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## ***Mycobacterium tuberculosis* expresses methionine sulfoxide reductases A and B that protect from killing by nitrite and hypochlorite**

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### **Abstract**

Methionine sulfoxide reductases (Msr's) reduce methionine sulfoxide to methionine and protect bacteria against reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI). Many organisms express both MsrA, active against methionine-(*S*)-sulfoxide, and MsrB, active against methionine-(*R*)-sulfoxide. *Mycobacterium tuberculosis* (Mtb) expresses MsrA, which protects  $\Delta msrA$ -*E. coli* from ROI and RNI (St. John *et al.*, 2001). However, the function of MsrA in Mtb has not been defined, and it is unknown whether Mtb expresses MsrB. We identified MsrB as the protein encoded by Rv2674 in Mtb and confirmed the distinct stereospecificities of recombinant Mtb MsrA and MsrB. We generated strains of Mtb deficient in MsrA, MsrB or both and complemented the mutants. Lysates of singly deficient strains displayed half as much Msr activity as wild type against *N*-acetyl methionine sulfoxide. However, in contrast to other bacteria, single mutants were no more vulnerable than wild type to killing by ROI/RNI. Only Mtb lacking both MsrA and MsrB was more readily killed by nitrite or hypochlorite. Thus, MsrA and MsrB contribute to the enzymatic defenses of Mtb against ROI and RNI.

### **Introduction**

ROI are critical to the control of Mtb infection in humans, judging from the high incidence of tuberculosis in people with chronic granulomatous disease, a specific deficiency in phagocyte oxidase (NOX2), the principal source of ROI in macrophages (Bustamante *et al.*, 2007). RNI are critical to the control of Mtb infection in mice, based on the failure of mice lacking nitric oxide synthase 2 (NOS2; iNOS) to restrain replication of Mtb or to survive Mtb infection for more than a few weeks (MacMicking *et al.*, 1997). In addition, the administration of NOS2 inhibitors to clinically stable, Mtb-infected wild type mice leads to rapid progression of infection and early death (MacMicking *et al.*, 1997; Scanga *et al.*, 2001). NOS2 deficiency states have not been identified in humans, but human macrophages from Mtb-infected organs have been repeatedly found to express NOS2 (Nicholson *et al.*, 1996; Wang *et al.*, 1998; Facchetti *et al.*, 1999; Wang and Kuo, 2001; Choi *et al.*, 2002;

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Schon *et al.*, 2004; Samarina, 2005), and killing of mycobacteria in vitro by NOS2-positive human macrophages was blocked by a NOS2 inhibitor (Nozaki *et al.*, 1997). Thus, it is likely that both ROI and RNI contribute to host defense against Mtb.

The persistence of Mtb in many and perhaps most infected people with normal immunity suggests that the microbe has mechanisms to resist ROI and RNI. Many such mechanisms have been identified. Some microbes encode enzymes that break down both ROI and RNI, such as a peroxynitrite reductase and peroxidase comprised of four proteins: alkylhydroperoxide reductase subunit C (AhpC), a thioredoxin-related oxidoreductase called AhpC-neighboring protein D (AhpD), dihydrolipoamide acyltransferase (DlaT) (formerly SucB), and lipoamide dehydrogenase (Lpd) (Bryk *et al.*, 2000; Bryk *et al.*, 2002; Tian *et al.*, 2005). Others help degrade macromolecules (or portions thereof) damaged by RNI, such as UV repair B (Darwin *et al.*, 2003; Darwin *et al.*, 2005) and, presumptively, the proteasomal protease (Darwin *et al.*, 2003; Gandotra *et al.*, 2007) aided by its associated ATPase, Mpa (Darwin *et al.*, 2003; Darwin *et al.*, 2005). Still another class of enzymatic defense of Mtb proteins against ROI and RNI can be postulated: the reversal of specific, potentially harmful oxidations by reduction. To our knowledge, the only precedent for this in Mtb lies in the cyclic oxidation and reduction of antioxidant enzymes themselves as part of their normal reaction mechanism.

Within proteins, the atom most susceptible to oxidation is sulfur, both in cysteine and methionine residues (Weissbach *et al.*, 2002). Exposure to ROI leads to disulfide bonding, the formation of higher oxides of cysteinyl sulfur (sulfenes, sulfines and sulfones), and the generation of methionine sulfoxide. Less well appreciated is that aerobic exposure of bacteria to nitrite or S-nitrosothiols can also lead to disulfide formation (Rhee *et al.*, 2005) and methionine oxidation, most likely via generation of peroxynitrite (St John *et al.*, 2001).

The oxidation of methionine to methionine sulfoxide, whether in proteins or as a free amino acid, results in the formation of equimolar amounts of its two epimers, methionine-(S)-sulfoxide and methionine-(R)-sulfoxide (Weissbach *et al.*, 2002). Many prokaryotes and eukaryotes express a family of methionine sulfoxide reductases (Msr), whose function is to reduce methionine sulfoxide to methionine using thioredoxin, thioredoxin reductase and NADPH as the reducing system (Boschi-Muller *et al.*, 2005). It has been proposed (Levine *et al.*, 1996) that the reversible oxidation of methionine acts as a sink for oxidants that might otherwise carry out less readily reversible reactions. Most organisms express at least one MsrA active on both free and peptidyl methionine-(S)-sulfoxide and one or more MsrBs active on peptidyl (but not free) methionine-(R)-sulfoxide. Additional Msr isoforms or activities have been described, some of which vary in subcellular localization and/or utilization of free and peptidyl methionine sulfoxide (Singh and Moskovitz, 2003; Lin *et al.*, 2007).

Deficiency of one Msr was sufficient to render *E. coli* (St John *et al.*, 2001), *S. aureus* (Singh and Moskovitz, 2003), *Helicobacter pylori* (Alamuri and Maier, 2004), and *Mycobacterium smegmatis* (Douglas *et al.*, 2004) hypersusceptible to killing by ROI, and in some cases, to RNI. Strains of *H. pylori* (Alamuri and Maier, 2004) and *M. smegmatis* (Nino, 2007) deficient in both MsrA and MsrB had little additional phenotype. There have been no reports in which it was necessary to delete both *msrA* and *msrB* in one bacterium in order to observe such a phenotype.

