INFECTION AND IMMUNITY, Aug. 2001, p. 4980–4987 Vol. 69, No. 8
0019-9567/01/$04.00+0 DOI: 10.1128/IAI.69.8.4980–4987.2001

Cu,Zn Superoxide Dismutase of *Mycobacterium tuberculosis* Contributes to Survival in Activated Macrophages That Are Generating an Oxidative Burst

DEBRA L. PIDDINGTON,[1] FERRIC C. FANG,[2] TRACEY LAESSIG,[2] ANDREA M. COOPER,[3] IAN M. ORME,[3] AND NANCY A. BUCHMEIER[1]*

Department of Pathology, University of California, San Diego, La Jolla, California,[1] and Departments of Medicine, Pathology, and Microbiology, University of Colorado Health Sciences Center, Denver,[2] and Department of Microbiology, Colorado State University, Fort Collins,[3] Colorado

Received 15 December 2000/Returned for modification 15 January 2001/Accepted 1 May 2001

Macrophages produce reactive oxygen species and reactive nitrogen species that have potent antimicrobial activity. Resistance to killing by macrophages is critical to the virulence of *Mycobacterium tuberculosis*. *M. tuberculosis* has two genes encoding superoxide dismutase proteins, *sodA* and *sodC*. SodC is a Cu,Zn superoxide dismutase responsible for only a minor portion of the superoxide dismutase activity of *M. tuberculosis*. However, SodC has a lipoprotein binding motif, which suggests that it may be anchored in the membrane to protect *M. tuberculosis* from reactive oxygen intermediates at the bacterial surface. To examine the role of the Cu,Zn superoxide dismutase in protecting *M. tuberculosis* from the toxic effects of exogenously generated reactive oxygen species, we constructed a null mutation in the *sodC* gene. In this report, we show that the *M. tuberculosis sodC* mutant is readily killed by superoxide generated externally, while the isogenic parental *M. tuberculosis* is unaffected under these conditions. Furthermore, the *sodC* mutant has enhanced susceptibility to killing by gamma interferon (IFN-γ)-activated murine peritoneal macrophages producing oxidative burst products but is unaffected by macrophages not activated by IFN-γ or by macrophages from respiratory burst-deficient mice. These observations establish that the Cu,Zn superoxide dismutase contributes to the resistance of *M. tuberculosis* against oxidative burst products generated by activated macrophages.

The virulence of *Mycobacterium tuberculosis* is dependent upon the establishment of an infection within human macrophages. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced by macrophages as part of their antimicrobial response. The production of ROS is initiated by NADPH oxidase, which catalyzes the reduction of molecular oxygen to superoxide (O_2^-). Superoxide can then be converted to H_2O_2 and hydroxyl radical (27). In addition, nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) can combine with superoxide to generate additional products with enhanced toxicity, such as peroxynitrite ($ONOO^-$) (4).

Despite the toxic effects of ROS and RNS, *M. tuberculosis* can survive and grow within macrophages. Several *M. tuberculosis* gene products have been associated with the detoxification of ROS and RNS. KatG is a catalase peroxidase (32) that protects *M. tuberculosis* from killing by hydrogen peroxide (26). KatG also has peroxynitritase activity (40). The alkylhydroperoxide reductase AhpC is capable of catalyzing the breakdown of peroxynitrite (5). Lipoarabinomannan can scavenge potentially toxic oxygen free radicals (8). *M. tuberculosis* also produces two superoxide dismutase (SOD) proteins, SodA and SodC. The enzymatic function of SOD is to convert O_2^- into molecular oxygen and hydrogen peroxide. This activity removes the toxic effects of O_2^- and prevents the formation of higher H_2O_2 levels by other reactions (39) and the synergism of ROS with RNS (25, 39). SodA is an Mn,Fe SOD and is one of the major extracellular proteins in *M. tuberculosis* (18, 44). SodC is a Cu,Zn SOD and is produced at much lower levels by *M. tuberculosis*. However, SodC contains a lipoprotein binding motif that may mediate its attachment to the outer membrane of the bacteria. Immunogold electron microscopy has detected SodC at the periphery of *M. tuberculosis* (42). The peripheral location of the Cu,Zn SOD suggests that it may protect the surface of *M. tuberculosis* against extracellular superoxide generated by host cells.

Because of the location of SodC on the surface of the bacteria, we examined the role of the Cu,Zn SOD in protecting *M. tuberculosis* from exogenous sources of ROS. A mutation in the *sodC* gene was constructed, and the *M. tuberculosis* mutant was used to assess the importance of SodC in protecting *M. tuberculosis* from the toxic effects of superoxide or a combination of superoxide and nitric oxide generated in vitro. In addition, we evaluated the contribution of the *sodC* gene to the survival of *M. tuberculosis* in macrophages. In this report, we show that the *sodC* gene of *M. tuberculosis* is necessary for resistance to killing by exogenously generated superoxide and toxic synergistic products of superoxide with RNS. Furthermore, Cu,Zn SOD contributes to survival of *M. tuberculosis* in gamma interferon (IFN-γ)-activated macrophages that are producing an oxidative burst.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study included *M. tuberculosis* strain Erdman (ATCC 35801), *Mycobacterium bovis* El Paso, *Mycobacterium africanum*, *Mycobacterium microti*, *M. bovis* BCG Pasteur, *Mycobacterium gordonii, Mycobacterium xenopi, Mycobacterium smegmatis* (ATCC 607), *Mycobacterium chelonae* subsp. *chelonae* (ATCC 35752), *Myco-*

* Corresponding author. Mailing address: University of California, San Diego, 9500 Gilman Dr., La Jolla, CA 92093-0640. Phone: (858) 534-6024. Fax: (858) 534-6020. E-mail: nbuchmeier@ucsd.edu.

