Methionine Sulfoxide Reductase A (MsrA) Deficiency Affects the Survival of *Mycobacterium smegmatis* within Macrophages

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Methionine sulfoxide reductase A (MsrA) is an antioxidant repair enzyme which reduces oxidized methionine to methionine. Since oxidation of methionine in proteins impairs their function, an absence of MsrA leads to abnormalities in different organisms, including alterations in the adherence patterns and in vivo survival of certain pathogenic bacteria. To understand the role of MsrA in intracellular survival of bacteria, we disrupted the gene encoding MsrA in *Mycobacterium smegmatis* through homologous recombination. The *msrA* mutant strain of *M. smegmatis* exhibited significantly reduced intracellular survival in murine J774A.1 macrophages compared to the survival of its wild-type counterpart. Furthermore, immunofluorescence and immunoblotting of phagosomes containing *M. smegmatis* strains revealed that the phagosomes with the *msrA* mutant strain acquired both p67^phox of phagocyte NADPH oxidase and inducible nitric oxide synthase much earlier than the phagosomes with the wild-type strain. In addition, the *msrA* mutant strain of *M. smegmatis* was observed to be more sensitive to hydroperoxides than the wild-type strain was in vitro. These results suggest that MsrA plays an important role in both extracellular and intracellular survival of *M. smegmatis*.

Although mononuclear phagocytes are specially equipped to produce superoxide (O_2^-·) radicals through the NADPH oxidase (phox) complex (32, 35), most living cells also generate superoxide as a by-product of normal aerobic metabolism. This occurs due to incomplete reduction of oxygen at the level of the cytochrome oxidase system, which accounts for approximately 4% of the oxygen that enters into this system (50). Superoxide reacts with a variety of organic and inorganic compounds and generates toxic reactive oxygen intermediates (ROI), such as H_2O_2, HO·⁻, HOCl, and OONO·⁻ (25, 52). Toxic ROI oxidize cellular macromolecules like proteins, lipids, and nucleic acids, which accelerates aging processes and disease in living organisms (4).

In proteins, oxidation occurs mainly at the sulfur-containing amino acids cysteine and methionine (40). Oxidation of methionine leads to the formation of methionine sulfoxide, and methionine sulfoxide reductase A (MsrA), which was first identified in *Escherichia coli* (5), has long been implicated in the reduction of methionine sulfoxide in proteins. However, recent studies have demonstrated that oxidation of methionine leads to two stereomeric forms of methionine sulfoxide, methionione *S*-sulfoxide and methionine *R*-sulfoxide (21, 50); the reduction of these compounds is catalyzed by MsrA and MsrB, respectively, (21, 30). Analysis of the sequences of different genomes has revealed that the genes coding for both MsrA and MsrB are present in almost all living organisms, from prokaryotes to humans (11, 18, 21). Interestingly, however, MsrB from different organisms shows no sequence identity with

MsrA (17). Also, in contrast to MsrA, MsrB requires the metal cofactor selenium or zinc (18) for reactivity.

Since the oxidation of methionine in proteins impairs the function of proteins, the absence of MsrA has been implicated in a variety of abnormalities in different organisms. Mice lacking MsrA have been reported to develop atypical walking patterns (tip-toe) and have reduced life spans (26). In addition, prokaryotes like *Streptococcus pneumoniae*, *Neisseria gonorrhoeae*, and *Escherichia coli* deficient in MsrA show altered adherence patterns (51). An absence of MsrA also affects the adherence of the urogenital pathogen *Mycoplasma genitalium* to sheep red blood cells (8). Furthermore, the yeast *Saccharomyces cerevisiae* (27) and the prokaryotes *E. coli* (29), *Erwinia chrysanthemi* (13), *M. genitalium* (8), and *Staphylococcus aureus* (37) lacking the *msrA* gene show increased sensitivity to H_2O_2 stress. A recent study demonstrated that an *msrA* mutant strain of *E. coli* is hypersensitive to nitric oxide stress, and introduction of a plasmid containing *msrA* of *Mycobacterium tuberculosis* could correct this defect (41). Moreover, human T cells (28) and yeast cells (28) overexpressing MsrA and transgenic *Drosophila* overexpressing MsrA are more resistant to oxidative stress (34). Finally, MsrA has been shown to be important for the survival of *E. chrysanthemi* (13) and *M. genitalium* (8) inside the host.

This study was undertaken to determine whether MsrA had any effect on the intracellular survival of bacteria. We used the fast-growing nonpathogenic bacterium *Mycobacterium smegmatis* in this study. *M. smegmatis* can survive inside mononuclear phagocytes, including adherent human peripheral blood monocytes (3), for several days, although it cannot replicate inside macrophages like its pathogenic counterparts, such as *M. tuberculosis* and *Mycobacterium leprae*. This property of *M. smegmatis* has been exploited to identify genes of *M. tuberculosis* that are important for intracellular survival (23, 24, 31,

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49). We show here that an *M. smegmatis msrA* mutant (strain MSΔ*msrA*) has a decreased ability to survive in macrophages compared to wild-type *M. smegmatis* (strain MSWt). We also demonstrate that phagosomes containing the MSΔ*msrA* strain acquire oxidative markers of macrophages earlier than phagosomes containing the MSWt strain acquire them.

MATERIALS AND METHODS

Materials and reagents. Restriction enzymes and DNA modification enzymes used in this study were purchased from either New England Biolabs (Beverly, Mass.) or GIBCO-BRL (Rockville, Md.). Taq polymerase and deoxynucleoside triphosphates were purchased from Perkin-Elmer (Foster City, Calif.). Qiaprep columns were obtained from Qiagen Inc. (Valencia, Calif.). Hydrogen peroxide, methyl viologen, *t*-butyl hydroperoxide, and cumene hydroperoxide were purchased from Sigma Chemical Co. (St. Louis, Mo.). *S*-Nitrosoglutathione (GSNO) and papanonate were purchased from Alexis (San Diego, Calif.). Nitrocellulose membranes were obtained from Schleicher & Schuell (Keene, N.H.).

Bacterial strains, media, and growth conditions. *M. smegmatis* mc²155 (= MSWt) was grown either in Middlebrook 7H9 broth containing 0.2% glycerol, albumin dextrose complex (ADC) (5 g of bovine serum albumin per liter, 0.85 g of NaCl per liter), and 0.05% Tween 80 (7H9-TW-ADC) or on Middlebrook 7H10 agar containing 0.2% glycerol, ADC, and 0.05% Tween 80 (7H10-TW-ADC). *M. smegmatis* harboring a plasmid was grown in 7H9-TW-ADC or 7H10-TW-ADC containing the antibiotic kanamycin (25 μg/ml) or hygromycin (50 μg/ml) or both. *M. tuberculosis* H37Rv was grown in 7H9 broth containing 0.05% Tween 80 and the oleic acid-albumin-dextrose-catalase supplement. All subcloning procedures were performed in *E. coli* strain Inv-α (Invitrogen, Carlsbad, Calif.). Plasmid-containing *E. coli* was grown in Luria-Bertani broth or agar with the appropriate antibiotic (100 μg of ampicillin per ml, 25 μg of kanamycin per ml, or 100 μg of hygromycin per ml). Unless indicated otherwise, all bacteria were grown at 37°C.

