in fresh medium and passed several times through a 22-gauge needle to break up cell clumps before plating on Middlebrook 7H9 agar (containing 0.05% Tween 80 and 0.4% glucose) with 1 mM diamide added. Plates were incubated for 9 days at 37°C in a humidified incubator, and scored daily for the appearance of new colonies. Individual colonies were marked according to the day of appearance. Treatment with MNNG reduced survival to 20% of that of the untreated control. Slow-growing colonies were replated in duplicate as described above but without diamide using a grid layout in 100 mm culture dishes. After colony development, one plate was used to screen for MSH-deficient mutants using a membrane-based immunoassay highly specific for MSH (10). Briefly, this assay involves transfer of colonies to a nitrocellulose membrane coated with bovine serum albumin linked to *N*-ethylmaleimide, lysing of cells with *N*-acetylglucosaminidase to release MSH and permit its reaction with the maleimide residue, washing unbound material from the membrane, and detection of MSH using a rabbit antibody specific for bound MSH. Cells not reacting with MSH antibody were identified as potential mutants in MSH production and were further characterized.

Cosmid libraries for complementation of *M. smegmatis* mc^{2}155 strain 49 have been constructed from *M. tuberculosis* H37Rv using shuttle plasmid pYUB412, kindly provided by W. R. Jacobs, essentially as described by Jacobs *et al.* (12) for pYUB18 constructs. Genomic DNA from *M. tuberculosis* H37Rv was partially digested with *Sau*3A for 45 min at 37°C. The digestion mix was ethanol precipitated and ligated to *Bc*lI-cut vector. The pYUB412::H37Rv library was packaged in lambda phage and used to transfect maltose-treated Stratagene *Escherichia coli* XL-1 Blue MRF' according to the manufacturer's instructions. The *E. coli* cosmid library was grown on LB agar with ampicillin (100 μg per ml). The plasmid DNA form of the mycobacterial library was generated by standard alkaline lysis of the pooled *E. coli* clones. This plasmid DNA was purified by treatment of the plasmid with DNAse-free ribonuclease followed by extraction with chloroform-phenol. It was then used to transform *M. smegmatis* strain 49 by electroporation as previously described (13). The cells were plated on 7H9 agar containing hygromycin (100 U per ml) and the transformants were manually picked and gridded on the same media for screening of mycothiol production by immunoblotting as described above. Transformants that appeared to be MSH positive by immunoblotting were grown in Middlebrook 7H9 medium and the thiol content determined by monobromobimane labeling and HPLC as described below.

For growth rate studies in liquid medium an overnight starter culture of each *M. smegmatis* strain was prepared in Middlebrook 7H9 with glucose (0.4%) and Tween 80 (0.05%) producing A_{600} = 1 - 2. From this starter culture duplicate 50 ml cultures were inoculated in the same medium in 125 ml Erlenmeyer flasks to A_{600} = 0.1 and shaken at 225 rpm at 37°C. Aliquots of 1 ml were removed from one flask for absorbance (A_{600}) measurements using a Beckman model DU640 spectrometer. At A_{600} ~ 0.8 the second culture was chilled on ice and pelleted by centrifugation at 4°C for amine and thiol analysis (see below). The log phase doubling time (T_D) in liquid culture was assessed from a plot of log A_{600} versus time at $A_{600} \leq 0.8$.

For peroxide toxicity studies a 24 h culture in Middlebrook 7H9 with glucose (0.4%) and Tween 80 (0.05%) was prepared and adjusted to 2.5 × 10^7 CFU per ml based upon the A_{600} value. Hydrogen peroxide (Fisher) was diluted in MilliQ water to prepare 1 M or 0.2 M stock solutions, which were filter-sterilized and stored on ice. The required amount of H_2O_2 stock solution (at least 10 levels) was added to 1 ml of cell suspension in a 15 ml culture tube and the suspension was shaken at 37°C for 2 h. Cells were syringe resuspended, diluted into fresh medium, and plated on 7H10 agar. Plates were incubated at 37°C until colonies were approximately 1-2 mm across. The time in days (T_{CF}) for aerobic growth of *M. smegmatis* strains on 7H10 agar was assessed using the peroxide-free controls in these experiments.

