JOURNAL OF BACTERIOLOGY, Nov. 2003, p. 6736–6740 Vol. 185, No. 22
0021-9193/03/$08.00+0 DOI: 10.1128/JB.185.22.6736–6740.2003

Mycothiol Is Essential for Growth of *Mycobacterium tuberculosis* Erdman

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Received 15 May 2003/Accepted 19 August 2003

Mycothiol (MSH) is the major low-molecular-mass thiol in mycobacteria and is associated with the protection of *Mycobacterium tuberculosis* from toxic oxidants and antibiotics. The biosynthesis of MSH is a multistep process, with the enzymatic reaction designated MshC being the ligase step in MSH production. A targeted disruption of the native *mshC* gene in *M. tuberculosis* Erdman produced no viable clones possessing either a disrupted *mshC* gene or reduced levels of MSH. However, when a second copy of the *mshC* gene was incorporated into the chromosome prior to the targeted disruption, multiple clones having the native gene disrupted and the second copy of *mshC* intact were obtained. These clones produced normal levels of MSH. These results demonstrate that the *mshC* gene and, more generally, the production of MSH are essential for the growth of *M. tuberculosis* Erdman under laboratory conditions.

Mycothiol (MSH; AcCys-GlcN-Ins) is a conjugate of *N*-acetylcysteine (AcCys) with 1-D-*myo*-inosityl 2-amino-2-deoxy-α-D-glucopyranoside (GlcN-Ins) and is the major low-molecular-mass thiol in most actinomycetes (7). MSH is the functional equivalent of glutathione in mycobacteria (8) and is associated with the protection of *Mycobacterium tuberculosis* from toxic oxidants and antibiotics (4). The biosynthesis of MSH is a multistep process involving four enzymatic reactions designated MshA, MshB, MshC, and MshD (Fig. 1). An *M. tuberculosis mshB* mutant demonstrated a heightened sensitivity to the toxic oxidant cumene hydroperoxide and to the antibiotic rifampin even though the *mshB* mutant produced approximately 20% of the wild-type levels of MSH (4). A compensating deacetylase activity was apparently sufficient for the production of moderate levels of MSH in the *mshB* mutant. In *Mycobacterium smegmatis*, mutants have been isolated with mutations in either the *mshA* gene (9, 10) or the *mshC* gene (13) that are devoid of detectable levels of MSH. The MshC activity in *M. tuberculosis* was recently identified as being encoded by open reading frame Rv2130c (14). The *mshC* gene or Rv2130c had originally been annotated as a cysteinyl-tRNA synthetase gene called *cysS2* (5). In order to examine the effect upon *M. tuberculosis* of very low levels of MSH, the construction of a targeted gene disruption in the *mshC* gene of *M. tuberculosis* was attempted.

The production of targeted gene disruptions within the chromosome of *M. tuberculosis* Erdman was carried out via allelic exchange by using the conditionally replicating mycobacteriophage phAE87 (generous gift of J. S. Cox) (2). phAE87 is a temperature-sensitive shuttle phasmid that replicates at 30°C but not at 37°C. The method for specialized transduction to generate targeted gene disruptions in *M. tuberculosis* was recently described by Bardarov et al. (1). For construction of the *mshC* knockout phage, an ~500-bp fragment comprising 102 bp of the N-terminus of the *mshC* gene plus the adjacent 370 flanking bases was amplified by PCR. A second fragment containing residues 195 to 708 of the *mshC* gene was also amplified by PCR. A 93-bp section within the *mshC* gene that encodes the active HLGH region of the MshC protein was not included within the amplified fragments (14). Each PCR fragment included suitable endonuclease sites for directional cloning into pJSC284 (gift of J. S. Cox). pJSC284 is a cosmid containing a *Pac*I site and a *res-hyg-res* cassette (2) flanked by multiple cloning sites. The *mshC* fragments were sequentially cloned on either side of the hygromycin resistance cassette in pJSC284 to produce a mutated copy of *mshC* interrupted by the Hyg^r gene. This *mshC* knockout cosmid was digested with *Pac*I and ligated into the *Pac*I site of the specialized transducing phage phAE87 to generate a phasmid capable of replicating as a cosmid in *Escherichia coli* and as a temperature-sensitive phage in mycobacteria. The ligated DNA was packaged into phage λ with Gigapack III Gold packaging extract (Stratagene), and *E. coli* HB101 cells grown on maltose were infected with the phage. Colonies were selected for growth on LB plates containing 200 μg of hygromycin/ml. Cosmid DNA was extracted from two of the Hyg^r colonies, and the DNA was electroporated into *M. smegmatis* mc^{2}155. The transformation plates were incubated at 30°C until plaques appeared (2 to 3 days later). Several plaques were picked, and a high-titer phage stock (10^{10} to 10^{11} PFU/ml) was prepared with *M. smegmatis* mc^{2}155.

