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Methionine sulfoxide reductase B (MsrB) of *Mycobacterium smegmatis* plays a limited role in resisting oxidative stress

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summary

Pathogenic mycobacteria including *Mycobacterium tuberculosis* resists phagocyte generated reactive oxygen intermediates (ROI) and this constitutes an important virulence mechanism. We have previously reported, using *Mycobacterium smegmatis* as a model to identify the bacterial components that resist intracellular ROI, that an antioxidant methionine sulfoxide reductase A (MsrA) plays a critical role in this process. In this study, we report the role of methionine sulfoxide reductase B (MsrB) in resistance to ROI by constructing a *msrB* mutant (MSΔ*msrB*) and MsrA/B double mutant (MSΔ*msrA/B*) strains of *M. smegmatis* and testing their survival in unactivated and interferon gamma activated mouse macrophages. While *msrB* mutant exhibited significantly lower intracellular survival than its wild type counterpart, the survival rate seemed to be much higher than *msrA* mutant (MSΔ*msrA*) strain. Further, the *msrB* mutant showed no sensitivity to oxidants in vitro. The *msrA/B* double mutant (MSΔ*msrA/B*), on the other hand, exhibited a phenotype similar to that of *msrA* mutant in terms of both intracellular survival and sensitivity to oxidants. We conclude, therefore, that MsrB of *M. smegmatis* plays only a limited role in resisting intracellular and in vitro ROI.

Keywords

Macrophage; Reactive oxygen intermediates; Infection; Evasion; Mycobacteria

1. Introduction

Mycobacterium tuberculosis and other human pathogenic mycobacteria are well known for their ability to survive and replicate within mononuclear phagocytes, the host defense cells of the body¹. This ability of pathogenic mycobacteria is primarily due to their potential to modulate and defend the antimicrobial responses of phagocytes that include reactive oxygen species (ROS) and reactive nitrogen species (RNS) (collectively called reactive oxygen intermediates [ROI]), generated by NADPH oxidase and iNOS, respectively. However, the mechanisms by which pathogenic mycobacteria and other intracellular bacteria evade ROI still remain elusive, although evidence suggests that bacterial antioxidants like Cu,Zn-

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superoxide dismutase (Cu,Zn-SOD)², catalase-peroxidase³ and other related enzymes⁴ contribute to this process.

Methionine sulfoxide reductases (Msr) are antioxidant repair enzymes that have a role in the detoxification of ROI^{5,6}. Msr catalyze the reduction of oxidized methionine (Met-O) to methionine (Met) in free and protein-bound forms⁵. Although two kinds of Msr namely MsrA and MsrB exist in both prokaryotes and eukaryotes, they share little identity between them either at primary sequence level or at structural level^{7–10}. In the majority of organisms, the genes encoding MsrA and MsrB exist independently of each other. However, a single gene encoding MsrA/MsrB as a fused protein has also been noticed in a few organisms¹¹. It has been reported that MsrA is specific to Met-S-O, while MsrB is specific to Met-R-O^{5,6,9,12}. Further, it has been speculated that methionine residues in proteins serve as sinks for the ROI in the surroundings¹³. The redox cycle involving the chemical oxidation of Met to Met-O and enzymatic reduction of Met-O to Met is considered as an alternate mechanism to protect cells against oxidative stress. Interestingly, both prokaryotic and eukaryotic cells lacking Msr are sensitive to oxidative stress^{11,14,15}. Further, *msrA* has been shown to be critical for the survival of *Erwinia chrysanthemi*¹⁶, *Mycoplasma genitalium*¹⁷ and *Helicobacter pylori*¹⁸ in their hosts.

