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OsmC proteins of *Mycobacterium tuberculosis* and *M. smegmatis* protect against organic hydroperoxide stress

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Summary

Bacterial antioxidants play a critical role in the detoxification of endogenously and host derived oxidative radicals during host-pathogen interactions. Recently, the osmotically induced bacterial protein C (OsmC) is included in the antioxidant category of enzymes as it shows structural and functional relationships with organic hydroperoxide reductase (Ohr) enzyme. A copy of the gene encoding OsmC is conserved across mycobacterial species, including *Mycobacterium tuberculosis* (*Rv2923c*) and *M. smegmatis* (*MSMEG2421*), but its role in protecting these species against oxidative stress is unknown. To determine the role of OsmC in mycobacterial oxidative stress, we overexpressed and purified OsmCs of *M. tuberculosis* and *M. smegmatis* and assessed their ability to reduce peroxide substrates like hydrogen peroxide (H₂O₂), cumene hydroperoxide (CHP) and *t*-butyl hydroperoxide (*t*-BHP) in Ferrous Ion Oxidation in Xylenol (FOX) assay. This revealed that OsmCs from both species were capable of reducing both inorganic (H₂O₂) and organic (CHP and *t*-BHP) peroxides. Further, an *M. smegmatis* mutant (*MSΔosmC*) deficient in OsmC exhibited reduced reduction of CHP and *t*-BHP than the parental wild type strain, indicating that OsmC protein contributes significantly for the total peroxide reductase activity of mycobacteria. The *MSΔosmC* strain was also sensitive to organic hydroperoxides, which could be reversed by complementing with a plasmid borne *osmC*. Plasmid borne *osmC* also increased the resistance of *M. smegmatis* wild type strain to isoniazid (INH) but at a relatively lower level than *ahpC*, an organic hydroperoxide reductase. These results suggest that OsmC plays an important role in peroxide metabolism and protecting mycobacteria against oxidative stress.

Keywords

macrophage; oxidative stress; evasion; antioxidants; organic hydroperoxides; Ohr; OsmC; INH; mycobacteria

1. Introduction

The success of *M. tuberculosis* as a formidable pathogen partly depends upon its ability to survive and replicate within mononuclear phagocytes that include macrophages¹. It is well known that phagocytes are the host defense cells and are equipped with antimicrobial

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Competing interests

The authors have no competing interests

responses². One of the mechanisms by which macrophages/phagocytes kill invading pathogen is by the generation of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI)³. Phagocyte ROIs are produced by phagocyte oxidase, which has subunits in the cytoplasm and in the membrane⁴. Phagocytosis of pathogen induces signaling cascades that leads to the assembling of the cytoplasmic subunits of phagocyte oxidase (NOX2/gp91^{phox}) to the subunits on the phagosomal membrane, which converts molecular O₂ to superoxide (O₂^{•-})^{5,6}. This dismutates to become hydrogen peroxide (H₂O₂) and this is further converted to hydroxyl ions (HO[•]), organic hydroperoxides (–OOH[•]) and others by enzymatic and non-enzymatic (inorganic or organic) reactions. RNIs are mainly produced by the inducible nitric oxide synthase (iNOS) by IFN-γ activated phagocytes/macrophages³. iNOS (NOS2) produces nitric oxide (NO), which later gets converted to nitrous acid and nitrite. Importantly, the superoxide generated by the NOX pathway can react with NO produced by iNOS to yield the most potent peroxynitrite (ONOO[•])⁷⁸. In short, both ROI and RNI generated by phagocytes/macrophages are toxic to *M. tuberculosis*, as they damage macromolecules, and how this pathogen inactivates or evades this toxicity by ROI and RNI continues to remain an important area of research.

Although the low permeability of Mycobacterial envelope and surface components like lipoarabinomannan (LAM) and phenolic glycolipid (PGL) resist ROI/RNI to some extent⁹, increasing evidences indicate that antioxidant enzymes of *M. tuberculosis* play critical role in this process. For instance, Piddington et al¹⁰ have shown that *M. tuberculosis* Cu, Zn-superoxide dismutase (Cu, Zn-SOD), also known as SodC and located on the cell envelope¹¹, resists ROI and RNI. They observed that *M. tuberculosis* mutant strain lacking Cu, Zn-SOD was more susceptible to killing by superoxide and nitric oxide, and murine macrophages activated by IFN-γ. This mutant strain was not killed by resting macrophages and macrophages deficient in NOX2, thus providing additional support for the role of Cu, Zn-SOD in the detoxification of ROI/RNI. The other superoxide dismutase of *M. tuberculosis*, also known as SodA and a secreted protein essential for in vitro growth, has also been shown to be important in resisting oxidative stress¹². *M. tuberculosis* deficient in SodA was more susceptible to H₂O₂ in vitro and markedly attenuated for growth in mice. *M. tuberculosis* catalase/peroxidase (KatG), which catalyzes H₂O₂ into H₂O and O₂ and again a secreted protein, also displays a significant role in resisting oxidative stress¹³. Similar to that of *sodA* mutant strain of *M. tuberculosis*, strains lacking KatG showed hypersusceptibility to H₂O₂ in vitro. Further, this mutant strain exhibited usual growth in macrophages of NOX2 deficient mice but failed to grow in macrophages of normal wild type mice and macrophages of iNOS deficient mice, indicating that KatG provides specific protection against ROI but not RNI. In addition, *M. tuberculosis* alkylhydroperoxide reductase subunit C (AhpC) has also been implicated^{14,15}. Initially an *ahpC* deletion mutant of *M. tuberculosis* was shown to have no sensitivity towards peroxides in aerated cultures but was sensitive only at static conditions of growth¹⁴. This discrepancy was attributed to growth related expression of *ahpC*. However, *ahpC* was later linked to nitrosative stress in *M. tuberculosis* and has been shown to function as an NADH peroxidase and peroxynitrite reductase along with alkyl hydroperoxide reductase D (AhpD), dihydrolipoamide acyltransferase (DlaT) and lipoamide dehydrogenase (Lpd)¹⁶.