DNA manipulations. Chromosomal DNA of mycobacteria was isolated by using cetyltrimethylammonium bromide as described previously (43). Plasmid DNA from *E. coli* was isolated by using a Qiaperp kit (Qiagen Inc.). Southern hybridization and PCR were performed as described by Ausubel et al. (1). Oligonucleotide primers were synthesized at the Center for DNA Technology, University of Texas Health Science Center at San Antonio.

Disruption plasmid for *M. smegmatis msrA*. In order to construct an *msrA* disruption plasmid for *M. smegmatis*, we first cloned the *msrA* gene of *M. smegmatis*. We used The Institute for Genome Research database to obtain the *M. smegmatis* genome sequence for this. We analyzed the *M. smegmatis* genome sequence by BLAST analysis using the *msrA* gene of *M. tuberculosis*, and we identified the sequence containing the *M. smegmatis msrA* gene. The information was downloaded from the database, and based on the sequence, primers to amplify an approximately 2.7-kb region were synthesized. In fact, four primers were synthesized, as shown in Fig. 1B. Primers MSMSRA1 (5′-ACGTGCGAT CCCAGGAATCC-3′) and MSMSRA4 (5′-CGTCGGATGGTCGTTC A GGCC TCCGGTGTAGCC-3′) were used to amplify the 5′ portion of *msrA* and its upstream region (1,097 bp), and primers MSMSRA3 (5′-GGCTACACCGGA GGCC T GAACGACCATCCGACG-3′) and MSMSRA2 (5′-AATGTCCAGCC GAACCTCAGCC-3′) were used to amplify the 3′ portion of *msrA* and its downstream region (1575 bp). The sequences of primers MSMSRA3 and MSMSRA4 were modified (underlined nucleotides) to create an StuI site within the *msrA* gene. The amplified 1,097- and 1,575-bp fragments were separately cloned in pCR2.1 to create plasmids pMSMSRA1 and pMSMSRA2. Plasmid pMSMSRA2 was cut with StuI and BamHI to release the 1,575-bp fragment, and this fragment was ligated to StuI- and BamHI-cut pMSMSRA1 to create plasmid pMSMSRA3. This step was necessary to create a unique StuI site within the *msrA* gene. Plasmid pMSMSRA3 was cut with EcoRI to release the 2.6-kb fragment, and this fragment was cloned into EcoRI-cut pUC18 to create plasmid pMSMSRA4. Finally, plasmid pMSMSRA4 was cut with StuI and ligated to the 1.2-kb kanamycin resistance gene from pMV206 (42) to create plasmid pMSMSRA5. This plasmid served as the *M. smegmatis msrA* disruption construct, which was electroporated into wild-type *M. smegmatis* to obtain *M. smegmatis msrA* mutant strains.

Construction of other plasmids. Several other plasmids were created to enable complementation of MsrA activity in the *M. smegmatis msrA* mutant strains or expression of green fluorescent protein in *M. smegmatis* or for other purposes. Plasmid pHG361 was constructed by deleting the 1.2-kb *aph* gene (kanamycin resistance gene) in pMV261 (42) by cutting with NheI and SpeI and ligating the remaining fragment (blunt ended with the Klenow fragment) with the 1.7-kb
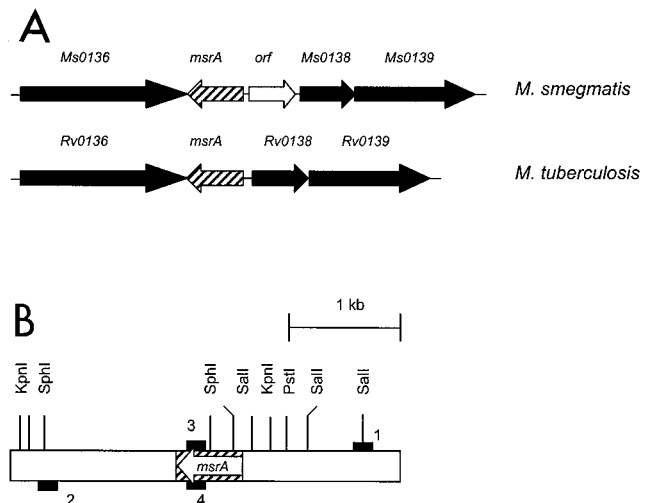

FIG. 1. (A) Organization of *msrA* in the genomes of *M. smegmatis* and *M. tuberculosis*. *M. smegmatis* open reading frames showing similarity to *M. tuberculosis* open reading frames, other than the MsrA open reading frame, are designated *Ms0136*, *Ms0138*, and *Ms0139*. (B) Schematic representation of the *msrA* locus in the chromosome of *M. smegmatis*. The open boxes represent the flanking regions, the striped box represents the *msrA* gene, and the solid boxes represent the primers. 1, 2, 3, and 4 indicate primers MSMSRA1, MSMSRA2, MSMSRA3, and MSMSRA4, respectively. The arrow indicates the direction of *msrA* transcription. KpnI, PstI, SalI, and SphI indicate the restriction sites in and around *msrA*.

hygromycin resistance gene (cut with BamHI and PstI and blunt ended with the Klenow fragment) from pIJ963 (10). Similarly, plasmid pHG206 was constructed by deleting the 1.2-kb *aph* gene (kanamycin resistance gene) in pMV206 by cutting the sequence with NheI and SpeI and ligating the remaining fragment (blunt ended with the Klenow fragment) with the 1.7-kb hygromycin resistance gene (cut with BamHI and PstI and blunt ended with the Klenow fragment) from pIJ963 (10). Plasmid pMSMSRA6 was generated by cloning the 1.2-kb *M. smegmatis msrA*-containing PCR fragment (blunt ended) into pHG361. Plasmid pMTSIGHGFP3 was generated by cloning the DNA containing the *M. tuberculosis sigH* promoter and the *gfpuv* gene region (*sigH::gpuv*) in the EcoRV site of pOLYG. The *sigH::gpuv* region was obtained by cutting plasmid pMTSIGHGF PUV1 (unpublished data) with EcoRI and NotI.

Electroporation. Transformation of *M. smegmatis* with plasmids containing the mycobacterial origin of replication or plasmids without the mycobacterial origin of replication was achieved by electroporation (16).