Msrs from Mtb have only recently come under study. Recombinant Mtb MsrA was shown to reduce *N*-acetyl methionine sulfoxide (St John *et al.*, 2001), and its crystal structure has been solved (Taylor *et al.*, 2003). However, the generation of a strain of Mtb deficient in *msrA* has not been reported, and Mtb MsrB has not yet been identified or characterized. In this

work, we have identified Mtb MsrB as the product of the gene Rv2674, confirmed the stereospecificity of Mtb's MsrA and MsrB as recombinant proteins and generated single and double deletion mutants of Msr A and Msr B.

## Results

### Identification of Mtb MsrB and construction of Msr mutants

Rv2674 is a 411 bp gene annotated as encoding a conserved hypothetical protein. We performed a multiple sequence alignment against MsrB proteins from *E. coli* (Grimaud *et al.*, 2001) and *N. gonorrhoeae* (Skaar *et al.*, 2002) (both of which have been expressed and proven to have Msr activity) as well as putative MsrB sequences from numerous other bacteria (Fig. 1). Rv2674 is 87% identical in amino acid sequence to MsrB from *M. smegmatis* and 64% identical to MsrB from *E. coli* and includes two conserved cysteine residues (Cys 67 and Cys 123) as well as adjacent clusters of residues that form the presumptive active site (Lowther *et al.*, 2002).

The Rv2674 (*msrB*) single knockout mutant (H37Rv $\Delta$ *msrB*) was constructed in Mtb strain H37Rv by single-step homologous recombination using a specialized transducing phage system. Replacement of the gene by a hygromycin resistance cassette was verified by Southern blotting (Fig. 2A-B). The loss of protein expression was confirmed by Western blotting (Fig. 2C). Although Mtb *msrA* has been cloned (St John *et al.*, 2001), the knockout has not been reported. Accordingly, the *msrA* single knockout mutant (H37Rv $\Delta$ *msrA*) was constructed in Mtb strain H37Rv and confirmed by the same approaches (Fig. 3A-B). The loss of protein expression was confirmed by Western blotting (Fig. 3C).

A double knockout mutant lacking both *msrA* and *msrB* (H37Rv $\Delta$ *msrAB*) was subsequently constructed in H37Rv $\Delta$ *msrA* by single-step homologous recombination to replace *msrB* with a streptomycin resistance cassette. Deletion of *msrB* in H37Rv $\Delta$ *msrA* was confirmed by both Southern and Western blotting (Fig. 3D-E). Transformation of H37Rv $\Delta$ *msrAB* with an integrative plasmid containing both the *msrA* and *msrB* genes and their native promoters (pMV306-*msrA/msrB*) was successful in restoring expression of both proteins to approximately wild type levels (Fig. 3F-G).

### Biochemical characterization of Msr proteins and mutants

We expressed and purified recombinant MsrB from Mtb and compared it to the MsrA previously purified (St John *et al.*, 2001). MsrA was active against the *S* epimer of free methionine sulfoxide (but not the *R* epimer) with Michaelis-Menten kinetics, with a  $K_m$  of  $4.3 \pm 0.6$  mM and a  $V_{max}$  of  $2.4 \pm 0.1$  sec<sup>m=-1</sup> ( $V_{max}/K_m$   $558 \pm 81$  M<sup>-1</sup> s<sup>-1</sup>) (Fig. 4A). This is comparable to a  $K_m$  of 1.9 mM reported for MsrA from *E. coli* (Boschi-Muller *et al.*, 2005). MsrB displayed no activity against either epimer of free methionine sulfoxide either in the presence or absence of 10  $\mu$ M zinc (data not shown). MsrA and MsrB each displayed activity against *N*-acetyl methionine sulfoxide, used as a surrogate for peptidyl methionine sulfoxide, in a stereospecific manner. Thus, MsrA reduced *N*-acetyl methionine-(*S*)-sulfoxide with a  $K_m$  value of  $1.6 \pm 0.4$  mM and a  $V_{max}$  of  $2.6 \pm 0.2$  s<sup>-1</sup> ( $V_{max}/K_m$   $1625 \pm 425$  M<sup>-1</sup> s<sup>-1</sup>), while MsrB reduced *N*-acetyl methionine-(*R*)-sulfoxide with a  $K_m$  of  $1.7 \pm 0.6$  mM and a  $V_{max}$  of  $0.8 \pm 0.1$  s<sup>-1</sup> ( $V_{max}/K_m$   $470.6 \pm 176$  M<sup>-1</sup> s<sup>-1</sup>) (Fig. 4A). The stereospecificity of MsrA and MsrB for peptidyl methionine sulfoxide was confirmed using the oxidized Met(R,S)O form of the opioid pentapeptide Met-enkephalin as a substrate (Supplemental Figure 1).

To determine if native MsrA and MsrB also functioned to reduce oxidized methionine and if they accounted for all detectable Msr activity in Mtb lysates, we tested lysates from wild type and mutant strains for activity against *N*-acetyl methionine sulfoxide. The single

knockout strains had intermediate levels of Msr activity, while activity was undetectable in the strain deficient in both MsrA and MsrB. Transformation of the double knockout strain with *msrA* and *msrB* restored Msr activity to approximately wild type levels (Fig. 4B).

### In vitro growth and susceptibility of Msr mutants to oxidative and nitrosative stresses

H37Rv $\Delta$ *msrB* displayed a rate of growth indistinguishable from wild type (not shown). The growth of H37Rv $\Delta$ *msrA* displayed a mild lag, while H37Rv $\Delta$ *msrAB* exhibited a greater lag in growth than H37Rv $\Delta$ *msrA*. This defect was fully corrected in the complemented strain (H37Rv $\Delta$ *msrAB*-pMV306:*msrAB*) (Fig. 5A). All strains exhibited similar post-lag growth rates, grew to the same final optical density, and upon plating, yielded approximately the same number of colonies (not shown). The minimal growth phenotypes suggested that the mutants were not severely compromised under standard growth conditions.