We have earlier reported that an *msrA* deletion mutant (MSΔ*msrA*) of *Mycobacterium smegmatis* was less able to survive within unactivated and IFN-γ-activated mouse macrophage cell line¹⁹. We have also reported that phagosomes containing *msrA* mutant (MSΔ*msrA*) of *M. smegmatis* acquired p67^{phox} component of phagocyte NADPH oxidase and inducible nitric oxide synthase (iNOS) much earlier than the phagosomes with wild-type *M. smegmatis* strain. Here, we show that MsrB has only a limited role in the intracellular survival of *M. smegmatis*. Further, we demonstrate that *M. smegmatis* deletion mutant lacking both MsrA and MsrB (MSΔ*msrA/B*) exhibits a phenotype similar to that of *msrA* mutant (MSΔ*msrA*) in terms of both intracellular survival and sensitivity towards in vitro oxidative stress. These results differ significantly from *msrA/msrB* mutant (MTΔ*msrA/B*) of *M. tuberculosis* which showed no resistance to hydroperoxides²⁰.

2. Material and methods

2.1. Bacterial strains, media and growth conditions

Escherichia coli strain INV-α (invitrogen) was grown in LB broth or LB Agar plates. *E. coli* harboring plasmid was grown in the presence of ampicillin (100 µg/ml) or kanamycin (25 µg/ml) or hygromycin (100 µg/ml) depending upon the resistance gene in the plasmid. *M. smegmatis* mc² 155 (wild type) was grown in 7H9 broth or 7H10 agar supplemented with 10% albumin dextrose complex (ADC) and 0.05% Tween 80 (TW).

2.2. DNA manipulations and creation of plasmids

Isolation of genomic DNA from mycobacteria, Southern hybridization, radiolabelling and polymerase chain reaction (PCR) were performed as described by Ausubel et al.²¹. Qiaprep kit (Qiagen Inc) was used to isolate plasmid DNA. Oligonucleotides were synthesized at DNA core facility of University of Texas Health Science Center at San Antonio. To construct *msrB* disruption plasmid, we amplified 2.27 kb DNA containing *msrB* and adjacent region from *M. smegmatis* genomic DNA using primers MSMSRB1 (5'-CTGCGGATCTTCGACTACAC-3') and MSMSRB2 (5'-GCCGACTTCATGATCTGGAC-3'). The PCR fragment was initially cloned in pCR2.1 vector, cleaved as EcoRI fragment and cloned into EcoRI site of pUCH (pUC18 plasmid lacking HindIII site). The resulting plasmid was cut with HindIII, blunt ended with klenow treatment and ligated to a blunt-ended 1.9 kb DNA, obtained from pIJ963 by BamHI/PstI

digestion and klenow treated, that has the gene encoding hygromycin resistance. This plasmid pMSMRBK, which has *M. smegmatis* *msrB* interrupted with hygromycin resistance gene, served as the disruption construct for *msrB*. In order to disrupt *msrA* in *msrB* mutant (MSΔ*msrB*) strain of *M. smegmatis*, we created another plasmid construct as follows. First, the plasmid pMSMSRA5¹⁹ was cut with EcoRI to release the DNA fragment containing *M. smegmatis* *msrA* gene disrupted with kanamycin resistance gene. This fragment was ligated to similarly cut p1NIL plasmid²² to create p1MSMSRA1. A DNA fragment that contained *lacZ* and *sacB* genes, from pGOAL17²², was later added to this plasmid at PacI site to obtain plasmid p1MSMSRA2. This plasmid was used to disrupt *msrA* in the *msrB* mutant (MSΔ*msrB*) strain. We have earlier described the construction of pET16b based overexpression plasmid pTBMSRAEX for the expression of *M. tuberculosis* MsrA²³. To generate MsrB overexpression construct, we amplified 585 bp *msrB* gene containing DNA fragment of *M. tuberculosis* by PCR with primers TBMSRBE1 (5'-GGACATATGACGCGCCCAAGCTAGAACTG-3') and TBMSRBE2 (5'-TGGAGCGGATCCGGGCGATTAAGCCGRGGC-3'). In these, primer TBMSRBE1 was designed to have an NdeI restriction site and primer TBMSRBE2 was designed to have a BamHI restriction site. The DNA fragment obtained was cut with *NdeI* and *BamHI* and ligated to a similarly cut pET16b vector to create plasmid pTBMSRBEX.