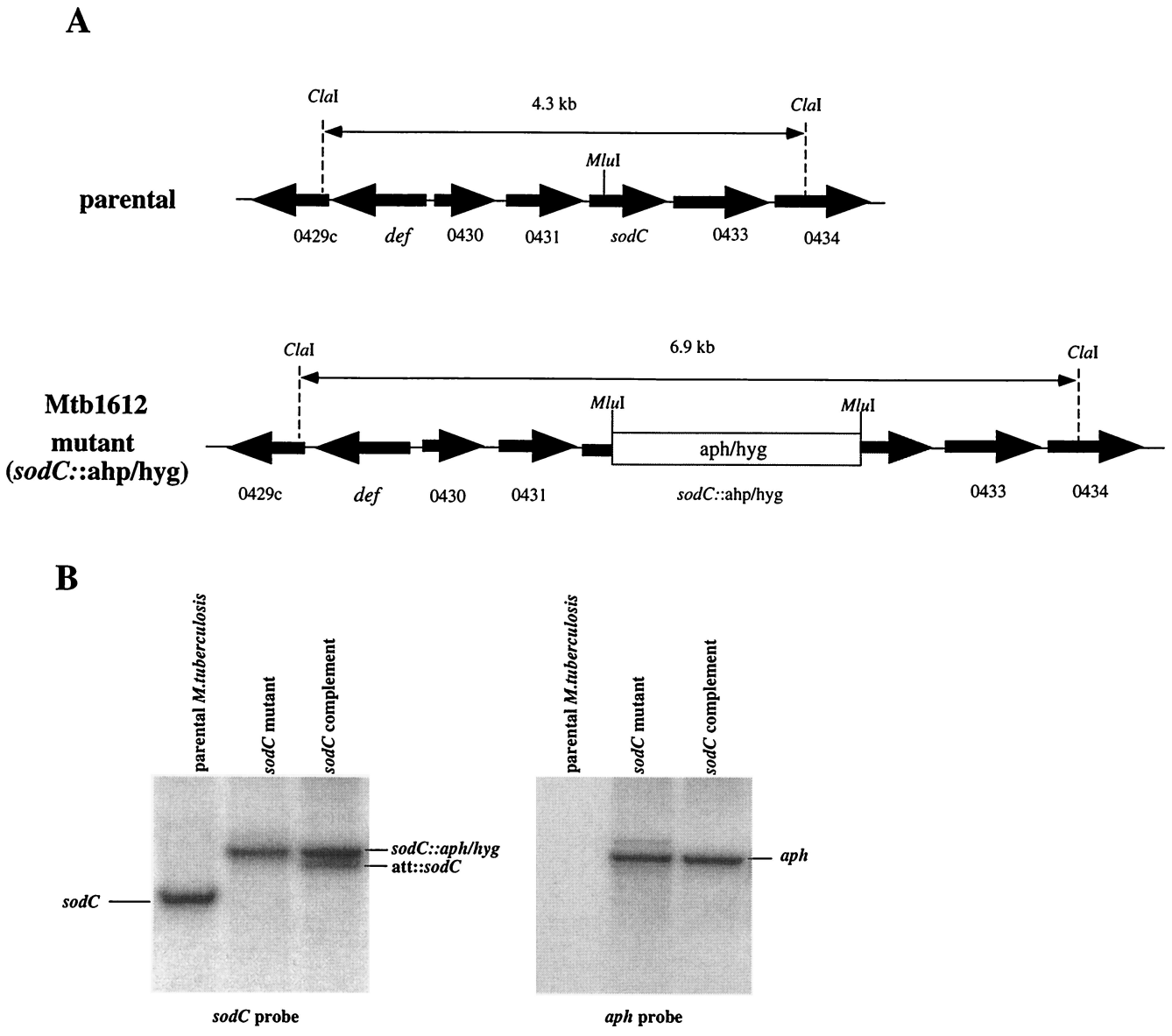

FIG. 1. (A) Restriction map of the *sodC* region in parental and *sodC* mutant strains of *M. tuberculosis.* (B) Southern blot of chromosomal DNAs from the parental, mutant, and complemented strains. DNA was digested with *Eco*RV and probed with DNA containing the gene for *sodC* or for *aph*. The *sodC*-hybridizing fragment in mutant Mtb1612 is larger than the unmutated fragment due to insertion of the drug resistance cassette. This fragment also hybridizes with the *aph* probe, confirming that Mtb1612 contains the kanamycin resistance gene that produced the mutation.

bacterium fortuitum subsp. *peregrinum* (ATCC 14467), *Mycobacterium kansasii* (clinical isolate), *Mycobacterium scrofulaceum* (clinical isolate), *Mycobacterium marinum* (ATC 927), *Mycobacterium avium* (clinical isolate), and *Mycobacterium intracellulare* (clinical isolate). Bacteria were grown in Middlebrook 7H9 medium supplemented with albumin-dextrose complex (ADC) and 0.05% Tween 80 (20). Hygromycin (50 μg/ml) and kanamycin (25 μg/ml) were added during the selection for the *sodC* mutant. Growth of bacteria was measured by monitoring the optical density at 580 nm (OD_{580}) in triplicate cultures.

Construction of a *sodC* mutant of *M. tuberculosis*. The *sodC* gene of *M. tuberculosis* was cloned from cosmid Y22G10, which was obtained from S. Cole (9). A 4.3-kb *Cla*I restriction fragment containing the 719-bp *sodC* gene flanked by 1.1 and 2.6 kb of DNA was subcloned into pBluescript KS (Stratagene) using standard protocols (33). The *sodC* gene was disrupted by cloning a 2.7-kb cassette containing the genes for resistance to kanamycin (*aph*) and to hygromycin (*hyg*) into the *Mlu*I site within the *sodC* gene (Fig. 1A.). DNA containing the disrupted *sodC* gene was linearized and electroporated into *M. tuberculosis* Erdman. Transformants were selected on 7H11 plates containing both kanamycin and hygromycin. Transformants that had undergone homologous recombination within the *sodC* gene were identified by Southern hybridization using probes corresponding to the genes for *sodC* and *aph* (6). One of the *sodC* mutants was designated Mtb1612 and was used in these studies.

Complementation of the *sodC* mutant. To genetically complement the *sodC* mutant, a wild-type copy of the *sodC* gene was introduced into the *sodC* mutant Mtb1612 using the integrative vector pCV125 (a gift from MedImmune) (6). A PCR fragment carrying the complete coding sequence of the *sodC* gene was amplified from *M. tuberculosis* Erdman DNA using forward primer 5′TTGATA TCTTTGATAAACGCCAGGTTAGCTCTC3′ and reverse primer 5′TACTAG TTTATCACTAGCCGGAACCAATGAC3′. The forward primer contains an *Nru*I restriction site, two stop codons, and the sequence immediately upstream of the *sodC* start codon. The reverse primer corresponds to the 3′ end of the *sodC* gene with an additional stop codon and an *Spe*I restriction site. The *sodC* PCR fragment was digested with *Nru*I and *Spe*I and cloned between the *Nru*I and *Spe*I sites in pCV125 (Sp/Sm). The *sodC* gene in this vector is transcribed from the *aph* promoter. DNA sequencing confirmed that no mutations were present in the PCR-amplified *sodC* gene compared to the published sequence (9). pCV125 (Sp/Sm)::*sodC* was electroporated into the *sodC* mutant Mtb1612, and transfor-

mants were selected on 7H11 plates containing streptomycin (30 μg/ml). As a control, plasmid pCV125 was electroporated into additional preparations of Mtb1612. Integration of pCV125::*sodC* into the chromosome of Mtb1612 was confirmed by Southern hybridization.

In vitro superoxide and nitric oxide susceptibility assays. In vitro superoxide killing assays were performed using a hypoxanthine/xanthine oxidase system to generate superoxide (11). Mid-log-phase cultures of bacteria grown in 7H9 medium were washed and adjusted to a density of approximately 10^6 CFU/ml. Bacteria were exposed to superoxide generated by combining 250 μM hypoxanthine with 0.1 U of xanthine oxidase (Sigma) per ml in phosphate-buffered saline. Catalase (1 U/ml) was added to prevent killing by H_2O_2 during the assay. Percent survival was determined at 0, 1, and 3 h postexposure by plating serial dilutions of the bacteria on 7H10 plates. The means from triplicate tubes were calculated, and the data were expressed as a percentage of the value at time zero. Sensitivity to nitric oxide was tested using 1 mM 2,2′-(hydroxynitrosohydrazono)bisethanamine prepared in NaOH (SPER/NO; Alexis Biochemicals, San Diego, Calif.) (11). NO is generated from this compound under slightly basic conditions. The NO assays were carried out in a manner similar to that described for the superoxide assays. To test for sensitivity to synergistic interactions of ROS and RNS, superoxide and nitric oxide were generated in the same tubes (11).