For infection of *M. tuberculosis* Erdman cells, 10 ml of mid-log to late log phase bacteria was washed with MP buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 2 mM $CaCl_2$, 10 mM $MgCl_2$) and resuspended in 1 ml of MP buffer at 39°C. Phage were added at a multiplicity of infection of 10, and the mixture was incubated at 39°C for 4 h to allow for phage infection. The bacteria were then pelleted by centrifugation, resuspended in 500 μl of MP buffer, and plated on Middlebrook 7H11 plates supplemented with OADC (oleic acid, albumin, dextrose, catalase; BBL) and containing hygromycin (50 μg/ml). Hygromy-

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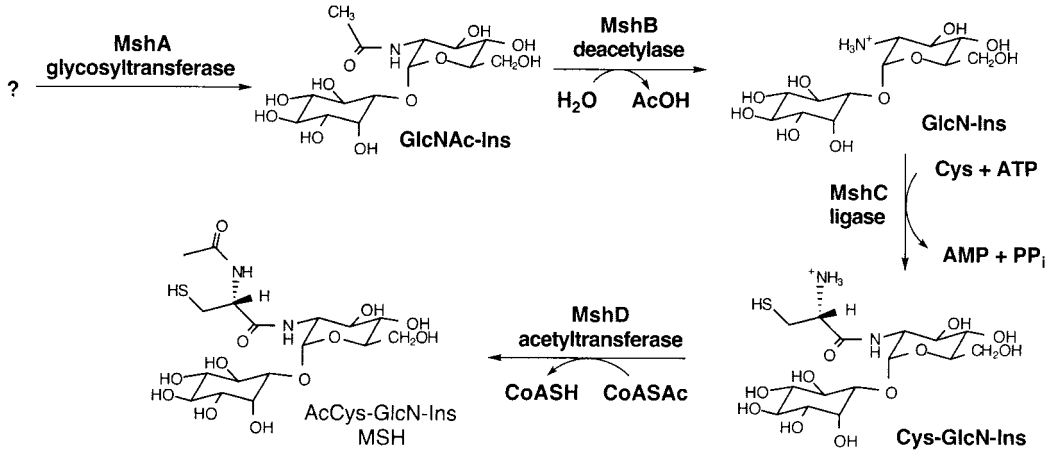

FIG. 1. Key enzymes in the biosynthesis of MSH in *M. tuberculosis* include the glycosyltransferase (MshA, encoded by Rv0486), the GlcNAc-Ins deacetylase (MshB, encoded by Rv1170), the ATP-dependent Cys:GlcN-Ins ligase (MshC, encoded by Rv2130c), and the acetyl-CoA (CoASAc): Cys-GlcN-Ins acetyltransferase (MSH synthase; MshD, encoded by Rv0819).

cin-resistant colonies appeared in 3 to 5 weeks. Individual colonies were cultured for analysis by Southern hybridization to identify clones in which allelic exchange had occurred within the *mshC* gene and to measure MSH content (4).