2.3. Construction of *msr* mutants

For the generation of *msrB* mutant (MSΔ*msrB*), wild-type *M. smegmatis* was electroporated with pMSMRBK and transformants selected on 7H10-ADC-TW plates containing hygromycin (50 µg/ml). MSΔ*msrB* was identified by screening the transformants in Southern using *msrB* and hygromycin resistance gene specific probes. However, for the generation of *msrA/msrB* double mutant (MSΔ*msrA/B*), MSΔ*msrB* strain of *M. smegmatis* was electroporated with plasmid p1MSMSRA2 and transformants obtained were screened by a two-step selection method reported by Parish and Stoker²². In this method, transformants were initially selected in 7H10-ADC-TW plates containing the antibiotic kanamycin (25 µg/ml) and X-gal (40 µg/ml). Blue colonies representing single cross-overs were selected and streaked onto 7H10-ADC-TW plates. After growth, a loopful of bacteria was serially diluted and screened for white colonies on 7H10-ADC-TW plates containing 2% sucrose and X-gal. Chromosomal DNA from the white colonies was later screened for disruption of *msrA* by Southern using *msrA* gene as probe.

2.4. Intracellular survival

Mouse macrophage cell line J774A.1 grown in Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum was used to determine the intracellular survival of *M. smegmatis* strains. Methods for macrophage infection and CFU determination are detailed in our previous paper¹⁷. Briefly, monolayer cultures were established in 24-well plates and infected with *M. smegmatis* strains by incubating the bacteria with macrophages at the ratio of 1:1 at 37°C. Each strain was infected in quadruplicate wells. After 4 h of incubation, the wells were washed with 1 ml of warm DMEM medium to remove the non-phagocytosed bacteria. The plates were incubated at 37°C with 5% CO₂. Cells were harvested at 0, 1 and 3 days of infection by lysing with 0.15% SDS for 15 min at room temperature. The lysed suspensions were serially diluted and plated on 7H10-ADC-TW plates, incubated for three days and colonies counted.

2.5. Survival under oxidative stress

The ability of *msr* mutant strains of *M. smegmatis* to defend against in vitro oxidative stress was tested as described previously¹⁷. Freshly grown *M. smegmatis* strains were diluted to 0.3 OD at 595 nm in 7H10-ADC-TW and 1 ml of each of diluted culture was incubated with either hydrogen peroxide (5 mM) or cumene hydroperoxide (5 mM) or *t*-butyl

hydroperoxide (5 mM) or methyl viologen (5 mM) for 1 h at 37°C. Afterwards, cultures were serially diluted tenfold, plated onto 7H10-ADC-TW plates, incubated at 37°C and colonies counted after 3 days. Experiments with nitric oxide donors like GSNO (5 mM), sodium papanonate (1 mM) were also conducted similarly except the pH of the 7H10-ADC-TW medium was kept at 5.0.

2.6. Overexpression of *M. tuberculosis* Msr in *E. coli* and production of antisera

To overexpress MsrA and MsrB in *E. coli*, we used the T7 promoter-based pET16b overexpression system. This produces fusion proteins with 10 histidine amino acids (His₁₀-tag) at the N-terminus. We have previously described methods to express and purify proteins using this system²⁴, and the constructs for the expression of *M. tuberculosis* MsrA and MsrB are described in the previous section. The constructs were transformed into *E. coli* strain BL21(DE3) and log phase cultures of *E. coli* cells bearing the plasmid constructs were induced with 0.1 mM IPTG to overexpress the MsrA and MsrB proteins. Analysis of *E. coli* proteins extracts in SDS-PAGE 3 h post induction revealed overexpression of 22 kDa and 15 kDa proteins for constructs pTBMSRAEX and pTBMSRBEX, respectively (Fig. 1). The sizes of these proteins corresponded to the expected sizes of 20.4 kDa and 13.3 kDa for MsrA and MsrB, respectively. Using Ni-NTA agarose column, we purified the His₁₀-MsrA and His₁₀-MsrB proteins and these proteins were used to immunize rabbits for the production of antisera according to standard protocols. The antisera reacted with MsrA and MsrB from a wide variety of mycobacteria including *M. smegmatis* (Fig. 2).