Macrophage killing assays. Mouse peritoneal macrophages were used for these studies because they can be stimulated to produce significant quantities of both ROS and RNS in vitro. Cells were harvested from C57BL/6 mice (Jackson Laboratories) to obtain macrophages that produce a respiratory burst or from gp91$^{phox-/-}$ mice (C57BL/6) or iNOS$^{-/-}$ mice (C57BL/6) to obtain macrophages defective in the production of ROS (31) or RNS (24). Macrophages were elicited by injection of 1 ml of 5 mM sodium periodate into the peritoneal cavity (11). After 4 days, mice were sacrificed and macrophages were harvested by peritoneal lavage. Macrophages (2×10^5 per well) were seeded in wells of a 48-well plate and were incubated overnight with murine recombinant IFN-γ (100 U/ml). Macrophages were infected with wild-type *M. tuberculosis*, the *sodC* mutant (Mtb1612), or the *sodC*-complemented strain (Mtb1623) at a multiplicity of infection of 10:1. Bacteria were opsonized by incubation with normal mouse serum for 30 min at 37°C prior to infection. After the addition of bacteria, the cells were incubated at 37°C for 1 h to allow the bacteria to be phagocytized. Nonadherent bacteria were removed by being washed three times with RPMI plus 2% fetal calf serum. After washing, fresh RPMI with 10% fetal calf serum was added and the cultures were further incubated. Macrophages were lysed at 0, 2, 6, 24, and 36 h, and numbers of surviving intracellular bacteria were determined by plating on 7H10 medium (6). Data are expressed as means and standard errors of the means from triplicate (C57BL/6 and gp91$^{phox-/-}$) or quadruplicate (iNOS$^{-/-}$) wells at each time point.

Detection of ROS and RNS. The production of ROS and RNS was measured in macrophages from C57BL/6 mice used in the macrophage killing assays. Macrophages (2.5×10^5 per well) were seeded in wells of a 48-well plate and were incubated overnight with murine recombinant IFN-γ (100 U/ml). Macrophages were either infected with wild-type *M. tuberculosis* as described above, stimulated with phorbol myristate acetate (PMA) (100 ng/ml), or left unstimulated. The production of superoxide was quantified by detecting the reduction of nitroblue tetrazolium (NBT) within the macrophages (1). For each measurement, culture medium was removed from triplicate wells of macrophages, 0.5 ml of 0.1% NBT in phosphate-buffered saline was added to each well, and the cells were incubated at 37°C for 15 min. The NBT solution was then removed, and the cells were resuspended in 1 ml of dimethyl sulfoxide to solubilize the formazan precipitate that resulted from the reduction of NBT. Formazan amounts were quantitated by measuring the optical density at 570 nm against a dimethyl sulfoxide reference. Triplicate samples were examined at 0, 2, 6, 24, and 36 h posttreatment in two independent experiments, and the values were then combined and expressed as the mean and standard error of the mean.

The production of RNS was detected by quantitating the amount of nitrite released by macrophages, using the Griess reagent (37). Culture supernatants removed from cells used in the macrophage killing assay were filtered through a 0.22-μm-pore-size filter to remove infectious bacteria. One hundred microliters of supernatant was mixed with 100 μl of the Griess reagent and incubated at 25°C for 10 min. The OD_{550} was read, and the nitrite values were calculated using a standard curve prepared by using $NaNO_2$. Triplicate samples were examined at 0, 2, 6, 24, and 36 h posttreatment in two independent experiments, and the values were then expressed as means and standard errors of the means.

Southern hybridization analysis. Southern blot analysis was carried out as previously described (6). Briefly, chromosomal DNA was digested with *Not*I, electrophoresed through 0.8% agarose gels, and transferred overnight onto a nylon membrane. The membrane was probed with the entire open reading frame of the *sodC* gene which had been amplified by PCR from *M. tuberculosis* Erdman DNA.

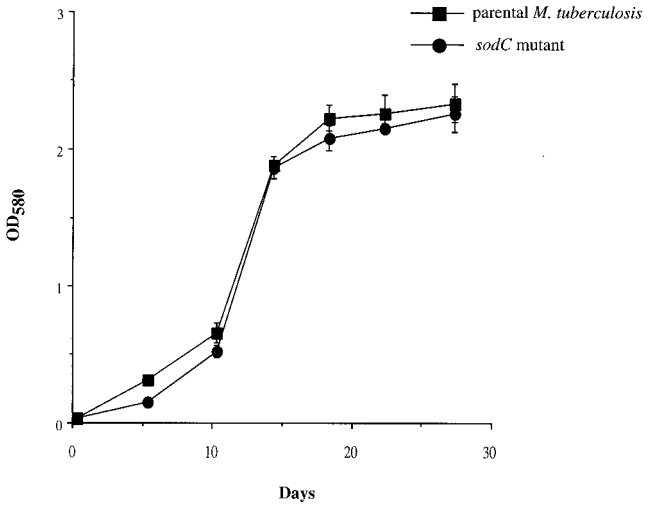

FIG. 2. Growth of the *sodC* mutant Mtb1612 and parental *M. tuberculosis* Erdman in 7H9 broth. Growth was monitored by reading the OD_{580} and is expressed as the mean and standard error of the mean for triplicate samples.

RESULTS

Construction of a *sodC* mutant of *M. tuberculosis*. To evaluate whether the Cu,Zn SOD is essential for the protection of *M. tuberculosis* from the toxic effects of ROS and from killing by macrophages, we constructed an *M. tuberculosis* mutant defective in the *sodC* gene. In *sodC* mutant Mtb1612, the *sodC* gene has been disrupted by the insertion of the *aph* and *hyg* genes (Fig. 1A.). This additional DNA increases the size of the *sodC*-containing fragment, as shown in Fig. 1B. A similar-sized fragment hybridizes with the *aph* probe, confirming that the *sodC* mutant contains the kanamycin resistance gene that produced the mutation (Fig. 1B.). The *sodC*-complemented strain (Mtb1623) carries both a mutated copy and a wild-type copy of *sodC* (Fig. 1B).

The *sodC* mutant of *M. tuberculosis* grows normally in 7H9 medium. Toxic ROS are generated within cells during aerobic respiration (27). To address whether SodC is essential for maintaining normal growth of *M. tuberculosis* in laboratory medium, we compared growth of parental *M. tuberculosis* with that of the *sodC* mutant in 7H9 broth. Cultures were grown at 37°C in an atmosphere of 19 to 20% O_2 and 5% CO_2 and were monitored for 30 days by reading the OD_{580}. As shown in Fig. 2, the *sodC* mutant exhibited no defect in growth under these conditions.