Intracellular survival of *M. smegmatis*. The adherent mouse macrophage cell line J774A.1 grown in Dulbecco modified Eagle medium with 10% fetal bovine serum was used to determine the intracellular survival of *M. smegmatis* strains. Freshly grown suspensions of *M. smegmatis* strains (viability, more than 90%) were gently sonicated at 4 W for 15 s and matched to a McFarland no. 1 standard suspension. J774A.1 macrophages were infected at various multiplicities of infection (MOI) ranging from 1 to 10 during phagocytosis. After 4 h of phagocytosis, macrophages were washed to remove nonphagocytosed bacteria and stained with fluorescent dyes to determine the number of bacteria per macrophage. Triplicate wells containing macrophages (10^6 macrophages per well) were examined for each MOI in three independent experiments to determine the optimal uptake. Strains MSWt, MSΔ*msrA*, and MSΔ*msrA*/c (a mutant strain complemented for *msrA*) were taken up equally well by macrophages, and there was no significant difference in their abilities to phagocytose into macrophages. An MOI of 1 was chosen for all further experiments since most macrophages contained 1 CFU and this value correlated with the natural mode of infection of macrophages. For viability experiments, monolayer cultures of J774A.1 cells were established in 24-well plates and then were infected with bacteria. After nonphagocytosed bacteria were washed with warm Dulbecco modified Eagle medium, 1 ml of fresh medium was added to each well, and the plates were incubated at 37°C in the presence of 5% CO_2. Macrophages in quadruplicate wells for each strain for each time point were harvested at zero time and at 1 and 3 days and lysed with 0.05% sodium dodecyl sulfate (SDS) for 15 min at room

temperature. The lysed suspensions were diluted 10-fold, and 100 μl of each dilution was plated in triplicate on 7H10-TW-ADC plates. The plates were incubated at 37°C for 3 days, and colonies were counted. The viability assays were repeated three times.

Immunofluorescent localization of phagosomal oxidative markers. For this study, we used sonicated and well-dispersed green fluorescent protein-expressing *msrA* mutant (MSΔ*msrA*-gfp) and wild-type (MSWt-gfp) *M. smegmatis* strains. These strains were produced by transforming the *msrA* mutant (MSΔ*msrA*) and wild-type (MSWt) *M. smegmatis* strains with plasmid pMTSIGHGFP3. Macrophages were infected (MOI, 1) in eight-well slide chambers for 4 h at 37°C in the presence of 5% CO_2, washed, and incubated in fresh medium for different times up to 72 h. At 24-h intervals, cells were washed with warm phosphate-buffered saline once, fixed in ice-cold methanol for 15 s, and then fixed for 30 min in 2.7% paraformaldehyde. Cells were permeabilized for 30 min with a staining buffer containing 1% saponin, 0.1% glycine, and 5% heat-inactivated normal mouse or goat antiserum (depending upon the primary antibody) in phosphate-buffered saline, and then they were washed and incubated at 4°C for 18 h with 1/500 to 1/1,000 dilutions of rabbit anti-mouse inducible nitric oxide synthase (iNOS), antibody to nitrotyrosine (NT), and antibody to mouse p67[phox] (Santa Cruz Biotechnology, Santa Cruz, Calif.). A monoclonal anti-iNOS antibody (Sigma Chemical Co.) was also used. Macrophages were washed four times with the staining buffer and then counterstained with Texas red conjugated with goat anti-rabbit or goat immunoglobulin G (Jackson Immunochemicals, West Grove, Pa.) at room temperature for 2 h. After further washing, cells were mounted in elvanol mountant and examined with a Nikon fluorescence microscope. Green fluorescent and Texas red images were acquired, and the images were merged by using the metaview software to examine colocalization of stains. A positive control consisting of gamma interferon (IFN-γ)-activated and uninfected macrophages and a negative control consisting of untreated and uninfected macrophages were stained with isotype controls, followed by conjugates. At least 100 microscopic fields were scored for each macrophage preparation. Usually, each microscopic field contained one to five macrophages containing fluorescent organisms. Three independent experiments for each time point were performed, and the results are presented below as mean percentages. Colocalization was confirmed by using a deltavision laser scanning microscope.

Immunoblot analysis of phagosomal maturation and trafficking markers. J774A.1 cells, grown in 75-ml flasks, were infected with *M. smegmatis* strains or latex bead controls at an MOI of 1, the cells were mixed for 4 h to enable uniform infection, and monolayers were then washed and incubated for another 72 h. Phagosomes were purified by sucrose gradient centrifugation as described previously (9, 46). Phagosome pellets were separated by SDS—10% polyacrylamide gel electrophoresis (PAGE) and transferred to Immobilon membranes. Phagosomal contamination with endoplasmic or Golgi membranes was monitored by staining for the Golgi-derived grp28 protein or calnexin (Santa Cruz Biotechnology) and was found to be less than 2%. The membranes were then probed with antibodies to free radical markers like p67[phox] and iNOS. The membranes were also probed with Rab5b antibodies to determine the maturation of phagosomes. The blots were developed with an ECL kit (Amersham, Piscataway, N.J.).

SDS-PAGE and immunoblotting. SDS-PAGE and immunoblotting for *M. smegmatis* proteins were performed as described previously (1). Protein extracts were separated by SDS—12% PAGE. Protein concentrations in extracts were determined by using the bicinchoninic acid reagent (38), and 75 μg of protein was loaded in each lane. Separated proteins were transferred to nitrocellulose membranes and probed with the appropriate antiserum. The blots with anti-MsrA antiserum were developed with the 5-bromo-4-chloro-3-indolylphosphate (BCIP)—nitroblue tetrazolium substrate.

Survival under oxidative stress conditions. Survival of *M. smegmatis* strains after exposure to oxidative stress was examined as follows. *M. smegmatis* strains grown in 7H9-TW-ADC were diluted to obtain an optical density at 595 nm of 0.300 in the same medium. Aliquots (1 ml) of each diluted culture were exposed to different oxidants (H_2O_2, *t*-butyl hydroperoxide, cumene hydroperoxide, and methyl viologen) for 1 h at 37°C. Control cultures were treated similarly but without oxidants. For experiments with the nitric oxide (NO) donors GSNO and sodium papanonoate, the pH of 7H9-TW-ADC was kept at pH 5.0. Cultures were serially diluted to 10-fold, and 100-μl portions of the diluted cultures were plated on 7H10-TW-ADC plates. Colonies were counted after 3 days of incubation at 37°C, and the results are reported below as log_{10} values.