2.7. SDS-PAGE and immunoblotting

Standard methods described by Ausubel et al.²¹ were followed for SDS-PAGE and immunoblotting. Protein extracts of *M. smegmatis* strains were obtained by bead-beating technique and total protein content of the extracts was determined by using BCA (bicinchoninic acid) method. Soluble and membrane fractions of *M. smegmatis* were prepared as described previously²⁵. Fifteen percent SDS-PAGE was used to separate the proteins and each lane had 150 µg total protein. SDS-PAGE-separated proteins were transferred to nitrocellulose membranes and probed with anti-*M. tuberculosis* MsrA (anti-MsrA) and anti-*M. tuberculosis* MsrB (anti-MsrB) antisera.

3. Results

3.1. Organization of *msrB* in the chromosome of *M. smegmatis*

A blast search with *Neisseria gonorrhoeae* MsrB sequence²⁶ led to the identification of gene *MSMEG2784* in the *M. smegmatis* genome sequence (J. Craig Venter Institute [JCVI], earlier known as The Institute for Genome Research [TIGR], database) as the gene encoding MsrB of *M. smegmatis*. The product of this gene exhibits extensive homology and identity with a variety of prokaryotic organisms including *msrB* gene of *M. tuberculosis*, for which it showed 87% identity. *msrB* gene of *M. smegmatis* shows complementary strand-based transcription, and analysis of the flanking regions revealed that it is the terminal gene of a cluster of genes showing transcription towards this direction (Fig. 3A). Gene *MSMEG2783* which is upstream to *msrB* shows transcriptional direction that is opposite to *msrB*, hence disruption of *msrB* in *M. smegmatis* is not expected to have any polar effect on adjacent genes.

3.2. Expression of MsrB and its localization

In order to understand the expression of MsrB in *M. smegmatis* and its localization within the cell, we analyzed the whole, soluble and membrane fractions of *M. smegmatis* lysates in immunoblot. Although MsrB protein was readily detectable with anti-MsrB antibodies in the

whole extract and soluble fraction, there was no detectable MsrB protein in the membrane fraction. We also probed these fractions against anti-MsrA antiserum to understand its localization. This also showed a pattern similar to that of MsrB (Fig. 3B). In contrast, *M. tuberculosis* has been reported to have MsrA in both soluble and membrane fractions and MsrB only in the soluble fraction²⁷. The non-membrane association of Msr enzymes in *M. smegmatis* may suggest that they have limited role in the reduction of Met-O on the membrane/surface of the bacterium.

3.3. Construction of *msrB* and *msrA/B* mutants

A *msrB* mutant (MS Δ *msrB*; MS20) strain was constructed through allelic replacement by electroporating the plasmid pMSMSRBK into wild-type *M. smegmatis* and subsequent screening of the hygromycin-resistant transformants by Southern analysis (Figs. 4A,B). The disruption of expression of MsrB in this strain was confirmed by the absence of MsrB using anti-MsrB antiserum (Fig. 5A).

We have earlier reported the disruption of *msrA* (MSMEG6477) gene in *M. smegmatis* through homologous recombination. A *msrA/B* double mutant (MS Δ *msrA/B*) was created by electroporating the plasmid p1MSMSRA2 in MS Δ *msrB* (MS20) and subsequent screening for sucrose-resistant white colonies. Transformant MS441 was identified by Southern analysis as a double mutant that had disruption in both *msrA* and *msrB* (data not shown). Immunoblot analysis showed that this strain lacked the expression of both MsrA and MsrB (Fig. 5A).