The *M. tuberculosis sodC* mutant is sensitive to superoxide-dependent killing. The sensitivity of the *sodC* mutant to superoxide was tested using hypoxanthine/xanthine oxidase to generate superoxide externally (11). Following a 3-h exposure to superoxide, there was greater than a 90% decrease in survival of the *sodC* mutant (Fig. 3A). The majority of this killing took place during the first hour of exposure to superoxide. Sensitivity of the *sodC* mutant to superoxide was significantly greater than that of parental *M. tuberculosis,* which declined in viability by 15% over the 3-h period. In the *sodC*-complemented strain,

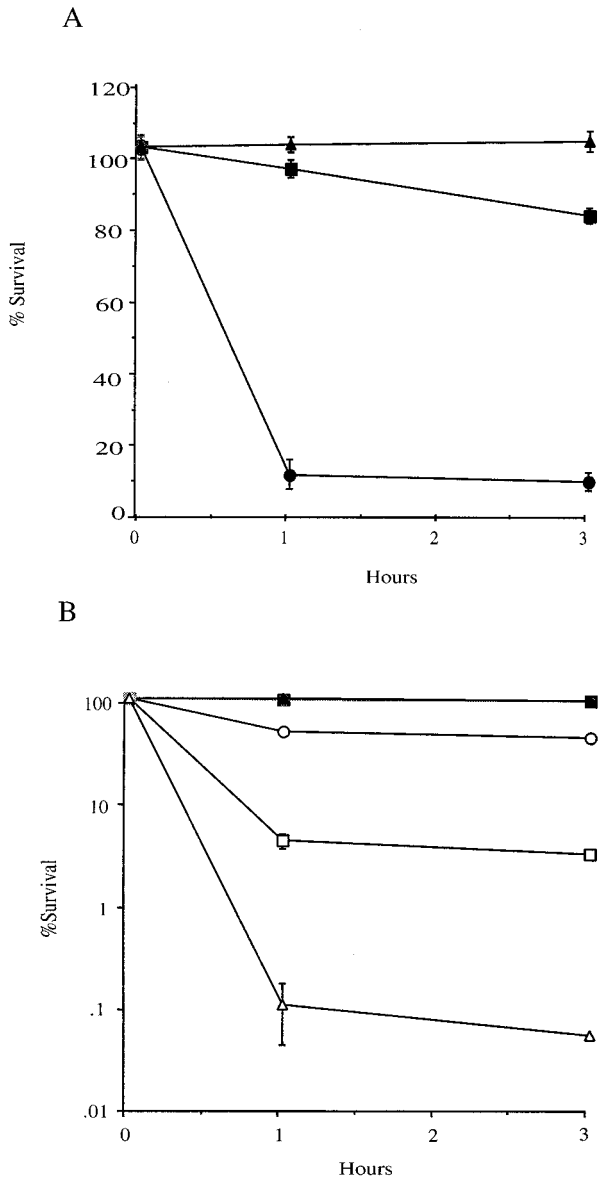

FIG. 3. (A) Survival of the *sodC* mutant Mtb1612 (circles) and the *sodC*-complemented strain Mtb1623 (triangles) was compared to survival of parental *M. tuberculosis* (squares) using hypoxanthine/xanthine oxidase to generate superoxide. The number of surviving bacteria was determined at 0, 1, and 3 h after exposure to superoxide by plating dilutions of the bacteria on 7H10 plates. The means from triplicate tubes were calculated, and the data are expressed as percentages of the time zero value. Results of a representative assay from six experiments are shown. (B) Survival of the *sodC* mutant Mtb1612 (open symbols) was compared to that of parental *M. tuberculosis* (closed symbols) using hypoxanthine/xanthine oxidase and SPER/NO (Alexis Biochemicals) to generate superoxide (squares), nitric oxide (circles), or a combination of both (triangles). The number of surviving bacteria was determined at 0, 1, and 3 h after exposure to the compounds by plating dilutions on 7H10 medium. The means from triplicate tubes were calculated, and the data are expressed as percentages of the time zero value. Results of a representative assay from three experiments are shown.

resistance to superoxide was restored to levels slightly higher than in parental *M. tuberculosis*. The higher level of resistance in the complemented strain may be due to differences in levels of expression of the *sodC* gene in these two strains. In the complemented strain, the *sodC* gene is expressed from the *aph* promoter, whereas in the parental strain, *sodC* is expressed from its own promoter.

Superoxide can interact with nitric oxide to produce peroxynitrite or other synergistic toxic products that have greater toxic activity than superoxide alone. Therefore, we compared the sensitivities of the *sodC* mutant to superoxide, to nitric oxide, or to a combination of superoxide and nitric oxide. Parental *M. tuberculosis* retained full viability after a 3-h exposure to superoxide, to nitric oxide, or to the combination of superoxide and nitric oxide (Fig. 3B). In contrast, the viability of the *sodC* mutant was reduced by 90% in the superoxide-generating cultures and by 10% in the nitric oxide-generating cultures. The combination of superoxide and nitric oxide proved extremely toxic to the *sodC* mutant, with a 1,000-fold decrease in viability. These data establish that *M. tuberculosis* requires the Cu,Zn SOD to maintain full resistance to the toxic effects of exogenously generated superoxide or its synergistic products.

Cu,Zn SOD contributes to the survival of *M. tuberculosis* in macrophages that are generating an oxidative burst. Production of ROS and RNS by macrophages is a major component in the host's antimicrobial defense. To determine if Cu,Zn SOD contributes to the resistance of *M. tuberculosis* to killing by macrophages, survival of the *sodC* mutant after phagocytosis by macrophages was assessed. Murine peritoneal macrophages were used in these assays because they can be stimulated to produce large amounts of ROS and NOS in vitro. Although human macrophages produce both ROS and RNS in vivo, human macrophages in general produce lower levels of RNS in vitro (29) and are thus less informative for in vitro studies to examine the effect of RNS. Macrophages from phagocyte oxidase-deficient mice ($gp91^{phox-/-}$) were used to identify intracellular killing that was dependent upon the generation of ROS. Macrophages deficient in the iNOS gene ($iNOS^{-/-}$) were used to examine killing that was dependent upon RNS production. Macrophages were elicited with sodium periodate, which activates the cells to produce both ROS and RNS (12). A portion of the macrophages were further activated by incubation with IFN-γ for 20 h prior to infection. In macrophages not activated with IFN-γ, we observed no killing with any of the bacterial strains (data not shown). Therefore, all further experiments were performed with IFN-γ-activated cells. There was significant killing of the *sodC* mutant in macrophages from C57BL/6 mice (Fig. 4A). By 6 h after infection, there was a 43% decrease in survival of the *sodC* mutant, while the numbers of parental *M. tuberculosis* cells and the *sodC*-complemented mutant cells declined only slightly. Sensitivity of the *sodC* mutant to killing by macrophages was abolished in cells from phagocyte oxidase-deficient mice ($gp91^{phox-/-}$) (Fig. 4B), indicating that killing of the *sodC* mutant was due to the oxidative burst. Interestingly, overall killing of the *sodC* mutant in the iNOS-deficient macrophages was similar to killing in the normal macrophages, with a 46% decrease in viability at 6 h (Fig. 4C). This suggests that the *sodC* mutant was not exposed to synergistic products formed by the interaction of ROS with RNS during the in vitro macrophage assay.