Next, we tested the resistance of the mutant strains to oxidant stresses. Hypochlorite is thought to preferentially oxidize methionine residues in proteins (Vogt, 1995). For H37Rv $\Delta$ *msrA*, the minimal inhibitory concentration (MIC) of sodium hypochlorite (NaOCl) was identical to that of the wild type (0.094%). Deficiency of *msrB* alone caused a two-fold decrease in MIC to NaOCl. Lack of both *msrA* and *msrB* caused further sensitivity, with a four-fold decrease in MIC compared to wild type (Table 1). Nonetheless, none of the knockout strains displayed increased sensitivity to H<sub>2</sub>O<sub>2</sub> or cumene hydroperoxide (Fig. 5B and data not shown). To test if hypersusceptibility of H37Rv $\Delta$ *msrAB* to hypochlorite was associated with accumulation of peptidyl methionine sulfoxide, we exposed Mtb strains to NaOCl for 15 minutes, washed them and allowed 2 hours for recovery before preparing lysates and quantifying the amount of peptidyl methionine sulfoxide. In keeping with the MIC data, we observed a significant increase in methionine sulfoxide levels only in proteins from the double knockout strain relative to the wild type and complemented strains (Figure 6).

To address the hypothesis that Msr enzymes protect Mtb against nitrosative stress, we exposed wild type and mutant strains to acidified nitrite. H37Rv $\Delta$ *msrAB* was killed to a 40-fold greater extent by 3 mM nitrite (pH 5.5) than wild type H37Rv, while sensitivity of the single knockout strains was similar to wild type. Complementation of *msrA* and *msrB* restored the sensitivity of the double knockout strain to wild type levels (Fig. 5C).

In order to study the in vivo effect of the double mutation, groups of five C57BL/6 mice per strain were infected by inhalation. The number of colony forming units recovered from the lungs 21 weeks later was 0.75 log<sub>10</sub> lower for the Mtb H37Rv $\Delta$ *msrAB* than for wild type H37Rv, but this phenotype was not complemented (Fig. 7). Failure of complementation was not due to loss of the plasmid, because Mtb grown from the lungs of mice infected for 21 weeks with the complemented strain were fully Msr-positive by western blot (data not shown). Thus we can draw no conclusions about the role of MsrA and B in the pathogenesis of Mtb in the mouse.

## Discussion

This work establishes that Mtb, like many other organisms, expresses at least two Msrs, each stereospecific for one of the epimers of methionine sulfoxide. Unlike in other organisms, the collective contribution of MsrA and MsrB to the resistance of Mtb to ROI is limited, in that their dual deficiency increased sensitivity to ROI only four-fold and only to a very strong oxidant, hypochlorite, but not to hydrogen peroxide or an organic peroxide. Deficiency of MsrB alone only mildly increased sensitivity to hypochlorite and the MsrA-deficient strain was indistinguishable in this respect from wild type. The ability of MsrA and MsrB to protect Mtb from hypochlorite may help the organism resist being killed by

myeloperoxidase-expressing cells, such as neutrophils and monocytes (Borelli *et al.*, 1999). The lack of sensitivity of Msr-deficient Mtb to cumene hydroperoxide is in contrast to the marked vulnerability to this oxidant exhibited by MsrA-deficient *Mycobacterium smegmatis* (Douglas *et al.*, 2004). Differences between the antioxidant defenses of Mtb and non-pathogenic mycobacteria are not without precedent (Lama *et al.*, 2006; Stewart, 2000) and may reflect adaptations to the different levels of oxidative stress faced by the organisms or difficulties in using gene disruption to evaluate roles of pathways that are to some extent redundant. The same explanation may account for the greater sensitivity of MsrA-deficient *E. coli* to oxidants, which was rescued by complementation with *msrA* from Mtb (St John *et al.*, 2001). In contrast to other organisms (including *E. coli* and *M. smegmatis*), Mtb persists inside phagosomes which are highly oxidative and nitrosative environments (Schnappinger *et al.*, 2003). Thus, the lack of impact of Msr deficiency on susceptibility to peroxides may reflect the diversity and effectiveness of other antioxidant defenses of Mtb. These include catalase (Ng *et al.*, 2004), mycothiol and mycothiol reductase (Buchmeier *et al.*, 2003), several thioredoxins (Jaeger *et al.*, 2004) and the peroxiredoxin system (Bryk *et al.*, 2002). In previous work, neither *msrA* nor *msrB* were induced in Mtb upon exposure to various stresses, including hydrogen peroxide, hypoxia, thermal stress, and nitric oxide (Schnappinger *et al.*, 2003).

However, our data demonstrate that MsrA and MsrB collectively contributed to the defense mechanisms of Mtb against mildly acidified nitrite. This was so notwithstanding that Mtb encodes many other potential defenses against RNI, including those cited in the introduction and a truncated hemoglobin (Pathania *et al.*, 2002). Unexpectedly, only the absence of both MsrA and MsrB resulted in sensitivity to RNI. If RNI such as peroxynitrite oxidize methionine residues with a potentially lethal effect on Mtb, it would be expected that the resulting methionine sulfoxide would be comprised of 50% of each epimer. In Mtb lacking only MsrA, the methionine-(*R*)-sulfoxides should be reduced but the methionine-(*S*)-sulfoxides should accumulate, impacting protein function (Sun *et al.*, 1999). Conversely, in Mtb lacking only MsrB, the methionine-(*S*)-sulfoxides should be reduced but the methionine-(*R*)-sulfoxides should accumulate, again with potentially adverse functional consequences. In both cases, sensitivity to RNI should be greater than in wild type Mtb. That it required deletion of both *msrA* and *msrB* to see this phenotype suggests that the two enzymes are functionally redundant for defence against nitrite, although we have demonstrated that they act on stereochemically distinct substrates. Perhaps a phenotype resulting from deficiency of only one Msr escaped detection under the conditions of our tests. As a related possibility, there may be a threshold of functionally consequential damage resulting from the oxidation of methionine in proteins that was only exceeded in Mtb when both Msr enzymes were deficient.