In vitro growth of *msr* mutant strains indicated that the growth of MS Δ *msrA/B* strain was slightly lower than MS Δ *msrA*, MS Δ *msrB* and *M. smegmatis* wild-type (MSWt) strains only during the exponential phase (Fig. 5B).

3.4. Intracellular survival of *msr* mutants

Since our goal was to determine the role of MsrB in the intracellular survival, we infected J774A.1 cell line with MSWt, MS Δ *msrA*, MS Δ *msrB* and MS Δ *msrA/B* strains of *M. smegmatis* and examined the survival of these strains within macrophages for 3 days. In the unactivated macrophages the survival of MS Δ *msrB* was considerably lower than that of MSWt 1 day post infection but higher than that of MS Δ *msrA* and MS Δ *msrA/B* strains (Fig. 6). This trend continued even 3 days post infection and the survival of MS Δ *msrB* was noticed to be significantly lower than MSWt. In contrast, MS Δ *msrA* and MS Δ *msrA/B* strains showed almost no viability 3 days post infection. In the IFN- γ -activated macrophages, MSWt and MS Δ *msrB* showed a moderate but more or less similar decline 1 day post infection, while MS Δ *msrA* and MS Δ *msrA/B* strains exhibited no survival at this time point. Although a further decline was noticed with MSWt and MS Δ *msrB* 3 days post infection, they still had some survival at this time point. These results indicate that MsrB protects only moderately against killing by macrophages, although MsrA plays a major role¹⁹. Further, there seems to be no synergistic effect with regard to protection against macrophage killing by MsrA and MsrB, since MS Δ *msrA/B* mutant exhibited a phenotype very much similar to that of MS Δ *msrA* strain. In order to understand whether the observed moderate protection by MS Δ *msrB* strain was really due to lack of MsrB, we determined the survival of *msrB* merodiploid strain (MS19) in macrophages. This showed a phenotype (data not shown) similar to that of MSWt in both unactivated and activated macrophages, indicating that MsrB does play a role in the intracellular survival. Although it would be of interest to check the intracellular survival of complemented MS Δ *msrA/B* strain, the similarity of its phenotype to that of MS Δ *msrA* strain discouraged such an effort. We have earlier reported that complemented MS Δ *msrA* strain had a phenotype similar to that of MSWt¹⁹.

3.5. Sensitivity of *msr* mutant strains for in vitro oxidative stress

Absence of MsrA in bacteria has been reported to lead to hypersensitivity to peroxide, nitric oxide and hydroperoxide stress^{16–19,28–32}. We have previously reported that *MSΔmsrA* strain of *M. smegmatis* was resistant to hydrogen peroxide, superoxide donors and nitric oxide donors but sensitive to hydroperoxides. To understand the role of MsrB in protection against oxidative stress, we determined the susceptibility of *M. smegmatis* mutant strains to different oxidants. *MSΔmsrB* strain showed no sensitivity to hydrogen peroxide, superoxide and nitric oxide stress as noticed for *MSΔmsrA* strain, hence the data are not shown here. Surprisingly, however, *MSΔmsrB* strain also did not exhibit any sensitivity to hydroperoxides (*t*-butyl hydroperoxide and cumene hydroperoxide) for which *MSΔmsrA* strain was highly susceptible (Fig. 7). *MSΔmsrA/B* strain, on the other hand, showed sensitivity similar to that of *MSΔmsrA* strain¹⁹, indicating that sensitivity to hydroperoxides in *MSΔmsrA/B* strain was primarily due to the absence of MsrA.