To examine whether the kinetics of killing of the *sodC* mutant in the C57BL/6 macrophages correlated with the production of respiratory burst products in these cells, we measured the production of superoxide and nitrite following infection

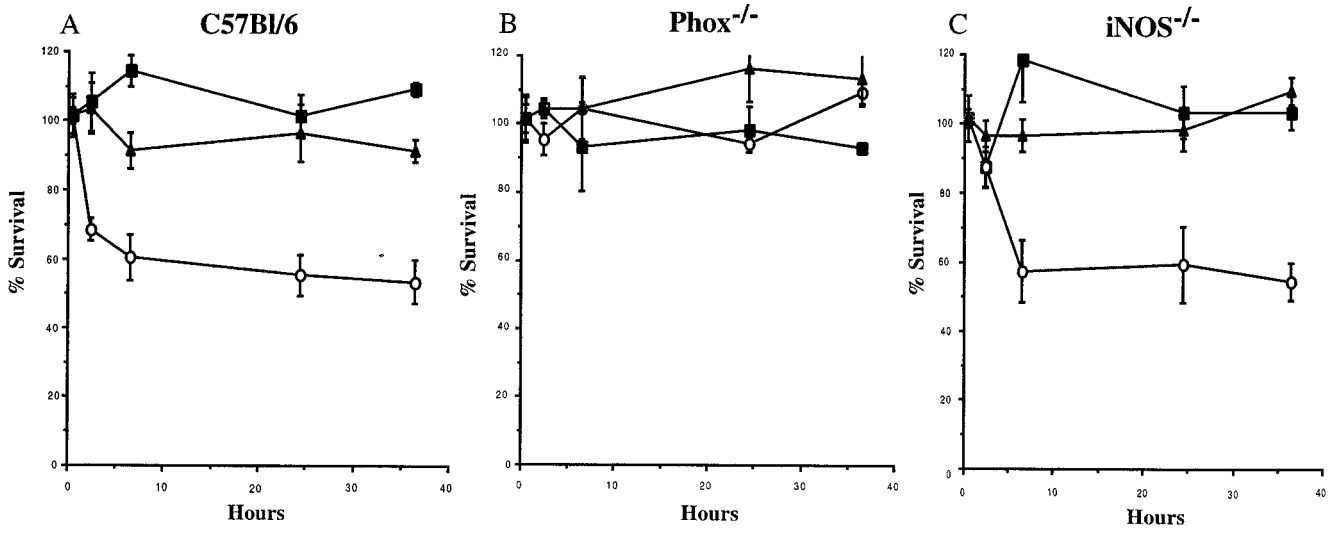

FIG. 4. Survival of parental *M. tuberculosis* (squares), the *sodC* mutant Mtb1612 (circles), or the *sodC*-complemented strain Mtb1623 (triangles) in peritoneal macrophages from C57BL/6 mice (A), gp91$^{phox-/-}$ mice (B), or iNOS$^{-/-}$ mice (C). Macrophages were activated with IFN-γ (100 U/ml) overnight and were then infected with bacteria at a multiplicity of infection of 10:1. The number of surviving bacteria was determined by plating dilutions of the macrophage lysate on 7H10 plates. The data are expressed as the mean and standard error of the mean from triplicate wells (C57BL/6 and iNOS$^{-/-}$) or quadruplicate wells (gp91$^{phox-/-}$) at each time point.

with parental *M. tuberculosis*. Superoxide was detected by measuring the reduction of NBT, while the production of nitrite was detected with the Griess reagent. Significant levels of superoxide were detected at 2 h after infection in the *M. tuberculosis*-infected cells, and by 6 h, superoxide levels had peaked (Table 1). PMA stimulated the production of superoxide at 2 h in parallel cultures of macrophages, and superoxide levels remained elevated for the 36-h assay. Production of superoxide in uninfected macrophages was negligible. Generation of significant levels of nitrite in the *M. tuberculosis*-infected cells occurred significantly later than with superoxide and was not detected until the 24-h time point. Thus, inactivation of the *sodC* mutant within C57BL/6 macrophages corresponds with the initial detection of superoxide production in the infected cells.

The *sodC* gene is present in most mycobacterial species. To evaluate the distribution of the *sodC* gene in mycobacterial species other than *M. tuberculosis*, chromosomal DNAs from 15 mycobacterial species were probed with the *sodC* gene from *M. tuberculosis.* By Southern blot analysis, the *sodC* gene was detected in the majority of mycobacteria assayed. These include members of the tuberculosis complex (*M. tuberculosis* [Erdman], *M. africanum*, *M. bovis*, *M. microti*, and *M. bovis* BCG) (Fig. 5, lanes 1 to 5), two of three rapid-growing species of mycobacteria (*M. fortuitum* and *M. smegmatis*) (Fig. 5, lanes 6 and 8), and seven slow-growing mycobacteria (*M. xenopi*, *M. gordonae*, *M. marinum*, *M. scrofulaceum*, *M. kansasii*, *M. intracellulare,* and *M. avium*) (Fig. 5, lanes 9 to 15). A *sodC*-hybridizing sequence was not detected in *M. chelonae* in two different preparations of DNA. Thus, it appears that the *sodC* gene is widely distributed among mycobacteria.

DISCUSSION

The phagocyte respiratory burst is important for controlling infections caused by many pathogens, as evidenced by clinical observations of patients with chronic granulomatous disease (28). This is true for mycobacterial infections, including BCG and *M. tuberculosis,* which exhibit an increased incidence in chronic granulomatous disease patients (19, 23, 28). In addition, experiments performed with phagocyte oxidase knockout mice have established that the respiratory burst of the host contributes to the control of *M. tuberculosis* during experimental infections (2, 10). However, *M. tuberculosis* can persist in the macrophage despite the activity of the macrophage NADPH

TABLE 1. Superoxide and nitrite production by IFN-γ-activated C57BL/6 macrophages

h	NBT reduction (OD_{570})[a] in:			Nitrite production (μM)[b] in:		
	Uninfected macrophages	Macrophages treated with PMA (100 ng/ml)	*M. tuberculosis*-infected macrophages	Uninfected macrophages	Macrophages treated with PMA (100 ng/ml)	*M. tuberculosis*-infected macrophages
0	0	0	0	0	0	0
2	0.009 ± 0.001	0.093 ± 0.013	0.061 ± 0.010	9.8 ± 0.100	9.7 ± 0.150	9.7 ± 0.100
6	0.015 ± 0.002	0.225 ± 0.029	0.274 ± 0.015	9.7 ± 0.100	9.7 ± 0.100	9.7 ± 0.050
24	0.017 ± 0.009	0.254 ± 0.024	0.052 ± 0.003	2.1 ± 0.150	23.5 ± 0.300	113.0 ± 1.50
36	0.021 ± 0.004	0.114 ± 0.018	0.060 ± 0.006	2.1 ± 0.001	21 ± 0.008	165.0 ± 3.00

[a] Superoxide was measured by reduction of NBT. Data are means ± standard errors of the means six wells in two independent assays.
[b] Nitrite production was measured using the Griess reagent. Data are means ± standard errors of the means (per well) from six wells in two independent assays.