In our first experiment, few hygromycin-resistant clones grew after transduction of the wild-type *M. tuberculosis* Erdman cells with the *mshC* allelic exchange phage. Of the nine clones recovered, none had undergone allelic exchange within the *mshC* gene, and all had normal levels of MSH (data not shown). In a parallel experiment with a similar allelic exchange phage for the *corA* gene (which encodes a putative magnesium transport protein), multiple *corA* mutants were identified. The failure to identify *mshC* mutants suggested that either the region encompassing the *mshC* gene in *M. tuberculosis* is refractive to homologous recombination or that a functional *mshC* gene is essential to the growth of *M. tuberculosis.*

In order to determine whether the *mshC* gene is required for the viability of *M. tuberculosis*, a second copy of the *mshC* gene was introduced into the chromosome of *M. tuberculosis*, generating strain 2X-*mshC*, prior to transduction with the *mshC* allelic exchange phage. This strategy had been employed by Parish and Stoker (11) to establish the essential nature of the *glnE* gene. If allelic exchange within the *mshC* gene could be demonstrated in the 2X-*mshC* strain but not in the wild-type *M. tuberculosis*, this result would strongly suggest that a functional *mshC* gene is essential for growth of *M. tuberculosis.*

A second copy of the *M. tuberculosis mshC* gene and its ribosomal binding site were incorporated into the genome by using integrative vector pCV125. pCV125 had been previously modified to include the streptomycin and spectinomycin resistance genes (3). The *mshC* open reading frame plus its ribosomal binding site (71 bp upstream of the ATG start codon) were amplified by PCR with genomic *M. tuberculosis* Erdman DNA. For directional cloning, a forward primer, 5′-TCCCCC GGGACGCGTGGCGCTGAT-3′, containing an *Sma*I restriction site and a reverse primer, 5′-GGACTAGTCTACAG GTCCACCCCGAGCAG-3′, containing an *Spe*I restriction site were used. The PCR fragment was cloned into pCR 2.1 (Invitrogen), and the fragment's sequence was confirmed by restriction analysis and sequencing. The *Sma*I/*Spe*I fragment containing the *mshC* gene was then cloned between the *Nru*I and *Spe*I sites within the *aph* gene in pCV125. This process resulted in a vector (pCV125::*mshC*) containing a copy of the *mshC* gene that is transcribed from the *aph* promoter. pCV125::*mshC* DNA was introduced into wild-type *M. tuberculosis* Erdman by electroporation with selection on Middlebrook 7H11 plates containing streptomycin (30 μg/ml). Streptomycin-resistant colonies were grown, chromosomal DNA was extracted, and the presence of two copies of the *mshC* gene was confirmed by Southern hybridization. One of the 2X-*mshC* clones was named Mtb1682 and was used in further experiments.

To assess the effectiveness of pCV125::*mshC* in providing a functioning MshC protein during MSH biosynthesis, pCV125::*mshC* was introduced into an *MshC* chemical mutant of *M. smegmatis* (strain I64) which produces about 1% of the parental level of MSH (13). Incorporation of the *M. tuberculosis mshC* gene resulted in the production of 150% of the parental level of MSH in this MSH mutant (results not shown).

In three separate experiments, the *mshC* allelic exchange phage was used to infect either wild-type *M. tuberculosis* Erdman or the 2X-*mshC* strain Mtb1682. As a positive control for the transduction procedures, wild-type *M. tuberculosis* Erdman was infected with the *corA* allelic exchange phage construct. Several hundred hygromycin-resistant clones resulted from these transductions, and each clone was transferred into Middlebrook 7H9 broth supplemented with OADC and containing hygromycin (50 μg/ml) and, for the 2X-*mshC* strain transductants, also streptomycin (30 μg/ml). Approximately one-third of the hygromycin-resistant clones grew sufficiently for further analysis. These clones were tested for homologous recombination within the *mshC* gene (by using Southern hybridization after digestion with *Nco*I- or *Sac*I-digested chromosomal DNA and, as a probe, the PCR fragment from residues 195 to 708 within the *mshC* gene) and for MSH production. With the wild-type *M. tuberculosis* Erdman strain used as the transduction recipient, 0 out of 67 hygromycin-resistant clones had undergone homologous recombination