Several other *M. tuberculosis* components that do not play a direct role in the detoxification of oxidative radicals were also implicated in resistance to ROI and RNI. This includes mycothiol¹⁷, sulfate reductase¹⁸, and methionine sulfoxide reductase (Msr). In *M. tuberculosis*, mycothiol (MSH) is considered an equivalent of glutathione. *M. tuberculosis* mutants with reduced MSH showed increased sensitivity to oxidative stress^{19,20}. Similarly, deletion of *cysH*, which encodes 5'-adenosine phosphosulfate reductase showed increased susceptibility to H₂O₂ and peroxynitrite¹⁸. Further, *M. tuberculosis* has two genes encoding for Msr, *msrA* and *msrB*. Knock out mutants of *M. tuberculosis* lacking both *MsrA* and

MsrB were readily killed by acidified nitrite and hypochlorite but not by H₂O₂ or cumene hydroperoxide (CHP) ²¹. In contrast, *M. smegmatis* that lacks MsrA has been sensitive to CHP but not to nitric oxide donors ²². In addition, biochemical evidences indicate that *M. tuberculosis* truncated hemoglobin ²³ and coenzyme F-420 ²⁴ reduce NO and thereby protect *M. tuberculosis* from nitric oxide stress, although their physiological roles remain to be established.

This study was undertaken to functionally evaluate the role of OsmC proteins of *M. tuberculosis* and *M. smegmatis* in organic hydroperoxide reduction and protection against oxidative stress. OsmC homolog was initially identified in *Escherichia coli* as a protein responding to osmotic stress ²⁵. Recently, this protein has been shown to share structural and functional identity with organic hydroperoxide reductase (Ohr) ²⁶. Also, the OsmC enzyme acts as a thiol-dependent peroxide reductase, like Ohr protein, has experimentally been verified in some bacterial species ^{27, 28}. Currently, OsmC and Ohr are considered as two subfamilies of the Ohr/OsmC superfamily ²⁶. However, the genes encoding OsmC and Ohr have limited occurrence in bacterial species and only few species have genes coding for both enzymes. Bioinformatics analysis revealed that a copy of the gene encoding OsmC is conserved across mycobacterial species, including *M. tuberculosis* (Rv2923c) and *M. smegmatis* (MSMEG 2421), while the gene encoding Ohr is present only in *M. smegmatis* (MSMEG408) and not in other mycobacteria. Here, we focus on the role of OsmC proteins in protection of mycobacteria against oxidative stress.

2. Material and Methods

2.1 Bacterial strains and culture conditions

The bacterial strains and plasmids that were in this study are given in Table 1. Middlebrook 7H9 broth or Middlebrook 7H10 agar plates supplemented with 0.05% Tween 80 (TW), 0.2% glycerol and OADC enrichment (7H9-TW-OADC; for *M. tuberculosis*) or ADC enrichment (7H9-TW-ADC; for *M. smegmatis*) were used for growing the mycobacteria. Mycobacteria harboring plasmids or mutant strains disrupted with antibiotic resistance gene were grown in 7H9-TW-OADC or 7H9-TW-ADC containing the antibiotic kanamycin (25 µg/ml) or hygromycin (50 µg/ml) or both. *Escherichia coli* strain DH5-α (Invitrogen) was used for sub cloning experiments and *E. coli* strain BL-21 was used to overexpress recombinant proteins (Invitrogen). Luria-Bertani (LB) broth or agar with appropriate antibiotics (100 µg/ml ampicillin or 25 µg/ml kanamycin or 100 µg/ml of hygromycin) was used to grow *E. coli*. Growth condition for all bacteria was 37°C.

2.2 DNA manipulations

Plasmids from *E. coli* were isolated using QIAprep Spin kit (Qiagen). Genomic DNA from mycobacterial strains were isolated by using cetyltrimethylammonium bromide as described previously ²⁹. Primers for the amplification of genes (Table 2) and real-time reverse transcription-PCR (RT-PCR) were synthesized at the DNA core facility, The University of Texas Health Science Center at San Antonio (UTHSCSA). The *osmC* region of *M. tuberculosis* was amplified with primers RV2923A and RV2923B (Table 2) and cloned into pCR2.1. The HindIII and XbaI fragment of this plasmid was cloned in plasmid pMV206 ³⁰ cut with similar enzymes. The resultant plasmid pMRV2923 was used to complement *osmC* expression in *MSΔosmC*. This plasmid was also used in multicopy mediated overexpression of OsmC in *M. tuberculosis* and *M. smegmatis* wild type strains. In addition, the *osmC* regions of *M. tuberculosis* and *M. smegmatis* were amplified with primers RV2923EX1 and RV2923EX2, and MSOSMCEX1 and MSOSMCEX2, respectively. The amplified fragments were cloned into pCR2.1 to result in plasmids pMTBOSMCEX and pMSOSMCEX. The NdeI and BamHI cut DNA fragments obtained from these plasmids

were cloned in pET16b to create overexpression plasmids p16MTBOSMCEX and p16MSOSMCEX, respectively.