The amine components of MSH (GlcN and GlcN-Ins) were determined by HPLC analysis of the 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AccQ-Fluor) derivatives and the thiol components were measured as the 4-bromomethyl-3,6,7-trimethyl-1,5-diazabicyclo[3.3.0]octa-3,6-diene-2,8-dione (monobromobimane, mBBr) derivatives as described previously (9).

Cys/GlcN-Ins ligase activity was measured on crude, undialyzed supernatants at a final concentration of 1 mg protein per ml (A_{280} = 1.0) as described earlier (9) with the following changes. Reaction mixtures (25 μl) were reacted for 0 and 45 min using 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.5, in place of phosphate buffer to eliminate interfering peaks in the HPLC analysis. An equal volume of 8 mM mBBr in warm acetonitrile was then added and the mixture heated 3 min at 60°C. The derivatization reaction was quenched by 4-fold dilution in 10 mM methanesulfonic acid and samples analyzed by HPLC (9). Direct comparison revealed that the change in buffer produced no change in reaction rate.

For drug toxicity studies, filter sterilized stock solutions of rifampin (RIF) (in 50% DMSO in water) and isoniazid (INH, aqueous) were diluted into warm (~45°C) Middlebrook 7H9 agar at not less than 15 different levels between 0.01 μg/ml and 100 μg/ml and allowed to solidify overnight in the dark prior to use. Log phase cultures were plated as above for the peroxide sensitivity studies to give about 300 colonies on control plates. Colonies were counted after ~4 days for mc^{2}155, ~5 days for strains 5, 6 and A18 and 8-10 days for strain 49 in control experiments. Experiments with the highest levels of RIF or INH took an additional ~5 days for colony formation. The MIC_{50} value is defined as the drug concentration that gives 50% of the control survival level.

TABLE 1

Mycothiol Component Levels in Early Log Phase *M. smegmatis* Strains, Mutants and Mutant 49::*M. tuberculosis* H37Rv Complements

	Cellular Mycothiol Component Content[a] (μmol g^{-1} residual dry weight)				
Strain/Complement	GlcN	GlcN-Ins	Cys	Cys-GlcN-Ins	MSH
mc^2155	0.02	0.2	0.16	<0.002	10.6
Mutant 49	0.042	<0.004	0.20	<0.001	<0.004
Mutant 5	0.023	0.19	0.13	<0.002	2.8
Mutant 6	0.023	0.15	0.13	<0.001	3.3
49::H37Rv A18	0.027	0.026	0.24	<0.001	3.3
49::H37Rv F17	0.044	0.006	0.25	<0.001	0.66

[a] Levels listed as "<" showed no discernable peak larger than found in the NEM-mBBr control sample.

RESULTS

An approach analogous to that used to obtain mutants defective in glutathione biosynthesis (14) was adopted here for MSH-deficient mutants. *Mycobacterium smegmatis* mc^2155 was mutagenized with MNNG and colonies developed on medium containing 1 mM diamide. Diamide penetrates cells and oxidizes intracellular thiols (15). A concentration of 0.15 mM diamide served for selection of *E. coli* mutants in glutathione metabolism (14) but 1.0 mM was needed with *M. smegmatis* which was found to be more resistant to diamide. *M. smegmatis* colonies were selected which were apparent only after 7-11 days of incubation. These were classified as slow-growing on diamide medium since control cells all developed within 3-4 days on this medium. A total of 415 slow growing colonies, ~7% of the total colonies, was selected. These were replated on medium without diamide and the resulting colonies screened for MSH production by immunoblotting (10). Immunoblotting identified 29 colonies with apparent low or no MSH production and these were grown in bulk culture for full thiol analysis by fluorescent labeling with mBBr and HPLC (9,16). Most of these mutants proved to have normal or near-normal MSH content when grown in liquid medium. Why these mutants failed to give positive immunoblots is unclear, but may reflect a difference in MSH production between culture on solid versus liquid medium or a difference in membrane or cell wall properties which resulted in poor transfer or lysis in the immunoblotting protocol. However, three mutants proved to have measurably low MSH content when grown on liquid medium. Two of these, designated strains 5 and 6, had low but measurable MSH content and a third, designated strain 49, was essentially devoid of MSH (Table 1).