RESULTS

***msrA* gene and its organization in the genome of *M. smegmatis*.** The sequence encoding MsrA of *M. smegmatis* and its flanking regions was obtained from The Institute for Genome Research database for the *M. smegmatis* genome sequence. Although in some species of bacteria multiple genes code for MsrA (36), the *M. smegmatis* genome has only one gene encoding MsrA. The amino acid sequence of *M. smegmatis* MsrA (170 amino acids) exhibited 80 and 77% identity with the sequences of MsrAs of *M. tuberculosis* (182 amino acids) and *M. leprae* (176 amino acids), respectively. It also showed significant identity with several prokaryotic and eukaryotic MsrAs. Furthermore, MsrA of *M. smegmatis* also possesses the critical N-terminal GCFWG residues at positions 12 to 16 which are a prerequisite for MsrA activity (20). Analysis of the flanking regions (4 kb) of *M. smegmatis msrA* revealed that the organization is more or less similar to that of *msrA* (*Rv0137c*) of *M. tuberculosis*. The flanking regions of *M. smegmatis msrA* have homologues for the *Rv0136*, *Rv0138*, and *Rv0139* genes of *M. tuberculosis*, which are located in the flanking regions of *M. tuberculosis msrA* (Fig. 1A). We designated these genes *Ms0136*, *Ms0138*, and *Ms0139*, respectively. However, the intergenic region between *msrA* and *Ms0138* in the *M. smegmatis* genome is longer (511 bp) than the intergenic region between *msrA* and *Rv0138* in the *M. tuberculosis* genome (69 bp). In fact, this region of *M. smegmatis* has a putative open reading frame with a coding capacity of 130 amino acids. However, the function of the protein encoded by this open reading frame is not known, although it has some identity with the C-terminal region of a putative *Streptomyces coelicolor* large membrane protein (SC6G10.33).

Disruption of *M. smegmatis msrA*. To examine the role of MsrA in intracellular survival, we first disrupted the *msrA* gene in *M. smegmatis* through homologous recombination. We electroporated plasmid pMSMSRA5 into wild-type *M. smegmatis* and plated the organisms onto 7H10-TW-ADC plates containing 25 μg of kanamycin per ml. Several electroporations resulted in approximately 200 kanamycin-resistant colonies. Genomic DNA isolated from these colonies was cut with KpnI and probed in Southern blots with a 1.2-kb kanamycin resistance gene (*aph*), a 2.2-kb *M. smegmatis msrA* gene, and 2. 7-kb plasmid pUC18. We predicted that an *msrA* mutant resulting from a double-crossover event should produce a single signal band (3.4 kb) with the *msrA* and *aph* probes and no signal with the pUC18 probe. Transformants MS97 and MS105 exhibited the expected results (Fig. 2A and B); hence, the *msrA* gene in these transformants was considered disrupted. To confirm the disruption of *msrA* expression in strains MS97 and MS105, we probed the protein extracts of these strains by Western blotting by using anti-MsrA antiserum produced against the overexpressed and purified MsrA protein of *M. tuberculosis* (41). As expected, an *msrA* mutant strain of *M. smegmatis* (MSΔ*msrA*) produced no band for MsrA, although wild-type *M. smegmatis* and *M. tuberculosis* produced strong bands (Fig. 2C). This indicated that MsrA expression was completely disrupted in the MSΔ*msrA* strain. In addition to the cross-reactivity with the MsrA protein, the anti-MsrA antiserum showed strong cross-reactivity with some other proteins of both *M. tuberculosis* and *M. smegmatis*, which is a common problem for many polyclonal antisera. However, the cross-reactivity did not prevent use of this antiserum against MsrA in immunoblotting since the MsrA band was very distinct (Fig. 2C). The MSWt and
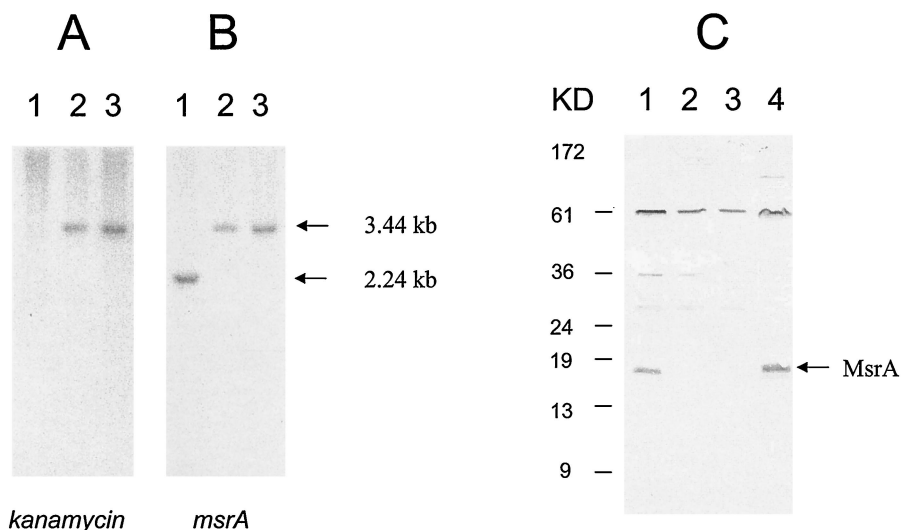
FIG. 2. Southern and Western analysis of *M. smegmatis* strains. (A and B) Genomic DNA of *M. smegmatis* strains probed with the 1.2-kb kanamycin resistance gene (A) and the 2.2-kb *M. smegmatis msrA* gene (B). The results of a Southern analysis with the pUC18 probe are not shown as there were no signals in any of the lanes. Lane 1, wild-type *M. smegmatis* strain; lane 2, *M. smegmatis msrA* mutant 97; lane 3, *M. smegmatis msrA* mutant 105. The arrows on the right indicate the positions of the DNA fragments. (C) Immunoblot showing the reactivity of mycobacterial proteins with anti-MsrA antiserum. Lane 1, *M. smegmatis* wild-type strain; lane 2, *M. smegmatis msrA* mutant 97; lane 3, *M. smegmatis msrA* mutant 105; lane 4, *M. tuberculosis* H37RV (wild-type strain). The arrow indicates the position of the MsrA protein.

MSΔ*msrA* strains showed similar in vitro growth patterns (data not shown).