2.3 Disruption plasmid for *M. smegmatis* *osmC*

We created an *osmC* (*MSMEG2421*) gene deletion in *M. smegmatis* wild type strain through homologous recombination using the plasmid pMSOSMCH. This plasmid was constructed as follows. First, we synthesized four oligonucleotide primers namely MSOSMC1, MSOSMC2, MSOSMC3 and MSOSMC4 (Table 2) based on DNA sequences of *osmC* gene and its adjacent regions. Primers MSOSMC1, MSOSMC2 were designed to amplify the 5' prime region of *osmC* and its upstream 1270 bp fragment (Fragment I); while primers MSOSMC3 and MSOSMC4 were designed to amplify the 3' region of *osmC* and its downstream 864 bp fragment (Fragment II). PCR was performed to amplify the DNA fragments, using these primers and *M. smegmatis* genomic DNA template, and these fragments were cloned into pCR2.1 Vector (Invitrogen) to result in plasmids pMSOSMC1 and pMSOSMC2, respectively. The plasmid pMSOSMC1 was cut with HpaI and HindIII to release fragment I and cloned into similar sites of pMSOSMC2 to generate plasmid pMSOSMC3. This plasmid was then cut with HpaI, and ligated to hygromycin gene fragment from pIJ963 (PstI and BamHI cut and blunt ended by Klenow treatment) to result in plasmid pMSOSMCH.

2.4 Southern hybridization

Southern hybridization was performed to confirm the recombination event at *osmC* locus. For this, we cut the chromosomal DNA from *M. smegmatis* strains with BamHI and PstI and separated the DNA fragments in 1% agarose gels. The DNA fragments were transferred to Zeta probe membranes (Bio-Rad) by Southern blotting. After crosslinking the DNA with UV treatment, membranes were prehybridized for 4 h in a prehybridization solution (50% formamide, 0.12 M Na₂HPO₄, 0.25 M NaCl, 7% (wt/vol) sodium dodecyl sulfate (SDS), and 1 mM EDTA). The membranes were later hybridized with a [α -³²P] dCTP labeled DNA (PCR fragment II, described above). After hybridization, membranes were washed three times (each wash for 15 min) at 42°C with 2X SSC (1X SSC is 0.15 M NaCl and 0.015 M sodium citrate) with 0.1% SDS, 0.5X SSC with 0.1% SDS and 0.1X SSC with 0.1% SDS. The membranes were then exposed to X-ray film for autoradiography.

2.5. Overexpression of *M. tuberculosis* and *M. smegmatis* OsmC in *E. coli*

We have recently described the overexpression of protein in *E. coli* using pET16b based vector and purification of the overexpressed protein with Ni-NTA affinity chromatography³¹. We followed the same procedures to overexpress and purify His₁₀OsmC proteins of *M. tuberculosis* and *M. smegmatis* in this study by using *E. coli* strain BL21 (DE3) harboring the plasmid constructs p16MTBOSMCEX and p16MSOSMCEX. *E. coli* extracts and purified proteins were separated on 12% SDS-PAGE as described²⁹.

2.6. Preparation of crude lysates for enzymatic assay

Cell pellets from *M. smegmatis* strains and *E. coli* DH5 α (OD₆₀₀= 0.6–1.0) were resuspended in phosphate buffer (50 mM, pH 7.4) containing NaCl (50 mM), washed once with the same buffer and disrupted by sonication. Total protein concentration in extracts was determined by standard BCA (Pierce) method.

2.7. Enzyme assay

Peroxidase activity was measured by the colorimetric ferrous ion oxidation in xylenol orange (FOX) assay³². Incubation mixture (50 μ l of total volume) contained the following: Substrate 200 μ M (hydrogen peroxide H₂O₂ or cumene hydroperoxide (CHP) or *t*-butyl

hydroperoxide (*t*-BHP)), 1 mM DTT, and 100 mM potassium phosphate pH 7.0. The reactions were initiated by addition of 2 µg of protein (purified OsmC protein or cell lysate) preincubated with 5 mM dithiothreitol (DTT) for 1 h on ice. After 30 minutes incubation at room temperature, the reaction was stopped by the addition of ice cold Fox reagent and absorbance was measured at 560 nm. The peroxide reduced was calculated by measuring the concentration of peroxide in each reaction (nmole peroxide /ml) using appropriate standard curves.

2.8. Survival under oxidative stress conditions

To understand if *M. smegmatis* *osmC* mutant (*MSΔosmC*) strain was sensitive to peroxides, we assessed the survival of this strain and wild type strain after exposure to peroxide stress. For this one ml each of *M. smegmatis* strains, grown in 7H9-TW-ADC and diluted to OD₆₀₀ of 0.3 in the same medium, was exposed to 5 mM H₂O₂ (hydrogen peroxide), 5 mM *t*-BHP (*t*-butyl hydroperoxide), and 5 mM CHP (cumene hydroperoxide) for 1 h at 37°C. Control cultures were prepared in a similar fashion but without peroxides. Serial dilutions of these cultures were plated onto 7H10-TW-ADC plates. After 3 days of incubation at 37°C, the colonies were counted and the results reported as log₁₀ values.

2.9. Stress response of Rv2923c and MSMEG2421

Stress responses of Rv2923c and MSMEG2421 were studied as described previously³³. *M. tuberculosis* and *M. smegmatis* cultures were exposed for 1 h to determine the effect of each stress condition. Other than physical stresses like heat and cold, which were studied by incubating the cultures at 52°C and 4°C, respectively, stresses by chemicals (H₂O₂, CHP, *t*-BHP, NaCl) and ethanol were studied by incubating the cultures at 37°C.