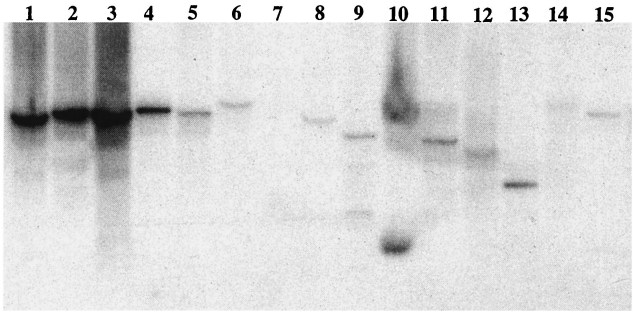

FIG. 5. Southern blot of chromosomal DNAs from 15 mycobacterial species. DNA was digested with *Not*I and probed with the *sodC* gene from *M. tuberculosis*. Lane 1, *M. tuberculosis;* lane 2, *M. africanum;* lane 3, *M. bovis;* lane 4, *M. microti;* lane 5, *M. bovis* BCG; lane 6, *M. fortuitum;* lane 7, *M. chelonae;* lane 8, *M. smegmatis*; lane 9, *M. avium;* lane 10, *M. intracellulare;* lane 11, *M. gordonii;* lane 12, *M. marinum;* lane 13, *M. scrofulaceum*; lane 14, *M. kansasii;* and lane 15, *M. xenopi*.

oxidase and iNOS, indicating that *M. tuberculosis* has defenses to protect it from the toxic effects of ROS and RNS.

In this report, we establish that Cu,Zn SOD of *M. tuberculosis* is protective against the toxic effects of superoxide generated by hypoxanthine/xanthine oxidase and contributes to resistance to killing by oxidative products generated by activated macrophages. Recently it was reported that Cu,Zn SOD mutants of *M. tuberculosis* and of BCG are only moderately sensitive to superoxide generated in vitro using the superoxide-generating agent menadione or plumbagin and that the BCG *sodC* mutant was unaffected in activated murine bone marrow macrophages or in guinea pig tissues (13). Although differences in methodology make it difficult to compare the present data and results in the previous report, the *sodC* mutant appears to have greater sensitivity to superoxide that is generated extracellularly using hypoxanthine/xanthine oxidase. Both plumbagin and menadione are known to increase intracellular levels of superoxide (3, 15, 21). If the Cu,Zn SOD protects the surface of the bacteria from externally generated superoxide, the influence of a *sodC* mutation is expected to be more pronounced when an extracellular superoxide-generating agent such as hypoxanthine/xanthine oxidase is used. We also predict that the contribution of the Cu,Zn SOD to survival of *M. tuberculosis* in macrophages is dependent upon the quantity of ROS generated. In our study, murine peritoneal macrophages activated with IFN-γ were used because they produce large amounts of ROS in response to infection. In previous studies, we have noted that bone marrow-derived macrophages produce a less robust oxidative burst than peritoneal macrophages. In the present study, the *sodC* mutant was sensitive to killing by peritoneal macrophages from normal mice, and this sensitivity was abolished in peritoneal macrophages from gp91$^{phox-/-}$ mice. These results indicate that Cu,Zn SOD contributes to the resistance of *M. tuberculosis* to phagocyte-derived oxidative burst products.

Inactivation by macrophages of the *sodC* mutant corresponded with the initial detection of superoxide production in these cells. From the kinetics of inactivation of the *sodC* mutant in macrophages, it appears that the initial interaction with superoxide is most critical in determining whether the mutant bacteria are killed. After the first 6 h of infection, we observed significantly less death of the *sodC* mutant even though elevated levels of superoxide were detected in the macrophages. Possible reasons for this later stabilization of *sodC* mutant viability are alterations in the sensitivity of the bacteria to superoxide during the later stages of infection due to changes in expression of other gene products or changes in the localization of ROS products during infection. These questions will be addressed in future studies

In concert with the phagocyte respiratory burst, macrophages generate toxic NOS. Synergism between the NADPH oxidase and iNOS pathways generate products with enhanced toxicity. Peroxynitrite as well as other synergistic intermediates formed by the reaction of ROS with nitric oxide have potent antimicrobial activity (29, 30). In our studies, parental *M. tuberculosis* was resistant to a combination of superoxide and nitric oxide when generated in vitro. This is consistent with a previous report that virulent *M. tuberculosis* is relatively resistant to peroxynitrite (43). The *sodC* mutant was, however, extremely sensitive in vitro to a combination of superoxide and nitric oxide. Despite this sensitivity in vitro, inactivation of the *sodC* mutant was unchanged in iNOS-deficient macrophages compared to normal macrophages. We also cultured macrophages from wild-type mice in medium deficient in L-arginine, which prevents the production of RNS, and did not observe a change in virulence of the *sodC* mutant (data not shown). Therefore, it appears that the production of RNS does not contribute to killing of the *sodC* mutant during the in vitro macrophage assay. This corresponds with our observations that the production of NO by the *M. tuberculosis*-infected macrophages does not occur until after killing of the *sodC* mutant has subsided and that there is no additional inactivation of the mutant with the appearance of NO at 24 h. It is clear that the simultaneous addition of exogenous superoxide and nitric oxide has different consequences for the *sodC* mutant than exposure to these products by macrophages cultured in vitro. Presumably, the kinetics for production of superoxide and nitric oxide in vivo may be different from those in our macrophage system, as activation of both the NADPH oxidase and the iNOS is dependent on the complex cytokine response generated by *M. tuberculosis* infection.

Cu,Zn SOD genes have been detected in a number of other bacteria, including *Haemophilus* (34), *Neisseria* (41), *Escherichia* (17), *Legionella* (36), and *Salmonella* (7). In some pathogenic bacteria, such as *Neisseria meningitidis* (41), *Salmonella enterica* serovar Typhimurium (11, 16), and *Haemophilus ducreyi* (34), the Cu,Zn SOD has been associated with virulence. In fact, virulent strains of *Salmonella* produce two distinct Cu,Zn SODs, each of which contributes to the virulence of *Salmonella* (14). In other pathogenic bacteria, an association of the Cu,Zn SOD with virulence is unclear. In the swine pathogen *Actinobacillus pleuropneumoniae,* a *sodC* mutant was not attenuated after intratracheal infection (35), and one of two studies using a *sodC* mutant of *Brucella abortus* found no attenuation of the mutant (22, 38). This suggests that the role of Cu,Zn SOD during infection may depend upon a variety of factors, including the infecting organism, host, route of acquisition, and site of infection.

In addition to the Cu,Zn SOD, *M. tuberculosis* carries an Mn,Fe SOD, SodA. Interestingly, SodA is one of the major

secreted proteins of *M. tuberculosis* (44). We observed a disproportionate production of SodA versus SodC on SOD activity gels, making it impossible to visualize SodC activity in this manner (data not shown). Although SodC is produced in much smaller amounts than SodA, the phenotype of the *M. tuberculosis sodC* mutant was very dramatic in the in vitro assays. Thus, despite the presence of large amounts of SodA, Cu,Zn SOD is essential for protecting *M. tuberculosis* from the toxic effects of superoxide.