TABLE 1. Summary of targeted gene disruptions in *M. tuberculosis* Erdman

Gene target	Second copy of gene present before mutagenesis	No. of clones screened	No. of clones with disruption in targeted gene	Clones (%) with disruption in targeted gene
mshC	No	67	0	0
mshC	Yes	12	6	50
corA	No	18	6	33

within the *mshC* gene according to Southern analysis (Table 1). The *Nco*I fragment containing the entire *mshC* gene is predicted to be 3.0 kb, as found for the native strain (Fig. 2). If homologous recombination had occurred, the predicted *Nco*I fragmentation pattern would lack the 3.0-kb fragment and include a 2.4-kb fragment generated from *Nco*I sites at the ends of the allelic exchange substrate. The Southern blots (Fig. 2) revealed that in some of the clones, the *mshC* knockout substrate had been incorporated in a nonhomologous region of the chromosome, while the native copy of the *mshC* gene remained unchanged (Fig. 2, clones 151, 226,182, and 135). Other clones contained only a single copy of the *mshC* gene that was identical to wild-type *M. tuberculosis* (Fig. 2, clones 59, 214, 63, 318, 317, and 331). Thus, by Southern analysis and by MSH analysis (results not shown), no *mshC* mutants were produced when only a single copy of the *mshC* gene was present. In the control for the transduction procedures with the *corA* gene, Southern hybridization determined that 33% of the 18 tested clones had a disruption in the *corA* gene (data not shown). While this result demonstrates that the general methods were valid, it does not allow prediction of the success rate for disruption of the *mshC* gene, which may undergo homologous recombination at a substantially different frequency.

In contrast to the results with wild-type *M. tuberculosis* Erdman, multiple clones containing a disruption in the *mshC* gene were identified when the 2X-*mshC* strain Mtb1682 was used. Although fewer clones were cultured successfully from these transductions, 6 of 12 clones, or 50%, contained a mutated *mshC* gene (Table 1). The disruption of the *mshC* gene was most easily observed in Southern blotting with *Sac*I-digested chromosomal DNA and, as the probe, the PCR fragment from residues 195 to 708 within the *mshC* gene (Fig. 3). *Sac*I cuts within the *mshC* gene, producing two hybridizing pieces from wild-type DNA and four hybridizing fragments from the 2X-*mshC* strain Mtb1682. The predicted *Sac*I fragments from the *M. tuberculosis* H37Rv genome are 9.8 and 8.0 kb (Fig. 3B), as observed in *M. tuberculosis* Erdman (Fig. 3A). The predicted *Sac*I fragmentation pattern for the *mshC* knockout includes a 1.1-kb fragment generated from the *Sac*I site within the hygromycin resistance gene which replaces the 9.8-kb fragment (Fig. 3B). The smaller 8.0-kb *Sac*I fragment is unaffected by the knockout insertion and remains unchanged (Fig. 3AB). In clones 18, 157, 158, and 172, the larger fragment from the native copy of *mshC* is absent and has been replaced by a smaller *mshC* hybridizing fragment (Fig. 3A). In these clones, the two fragments from the second copy of *mshC* remained unchanged. In one instance, the second copy of the *mshC* gene was disrupted, while the native copy of *mshC* remained unchanged (Fig. 3A, clone 3). Clone 49 contains only a nondisrupted copy of *mshC* and resulted from a transduction with wild-type *M. tuberculosis*; it presumably represents an example of spontaneous hygromycin resistance.

The levels of MSH and two precursor molecules in the MSH biosynthetic pathway were analyzed by high-pressure liquid

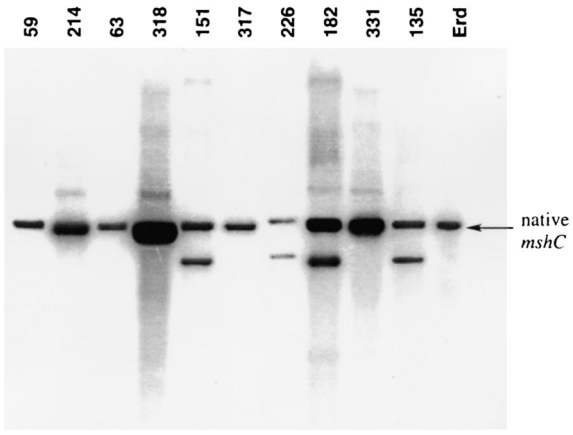

FIG. 2. Southern blot of chromosomal DNA digested with *Nco*I and probed with a 500-bp PCR fragment containing residues 195 to 708 from within the *mshC* gene. Erd, *M. tuberculosis* Erdman. Clones 59, 214, 63, 318, 317, and 331 have a single copy of *mshC* identical to that of the wild-type strain. Clones 151, 226, 182, and 135 have the native *mshC* intact and a component of the knockout substrate incorporated by nonhomologous recombination.

