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

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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 *Mycobacterium 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 (H_2O_2), 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 (H_2O_2) 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.

1. Introduction

The success of *Mycobacterium tuberculosis* as a formidable pathogen partly depends upon its ability to survive and replicate within mononuclear phagocytes that include macrophages.[1] It is well known that phagocytes are the host defense cells and are equipped with antimicrobial responses.[2] 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 membrane.[4] 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 O_2 to superoxide (O_2).[5,6] This dismutates to become hydrogen peroxide (H_2O_2) 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/macrophages.[3] 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).[7,8] 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

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glycolipid (PGL) resist ROI/RNI to some extent,[9] increasing evidences indicate that antioxidant enzymes of *M. tuberculosis* play critical role in this process. For instance, Piddington et al[10] have shown that *M. tuberculosis* Cu,Zn- superoxide dismutase (Cu,Zn-SOD), also known as SodC and located on the cell envelope,[11] 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 stress.[12] *M. tuberculosis* deficient in SodA was more susceptible to H_2O_2 in vitro and markedly attenuated for growth in mice. *M. tuberculosis* catalase/peroxidase (KatG), which catalyzes H_2O_2 into H_2O and O_2 and again a secreted protein, also displays a significant role in resisting oxidative stress.[13] Similar to that of *sodA* mutant strain of *M. tuberculosis*, strains lacking KatG showed hypersusceptibilty to H_2O_2 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 growth.[14] 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 peroxinitrite 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 mycothiol,[17] sulfate reductase,[18] 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′-adneosine phophosulfate reductase showed increased susceptibility to H_2O_2 and peroxinitrite.[18] 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 H_2O_2 or cumene hydroperoxide (CHP).[21] In contrast, *Mycobacterium 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 coenzme 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 used 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. *Esherichia coli* strain DH5-α (Invitrogen) was used for sub cloning experiments and *E. coli* strain BL-21 was used to overexpress recombinant proteins. 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 respectively. 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