2.10. Real time RT-PCR

Total RNA from treated and untreated *M. smegmatis* and *M. tuberculosis* strains were isolated using Aurum Total RNA Mini Kit (Bio-Rad). cDNA was synthesized using 50 ng of RNA by reverse transcriptase using iScript cDNA Synthesis Kit (Bio-Rad)

cDNA synthesized from total RNA isolated from *M. smegmatis* and *M. tuberculosis* exposed to different stresses was subjected to real time RT-PCR to determine the transcript level of each gene. Expression of genes was determined using Bio-Rad iQ5 Multicolor Real-Time PCR Detection System. We designed primers (MSOSMCRT3 and MSOSMCRT4; RV2923RT1 and RV2923RT2; Table 2) to amplify approximately 127bp and 130 bp fragments of *M. smegmatis* and *M. tuberculosis* *osmC* genes, respectively. Similarly, we designed primers (MSSIGART1 and MSSIGART2; TBSIGART1 and TBSIGART2; Table 2) to amplify approximately 70 bp and 67 bp fragments of *M. smegmatis* and *M. tuberculosis* *sigA* genes, respectively, which were used to normalize the expression of *osmC* genes from both species. Sso Fast EvaGreen Supermix (Bio-Rad) was used for detection of transcripts in iQ5 Bio-Rad detection system using 40 cycles of 95°C-5 sec, 55°C-10sec, 72°C-5 sec. Threshold cycle or CT values in the exponential phase of amplification were used to determine the transcript levels and CTs obtained with *sigA* was used to normalize the CT values of *osmC* transcripts. Finally, results were expressed as 'Normalized expression of genes'. To rule out the possibility of primer dimers, Melt down curve was performed in the same PCR program with linear increase of 1°C temperature from 55°C to 95°C with a hold for 30 sec per step. Primers for real time RT-PCR were checked for a single peak in the Melt down curve.

2.11. Disk Inhibition assay

Sensitivity of *M. smegmatis* strains to various oxidants and isoniazid (INH) was determined by disk inhibition assay. Briefly, *M. smegmatis* wild type, *MSΔahpC* and *MSΔosmC* strains were grown to OD₆₀₀ of 0.3. Two hundred microliters of each culture was mixed with 3 ml of 7H10 soft agar and plated onto 7H10 plates containing the appropriate antibiotics. Sterile filter paper disks saturated with 10 µl oxidants (200 µM) or INH (1 mg/ml) were placed on to the hardened top agar and incubated at 37°C for three days. The diameters of the zones of growth inhibition were recorded and analyzed statistically.

2.12. Sensitivity to isoniazid

Sensitivity of mycobacteria to isoniazid (INH) was performed as detailed before³⁴. Briefly, mycobacterial cultures were diluted with fresh medium to an OD₆₀₀ of 0.2. This was used to inoculate 3 ml cultures in 7H9-TW-ADC (or OADC) medium supplemented with serial dilutions of INH. The cultures were then incubated at 37°C for growth. *M. smegmatis* growth was assessed after 4 days of incubation and *M. tuberculosis* growth was assessed after three weeks of incubation. MIC was determined as previously described³⁴.

2.13. Statistical analysis

Paired t-test was performed using graphpad software.

3. Results

3.1. Overexpression and purification of OsmC

Both *M. tuberculosis* and *M. smegmatis* possess only a single copy of the gene coding for OsmC (Fig. 1A). To overexpress *osmC*, we constructed plasmids p16MTBOSMCEX and p16MSOSMCEX, respectively for *M. tuberculosis* and *M. smegmatis*, and transformed them in *E. coli* strain BL21. Log phase cultures of the resulting strains induced by IPTG showed overexpression of proteins around 18.0 kDa in SDS-PAGE. The cells sonicated and separated into soluble and insoluble fractions by centrifugation showed that OsmC proteins from both species were retained in the soluble fractions. This was subjected to affinity purification (Ni-NTA) and the purified recombinant OsmC proteins from both species showed a single band in SDS-PAGE. They were designated as His₁₀TBOsmC and His₁₀MSOsmC (Fig. 1B).

3.2. Recombinant OsmC proteins reduce peroxides

One of our goals was to ascertain whether the OsmC proteins of mycobacteria could reduce peroxides. To determine this, we performed FOX colorimetric assay as this has the convenience of using different peroxide substrates with a single reagent. Results obtained with this assay revealed that both His₁₀TBOsmC and His₁₀MSOsmC strongly reduce CHP, *t*-BHP and H₂O₂ in a concentration dependent manner (Fig. 2). Both enzymes showed relatively higher reduction of CHP than other substrates. Although OsmC comes under organic hydroperoxide reductase category, its ability to reduce H₂O₂ is little surprising. However, Ohr and AhpC, which are organic hydroperoxide reductases, have exhibited similar property of reducing H₂O₂ along with other peroxides^{35, 36}, suggesting that it is a general phenomenon for all organic hydroperoxide reductases.