Although mutant 49 appeared to be a mycobacterium, its slow growth and failure to produce mycothiol might be taken as evidence that it was actually a contaminant. This was excluded by sequencing a PCR-amplified internal fragment of the 16S rRNA which proved to be identical with that for the parent strain mc^2155 (data not shown). Mutant 49 has been repeatedly passaged in culture and found to retain its undetectable MSH content, indicating that the spontaneous reversion rate is quite low.

Complementation of the mycothiol-devoid mutant 49 was attempted to restore MSH production with a random *M. tuberculosis* library comprised of ~18 kb fragments cloned into the integrative shuttle plasmid pYUB412. Cells were transformed by electroporation and plated with hygromycin selection. Colonies were visible in 8-10 days; single colonies were picked, gridded on the same medium and screened for MSH production by immunoblotting with MSH-specific antibodies (10). Six colonies of 220 screened were found to be positive for mycothiol production by immunoblotting. These colonies were cultured in liquid media and were found to produce levels of mycothiol ranging from 6–31% of that for the parent strain (mc^2155). Results for analysis of the high (strain A18) and low (strain F17) MSH-containing complements are included in Table 1.

In an effort to determine the stage at which MSH biosynthesis was modified in the mutant strains, the cellular levels of GlcN-Ins and Cys-GlcN-Ins were determined using methods recently developed to characterize intermediates involved in MSH biosynthesis and degradation (9). Strains 5 and 6 had measureable levels of GlcN-Ins comparable to that of mc^2155, but Cys-GlcN-Ins was not detectable in any of the strains during early log phase growth (Table 1). However, strain 49 produced no detectable GlcN-Ins whereas complements F17 and A18 had GlcN-Ins levels corresponding to 3 and 13%, respectively, of the parent strain (Table 1), paralleling their relative MSH content which was respectively 6 and 31% of that for the parent strain mc^2155. Thus, the complementation of mutant 49 with *M. tuberculosis* genomic DNA partially restores the ability of 49 to produce GlcN-Ins, and thus also to make MSH.

TABLE 2

Characterization of *M. smegmatis* Parent, Mutant, and Complement Strains

Strain	Relative [MSH] %	T_{CF}[a] days	T_D[b] hrs	LC_{50} (mM) H_2O_2	MIC_{50} (μg/ml)[c] RIF	MIC_{50} (μg/ml)[c] INH
mc^{2}155	(100)	4-5	4.4 ± 0.1	12	1.4 ± 0.2	2 ± 1
5	26	5-6	4.8 ± 0.3	5	n.d.[d]	n.d.
6	31	5-6	4.4 ± 0.1	2.5	n.d.	n.d.
49	0	8-10	8.5 ± 0.5	1	0.07 ± 0.04	<50
49::H37Rv A18	31	5-6[e]	7.9 ± 1.0	1.8	0.3 ± 0.2	6 ± 1

[a] Time for colony formation (1-2 mm) after plating on 7H10 Middlebrook agar (n=11).
[b] Doubling time in 7H9 liquid culture, mean and range of duplicate cultures.
[c] Mean and range of 3 experiments.
[d] Not determined.
[e] With or without hygromycin selection.

Extracts of mc^{2}155 and mutant strains were examined for Cys/GlcN-Ins ligase activity, the ability to form Cys-GlcN-Ins from GlcN-Ins, cysteine and ATP. The assay was performed on a unfractionated, undialyzed supernatant fraction using a minor modification of the assay of Anderberg, *et al.* (9). Cys-GlcN-Ins was determined as the fluorescent mBBr derivative by HPLC (9). The extracts were analyzed for production of Cys-GlcN-Ins after incubation for 0 and 45 min at 30°C. Measured activities (nmol per min per mg protein) from duplicate determinations were 0.36 ± 0.03, 0.52 ± 0.05, 0.22 ± 0.01, and 0.20 ± 0.01 for strains mc^{2}155, 49, 5 and 6, respectively. Thus, mutant 49 is capable of forming Cys-GlcN-Ins, and presumably MSH, if supplied with a source of GlcN-Ins.