Intracellular survival of the *msrA* mutant. We infected unactivated (naïve) and IFN-γ-activated macrophage cell line J774A.1 with the MSWt and MSΔ*msrA* (MS97) strains of *M. smegmatis* and monitored the survival of the strains within the macrophages for 3 days (Fig. 3). Naïve and activated macrophages were deliberately used because phagosome maturation can occur independent of activation, although oxidative killing is regulated normally by IFN-γ in macrophages and IFN-γ is known to enhance certain stages of phagosome maturation. In the unactivated macrophages the survival of the MSΔ*msrA* strain decreased slightly 1 day postinfection, and this strain was almost completely nonviable by 3 days postinfection. In contrast, the survival of the MSWt strain was only moderately decreased even on day 3 postinfection. In the activated macrophages, both the MSΔ*msrA* and MSWt strains showed different profiles. While the survival of the MSWt strain was only slightly decreased on day 1 postinfection, the MSΔ*msrA* strain exhibited no survival on day 1 postinfection, which is similar to the survival profiles in naïve cells on day 3 postinfection. Furthermore, by 3 days postinfection, the MSWt strain was no longer viable in the activated macrophages. These results indicated that MsrA protected against killing in macrophages. To determine whether the relatively lower level of survival of the MSΔ*msrA* strain in macrophages was really due to the lack of MsrA, we performed complementation experiments. We transformed the MSΔ*msrA* strain (MS97) with plasmid
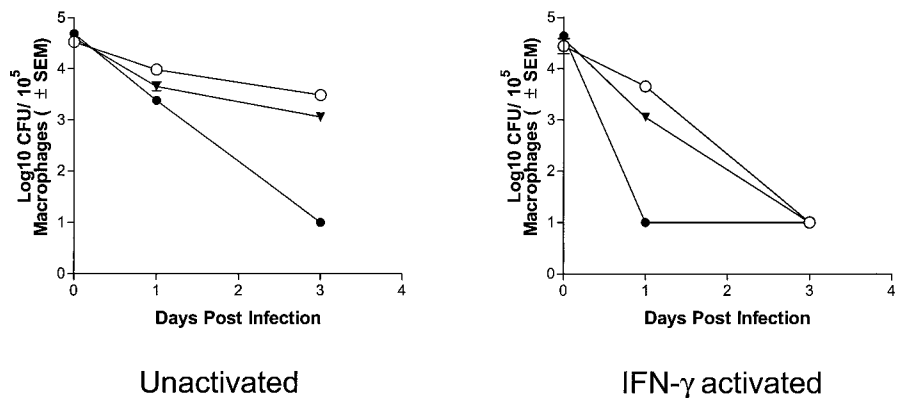

FIG. 3. Survival of *M. smegmatis* wild-type strain MSWt, *msrA* mutant 97 (= MSΔ*msrA*), and a complemented *msrA* mutant (MSΔ*msrA*/c) in murine macrophage-like J774A.1 cells. Naïve or IFN-γ-stimulated macrophages were infected at an MOI of 1 for 4 h, washed, lysed at intervals, and plated onto Middlebrook 7H10 agar plates to determine the number of CFU. ○, strain MSWt; ●, mutant MSΔ*msrA*; ▼, complemented strain MSΔ*msrA*/c. There are no error bars for some data points because of the small standard errors.
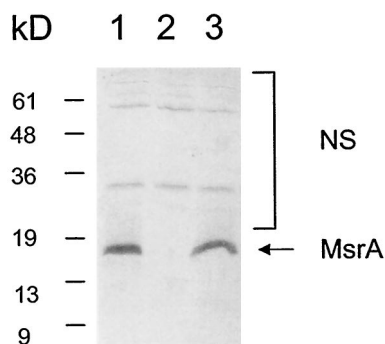
FIG. 4. Immunoblot showing expression of MsrA in an *msrA* mutant complemented with an integration plasmid-borne *msrA* gene. Lane 1, *M. smegmatis* wild-type strain MSWt; lane 2, *M. smegmatis msrA* mutant MSΔ*msrA*; lane 3, *M. smegmatis msrA* mutant complemented with the integration plasmid-borne *msrA* gene (MSΔ*msrA*/c). The arrow indicates the position of the MsrA protein. NS, nonspecific bands.

pMSMSRA6, which contained the *msrA* gene of *M. smegmatis* in the integration vector pHG306, and the resulting strain (MSΔ*msrA*/c) was examined for MsrA expression in immunoblots (Fig. 4) and was used to infect unactivated and IFN-γ-activated macrophages. As Fig. 3 shows, the survival rate of this strain was more or less similar to that of wild-type strain, confirming that the lower level of survival of the MSΔ*msrA* strain inside macrophages was due to the lack of MsrA. Although deletion of the *msrA* gene affects the adherence patterns in certain bacterial species (51), microscopic analysis revealed that the binding of the MSΔ*msrA* and MSΔ*msrA*/c strains to macrophages and the subsequent phagocytosis by macrophages were similar to the binding and phagocytosis of the MSWt strain. This may have been due to the availability of multiple receptors for entry into macrophages. Consequently, the reduced survival the MSΔ*msrA* strain was not due to defects in its entry into macrophages.

Immunofluorescence analysis of phagosomal oxidative stress markers. Since the level of intracellular survival of the MSΔ*msrA* strain of *M. smegmatis* was low, we hypothesized that this mutant was more susceptible to oxidative killing by macrophages than the MSWt strain was. Macrophages generate oxidative radicals, like superoxide ($O_2^{-\cdot}$) and nitric oxide ($NO^{\cdot-}$), by using the phox complex (NADPH oxidase) of proteins and iNOS, respectively (35). $O_2^{-\cdot}$ in turn combines with $NO^{\cdot-}$ to form the bactericidal radical peroxynitrite ($ONOO^{\cdot-}$). Peroxynitrite, an effective agent for Met oxidation in proteins, also reacts with the tyrosine residue of proteins and forms NT. Recently, colocalization of phagosomes with iNOS, p67[phox], and NT stains (45) has been used to determine the role of the oxidative mechanisms of macrophages in killing bacteria. We therefore infected J774A.1 macrophages with the MSWt and MSΔ*msrA* strains and labeled the phagosomes for iNOS, NT, and p67[phox] (Fig. 5). After 24 h of infection phagosomes containing the MSΔ*msrA* strain exhibited 76, 72, and 89% staining for iNOS, NT, and p67[phox], respectively. In contrast, phagosomes containing the MSWt strain showed only 11, 14, and 11% staining for iNOS, NT, and p67[phox], respectively, at the same time. The percentages of staining for iNOS and NT were greater in both the MSΔ*msrA*

strain (100% for iNOS and 100% for NT) and the MSWt strain (90% for iNOS and 78% for NT) containing phagosomes after 72 h of infection. However, the staining for p67[phox] was greater in the phagosomes containing the MSΔ*msrA* strain (100%) and not in the phagosomes containing the MSWt strain (20%) (Table 1).

Immunoblot analysis of free radical markers of phagosomes. To validate the confocal findings, purified phagosomes were also probed with anti-iNOS and anti-p67[phox] antibodies in immunoblots (Fig. 6), and the results largely confirmed the results of the phagosome colocalization experiments. Phagosomes with the MSΔ*msrA* strain indeed acquired iNOS and p67[phox] markers earlier than phagosomes with the MSWt strain acquired these markers. The fact that phagosomes containing the MSWt strain acquired less p67[phox] even after 72 h of infection was also evident. The MSΔ*msrA*/c strain also behaved like the MSWt strain with respect to p67[phox] and iNOS acquisition. These results together suggest that the MSWt strain has some components that naturally slow down the acquisition of phox components and iNOS by the phagosomal membrane. Nevertheless, the presence of the Rab5b phagosomal maturation marker indicated that initiation of phagosomal maturation started even before 6 h postinfection. It should be mentioned that assembly of phox and assembly of iNOS in the phagosomes occur independent of each other and probably independent of the phagosome maturation process.