3.3. Deletion of *osmC* gene in *M. smegmatis*

Since the recombinant His₁₀OsmC of both *M. tuberculosis* and *M. smegmatis* exhibited peroxide reductase activities, we wanted to know how significant is the activity of OsmC towards the total organic peroxide reductase activity in these species. In other words, we wanted to know if deletion of *osmC* leads to reduction in total organic peroxide reductase

activity. The other question we had was whether OsmC truly provides any protection against oxidative stress in these species. For these reasons, we sought to delete *osmC* gene from the chromosome of *M. smegmatis* through homologous recombination. We chose *M. smegmatis* because OsmC proteins from *M. smegmatis* and *M. tuberculosis* are highly identical (70%), and genetic manipulation is relatively easier in *M. smegmatis* as compared to *M. tuberculosis*. To generate the deletion mutant, we electroporated the plasmid pMSOSMCH into wild-type *M. smegmatis* and plated the cells onto 7H10-TW-ADC plates for growth. Hygromycin resistant colonies were screened for the mutant in Southern analysis by cutting the genomic DNA by BamHI and PstI and probing it with a labeled DNA fragment containing the *osmC* region. Although we obtained several single crossovers, colony #54 alone showed the expected signals for a double crossover (Fig. 3A). Further, probing of the DNA in Southern with a probe for hygromycin gene and by performing PCR analysis of this region (data not shown), we confirmed that this strain had a deletion in *osmC* gene. We named this strain as *MSΔosmC* and lack of *osmC* expression in this strain was further confirmed by real time RT-PCR (Fig. 3B). In fact, we developed an antiserum against His₁₀TBOsmC to detect the expression of OsmC protein in *M. tuberculosis* and *M. smegmatis* by immunoblot. Although, this serum reacted strongly with His₁₀TBOsmC and His₁₀MSOsmC, it did not detect a band in the whole extracts of both species. It is unclear at present whether this is due to the weakness of the antibody or due to the quick turnover of this protein in mycobacterial cells or due to its growth related expression. It may be recalled that AhpC expression of *M. tuberculosis* occurs only in static culture but not in regular oxygenated cultures¹⁴.

3.4. Peroxide reductase activity of the *osmC* mutant strain

To understand if OsmC contributes for the total organic peroxide reductase activity of mycobacteria, we assayed peroxide reductase using the total protein extracts of *M. smegmatis* wild type strain (*MSWt*), *M. smegmatis osmC* mutant strain (*MSΔosmC*), and *M. smegmatis ahpC* mutant strain (*MSΔahpC*) with different substrates. We also used *E. coli* DH5α strain as control. As shown in Fig. 4, *M. smegmatis* strains showed very little difference in activities between them when H₂O₂ was used as the substrate. However, a significant reduction in peroxide reduction activities was noticed when organic peroxides CHP and *t*-BHP were used as substrates. In comparison to *M. smegmatis* wild type strain, *MSΔahpC* strain showed highly reduced activity for CHP and *t*-BHP, while *MSΔosmC* displayed moderate but significant reduction in activity, particularly for the substrate CHP. This indicates that OsmC contributes to some extent for the total organic peroxide reductase activity of mycobacteria.

3.5. Sensitivity of *osmC* mutant strain for in vitro oxidative stress

To understand the role of *osmC* in defending mycobacteria against oxidative stress, we examined the susceptibility of *MSΔosmC* strain to different oxidative stresses. *MSΔosmC* strain showed no sensitivity to any of the peroxides (H₂O₂, CHP and *t*-BHP) tested in disk inhibition assay (data not shown). However, this strain showed significant inhibition by CHP and *t*-BHP in liquid growth as compared to wild type strain (Fig. 5). In contrast, there was no growth inhibition by these peroxides on *M. smegmatis* strain having a single crossover in *osmC* locus (having functional OsmC) (*MSosmC/SCO*) and on *MSΔosmC* strain complemented with *M. tuberculosis osmC* with a multicopy plasmid (*MS2923*). These results indicate that OsmC protects mycobacteria against oxidative stress.

3.5. Stress response of *osmC*

In bacteria, *ohr* responds to oxidative stress and *osmC* responds to osmotic stress and these genes are regulated by OhrR^{37–39} and RpoS^{25, 40}, respectively. To know whether *osmC* of mycobacteria responds to stress, we investigated the stress response of *osmC* from both *M.*

tuberculosis and *M. smegmatis*. Real time RT-PCR data presented in Fig. 6A and B indicate that *osmC* responds to only few stresses and the response varies between these two species. While *M. smegmatis osmC* responds to physical stresses like ethanol, heat and cold, *M. tuberculosis osmC* responds to H₂O₂ and cold stress. Interestingly, *osmC* from both species showed no response to NaCl stress, which actually creates osmotic stress.

3.6. Sensitivity of *osmC* mutant to isoniazid (INH)

We have previously reported that *MSΔahpC* strain was hypersensitive to the antituberculosis drug isoniazid (INH) and plasmid borne *ahpC* could increase the resistance of wild type *M. smegmatis* to INH^{34, 41}. Since OsmC has functions similar to that of AhpC, we sought to delineate the role of *osmC* in sensitivity or resistance to INH. Results presented in Table 3, show that, *MSΔosmC* has no significant inhibition by INH in Disk inhibition assay, although *MSΔahpC* did show significant inhibition. In contrast, plasmid borne *osmC* increased the resistance of wild type *M. smegmatis* (*MS2923*) slightly than its counterpart, which carries the vector (*MS206*) but less than the level of *M. smegmatis* bearing plasmid borne *ahpC* (*MSahpC*) (Table 3). However, plasmid borne *osmC* showed no such increase in resistance to INH in wild type *M. tuberculosis* (*MTB2923*) as compared to its control (*MTB206*). These results may indicate that *osmC* plays little role in the INH associated peroxide metabolism and there exists a difference between *M. smegmatis* and *M. tuberculosis* for sensitivity to INH.