## Experimental procedures

### Mycobacteria and culture conditions

Wild-type *M. tuberculosis* H37Rv and its mutants were grown at 37°C in Middlebrook 7H9 broth containing 0.2% glycerol, 0.5% bovine serum albumin, 0.05% Tween 80, 0.2% dextrose, and 0.085% sodium chloride (7H9-ADNaCl) or on Middlebrook 7H11 agar containing 10% oleic acid-albumin-dextrose-catalase (7H11-OADC). The antibiotics used were 50 µg/ml hygromycin for the H37RvΔ*msrA* and Δ*msrB* strains and 50 µg/ml hygromycin plus 30 µg/ml streptomycin for the H37RvΔ*msrA*/Δ*msrB* double knockout strain. Kanamycin (30 µg/ml) was added for the H37RvΔ*msrA*/Δ*msrB* double knockout strain that was complemented with pMV306-*msrA/msrB*.

### Sequence alignment of Rv2674 (*msrB*)

Multiple sequence alignment of Rv2674 with the sequences of MsrB proteins from representative bacteria was performed using ClustalW and analyzed using Jalview (Clamp *et al.*, 2004). Coloring of the alignment was performed following the ClustalX scheme for percent consensus for given residues or sets of residues.

### Construction, complementation and confirmation of mutants

H37Rv $\Delta$ *msrA* and H37Rv $\Delta$ *msrB* strains were created by single step homologous recombination using the temperature-sensitive mycobacteriophage phAE87 (Bardarov *et al.*, 2002). The single knockouts of  $\Delta$ *msrA* and  $\Delta$ *msrB* were generated in H37Rv wild type strains obtained from two different sources (designated WT-A [from ATCC] and WT-B [from the Trudeau Institute], respectively) while the double knockout,  $\Delta$ *msrA*/ $\Delta$ *msrB*, was generated in the  $\Delta$ *msrA* background. Flanking regions of *msrA* and *msrB* were amplified from Mtb genomic DNA by PCR. For *msrA*, primer pairs GSJ16 5' TCGGTACCAAGCCGTTTCGCGCCAATGAC and GSJ17 5' GTACTAGACTGTGAGCGCTTCGGAGTTCTC and GSJ18 5' CTGAAGCTTCGTGCGGCATCACCAC and GCJ 19 5' GTA GATCTGCCGGGCACGAAACCAC were used to amplify the upstream and downstream flanking regions, respectively. For *msrB*, primer pairs msrB15+ 5' GTCCACTAGTTTCTCAAACCCCTGCTCGG and msrB16-5' GTCCAAGCTTCGGACAGTTCTAGCTTTGGGC and msrB17+ 5' GTCCTCTAGATATTGCATCAACTCCATTTTCGC and msrB18- GTCCGGTACCGAGCAGTTGGTGCCTCGC were used to amplify the upstream and downstream flanking regions, respectively.

Fragments were cloned into pJSC284 such that they flanked the hygromycin cassette, generating the allelic exchange substrate (AES). For the *msrB* AES, *SpeI* and *HindIII* were used for the upstream amplicon and *KpnI* and *XbaI* for the downstream segment; for the *msrA* AES, *KpnI/XbaI* and *HindIII/BglII* were used. For generation of the  $\Delta$ *msrA*/ $\Delta$ *msrB* strain, a variant of pJSC284 containing a streptomycin cassette in place of hygromycin was used to create the *msrB* AES. The resulting cloning vector was digested with *PacI* and inserted into the *PacI* site of phAE287. After in vitro packaging and selective pressure, cosmids were used to transform *M. smegmatis*. Candidate phages were amplified and screened for the allelic exchange substrate by PCR and by restriction digest. Phage containing the AES was used to infect Mtb (wild type or  $\Delta$ *msrA*) at a multiplicity of infection of 10:1, which was then plated on antibiotic-impregnated agar for selection. Colonies of Mtb were screened by PCR and verification of the knockout of *msrA* or *msrB* (or both) was performed by Southern blotting, using an Amersham ECL kit in accordance with the manufacturer's instructions.

For complementation, the H37Rv $\Delta$ *msrA*/ $\Delta$ *msrB* strain was transformed with the plasmid pMV306 containing the Mtb *msrA* gene and a 351-bp upstream DNA sequence which may contain the promoter of the gene as well as the *msrB* gene and a 250-bp upstream sequence that may contain its promoter (pMV306-*msrA/msrB*). The H37Rv $\Delta$ *msrA*/ $\Delta$ *msrB* strain was grown to mid-log phase, collected by centrifugation, washed three times with 10% glycerol, and transformed by electroporation with pMV306-*msrA/msrB*. Transformants were selected on 7H11-OADC containing 50  $\mu$ g/ml hygromycin plus 30  $\mu$ g/ml kanamycin and streptomycin.

For confirmation of the targeted deletion of *msrB*, genomic DNAs were prepared from wild-type H37Rv and candidate strains, digested with *SmaI*, separated by agarose gel electrophoresis, and transferred to nylon membranes. The membrane was probed with a 425-

bp fragment containing the upstream *msrB* 5' flanking sequence, revealing a 1.1 kbp fragment for wild type Mtb and a 2.7 kbp fragment for the H37Rv $\Delta$ *msrB* mutant. For H37Rv $\Delta$ *msrA*, genomic DNA was digested with *XmnI* and *ApaI* and the membrane was hybridized with an upstream flank probe, revealing a 1.2 kbp fragment for the wild type strain and 3.7 kbp hybridization product for the  $\Delta$ *msrA* mutant.