3.6. Accumulation of oxidized proteins in *msr* mutant strains

Since lack of Msr makes bacterial proteins more susceptible to oxidation at methionine residue^{18,33}, we determined, qualitatively by oxyblot method, if oxidized proteins accumulate in *M. smegmatis msr* mutant strains. Oxidized protein profiles of exponentially growing mutant strains showed only negligible difference from that of oxidized protein profile of MSWt strain (data not shown). However, the intensities of oxidized proteins in *msr* mutant strains were distinctly different from that of MSWt during their stationary phase (Fig. 8). *MSΔmsrA* and *MSΔmsrA/B* displayed strongly oxidized polypeptides in the range of 36–79 kDa. Although *MSΔmsrB* strain displayed a profile similar to that of *MSΔmsrA* and *MSΔmsrA/B* strains, the intensity of the bands in this strain is relatively lower than for other *msr* mutants, indicating indirectly that MsrB plays only a limited role in protecting *M. smegmatis* against oxidative stress.

4. Discussion

Msr is a new family of antioxidant-related enzymes for which the role in defense against oxidative stress has been elucidated in few species^{16–19,28–32}. As mentioned previously, Msr can only detoxify Met residues that are already oxidized by ROI. Thus, Msr is distinct from conventional antioxidants like SOD, peroxidase and alkyl hydroperoxide reductase, which can directly detoxify the ROI. Nevertheless, the fact that absence of Msr makes prokaryotes and eukaryotes hypersensitive to ROI tends to indicate that the Met-O/Msr oxidoreduction mechanism is one of the major pathways to detoxify ROI in living cells.

In this study, we have shown that both MsrA and MsrB of *M. smegmatis* are localized only in the soluble fraction. This is contrary to the observations that were made in *M. tuberculosis*, *N. gonorrhoeae*²⁶ and *H. pylori*¹⁸. Whereas MsrA alone is reported to be present in both soluble and membrane fractions of *M. tuberculosis*, both MsrA and MsrB have been shown to be located in the membrane fractions of *N. gonorrhoeae*²⁶ and *H. pylori*¹⁸ as fused proteins. Whether or not the membrane-bound Msr in these species provides any additional advantages in combating ROI is not clear at present. However, it may be presumed that Msr in the soluble fraction can still be associated with the detoxification of ROI, since ROI from the environment can freely diffuse into cells as evident from in vitro studies¹⁹.

The observation that MsrB plays only a modest role in the survival of *M. smegmatis* within macrophages may indicate that MsrB is not very critical for the detoxification of ROI within macrophages. This is in sharp contrast to the reports that both MsrA and MsrB contribute to the in vivo survival of *H. pylori*¹⁸ and that *Lactobacillus reuteri*³⁴ lacking MsrB is less

efficient in survival inside the mouse gut. Although the reason for this discrepancy is not immediately apparent, one possibility could be the differences in their substrate specificity. Several studies^{5,6,9,12}, including studies on *M. tuberculosis*²⁰, have demonstrated that MsrA is very specific to the reduction of Met-S-O and MsrB is specific to the reduction of Met-R-O. It is, therefore, possible that oxidation of Met with R-epimers in *M. smegmatis* is more infrequent or insignificant as compared to oxidation of Met with S-epimers, thus requiring less or no MsrB to defend intracellular ROI. Since very limited studies describe the significance of *msrB* in the intracellular and in vivo survival, an extensive comparison seems to be difficult at this point. Recently, generation of a *msrB* mutant of *M. tuberculosis* has been reported but its role has been determined neither in intracellular nor in in vivo survival, although a *msrA/B* mutant has been shown to have a log less survival as compared to wild-type strain in a mouse model of infection²⁰.