4. Discussion

In this study, we have demonstrated that purified recombinant His₁₀OsmCs from both *M. tuberculosis* and *M. smegmatis* reduce organic and inorganic peroxides, although their ability to turn them over differs slightly. Similar results were noticed with purified recombinant OsmC proteins of *E. coli*²⁷ and *Thermococcus kodakarensis* KOD1²⁸. Recombinant Ohr proteins from *Pseudomonas aeruginosa*³⁵, *Xylella fastidiosa*⁴², *Mycoplasma gallisepticum*⁴³ have been reported to catalyze similar reactions. It has been proposed that cysteine residues, that are present in the middle and C-terminal portion of OsmC/Ohr, play critical role in peroxide reduction reaction^{35, 42}. These residues are highly conserved in both OsmC and Ohr proteins and involvement of these cysteines in the catalytic reaction is evidenced by mutagenic analysis of Ohr of *P. aeruginosa*³⁵. Further, crystal structures of Ohr and OsmC have shown that these cysteines are ideally positioned for the formation of an intramolecular disulfide bond^{27, 35}. Similar to OsmC/Ohr, peroxiredoxins have two cysteines and they independently act on organic peroxides⁴⁴. Therefore, this reaction is different from that of catalase-peroxidase, superoxide dismutase and glutathione peroxidase, all of which use a prosthetic group or cofactors for their enzymatic activity.

We have shown that *M. smegmatis MSΔosmC* strain has reduced organic peroxide reductase activity and this activity was much lower in *MSΔahpC* strain. These results indicate that OsmC and AhpC play major roles in the detoxification of organic peroxides. However, it is not known at present whether these two enzymes compete for the same substrates inside the cells. Also it is not known whether these two enzymes are localized in the same compartment of the cell. Even if they exist in the same compartment, whether expression of these enzymes occurs concurrently or at different growth phases remains unknown. Thus, any conclusion that relates to the functional relationship between these two enzymes needs an understanding of the points raised above. An earlier study has, however, demonstrated that Ohr, a paralog of OsmC, could functionally complement for AhpC in the bacterium *Xanthomonas campestris*⁴⁵.

The observation that *MSΔosmC* strain is hypersensitive to CHP and *t*-BHP and not to H₂O₂ is consistent with the observation in *osmC* mutant of *E. coli* and *ohr* mutants of *X. campestris*⁴⁶, *P. aeruginosa*⁴⁷, *Bacillus subtilis*⁴⁸, *Agrobacterium tumefaciens*³⁹ and *M. genitalium*⁴⁹. This, together with the fact that complementation of *MSΔosmC* strain with *M. tuberculosis osmC* gene reverses the sensitivity of this strain to organic peroxides suggest that *osmC* does protect against ROI. Yet, whether this in vitro protection of peroxides by OsmC can reflect the in vivo situation is currently uncertain. Our preliminary experiments on macrophage infections with *MSΔosmC* vs *MSWt* showed similar intracellular survival rates (data not shown), suggesting that *osmC* probably plays a less important role in the in vivo survival. Nonetheless, considering the fact that *M. smegmatis* and *M. tuberculosis* differ widely in terms of their intracellular survival, it is necessary to create an *osmC* mutant *M. tuberculosis* strain to explicitly confirm its role in intracellular and in vivo survival. On the other hand, previous studies have indicated that *M. tuberculosis* is more sensitive to RNI than ROI^{8, 50} and AhpC plays critical role in resistance against RNI¹⁵. Given the functional similarity between AhpC and OsmC, it will be interesting to determine if OsmC of *M. tuberculosis* plays any role in protection against RNI.

OsmC of mycobacteria did not respond to osmotic stress and *osmCs* of *M. tuberculosis* and *M. smegmatis* responded differently to physical and oxidative stresses. Particularly *osmC* of *M. tuberculosis* responded fourfold to H₂O₂ but not for CHP and *t*-BHP and *osmC* of *M. smegmatis* did not respond to any of the oxidants is astounding. Although this may be attributed to the cross-talks between stress responses, the normal expectation was the induction of *osmC*, as OsmC is associated with organic peroxide reduction, by organic peroxides. Nevertheless, previous studies have reported that *osmC* of *M. tuberculosis* is down regulated to 2–6 fold by erythromycin, tetracycline and streptomycin⁵¹ and upregulated about fourfold by palmitic acid (Liu and Schoolnik, unpublished result available with TBdatabase (tbd.org)). These results and our own observations in this study tend to suggest that this gene is transcriptionally controlled by some regulator. *osmC* gene in *E. coli* is regulated partially by RpoS^{25, 40} and in *B. subtilis* by RpoS and SigB⁵². Therefore, there is a possibility that *osmC* of mycobacteria is under the control of some sigma factor(s). In contrast, *ohr* gene in most bacteria is regulated by a transcriptional repressor *ohrR*^{37–39}.

In the current study, we obtained contradicting results with respect to INH resistance/sensitivity by OsmC. While it appears that plasmid borne OsmC increases the resistance of *M. smegmatis* to INH a little, no such increase in resistance to INH was noticed with *M. tuberculosis*. The *MSΔosmC* did not display any additional sensitivity to INH either. This situation forces us to speculate that OsmC, although it has peroxide reductase activity, has limited role in INH mediated peroxide metabolism. INH action in mycobacteria requires activation by catalase-peroxidase (KatG) and deletion of *katG* gene or point mutation in *katG* gene leads *M. tuberculosis* to become resistant to INH^{53, 54}. We and others have earlier reported that AhpC expression is upregulated, by point mutations in the promoter region of *ahpC*, in INH resistant strains of *M. tuberculosis*, to compensate for the deficiency of catalase-peroxidase^{34, 41, 55, 56}. Since OsmC performs catalytic functions more or less similar to that of AhpC and it has the ability to reduce H₂O₂ like catalase-peroxidase, it will not be a surprise if *osmC* expression is affected in some of these INH resistant strains.