In addition to Southern blotting, western blotting was used to confirm deletion and complementation, using the anti-MsrA antiserum reported (St John *et al.*, 2001) and anti-MsrB antiserum prepared analogously. Strains were grown to log phase and lysed by bead beating in cold buffer containing 50 mM Tris (pH 8.0) and 1 tablet of complete protease inhibitor cocktail (Roche) per 10 mL of buffer. Proteins were separated by 15% SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 5% low-fat milk, membranes were probed with 1:500 anti-MsrA or 1:1000 anti-MsrB. HRP-conjugated secondary antibodies were used at 1:10000 and blots were developed using the ECL kit from Amersham.

### Expression and purification of recombinant MsrA and MsrB

*MsrB* (Mtb Rv2674) was amplified from Mtb chromosomal DNA using primers PR5 5' GTCCCATATGACGCGCCCAAAGCTAGAAC and PR7 5' GTCCCTCGAGCACGCTACCGGGGACCAGG and cloned into the *NdeI-XhoI* sites of pET30b which adds 2 amino acids to the C-terminus and then a tag containing 6 histidine residues. Mtb *MsrA* was amplified from Mtb chromosomal DNA using primers PR10 5' GTCCCATATGACGAGCAATCAGAAAGCG and PR11 5' GTCCCTCGAGCCCGAGTTCGGGTGAAAGGC and also cloned into the *NdeI-XhoI* of pET30b. The final construct for MsrA expression contains a deletion of the final two amino acids, which did not affect the catalytic activity of the purified protein and was used for these studies. Both recombinant MsrA and MsrB were overexpressed in *E. coli* BL21(DE3) cells after a 4 hour induction with 1 mM IPTG. Approximately 15 mg/L each of MsrA and MsrB were obtained and purified to >95% purity by nickel affinity agarose chromatography.

### Substrate preparation

The 2 epimers of methionine sulfoxide were separated from free L-methionine-(*R,S*)-sulfoxide (Sigma) by a reported method (Lavine, 1947). Their purity was determined by HPLC; methionine-(*R*)-sulfoxide contained 6% contamination with the *S* epimer, while the *S* epimer contained no detectable *R* epimer.

*N*-acetylation of methionine-(*R,S*)-sulfoxide was based on the published procedure (Vieira Dos Santos *et al.*, 2005). One mmol of methionine sulfoxide was shaken with 4 mL of acetic glacial acid and 4 mL of acetic anhydride for 2 hours at room temperature. The reaction was quenched with 8 mL of water. The dried product was dissolved in water and applied to a Dowex 50H + cation exchange column to remove unacetylated products. The column was washed with 1 mL of water and the eluate combined with the flow through. The solution was lyophilized and the lyophilate dissolved in 500  $\mu$ L of water. We used NMR analysis to confirm acetylation of methionine sulfoxide and an enzymatic assay to determine the concentration of *N*-acetyl methionine sulfoxide. We assumed a 1:1 stoichiometric relationship between the amount of *N*-acetyl methionine sulfoxide added and the amount of *N*-acetyl-Met formed and that the *N*-acetyl methionine sulfoxide was a mixture containing 50% *S* and 50% *R* epimers. A fixed volume of substrate was added to a 150  $\mu$ L reaction mixture containing 50 mM Tris, pH 8.0, 40  $\mu$ M *E. coli* thioredoxin (Trx) ( $K_m = 8.9 \mu$ M  $\pm$  1.4) (Promega), 340 nM *E. coli* thioredoxin reductase (TrxR) (Sigma), 300  $\mu$ M NADPH (Sigma), and 40 nM of MsrA. Reactions were run to completion and the change in absorbance was used to determine the quantity of *N*-acetyl methionine sulfoxide converted

to *N*-acetyl methionine and back-calculations were done to determine the starting concentration of *N*-acetyl methionine sulfoxide. As a control, known concentrations of methionine sulfoxide were used to confirm the validity of the assay.

### Kinetic parameters and enzyme activity in Mtb lysates

Msr activity was measured by following the decrease in absorbance at 340 nm due to oxidation of NADPH at 37 °C. For every mole of methionine produced from *N*-acetyl methionine-(*R,S*)-sulfoxide, one mole of NADPH is oxidized. A typical reaction mixture (150  $\mu$ l) contained 50 mM Tris, pH 8.0, 40  $\mu$ M *E. coli* thioredoxin (Trx) ( $K_m = 8.9 \mu\text{M} \pm 1.4$ ) (Promega), 340 nM *E. coli* thioredoxin reductase (TrxR) (Sigma), 300  $\mu$ M NADPH (Sigma), 40 nM purified recombinant Mtb MsrA or MsrB, and varying concentrations of substrate. Under these conditions, initial rate measurements were proportional to the amount of enzyme added. Initial rate measurements were performed on an Uvikon XL spectrophotometer by monitoring the decrease in absorbance at 340 nm due to oxidation of NADPH ( $\epsilon = 6220 \text{ M}^{-1} \text{ cm}^{-1}$ ). Initial rates were fitted to:  $v = (V_{max} [S]) / (K_m + [S])$ . Background NADPH oxidation was subtracted from the values obtained with methionine-(*R,S*)-sulfoxide.  $V_{max}$  and  $K_m$  values were determined using nonlinear least-squares curve fitting program of SigmaPlot 2002 for Windows, version 8.0. To determine the amount of MsrA and B activity present in Mtb lysates, 200  $\mu$ g of lysate (Bradford assay) and a saturating concentration of *N*-acetyl methionine-(*R,S*)-sulfoxide (20 mM) was added to the reaction mixture.

### Susceptibility of Mtb strains to in vitro stresses

Strains were grown to log phase, centrifuged at 800 rpm for 10 min at room temperature to remove clumps, diluted in medium and exposed to 1-10 mM  $\text{H}_2\text{O}_2$  for 1-24 hours, followed by plating of serial dilutions on 7H11 agar. Alternatively, strains were grown to log phase, then washed with 7H9 medium that had been acidified to pH 5.5 with HCl, depleted of clumps as above, and diluted in 7H9-ADNaCl (pH 5.5) with or without 3.0 mM  $\text{NaNO}_2$ . Strains were then incubated at 37 °C for 4 days, serially diluted and plated. CFU were counted after 3 weeks. Where indicated, strains were grown to log phase, diluted in 7H9-ADNaCl to an optical density of 0.01 in a 96-well plate and exposed to serial (1:1) dilutions of sodium hypochlorite (0.00075%-0.375%) in medium. After 2-3 weeks, the lowest concentration of NaOCl that completely inhibited growth (measured by optical density at 600 nm) was recorded.