Sensitivity of *msrA* mutants to oxidants. Previous studies indicated that in both eukaryotes and prokaryotes, the absence of MsrA leads to hypersensitivity to oxidative stress (8, 13, 29, 37). To determine if MsrA of mycobacteria plays a similar role, we tested the MSΔ*msrA* strain with different oxidative stresses. The MSWt and MSΔ*msrA* strains exhibited similar sensitivities to H_2O_2, the superoxide generator methyl viologen, and the nitric oxide generators sodium nitrite, sodium papanonoate, and GSNO (Fig. 7). Neither an increase in the concentration of these chemicals nor prolongation of the incubation period (data not shown) had any additional effect. However, the MSΔ*msrA* strain was more sensitive to cumene hydroperoxide and *t*-butyl hydroperoxide (Fig. 8), and complementation of the mutant strain (strain MSΔ*msrA*/c) with *msrA* of *M. smegmatis* with the integration plasmid pMSMSRA6 completely reversed this sensitivity.

DISCUSSION

Macrophages and other mononuclear phagocytes are host defense cells that are capable of generating ROI and reactive nitrogen species (RNI) in addition to a variety of hydrolytic enzymes and antimicrobial peptides known as defensins (15, 32). Intracellular pathogens, including *M. tuberculosis*, are able to resist these responses of phagocytes. In order to understand the molecular mechanism of bacterial resistance to antimicrobial products, antioxidants of bacteria, such as superoxide dismutase (SOD), catalase-peroxidase (KatG), and alkyl hydroperoxide reductase (AhpC), have been investigated intensively. The investigations have been based on the premise that bacterial antioxidants can detoxify the ROI and RNI generated by phagocytes and thereby help the cells resist killing by macrophages. Although the precise role of bacterial antioxidants in the detoxification of phagocyte-derived ROI or RNI is
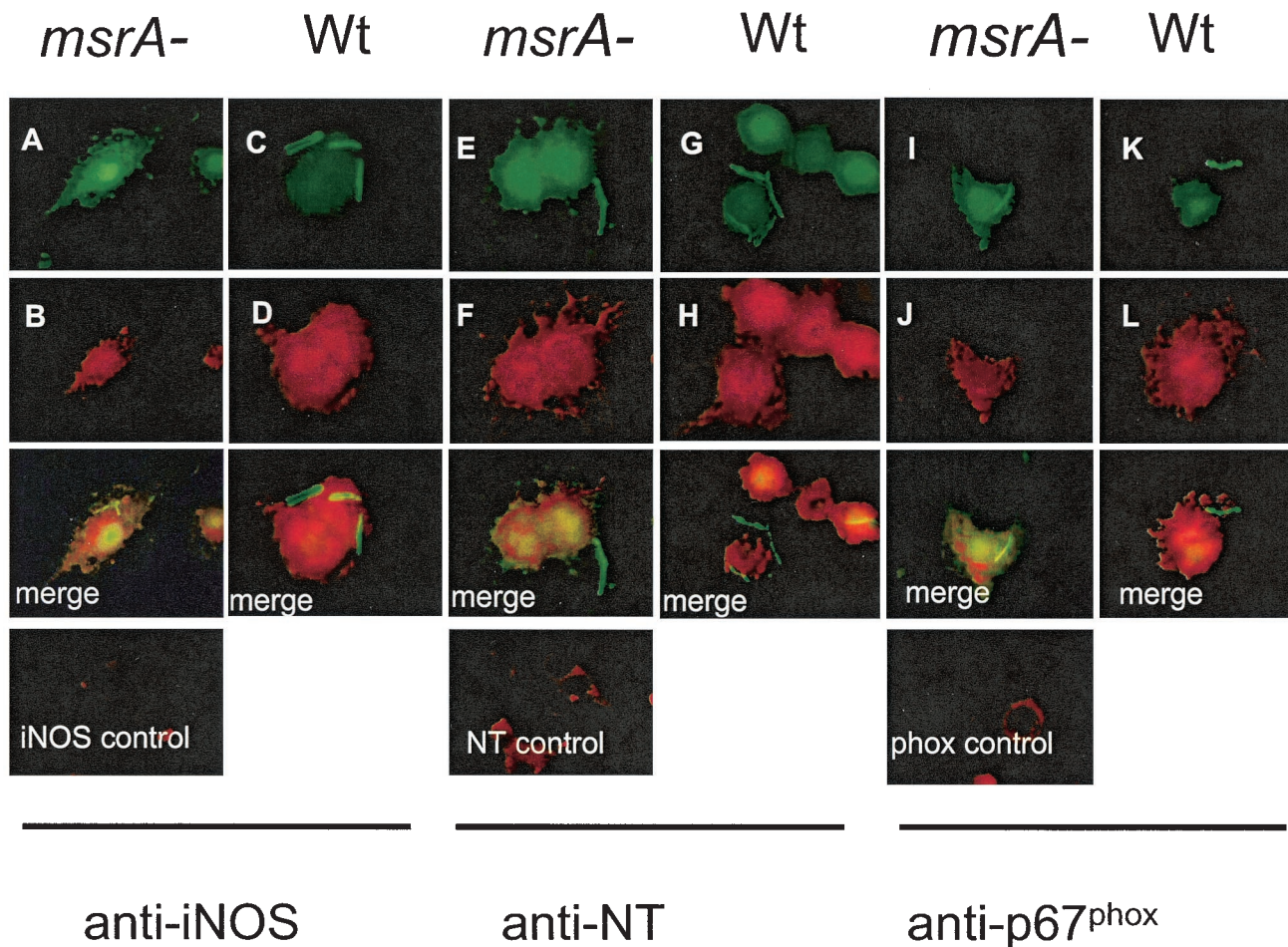
FIG. 5. Colocalization of iNOS, NT, and phagocyte oxidase protein (p67[phox]) with the strain MSWt and MS∆*msrA* phagosomes. J774A.1 macrophages were infected with *gfp*-expressing strains, washed, incubated for 24 h, fixed, and stained. (A and B) Strain MS∆*msrA* (*msrA*−) in macrophages stained with iNOS-specific antibodies; (E and F) strain MS∆*msrA* in macrophages stained with NT-specific antibodies; (I and J) strain MS∆*msrA* in macrophages stained with p67[phox]-specific antibodies; (C and D) strain MSWt (Wt) in macrophages stained with iNOS-specific antibodies; (G and H) strain MSWt in macrophages stained with NT-specific antibodies; (K and L) strain MSWt in macrophages stained with p67[phox]-specific antibodies. Fluorescein isothiocyanate and Texas red images are followed by merged images that showed colocalization of the mutant phagosomes with free radical-specific markers. The controls panels show naïve macrophages stained with primary antibody, followed by conjugates.