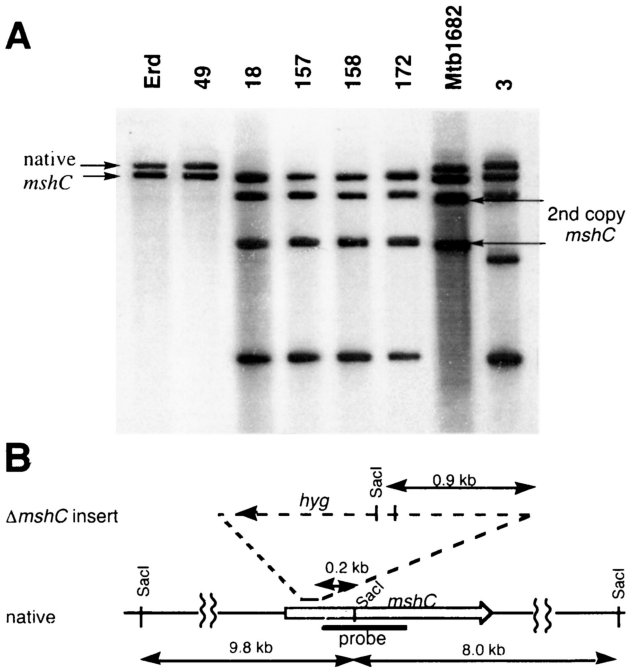

FIG. 3. (A) Southern blot of chromosomal DNA digested with *Sac*I and probed with a 500-bp fragment containing residues 195 to 708 from within the *mshC* gene. Erd, *M. tuberculosis* Erdman. Clone 49 is derived from wild-type *M. tuberculosis* Erdman, and clones 3, 18, 157, 158, and 172 are derived from Mtb1682 (2X-*mshC*). (B) Diagram showing location of *Sac*I sites within the *M. tuberculosis* H37Rv genome near *mshC* and within the insert of the *mshC* knockout.

TABLE 2. Levels of MSH and precursors in *M. tuberculosis mshC* disruption clones

Strain	Optical density (600 nm)	Cellular content (nmol/10^9 cells)[a]		
		GlcNAc-Ins	GlcN-Ins	MSH
Wild-type *M. tuberculosis* Erdman	0.50	1.7 ± 0.7	8.9 ± 0.2	13.7 ± 0.2
Mtb1682 (2X-*mshC*)	0.85	0.9 ± 0.1	3.2 ± 0.1	26.2 ± 1.2
Mtb1682 transformant				
Clone 3	0.41	≤0.71	7.3 ± 0.1	12.1 ± 1.3
Clone 14	0.36	≤1.4	14.1 ± 0.2	12 ± 0.3
Clone 16	0.37	≤0.6	5.7 ± 0.3	14.6 ± 0.1
Clone 18	0.43	≤1.2	12 ± 1	10.2 ± 0.1
Clone 157	0.58	1.5 ± 0.7	12 ± 1.4	10.2 ± 0.1
Clone 158	0.52	1.7 ± 0.4	15 ± 1	11.8 ± 0.1
Clone 172	0.49	1.0 ± 0.5	8.5 ± 0.2	11.3 ± 0.2

[a] Results are expressed as the means and ranges of duplicate samples.

chromatography (4). The MSH contents of all the clones containing a disruption in one of their *mshC* genes were very similar to the value in wild-type *M. tuberculosis* Erdman, 13.7 nmol/10^9 cells (Table 2). This level is in contrast to the reduced level of MSH of 2.9 nmol/10^9 cells previously observed in the *mshB* mutant (4). The levels of two precursor molecules, GlcNAc-Ins and GlcN-Ins, exhibited a wider variation but were generally of the same magnitude as found for the wild-type strain. Interestingly, the MSH level of Mtb1682 (2X-*mshC*) was nearly twice the level of the wild type, and the level of the immediate substrate of MshC, GlcN-Ins, was reduced by 64% from the wild-type level. This result may reflect the presence of two copies of *mshC* in Mtb1682 and is consistent with ligase substrate depletion due to a higher level of cellular MshC activity.