### Quantitation of peptidyl methionine sulfoxide from Mtb exposed to sodium hypochlorite

The various strains of Mtb were grown in 7H9 medium to to late log phase. The cultures were centrifuged and washed twice with PBS plus 0.02% Tyloxapol and resuspended in 10 mL of PBS plus 0.02% Tyloxapol to a final OD of 1 in the absence or presence of 250  $\mu$ M NaOCl. Treated and untreated cultures were incubated for 15 min at 37°C with shaking. Cold PBS (5mL) was added to each culture to dilute the NaOCl and the cultures were then centrifuged, washed twice with 5 mL of 7H9 medium, resuspended in 10 mL of 7H9 medium and allowed to recover for 2 hours at 37 °C with shaking. Cell lysates were prepared as previously described, but in the absence of protease inhibitor. An aliquot containing about 100  $\mu$ g of protein was dried in a vacuum centrifuge and the residue incubated for 1 hr at 80°C in a solution containing 0.1M CNBr in 70% formic acid. A duplicate sample was removed which was not subjected to CNBr treatment. All samples were hydrolyzed in vacuo in 6N HCl containing 1mM mercaptoethanol, for 24 hrs at 110°C, dried by vacuum centrifugation and the amino acids analyzed on a Beckman model 7300 amino acid analyzer. Under these conditions any methionine sulfoxide in the sample will be quantitatively converted to methionine during the hydrolysis. Because CNBr destroys only methionine, any methionine sulfoxide in the sample will be determined as methionine. The



duplicate tube that did not contain CNBr will yield the total methionine content of the sample. Thus % Met(O) is calculated as the methionine recovered after CNBr/total methionine. All samples were normalized by the recovery of Glu, Ala, Leu, Tyr and Phe.

### Mouse aerosol infections with wild-type and mutant *M. tuberculosis*

C57BL/6 mice were infected with logarithmic-phase cultures of *M. tuberculosis* by the aerosol route, using a Glas-Col inhalation exposure system (Glas-Col Inc., Terre Haute, IN), as described (Shi and Ehrt, 2006). Briefly, twenty mice were infected per strain of Mtb, and five mice were sampled at each of four time points. Animals were exposed for 40 min to an aerosol produced by nebulizing 5 ml of a bacterial suspension in phosphate-buffered saline at a concentration of  $2 \times 10^7$  bacilli/ml. This resulted in an inoculum of 100 CFU per lung, as determined by plating homogenized lungs onto enriched 7H11 plates at 24 h after the infection.

Mice were sacrificed by inhalation of CO<sub>2</sub> on day 1 and at 3, 9, and 21 weeks after the initial infection. Lungs were aseptically removed and homogenized in 4 ml phosphate-buffered saline containing 0.05% Tween 80. Serial dilutions of the lung homogenates were plated on 7H11-OADC plates. CFU were enumerated after 2 to 3 weeks incubation at 37 °C.

### Statistical analysis

Student's unpaired, two-tailed t test was used to compare results for different strains of Mtb.  $P < 0.05$  was considered significant.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgments

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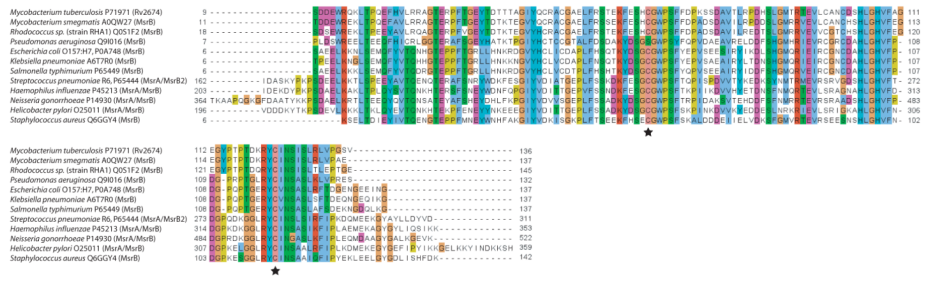
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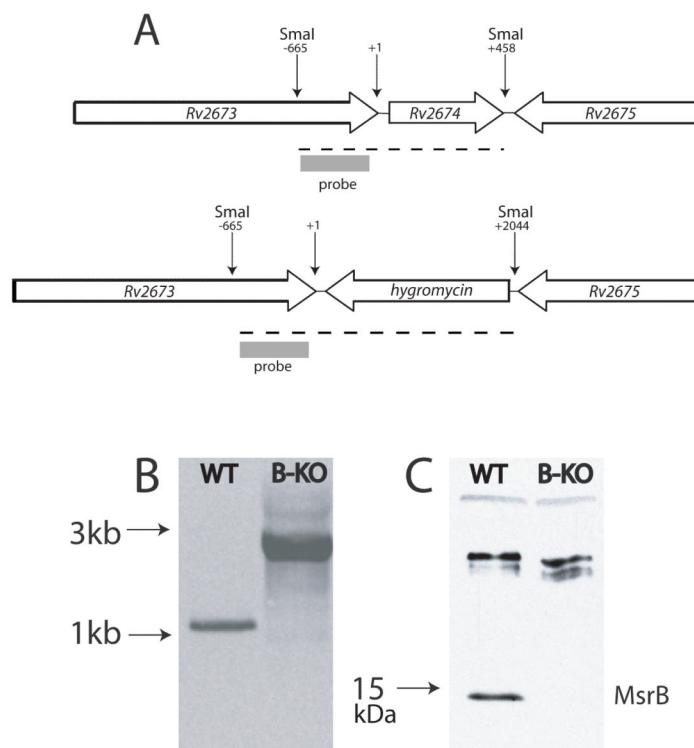
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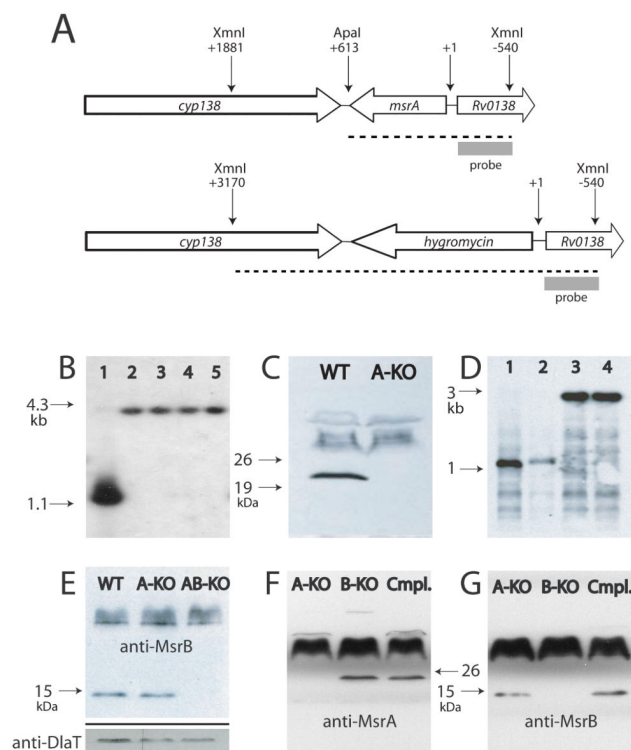
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**Fig. 1.** Partial amino acid sequence alignment of Rv2674 with MsrB proteins from selected bacteria. Alignment was performed with ClustalW and colored following the ClustalX scheme (only partial alignment shown). Stars indicate conserved cysteine residues involved in the reaction mechanism; note the conservation of surrounding residues as well.