not fully understood yet, the absence of some antioxidants had an effect on the intracellular and/or in vivo survival of bacteria. For example, a Cu,Zn-SOD mutant strain of *Salmonella enterica* serovar Typhimurium exhibited reduced survival in macrophages and attenuated virulence in mice (7). Likewise, a Cu,Zn-SOD mutant of the *M. tuberculosis* Erdman strain exhibited decreased survival in activated macrophages (33). A catalase-peroxidase (KatG) mutant of *M. tuberculosis* has also been implicated in intracellular survival and in survival and persistence in guinea pigs (14, 19, 22). Paradoxically, however, an *ahpC* mutant of *M. tuberculosis* showed no defect in intracellular or in vivo survival (39), suggesting that not all antioxidants play similar roles in promoting intracellular or in vivo survival. MsrA is probably different from the antioxidants mentioned above due to its unique specificity for oxidized methionine sulfoxide in proteins, and this study indicates that its role may be vital during intracellular survival. Disruption of the gene encoding MsrA of *M. smegmatis* led to a defect in survival

TABLE 1. Immunofluorescence colocalization of iNOS, NT, and phagocyte oxidase (p67[phox]) with *M. smegmatis* phagosomes in murine macrophages[a]

Strain[a]	Colocalization (%)					
	iNOS		NT		p67[phox]	
	24 h	72 h	24 h	72 h	24 h	72 h
MSWt-gfp	11 ± 4	90 ± 5	14 ± 4	78 ± 6	11 ± 4	20 ± 4
MS∆*msrA*-gfp	76 ± 3[b]	100	72 ± 7[b]	100	89 ± 6[b]	100

[a] J774A.1 macrophages were phagocytosed with green fluorescent protein-expressing strains MSWt (MSWt-gfp) and MS∆*msrA* (MS∆*msrA*-gfp) for 4 h, washed, incubated for 24 to 72 h, fixed, stained with primary antibodies against iNOS, NT, and p67[phox], and counterstained with Texas red conjugates. Colocalization of iNOS, NT, and the p67[phox] component of NADPH oxidase with *M. smegmatis* wild-type strain MSWt and *msrA* mutant strain MS∆*msrA* in macrophages is expressed as a mean percentage ± standard error for three independent experiments. Colocalization was confirmed by laser confocal microscopy. At least 100 microscopic fields were scored for each quadruplicate mouse macrophage preparation per experiment. Each microscopic field contained 5 to 10 macrophages, and at least 8 macrophages contained at least one fluorescent organism.
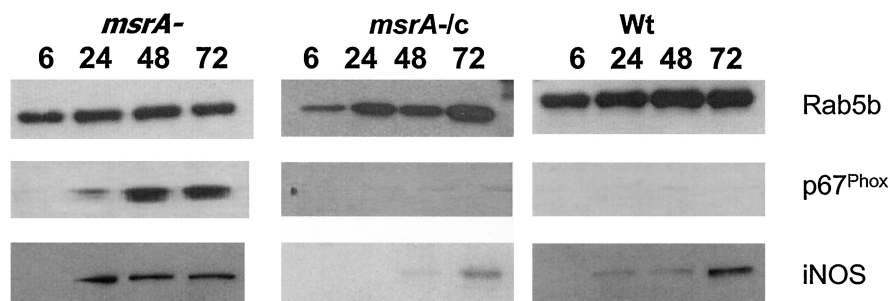
[b] $P < 0.05$ as determined by a *t* test.

FIG. 6. Immunoblot analysis of *M. smegmatis* phagosomes with anti-p67phox and anti-iNOS. Unactivated J774A.1 mouse macrophages were infected with MSΔ*msrA* (*msrA*−), MSΔ*msrA*/c (*msrA*−/c), and MSWt (Wt), and phagosomes were purified on sucrose gradients at different times (6, 24, 48, and 72 h) after infection. Rab5b, p67phox, and iNOS indicate reactivity of phagosomal proteins with the antibodies in the immunoblots.

of this organism inside macrophages, regardless of whether the macrophages were activated or naïve. This is consistent with previous reports which suggested the importance of MsrA in in vivo survival of *M. genitalium* and *E. chrysanthemi* (8, 13).

Within macrophages, oxidative damage due to free radicals is dependent upon two independent pathways, phagocyte oxidase (phox complex) and the iNOS enzyme, which produce $O^{\cdot-}$ and $NO^{\cdot-}$, respectively. The phox complex includes membrane-bound gp91phox and p22phox subunits, which together form the flavocytochrome b and cytosolic p40phox, p47phox, p67phox, Rac1, and Rac2 subunits (47). The compartmentalization of phox proteins prevents production of super-

oxide in the resting cells. However, upon activation of macrophages, cytosolic p47phox, p67phox, and Rac1 or Rac2 move towards the membrane and assemble with gp91phox and p22phox to form an active complex, and this process is mediated by protein kinase C isoforms. Since there is an invagination of the plasma membrane during phagocytosis, the assembly of the phox complex results in superoxide secretion into the lumen of the phagosome (2, 12). While superoxide is freely diffusible, many of the downstream reactive products of the reactive oxygen species cascade, like the $HO^{\cdot-}$ radicals, are not very stable; hence, localization of the phox complex becomes a critical event in targeting the microbicidal free radicals to the
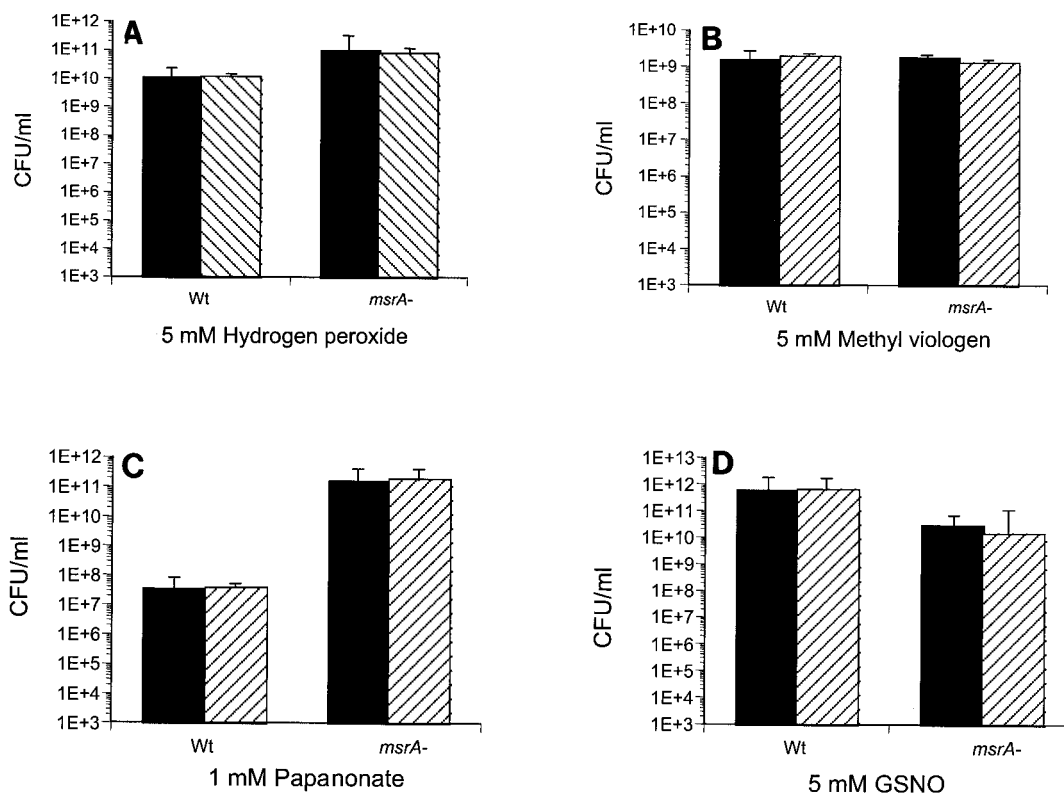