The Cu,Zn SOD may serve another function for mycobacteria in addition to protecting the bacteria from ROS generated by activated macrophages. The *sodC* gene was detected in 14 out of 15 mycobacterial species, which include rapid growers and nonpathogenic species. This suggests that the *sodC* gene product may play a role in detoxifying superoxide during growth of bacteria outside the host. Although we detected no defect in growth of the *sodC* mutant during culture in 7H9 broth, the Cu,Zn SOD may protect the bacteria from endogenously generated superoxide under specialized conditions. Gort et al. have suggested that the Cu,Zn SOD of *Escherichia coli* plays a role in protecting the bacteria from endogenously produced superoxide generated during specific phases in the growth cycle, such as the transition into stationary phase (17). Cu,Zn SOD may fulfill a similar role in mycobacteria.

This report demonstrates that Cu,Zn SOD of *M. tuberculosis* is protective against extracellular superoxide and against a combination of superoxide and nitric oxide. Furthermore, *sodC* mutant *M. tuberculosis* has increased sensitivity to hydrogen peroxide compared to the isogenic parental strain (data not shown). These results support the hypothesis that the Cu,Zn SOD protects *M. tuberculosis* from extracellular sources of ROS. However, despite the contribution of SodC to survival in macrophages in vitro, a preliminary study using low-dose aerosol infection suggests that the *sodC* gene is not essential for survival of *M. tuberculosis* in the lungs of mice during early stages of infection (data not shown). In this initial study, no difference in bacterial numbers between parental *M. tuberculosis* and the *sodC* mutant was observed in the lung up to 60 days postinfection. At 60 days, a slight difference in organism burden in the lungs was noted. This may indicate that in the absence of SodC, SodA is capable of protecting the bacteria from superoxide generated during the early stages of pulmonary infection. While performing the macrophage assays, we observed that *sodC* mutant *M. tuberculosis* was sensitive to killing by macrophages only when the macrophages were activated with IFN-γ. In nonactivated macrophages, which produce a less vigorous oxidative burst, the phenotype of the *sodC* mutant was lost. This suggests that a major role for the Cu,Zn SOD is to protect *M. tuberculosis* from large quantities of toxic reactive products produced by activated macrophages. During the early stages of infection, the lower levels of respiratory burst products generated by nonactivated macrophages may not require the presence of Cu,Zn SOD. Future experiments will evaluate the contribution of Cu,Zn SOD to *M. tuberculosis* infections in a long-term in vivo study.

In conclusion, we have established that *M. tuberculosis* Cu,Zn SOD is required for resistance to exogenous superoxide-dependent cytotoxicity, including the products of activated macrophages. Further work will address the role of the Cu,Zn SOD during infection within the host and how the SOD activity derived from *sodC* relates to the Mn,Fe SOD in *M. tuberculosis.*

ACKNOWLEDGMENTS

This work was supported by NIH grants AI40075 (to N.A.B.), AI39557 and AI44486 (to F.C.F.), and AI40488 (to I.A.O.) and by a Research Training Fellowship from the American Lung Association of California (to D.L.P.).

We thank Joshua Fierer for the gift of the gp91$^{phox-/-}$ mice and Stewart Cole for cosmid Y22G10.

REFERENCES

1. **Absolom, D.** 1986. Basic methods for the study of phagocytosis. Methods Enzymol. **132:**95–180.
2. **Adams, L. B., M. Dinauer, D. Morgenstern, and J. Krahenbuhl.** 1997. Comparison of the roles of reactive oxygen and nitrogen intermediates in the host response to *Mycobacterium tuberculosis* using transgenic mice. Tuber. Lung Dis. **78:**237–246.
3. **Archibald, F., and M. Duong.** 1986. Superoxide dismutase and oxygen toxicity defenses in the genus *Neisseria*. Infect. Immun. **51:**631–641.
4. **Bogdan, C., M. Rollinghoff, and A. Diefenbach.** 2000. Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. Curr. Opin. Immunol. **12:**64–76.
5. **Bryk, R., P. Griffin, and C. Nathan.** 2000. Peroxynitrite reductase activity of bacterial peroxiredoxins. Nature **407:**211–215.
6. **Buchmeier, N., A. Blanc-Potard, S. Ehrt, D. Piddington, L. Riley, and E. Groisman.** 2000. A parallel intraphagosomal survival strategy shared by *Mycobacterium tuberculosis* and *Salmonella enterica*. Mol. Microbiol. **35:** 1375–1382.
7. **Canvin, J., P. Langford, K. Wilks, and J. Kroll.** 1996. Identification of *sodC* encoding periplasmic [Cu, Zn]-superoxide dismutase in *Salmonella*. FEMS Microbiol. Lett. **136:**215–220.
8. **Chan, J., X. Fan, S. Hunter, P. Brennan, and B. Bloom.** 1991. Lipoarabinomannan, a possible virulence factor involved in persistence of *Mycobacterium tuberculosis* within macrophages. Infect. Immun. **59:**1755–1761.
9. **Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry III, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, B. G. Barrell, et al.** 1998. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature **393:**537–544.
10. **Cooper, A. M., B. Segal, A. Frank, S. Holland, and I. Orme.** 2000. Transient loss of resistance to pulmonary tuberculosis in p47$^{phox\ -/-}$ mice. Infect. Immun. **68:**1231–1234.
11. **De Groote, M. A., U. A. Ochsner, M. U. Shiloh, C. Nathan, J. M. McCord, M. Dinauer, S. J. Libby, A. Vazquez-Torres, Y. Xu, and F. C. Fang.** 1997. Periplasmic superoxide dismutase protects *Salmonella* from products of phagocyte NADPH-oxidase and nitric oxide synthase. Proc. Natl. Acad. Sci. USA **94:**13997–14001.
12. **Ding, A., C. Nathan, and D. Stuehr.** 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. J. Immunol. **141:**2407–2412.
13. **Dussurget, O., G. Stewart, O. Neyrolles, P. Pescher, D. Young, and G. Marchal.** 2001. Role of *Mycobacterium tuberculosis* copper-zinc superoxide dismutase. Infect. Immun. **69:**529–533.
14. **Fang, F. C., M. DeGroote, J. Foster, A. Baumler, U. Ochsner, T. Testerman, S. Bearson, J. Giard, Y. Xu, G. Campbell, and T. Laessig.** 1999. Virulent *Salmonella typhimurium* has two periplasmic Cu, Zn-superoxide dismutases. Proc. Natl. Acad. Sci. USA **96:**7502–7507.
15. **Farr, S., D. Natvig, and T. Kogoma.** 1985. Toxicity and mutagenicity of plumbagin and the induction of a possible new DNA repair pathway in *Escherichia coli*. J. Bacteriol. **164:**1309–1316.
16. **Farrant, J., A. Sansone, J. Canvin, M. Pallen, P. Langford, T. Wallis, G. Dougan, and J. S. Kroll.** 1997. Bacterial copper- and zinc-cofactored superoxide dismutase contributes to the pathogenesis of systemic salmonellosis. Mol. Microbiol. **25:**785–796.
17. **Gort, A. S., D. Ferber, and J. Imlay.** 1999. The regulation and role of the periplasmic copper, zinc superoxide dismutase of *Escherichia coli*. Mol. Microbiol. **32:**179–191.
18. **Harth, G., and M. A. Horwitz.** 1999. Export of recombinant *Mycobacterium tuberculosis* superoxide dismutase is dependent upon both information in the protein and mycobacterial export machinery. J. Biol. Chem. **274:**4281–4292.
19. **Jacob, C., A. Pastorino, A. Azavedo, H. Marques, H. Sato, L. Ferrazole, M. Aquino, P. Sakane, and A. Grumach.** 1996. Mycobacterium bovis dissemination (BCG strain) among immunodeficient Brazilian infants. J. Investig. Allergol. Clin. Immunol. **6:**202–206.
20. **Jacobs, W. R., Jr., G. V. Kalpana, J. D. Cirillo, L. Pascopella, S. B. Snapper, R. A. Udani, W. Jones, R. G. Barletta, and B. R. Bloom.** 1991. Genetic systems for mycobacteria. Methods Enzymol. **204:**537–555.