**Fig. 2.**  
 Generation of *MsrB*-deficient *Mtb*.  
 A. Genomic context of wild type *msrB* and organization of the disrupted allele. Dashed lines indicate *SmaI* restriction products.  
 B. Southern blot of *SmaI*-restricted DNA from wild type (WT) and *msrB*-deficient (B-KO) strains.  
 C. Western blot of lysates of WT and *MsrB*-deficient strains using rabbit antiserum raised against recombinant *MsrB* (75  $\mu$ g lysate/lane).

**Figure 3.**

Generation of *MsrA*- and *MsrA, B*-deficient *Mtbc*.

A. Genomic context of wild type *msrA* and organization of the disrupted allele. Dashed lines indicate products of restriction with *XmnI* and *ApaI*.

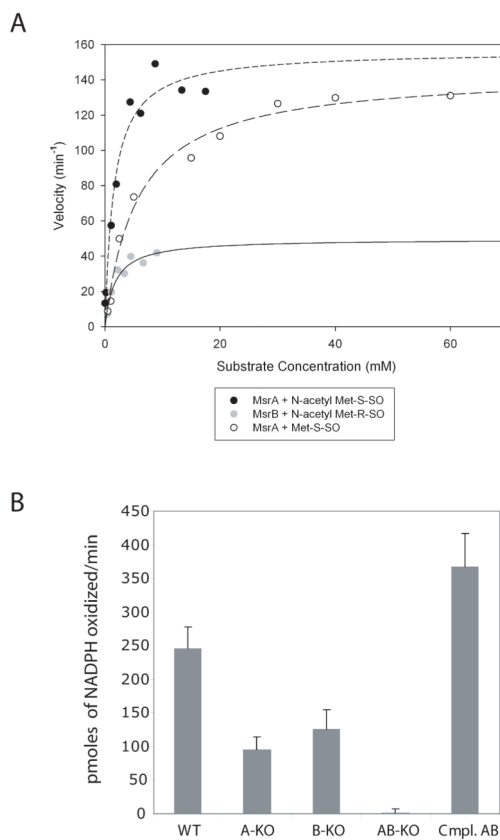
B. Southern blot of DNA restricted with *XmnI* and *ApaI* from wild type (lane 1) and single *msrA*-knockout clones (lanes 2-5).

C. Western blot of lysates of wild type (WT) and an *msrA*-knockout clone (A-KO) (30  $\mu$ g lysate/lane).

D. Southern blot of DNA restricted with *SmaI* from wild type *Mtbc* (lane 1), an *msrA* single knockout (lane 2), an *msrB* single knockout (lane 3), and an *msrA/msrB* double knockout strain (lane 4).

E. Western blot of lysates of wild type (WT), *MsrA*-deficient (A-KO) and *MsrA, B*-deficient (AB-KO) strains using anti-*MsrB* antiserum. Lower panel shows a loading control after the membrane shown in the main panel was stripped and reblotted with antiserum against dihydrolipoamide acyltransferase (50  $\mu$ g lysate/lane).

F., G. Western blots of lysates from *MsrA*-deficient (A-KO), *MsrB*-deficient (B-KO) and the doubly-deficient strain complemented with both wild type alleles (Cmpl.), using anti-*MsrA* (F) or anti-*MsrB* (G) antisera (65  $\mu$ g lysate/lane).

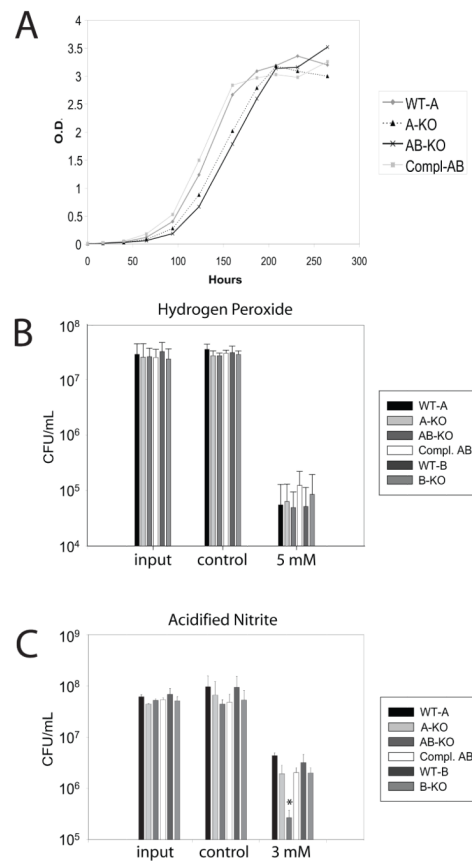
**Fig. 4.**

Enzyme activity of recombinant Msrs and cell lysates.