Our in vitro studies demonstrate that *msrB* mutant (*MSΔmsrB*) is insensitive to any of the oxidants tested and *msrA/B* double mutant (*MSΔmsrA/B*) is sensitive only to hydroperoxides, similar to *msrA* mutant (*MSΔmsrA*) strain. This is consistent with our hypothesis that *M. smegmatis* is relatively resistant to oxidation with Met-R epimer and possibly explains the better survival of this species in the intracellular environment. Similarly, a *M. tuberculosis* strain deficient in MsrB has also been reported to show no sensitivity to ROI. Interestingly, however, *msrA* mutant of *M. tuberculosis* has also been shown to be insensitive to oxidants-mediated ROI. Further, a *msrA/B* double mutant of *M. tuberculosis* has been shown to be sensitive to nitrite- and hypochlorite-mediated ROI. These results are not only in contrast to the observations that were made in *msrA* (*MSΔmsrA*) and *msrA/B* (*MSΔmsrA/B*) mutants of *M. smegmatis* but also with several other bacterial species deficient in MsrA. Existence of redundant pathways to defend against ROI in different species and diversity between species to adapt to different levels of ROI are considered the possible reason for these differences²⁰. This notion draws support from the fact that MsrB of *H. pylori*¹⁸ and *Campylobacter jejuni*³⁵ resist in vitro oxidative stress, although it shows little resistance in mycobacteria.

Moreover, our study reveals that *msrB* mutant (*MSΔmsrB*) strain accumulates relatively lower levels of oxidized protein carbonyls than *msrA* (*MSΔmsrA*) and *msrA/B* (*MSΔmsrA/B*) mutant strains during stationary phase with no added oxidants to the medium. This again reinforces our hypothesis that oxidation of Met with R-epimers is relatively lower. Further, this also provides additional evidence as to why MsrA is more critical in protecting *M. smegmatis* against intracellular ROI than MsrB. Protein carbonyl accumulation has also been noticed in the *msr* mutant strains of *H. pylori* that were exposed to H₂O₂ and GSNO (NO donor) and it has been demonstrated that proteins like catalase, GroEL chaperone and site-specific recombinase are the prominent proteins that undergo oxidation in the *msr* mutant strains of this species^{18,33}. Further, accumulation of oxidized proteins has also been reported from eukaryotic cells deficient in MsrA^{36,37}. It appears, therefore, that some of the oxidized proteins in *msr* mutant strains of *M. smegmatis* may be associated with important physiological functions, and identification of these proteins may provide additional insights into the role of Msr in protection against ROI.

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Fig. 1. SDS-PAGE protein profile showing overexpression and purification of *M. tuberculosis* MsrA and MsrB. Lane 1, Molecular marker. Lanes 2 and 3, extracts of *E. coli* strain BL21 carrying overexpression plasmid pTBMSRAEX in the absence (lane 2) and presence (lane 3) of 0.1 mM IPTG. Lanes 5 and 6, extracts of *E. coli* strain BL21 carrying overexpression plasmid pTBMSRBEX in the absence (lane 5) and presence (lane 6) of 0.1 mM IPTG. Lanes 4 and 7, His₁₀-MsrA and His₁₀-MsrB, respectively after Ni-NTA chromatography.

Fig. 2.

Immunoblot analysis of MsrA and MsrB from different mycobacteria. Whole extracts were prepared from mycobacteria grown in 7H9-ADC-TW medium. Each lane contained about 150 µg of protein. Anti-MsrA antiserum and anti-MsrB antiserum were used at 1:500 dilutions. Peroxidase conjugated anti-rabbit IgG was used as secondary antibody (1:10,000 dilution; Sigma). Bands were visualized by chemiluminescence method (Amersham).

Fig. 3.

(A) Organization of *msrA* and *msrB* in the genome of *M. smegmatis*. Black arrows in the upper and lower panels represent the genes in the flanking regions of *msrA* and *msrB*, respectively. The genes are named according to JVC1 (TIGR) annotations. Open arrows represent *msrA* (MSMEG6477) and *msrB* (MSMEG2784). (B) Immunoblot showing the expression of MsrA and MsrB in *M. smegmatis*. W, whole extract; S, soluble fraction; M, membrane fraction. Anti-MsrA antiserum and anti-MsrB antiserum were used at 1:500 dilutions. Peroxidase conjugated anti rabbit IgG was used as secondary antibody (1:10,000 dilution; Sigma). Bands were visualized by chemiluminescence method (Amersham).