In conclusion, OsmC is a new peroxide reductase that protects *M. tuberculosis* possibly from organic peroxides toxicity. Its role in intracellular and in vivo survival of this species, and resistance towards INH require additional investigations.

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Fig. 1. *osmC* gene organization and its overexpression. **A.** Schematic showing the organization of *osmC* gene in the genomes of *M. tuberculosis* and *M. smegmatis*. I, *M. tuberculosis*; II, *M. smegmatis*. Arrows represent genes and their orientations. Numbers above the arrows indicate the assigned number for each gene. **B.** SDS-PAGE protein profile showing overexpression and purification of *M. tuberculosis* and *M. smegmatis* OsmC. *E. coli* was grown in LB broth at 37°C, and lysates were prepared by sonication. Lane M, EZ-Run Rec Protein Ladder (Fisher Scientific); lanes 1 and 4, extracts of *E. coli* strain BL21 carrying the overexpression plasmids p16MTBOSMCEX and p16MSOSMCEX before the addition of 1 mM IPTG; lanes 2 and 5, extracts of *E. coli* strain BL21 carrying the overexpression plasmids p16MTBOSMCEX and p16MSOSMCEX after the addition of 1 mM IPTG; lanes 3 and 6, His₁₀TBOsmC and His₁₀MSOsmC after Ni-NTA affinity chromatography. The arrow points to the His₁₀OsmC bands.

Fig. 2.

Reduction of peroxides by His₁₀OsmC proteins in FOX assay. Reduction of peroxides was assayed by FOX assay. **A.** Reduction of peroxides by His₁₀TBOsmC. **B.** Reduction of peroxides by His₁₀MSOsmC. Each reaction contained 200 µM of substrates cumene hydroperoxide (CHP) or *t*-Butyl hydroperoxide (*t*-BHP) or hydrogen peroxide (H₂O₂) and 2 µg of the overexpressed His₁₀OsmC protein.

Fig. 3.

Southern and Real time RT-PCR analyzes of *M. smegmatis* strains. **A.** Southern analysis of genomic DNA. Genomic DNA was digested with BamHI and PstI, separated on 1% agarose gels and transferred to nitrocellulose membranes. Membranes were hybridized with [32 P] dCTP labeled 824 bp DNA fragment containing *osmC* region and signals captured by autoradiography. Arrows indicate the sizes of the signals MSWt, *M. smegmatis* wild type; $\Delta osmC$, *M. smegmatis osmC* mutant (*MS* $\Delta osmC$); SCO, *M. smegmatis osmC* single crossover (*MSosmC/SCO*). **B.** Expression of *osmC* by *M. smegmatis* strains. Determination of transcripts by real time RT-PCR and normalization are detailed in material and methods section. MSWt, *M. smegmatis* wild type; SCO, *M. smegmatis osmC* single crossover (*MSosmC/SCO*); $\Delta osmC$, *M. smegmatis osmC* mutant (*MS* $\Delta osmC$).

Fig. 4.

Reduction of peroxides by crude cell lysates of *M. smegmatis* strains. Reduction of peroxides was assayed by FOX assay. Each reaction contained 200 μ M of the substrates cumene hydroperoxide (CHP) or *t*-Butyl hydroperoxide (*t*-BHP) or hydrogen peroxide (H₂O₂) and 2 μ g of the protein extract. EC, *E. coli* DH5 α strain; MSWt, *M. smegmatis* wild-type strain; Δ osmC, *M. smegmatis* mutant (MS Δ osmC), Δ ahpC, *M. smegmatis* ahpC mutant (MS Δ ahpC). Asterisk indicates that the values are significantly ($P \leq 0.01$) lower than the values of MSWt strain.

Fig. 5.

Survival of *M. smegmatis* after exposure to hydroperoxides. Aliquots (1 ml) of cultures at OD₆₀₀ of 0.6 were exposed to 5 mM hydrogen peroxide (H₂O₂) or 5 mM cumene hydroperoxide (CHP) or 5 mM *t*-butyl hydroperoxide (*t*-BHP) for 1 h at 37°C. The cultures were serially diluted and plated onto 7H10-TW-ADC plates and the colonies counted after 3–4 days of incubation at 37°C. Solid grey bars, untreated cultures; Solid black bars, treated cultures. MSWt, *M. smegmatis* wild-type strain; $\Delta osmC$, *M. smegmatis* mutant (*MS* $\Delta osmC$); SCO, *M. smegmatis* *osmC* single crossover (*MSosmC*/SCO); $\Delta os/c$, *M. smegmatis* *osmC* mutant (*MS* $\Delta osmC$) harboring plasmid pMRV2923 (*MS* $\Delta osmC$ 2923). Asterisk indicates that the values are significantly ($P \leq 0.01$) lower than untreated cultures.

Fig. 6.