A. Kinetic analysis of recombinant MsrA and MsrB against peptidyl and free methionine sulfoxide epimers. Results are representative of 3 independent experiments.

B. Activity (pmoles of NADPH oxidized/min) of 200 µg of lysate from indicated strains against *N*-acetyl methionine-(*R,S*)-sulfoxide. Cmpl. AB refers to the strain deficient in both *msrA* and *msrB* after complementation with both genes. Results are the mean ± standard error from 3 independent experiments.



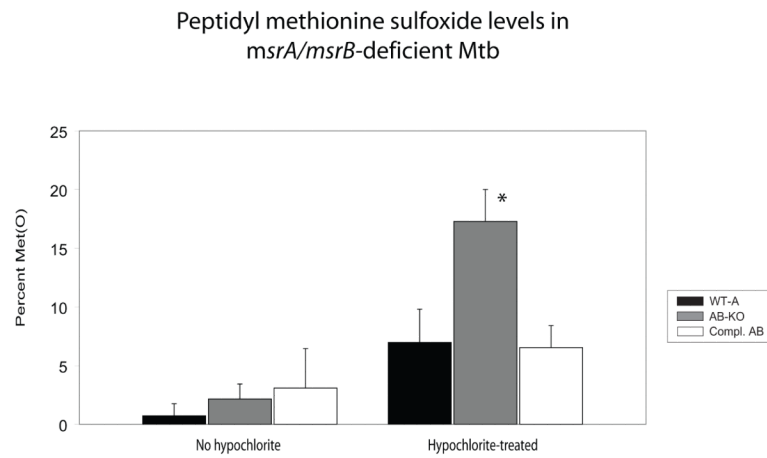
**Fig. 5.**

Phenotypes of *Msr*-deficient strains of *Mtb*.

A. Growth curve of indicated strains. Compl. AB refers to the strain deficient in both *msrA* and *msrB* after complementation with both genes. Curve is representative of 3 independent experiments.

B. Indicated strains were exposed to 5 mM H<sub>2</sub>O<sub>2</sub> or medium alone for 24 hours then plated for colony forming units (cfu). Data are means ± standard error from 4 independent experiments.

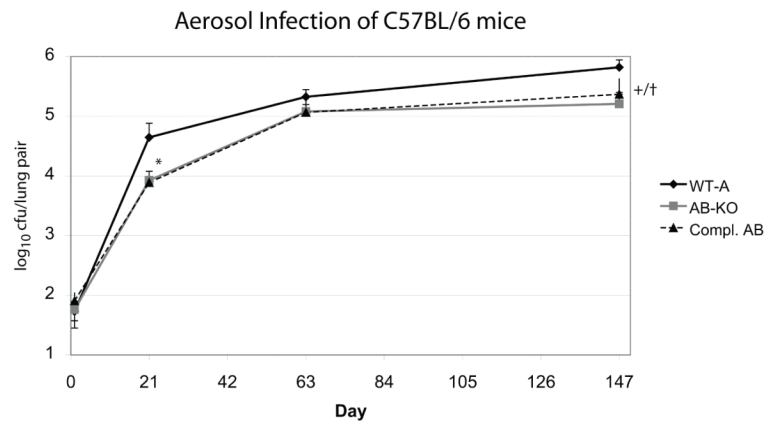
C. Indicated strains were exposed to 3 mM sodium nitrite or medium alone (control) at pH 5.5 for 4 days, then plated for cfu. Data are representative of 3 independent experiments. WT-A represents the wild-type strain from which the  $\Delta msrA$  strain was derived; WT-B represents the wild-type strain from which the  $\Delta msrB$  strain was derived. \**p*<0.01, AB-KO vs. WT-A



**Figure 6.**

Quantitation of methionine sulfoxide in proteins from *Mtb*

Wild-type (WT-A), *MsrA/MsrB*-deficient (AB-KO), and the *MsrA/MsrB*-deficient strain of *Mtb* complemented with both genes (Compl. AB) were exposed to sodium hypochlorite (or vehicle control) for 10 minutes then allowed to recover for 2 hours. Lysates from the different strains were analyzed for Met(O) levels. Values are means and standard deviations from two independent experiments, each in duplicate. \* $p < 0.05$  for comparison of hypochlorite-treated AB-KO and WT strains



**Figure 7.**

Aerosol infection of C57BL/6 mice with MsrA/MsrB-deficient Mtb.

C57BL/6 mice were infected with 100 CFU of wild-type (WT-A, diamonds) Mtb, MsrA/MsrB-deficient (AB-KO, grey squares) Mtb, or the MsrA/MsrB-deficient strain of Mtb complemented with both genes (Compl. AB, triangles). At the indicated times, five mice were sacrificed per group and lung homogenates were plated for CFU. Error bars indicate standard deviations. \* $p < 0.05$  for AB-KO vs. WT-A and for complemented strain (Compl. AB) vs. WT-A; † $p < 0.01$  for AB-KO vs. WT-A, ‡ $p < 0.05$  for complemented strain vs. WT-A

**Table 1**

|           | <b>M.I.C. to NaOCl</b> |
|-----------|------------------------|
| WT-A      | 0.094%                 |
| WT-B      | 0.094%                 |
| A-KO      | 0.094%                 |
| B-KO      | 0.047%                 |
| AB-KO     | <b>0.023%</b>          |
| Compl. AB | 0.094%                 |