21. **Kim, B., M. Han, and A. Chung.** 2001. Effects of reactive oxygen species on proliferation of Chinese hamster lung fibroblast (V79) cells. Free Radical Biol. Med. **30:**686–698.
22. **Latimer, E., J. Simmers, N. Sriranganathan, R. Roop, G. Schurig, and S. Boyle.** 1992. *Brucella abortus* deficient in copper/zinc superoxide dismutase is virulent in BALB/c mice. Microb. Pathog. **12:**105–113.
23. **Lau, Y., G. Chan, Y. Hui, and K. Yuen.** 1998. The role of the phagocytic burst in host defense against *Mycobacterium tuberculosis*. Clin. Infect. Dis. **26:**226–227.
24. **Laubach, V. E., E. G. Shesely, O. Smithies, and P. A. Sherman.** 1995. Mice lacking inducible nitric oxide synthase are not resistant to lipopolysaccharide-induced death. Proc. Natl. Acad. Sci. USA **92:**10688–10692.
25. **Liochev, S. I., and I. Fridovich.** 1994. The role of $O_2^{\cdot -}$ in the production of $HO^{\cdot}$: in vitro and in vivo. Free Radical Res. **16:**29–33.
26. **Manca, C., S. Paul, C. E. Barry III, V. H. Freedman, and G. Kaplan.** 1999. *Mycobacterium tuberculosis* catalase and peroxidase activities and resistance to oxidative killing in human monocytes in vitro. Infect. Immun. **67:**74–79.
27. **Miller, R., and B. Britigan.** 1997. Role of oxidants in microbial pathophysiology. Clin. Microbiol. Rev. **10:**1–18.
28. **Mouy, R., A. Fischer, E. Vilmer, R. Seger, and C. Griscelli.** 1989. Incidence, severity and prevention of infections in chronic granulomatous disease. J. Pediatr. **114:**555–560.
29. **Nathan, C., and M. Shiloh.** 2000. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. Proc. Natl. Acad. Sci. USA **97:**8841–8848.
30. **Pacelli, R., D. Wink, J. Cook, M. Krishna, W. DeGraff, N. Friedman, M. Tsokos, A. Samuni, and J. Mitchell.** 1995. Nitric oxide potentiates hydrogen peroxide-induced killing of *Escherichia coli*. J. Exp. Med. **182:**1469–1479.
31. **Pollock, J. D., D. A. Williams, M. A. C. Gifford, L. Li, X. Du, J. Fisherman, S. Orkin, C. Doerschik, and M. C. Dinauer.** 1995. Mouse model of X-linked chronic granulomatous disease, an inherited defect in phagocyte superoxide production. Nat. Genet. **9:**202–209.
32. **Rouse, D., J. A. DeVito, Z. Li, H. Byer, and S. L. Morris.** 1996. Site-directed mutagenesis of the *katG* gene of *Mycobacterium tuberculosis*: effects on catalase-peroxidase activities and isoniazid resistance. Mol. Microbiol. **22:**583–592.
33. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
34. **San Mateo, L., K. Toffer, P. Orndorff, and T. Kawula.** 1999. Neutropenia restores virulence to an attenuated Cu,Zn superoxide dismutase-deficient *Haemophilus ducreyi* strain in the swine model of chancroid. Infect. Immun. **67:**5345–5351.
35. **Sheehan, B., P. Langford, A. Rycroft, and J. S. Kroll.** 2000. [Cu,Zn]-superoxide dismutase mutants of the swine pathogen *Actinobacillus pleuropneumoniae* are unattenuated in infections of the natural host. Infect. Immun. **68:**4778–4781.
36. **St. John, G., and H. Steinman.** 1996. Periplasmic copper-zinc superoxide dismutase of *Legionella pneumophila*: role in stationary-phase survival. J. Bacteriol. **178:**1578–1584.
37. **Stuehr, D. J., and M. Marletta.** 1985. Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to *E. coli* lipopolysaccharide. Proc. Natl. Acad. Sci. USA **82:**7738–7742.
38. **Tatum, F., P. Detilleux, J. Sacks, and S. Hallig.** 1992. Construction of Cu-Zn superoxide dismutase deletion mutants of *Brucella abortus*: analysis of survival in vitro in epithelial and phagocytic cells and in vivo in mice. Infect. Immun. **60:**2863–2869.
39. **Teixeira, H. D., R. Schumacher, and R. Meneghini.** 1998. Lower intracellular hydrogen peroxide levels in cells overexpressing CuZn-superoxide dismutase. Proc. Natl. Acad. Sci. USA **95:**7872–7875.
40. **Wengenack, N. L., M. P. Jensen, F. Rusnak, and M. K. Stern.** 1999. *Mycobacterium tuberculosis* KatG is a peroxynitritase. Biochem. Biophys. Res. Commun. **256:**485–487.
41. **Wilks, K., K. Dunn, J. Farrant, K. Reddin, A. Gorringe, P. Langford, and J. S. Kroll.** 1998. Periplasmic superoxide dismutase in meningococcal pathogenicity. Infect. Immun. **66:**213–217.
42. **Wu, C. H. H., J. Tsai-Wu, Y. Huang, C. Lin, G. Lioua, and F. Lee.** 1998. Identification and subcellular localization of a novel Cu, Zn superoxide dismutase of *Mycobacterium tuberculosis*. FEBS Lett. **439:**192–196.
43. **Yu, K., C. Mitchell, Y. Xing, R. Magliozzo, B. Bloom, and J. Chan.** 1999. Toxicity of nitrogen oxides and related oxidants on mycobacteria: *M. tuberculosis* is resistant to peroxynitrite anion. Tuber. Lung Dis. **79:**191–198.
44. **Zhang, Y., R. Lathigra, T. Garbe, D. Catty, and D. Young.** 1991. Genetic analysis of superoxide dismutase, the 23 kilodalton antigen of *Mycobacterium tuberculosis*. Mol. Microbiol. **5:**381–391.

Editor: E. I. Tuomanen