Mutants 5 and 6 grew at rates comparable to that of the parent strain on solid medium and in liquid culture but growth of mutant 49 was a factor of two slower (Table 2). Complementation of strain 49 largely restored the growth rate on solid medium but not in liquid culture (Table 2 and data not shown). In Middlebrook 7H9 liquid medium, the mutant strains achieved maximal A_{600} values 30–50% below that of the parent strain whereas complement strains A18 and F17 reached maximal A_{600} values 15–20% below that of the parent strain. The same pattern in T_{CF} values shown in Table 2 for growth on Middlebrook 7H10 agar was also observed when cells were plated on Middlebrook 7H9 agar for the drug sensitivity controls (data not shown).

The hydrogen peroxide sensitivity of *M. smegmatis* strains was examined using a two hour exposure to various levels of H_2O_2 in Middlebrook 7H9 liquid media at 37°C. The cells were diluted, plated on 7H10 agar and monitored until colony formation had ceased (maximum 10 days). The parent strain mc^{2}155 tolerated H_2O_2 up to 12 mM with <10% loss in viability but was >95% killed by 12.5 mM H_2O_2, yielding a concentration for 50% lethality (LC_{50}) of ~12 mM (Table 2). In contrast, the LC_{50} for mutant 49 was 12-fold lower and 90% loss of viability occurred over a wider range of H_2O_2 concentration (0.5-3.5 mM).

Mutants 5 and 6 and complement A18 were found to have similar MSH contents, intermediate between that of the parent strain and mutant 49, during exponential growth (Table 1), and their sensitivity to H_2O_2 was also intermediate (Table 2). However, the LC_{50} for strain A18 is nearly 3-fold lower than that for strain 5 so sensitivity to peroxide is not quantitatively related to MSH content measured during exponential growth without added peroxide.

Sensitivity of strains mc^{2}155, 49, and A18 to RIF and INH was also examined by determining the concentrations required to inhibit growth by 50% (MIC_{50}) on Middlebrook 7H9 agar (Table 2). Mutant 49 was 20-fold more sensitive to RIF than the parent strain and complement A18 had a sensitivity largely restored to that of the parent strain. By contrast, mutant 49 showed a greater than 25-fold resistance to INH compared to the parent strain but this resistance was substantially eliminated in complement A18.

DISCUSSION

The isolation of mutant 49 fully blocked in GlcN-Ins and MSH biosynthesis leads to several conclusions. First, it demonstrates that production of measureable levels of these compounds is not essential for the survival of *M. smegmatis* under normal laboratory growth conditions. Second, it confirms that GlcN-Ins is an intermediate in the biosynthetic pathway leading to MSH and, coupled with the fact that complementation partially restores both GlcN-Ins and MSH biosynthesis, it demonstrates that the biosynthetic route via GlcN-Ins is the sole pathway leading to MSH. The specific defect in mutant 49 has not yet been identified. It might in principle involve a regulatory gene, but, if so, this gene does not also control expression of the Cys/GlcN-Ins ligase gene as this activity was present in mutant 49.

Mutant 49 exhibits characteristics which differ from those of its parent strain and these differences provide insight into the potential role of MSH and its precursors. The finding that mutant 49 grows more slowly on solid media than the parent strain suggests that MSH (and/or GlcN-Ins) plays a role in general mycobacterial metabolism enabling normal growth. The mutants were selected for slow growth and it is conceivable that mutant 49 has a defect in addition to that for GlcN-Ins production which is responsible for this phenotype. However, all six complements with partially restored MSH content had restored growth rates on solid medium (Table 2 and data not shown). This would require that both defects were complemented by the same DNA and therefore involve proximal mutations. Although the probability of multiple mutations is high, the chance of two random mutations occuring within a given ~18 kb region of the genome is quite low. Thus, the simplest and most probable explanation of the results is that the slow growth on solid medium is associated with the defect in MSH biosynthesis. The results suggest that restoration of MSH to as little as 6% of the level of the parent strain, as in strain F17, suffices to restore growth rates on solid medium (Table 1 and data not shown).