The only plausible explanation for the failure to detect any *mshC* gene knockouts among 67 clones from the transduction of native *M. tuberculosis* is that the *mshC* gene is essential. The rates of production of spontaneous hygromycin-resistant mutants and of illegitimate recombination mutants in the native strain and in the 2X-*mshC* strain are expected to be the same. For the 2X-*mshC* strain, the results show that the ratio of native *mshC* gene knockouts to spontaneous resistance plus illegitimate recombination mutants is 6:5, with one additional mutant (Fig. 3, clone 3) representing a disruption of the second copy of *mshC*. We would therefore expect that about half (6/11) of the clones examined from the transduction of native *M. tuberculosis* would have the *mshC* gene disrupted. The probability of finding 0 such mutants among the 67 clones tested is $\sim 0.5^{67}$, or about 1 in 10^{20}. Thus, we are forced to conclude that no *mshC* knockouts were found because such knockouts were unable to produce MSH and were thus prevented from replicating sufficiently to produce detectible colonies.

In studies with *M. smegmatis*, mutants in the *mshA*, *mshC*, or *mshD* genes produce ≤1% of wild-type levels of MSH during exponential growth (6, 9, 10, 13). In the late stationary phase, the Tn*5*::*mshD* mutant in *M. smegmatis* produces low levels of MSH and thus may have a compensatory acetyltransferase activity for MshD (T. Koledin, G. L. Newton, and R. C. Fahey, unpublished results). Thus, we consider *mshA* and *mshC* to be the primary genes for testing the essential nature of MSH in *M. tuberculosis*. The successful identification of a disruption in the *mshC* gene only when a second copy of *mshC* was present demonstrates that *mshC* and, by inference, MSH are essential for the growth of *M. tuberculosis*.

The absolute requirement for MSH by *M. tuberculosis* contrasts with observations from experiments with *M. smegmatis*, where we have isolated mutants that lack any detectable MSH. Although the MSH null mutants of *M. smegmatis* often grow poorly, these combined observations indicate that *M. tuberculosis* relies to a greater extent than *M. smegmatis* upon the detoxification activity of MSH for growth during normal metabolism. One key difference between these organisms is their growth rate, the doubling time for *M. tuberculosis* being ca. sixfold longer than that for *M. smegmatis*. It is conceivable that the rate of lethal oxidative damage is such that most *M. smegmatis* cells can replicate without incurring lethal damage sufficient to prevent normal growth but that for *M. tuberculosis* the ca. sixfold increase in damage per cell division is sufficient to prevent growth. Alternatively, *M. smegmatis* may have additional protective mechanisms, absent in *M. tuberculosis*, which supplement the role played by MSH. When the fully annotated genome for *M. smegmatis* becomes available, it may be possible to identify differences that could be responsible for this divergent dependence upon MSH.

M. smegmatis mutants devoid of MSH are much more sensitive to peroxide than the parent strain (10, 13), and we would therefore expect that MSH is of critical importance in protecting *M. tuberculosis* from oxidative compounds produced by mammalian host cells during infections. Current studies indicate that dormant (nonreplicating persistent) *M. tuberculosis* cells are metabolically active (16) and, therefore, must maintain a reducing intracellular redox environment. Since MSH and its disulfide reductase form the thiol redox buffer in mycobacteria (7, 12), we postulate that MSH biosynthesis drug targets may be particularly relevant to the treatment of dormant tuberculosis. The present experiments validate the MSH biosynthetic pathway as a source of potential drug targets in the treatment of tuberculosis and suggest that MshC is an especially attractive candidate.

Recently Sassetti et al. (15) reported an assessment of essential genes in *M. tuberculosis* by using transposon site hybridization. Although subject to some uncertainty, this method did identify Rv2130c (*mshC*/*cysS2*) as an essential gene, and the present results confirm that finding.

This research was funded by the National Institutes of Health (grant AI49174). R.C.F. was a consultant for eXegenics Inc. during the initial phase of these studies.

We thank J. S. Cox for the transducing mycobacteriophage strains, Teresa Koledin for technical assistance, and Yossef Av-Gay for helpful discussions.

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