FIG. 7. Survival of *M. smegmatis* after exposure to oxidants. Wt, *M. smegmatis* wild-type strain MSWt; *msrA*−, *M. smegmatis msrA* mutant MSΔ*msrA*. Solid bars, untreated cultures; striped bars, treated cultures. Aliquots (1 ml) of cultures were exposed to hydrogen peroxide (A), methyl viologen (B), papanonate (C), and GSNO (D) for 1 h at 37°C. The cultures were serially diluted and plated on 7H10-TW-ADC plates.
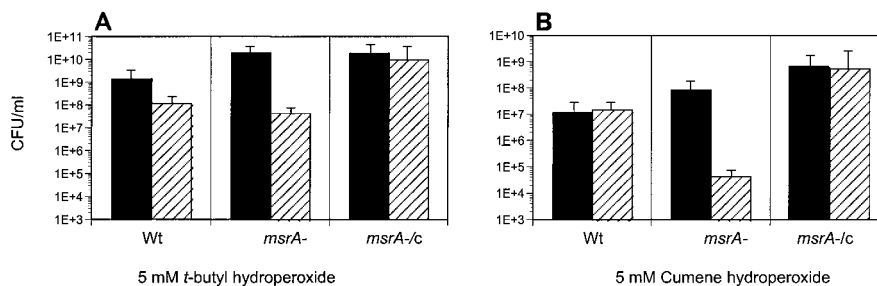
FIG. 8. Survival of *M. smegmatis* after exposure to hydroperoxides. Wt, *M. smegmatis* wild-type strain MSWt; *msrA−*, *M. smegmatis* mutant MSΔ*msrA*; *msrA−*/c, *M. smegmatis* complemented *msrA* mutant MSΔ*msrA*/c. Solid bars, untreated cultures; striped bars, treated cultures. Aliquots (1 ml) of cultures were exposed to *t*-butyl hydroperoxide (A) and cumene hydroperoxide (B) for 1 h at 37°C. The cultures were serially diluted and plated on 7H10-TW-ADC plates.

phagosomes (2, 12). Although localization of phox components with some bacterial phagosomes has been reported (45), this is the first report of localization of phox with mycobacterial phagosomes.

In contrast to the well-studied assembly of the phox complex, little is known about the mechanisms by which iNOS is targeted to phagosomes, although it has recently been found that IFN-γ induces the translocation of iNOS onto phagosomes containing either inert beads or mycobacteria through a Rab-dependent pathway (S. Daniel, G. Dai, C. Singh, D. Lindsey, S. Dhandayuthapani, R. Hunter, and C. Jagannath, submitted for publication). iNOS exists as a single 132- to 135-kDa polypeptide and has been reported to be present as a free or vesicle-bound form in the cytosol of IFN-γ-activated mouse macrophages (48). In this study, iNOS was present on MSΔ*msrA* phagosomes earlier than it was present on wild-type phagosomes. Since the association of NO · ⁻ with superoxide to form NT is well known, we suggest that the increased colocalization of phox and iNOS to form NT resulted in a rapid loss of viability of the MSΔ*msrA* mutant in macrophages compared to the loss of viability of the wild-type strain.

Enhanced colocalization of the phox subunit and iNOS with MSΔ*msrA* phagosomes led to speculation that wild-type *M. smegmatis* naturally possesses some molecules that either prevent or slow down the assembly of phox and iNOS in macrophages. Previously, *S. enterica* serovar Typhimurium pathogenicity island 2 (SPI2) proteins were found to affect the localization of phox and iNOS. Vazquez-Torres et al. (45) reported that salmonellae use SPI2 to exclude NADPH oxidase from *Salmonella*-containing phagosomes, and Chakravortty et al. (6) observed that SPI2 affects the colocalization of iNOS with *Salmonella* phagosomal vacuole. In both cases, it was suggested that the SPI2 products, which include host effector proteins (reviewed in reference 44), prevent localization of phox and iNOS to the phagosomes by interfering with the host cytoskeleton. By using the same line of logic, it is tempting to propose that MsrA somehow modulates the trafficking of phox and iNOS at least during the initial 24 h of infection in macrophages, although the mechanism remains unclear.

The disruption of *msrA* leading to hydroperoxide hypersensitivity in *M. smegmatis* is consistent with previous reports (8, 13, 29, 37) suggesting that MsrA plays a role in defending cells against oxidative stress (Fig. 8). An obvious difference between an *msrA* mutant of *M. smegmatis* and most other microbial

msrA mutants is the lack of sensitivity to H₂O₂. In addition, the MSΔ*msrA* strain of *M. smegmatis* showed no sensitivity to NO donors. In contrast, St. John and his colleagues (41) have reported that an *msrA* mutant of *E. coli* is sensitive to nitrosative stress and that complementation of the mutant with the *msrA* gene of *M. tuberculosis* reverses the sensitivity. This difference in sensitivities between the *msrA* mutants of *E. coli* and *M. smegmatis* may be due to differences in the regulation of oxidative stress in these two species. However, the in vitro sensitivity of an organism to a specific oxidant does not determine its susceptibility to killing by macrophages, because the phagosomal environment is rich in metals, metalloenzymes, cofactors, and various ligands that produce even more toxic species from superoxide as part of the cascade (2, 12).

Overall, our results indicate that the absence of MsrA in *M. smegmatis* leads to increased sensitivity to certain oxidants in vitro and reduced intracellular survival, probably as a consequence of enhanced localization of phox and iNOS components to phagosomes. At this point, it is not clear whether MsrA plays a similar role in other intracellular bacteria, including *M. tuberculosis*. However, this possibility cannot be totally excluded, since MsrA is critical to the normal function of proteins in both bacteria and eukaryotic organisms. It is likely, therefore, that studies of MsrA in pathogenic intracellular bacteria, which have an inherent ability to prevent localization of phox and iNOS to the phagosomal membrane, will provide additional insights into the role of MsrA.

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