A different pattern was found for growth in liquid medium. Complement A18 failed to exhibit a normal growth rate even though the MSH content was restored to a level similar to that of mutants 5 and 6 which did have normal growth rates (Table 2). Mutants 5 and 6 involve a different defect and have GlcN-Ins levels 6- to 7-fold greater than complement A18. If GlcN-Ins is not only a precursor to MSH but serves some other function important for growth, then the limited restoration of GlcN-Ins production in A18 could be insufficient to restore normal growth in liquid medium. Alternatively, a second mutation might be present in mutant 49 which is responsible for the slow growth in liquid medium and is not corrected by the complementation which partially restores MSH production.

Mutants 5, 6, and 49 were all significantly more sensitive to H_2O_2 than the parent strain mc^2155 which exhibited a marked resistance to peroxide (Table 2). The difference in peroxide concentration dependence suggests that a different mechanism of protection is involved in strains mc^2155 and 49. Strains 5, 6, and A18 have similar MSH content but vary 2- to 3-fold in peroxide sensitivity, indicating that factors other than MSH content are also important in determining peroxide sensitivity. Peroxide sensitivity correlates more simply with GlcN-Ins content (Table 1), the IC_{50} value (Table 2) increasing sharply for GlcN-Ins content above 0.15 μmol per g residual dry weight. No function for GlcN-Ins other than as a precursor of MSH has as yet been reported and how it might function to influence peroxide sensitivity is unclear.

The most striking results obtained with mutant 49 are those for sensitivity to RIF and INH. Mutant 49 is dramatically more sensitive to RIF than is the parent strain and complementation largely restores the sensitivity to that of the parent strain. This suggests that MSH or GlcN-Ins are involved in processes which reduce the activity of RIF. In mycobacteria RIF inhibits DNA-dependent RNA polymerase (17). Resistance to RIF has been associated with alterations of this enzyme (18,19) or with ribosylation of RIF (20,21). MSH might react with RIF, thereby adding a highly hydrophilic group to the molecule and, like ribosylation, make it unable to bind the polymerase.

Cell wall permeability has also been considered to be important in RIF sensitivity (22) and both GlcN and Ins play key roles in the biochemistry of the mycobacterial cell wall (23). Although no specific role in cell wall biochemistry has been demonstrated for α(1->1) linked GlcN-Ins, it is conceivable that this pseudodisaccharide plays an as yet unidentified function which influences uptake of RIF. Further studies are needed to clarify the role of MSH biosynthesis in RIF sensitivity.

The opposite pattern was found for sensitivity to INH. Mutant 49 was over 25-fold more resistant to INH than the parent strain and partial restoration of MSH by complementation largely restores the INH sensitivity (Table 2). This suggests that MSH might play a role in the activation of INH, most plausibly as a reductant. INH is a prodrug which is converted to an active form by an oxidative process catalyzed by KatG, but the structure of the active form of INH has not been identified (24,25). Reactive oxygen intermediates formed during INH oxidation may also function as potential toxins (26,27). The resting form of KatG must be reduced before it can oxidize INH to the active form (28) which then interferes with mycolic acid biosynthesis (29). One proposed mechanism involves inhibition of InhA, a fatty acyl enoyl reductase, by reaction with the thiol group of Cys243 (25). It is conceivable that MSH participates in the reductive activation of KatG. Alternatively, MSH may maintain Cys243 in its reactive reduced state and its absence could result in Cys243 being oxidized to an unreactive form. However, if reactive oxygen intermediates are important in the mechanism of action of INH, then the present result is difficult to understand since MSH would be expected to facilitate inactivation of these toxins.

We conclude that it is possible for cells to grow without producing MSH but that its absence markedly changes its response to its environment.

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