Fig. 4.

(A) Southern analysis of *M. smegmatis* strains. Genomic DNA of *M. smegmatis* wild-type strain (MSWt) and *msrB* mutant (MS20; MSΔ*msrB*) strains were cut with SalI and probed with 1.75 kb hygromycin-resistance gene (*hyg*) and 1 kb *M. smegmatis msrB* gene (*msrB*). (B) Schematic explaining *msrB* locus in the wild-type (WT) and MSΔ*msrB* (MS20) strain. White boxes represent the flanking region of *msrB*, stippled boxes represent the *msrB* gene, black box represents the hygromycin resistance gene. Arrows indicate the direction of transcription of genes. The sites for restriction enzymes in and around *msrB* gene are indicated. The lines below the boxes indicate the expected size of fragments from SalI-cut genomic DNA of WT and MS20 when probed with 1 kb *msrB* gene containing fragment.

Fig. 5.

(A) Immunoblot analysis of *M. smegmatis* strains. MSWt, wild-type *M. smegmatis*; MS97, *msrA* mutant ($MS\Delta msrA$); MS20, *msrB* mutant ($MS\Delta msrB$); MS441, *msrA/B* double mutant ($MS\Delta msrA/B$). Note the absence of MsrA, MsrB and both MsrA and MsrB in $MS\Delta msrA$, $MS\Delta msrB$ and $MS\Delta msrA/B$ strains, respectively. (B) Graph showing the growth of *M. smegmatis* strains in broth culture. MSWt, wild-type *M. smegmatis*; MS97, *msrA* mutant ($MS\Delta msrA$); MS20, *msrB* mutant ($MS\Delta msrB$); MS441, *msrA/B* double mutant ($MS\Delta msrA/B$). Cultures were grown in 7H9-ADC-TW broth for forty hours and their growth was assessed at different time points.

Fig. 6.

Survival of *M. smegmatis* strains in murine macrophage-like J774A.1 cells. Naive (unactivated) IFN- γ -activated macrophages were infected at an MOI of 1:1 for 4 h, washed, lysed at intervals and plated onto 7H10-ADC-TW plates for CFUs. Solid squares, wild-type (MSWt); open squares, *msrA* mutant (MS97; $MS\Delta msrA$); solid circles, *msrB* mutant (MS20; $MS\Delta msrB$); open circles *msrA/B* double mutant (MS441; $MS\Delta msrA/B$). Error bars are not seen for some time points because of the small SE.

Fig. 7.

Survival of *M. smegmatis* after exposure to hydroperoxides. MSWt, wild-type *M. smegmatis*; MS20, *msrB* mutant ($MS\Delta msrB$); MS441, *msrA/B* double mutant ($MS\Delta msrA/B$). Aliquots (1 ml) of culture were exposed to 5 mM H₂O₂, 5mM *t*-butyl hydroperoxide or 5 mM cumene hydroperoxide for 1 h at 37°C. Cultures were serially diluted and plated on 7H10-ADC-TW plates.

Fig. 8.

Oxyblot of *M. smegmatis* strains. MSWt, wild-type *M. smegmatis*; MS97, *msrA* mutant ($MS\Delta msrA$); MS20, *msrB* mutant ($MS\Delta msrB$); MS441, *msrA/B* double mutant ($MS\Delta msrA/B$). 7 μ g of protein extract was loaded in each lane. Protein extracts were treated with DNPH (dinitrophenyl hydrazine) and then with DTT (dithiothreitol) and mercaptoethanol to avoid further oxidation of proteins. Proteins separated on SDS-PAGE and transferred to nitrocellulose were detected with oxyblot protein oxidation detection kit (Chemicon).