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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.

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 body[1]. 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-superoxide dismutase (Cu,Zn-SOD)[2], catalase-peroxidase[3] and other related enzymes[4] 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 forms[5]. 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 organisms[11]. 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 surroundings[13]. 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 line[19]. 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

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limited role in the intracellular survival of *M. smegmatis*. Further, we demonstrate that *M. smegmatis* deletion mutant lacking both MsrA and MsrB (MS∆*msrA/B*) exhibits a phenotype similar to that of *msrA* mutant (MS∆*msrA*) in terms of both intracellular survival and sensitivity towards in vitro oxidative stress. These results differ significantly from *msrA/msrB* mutant (MT∆*msrA/B*) of *M. tuberculosis* which showed no resistance to hydroperoxides[20].

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* mc^{2}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 pMSMSRBK, 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 (MS∆*msrB*) strain of *M. smegmatis*, we created another plasmid construct as follows. First, the plasmid pMSMSRA5[19] 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 plasmid[22] to create p1MSMSRA1. A DNA fragment that contained l*acZ* and *sacB* genes, from pGOAL17[22], was later added to this plasmid at PacI site to obtain plasmid p1MSMSRA2. This plasmid was used to disrupt *msrA* in the *msrB* mutant (MS∆*msrB*) strain. We have earlier described the construction of pET16b based overexpression plasmid pTBMSRAEX for the expression of *M. tuberculosis* MsrA[23]. To generate MsrB overexpression construct, we amplified 585 bp *msrB* gene containing DNA fragment of *M. tuberculosis* by PCR with primers TBMSRBE1 (5′-GGACATATGACGCGCCCAAAGCTAGAACTG-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 *Nde*I and *Bam*HI and ligated to a similarly cut pET16b vector to create plasmid pTBMSRBEX.

2.3. Construction of msr mutants

For the generation of *msrB* mutant (MS∆*msrB*), wild-type *M. smegmatis* was electroporated with pMSMSRBK and transformants selected on 7H10-ADC-TW plates containing hygromycin (50 μg/ml). MS∆*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 (MS∆*msrA/B*), MS∆*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 Stoker[22]. 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 paper[17]. 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 quadraduplicate 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% CO_2. 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 previously[17]. 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 (His_{10}-tag) at the N-terminus. We have previously described methods to express and purify proteins using this system[24], 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).