Stress response of *osmC* of *M. tuberculosis* and *M. smegmatis*. **A.** Stress of response *osmC* of *M. tuberculosis*; **B.** Stress of response of *osmC* of *M. smegmatis*. Total RNA was isolated after exposure to stress conditions and real time RT-PCR was performed. *M. smegmatis* and *M. tuberculosis osmC* genes transcripts were normalized with their *sigA* genes transcripts. H₂O₂, 5 mM hydrogen peroxide; CHP, 5 mM cumene hydroperoxide; BHP, 5 mM *t*-butyl hydroperoxide; NaCl, 400 mM Sodium chloride; EtOH, 5% Ethanol; heat, 52°C; cold, 4°C.

Table 1**Mycobacterial strains and plasmids used in this study**

Strain or plasmid	Characteristics	Source or reference
Mycobacteria		
<i>M. tuberculosis</i> H37Rv	Wild type	ATCC#27294
MTB206	<i>M. tuberculosis</i> Wild type harboring plasmid pMV206	This study
MTB2923	<i>M. tuberculosis</i> Wild type harboring plasmid pMRV2923	This study
<i>M. smegmatis</i> mc ² 155	Wild type	ATCC#700084
MSΔ <i>osmC</i>	<i>M. smegmatis</i> <i>osmC</i> disrupted <i>hyg</i> ^r with marker	This study
MSΔ <i>ahp</i>	<i>M. smegmatis</i> <i>ahpC</i> disrupted with <i>kam</i> ^r marker	Zhang et al 1996
MS <i>osmC</i> /SCO	<i>M. smegmatis</i> with single crossover at <i>osmC</i> locus	This study
MS206	<i>M. smegmatis</i> harboring plasmid pMV206	This study
MS2923	<i>M. smegmatis</i> harboring plasmid pMRV2923	This study
MS <i>ahpC</i>	<i>M. smegmatis</i> harboring plasmid pMsahpC	This study
Plasmids		
pCR2.1	Ap ^r Km ^r ; TA cloning vector for PCR fragments	Invitrogen
pET16b	Overexpression vector carrying an N- terminal His-Tag	Novagen
pMV206	Mycobacteria replicating vector	Stover et al 1991
pMSOSMC1	pCR2.1 containing <i>osmC</i> PCR fragment I	This study
pMSOSMC2	pCR2.1 containing <i>osmC</i> PCR fragment II	This study
pMSOSMC3	pCR2.1 containing fused <i>osmC</i> PCR fragments I and II	This study
pMSOSMCH	pCR2.1 containing fused <i>osmC</i> PCR fragments with <i>hyg</i> ^r marker	This study
pMSOSMCEX	pCR2.1 containing <i>M. smegmatis</i> <i>osmC</i> coding region	This study
p16MSOSMCEX	pET16b containing <i>M. smegmatis</i> <i>osmC</i> coding region	This study
pMTBOSMC	pCR2.1 containing <i>M. tuberculosis</i> <i>osmC</i> coding region	This study
pMTBOSMCEX	pCR2.1 containing <i>M. tuberculosis</i> <i>osmC</i> coding region	This study
p16MTBOSMCEX	pET16b containing <i>M. tuberculosis</i> <i>osmC</i> coding region	This study
pMRV2923	pMV206 containing <i>M. tuberculosis</i> <i>osmC</i> coding region	This study
pMsahpC	pMV206 containing <i>M. smegmatis</i> <i>ahpC</i> coding region	Dhandayuthapani et al 1996

Table 2

Primers used in this study

Primer name	Primer sequence (5'-3')
MSOSMC1	TCCGGCATCGGCAACATCTA
MSOSMC2	GTAAACAGTGATAGAGATCGGTCATGCGCTAT
MSOSMC3	TACACTGTAAACATCGATCTGTCCGGTCTGTC
MSOSMC4	CCGAATCGCTCAGCAGTTCT
MSOSMCEX1	GCACATATGACCGATCTCTGGGTTGAGCGTACG
MSOSMCEX2	CTCGGATCCTCATTTCGGCAGGACGGTGTCGGC
MSOSMCRT3	GATCTCTGGGTTGAGCGTAC
MSOSMCRT4	AGTGCGATCTTCATGAGCTC
MSSIGART1	CCCACCGGAATTCGTAAG
MSSIGART2	TTGCCGGGCTTGCCTT
RV2923EX1	AGCACGCATATGACGCAACTGTGGGTCGAGCGCACC
RV2923EX2	GCGCTGGATCCCTCAGGCGCCGACATCGACGACCTC
RV2923A	ATCTTTAAATCTAGATTAAGGTGCCGGCGTCTGGGAGC
RV2923B	AGCTGGATCCATGGATTGGTTGGCCGCGTAACCGGTCA
RV2923RT1	GACTACCAGGCGGTGGTCAA
RV2923RT2	ACCACCAGCAGACGCTCCTT
TBSIGART1	GACGAGGAGATCGCTGAACC
TBSIGART2	TCGTCTTCATCCCAGACGAAA

Table 3Inhibition of *M. smegmatis* growth by INH in disk inhibition assay

Strains	Zone of inhibition (mm)
<i>M. smegmatis</i> wild type	26.84 ± 0.74
<i>MSΔahpC</i>	42.00 ± 0.92*
<i>MSΔosmC</i>	30.00 ± 1.00

Asterisk indicates value is significantly higher from *M. smegmatis* wild type ($P=0.0025$)

Table 4Plasmid borne *osmC* increases the MIC of INH in *M. smegmatis*

Strains	MIC for INH (µg/ml)
<i>MS206</i>	20
<i>MS2923</i>	30
<i>MSahpC</i>	40
<i>MSΔosmC</i>	20
<i>MTB206</i>	0.25
<i>MTB2923</i>	0.25