Bacillus anthracis-derived nitric oxide is essential for pathogen virulence and survival in macrophages

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Phagocytes generate nitric oxide (NO) and other reactive oxygen and nitrogen species in large quantities to combat infecting bacteria. Here, we report the surprising observation that *in vivo* survival of a notorious pathogen—*Bacillus anthracis*—critically depends on its own NO-synthase (bNOS) activity. Anthrax spores (Sterne strain) deficient in bNOS lose their virulence in an A/J mouse model of systemic infection and exhibit severely compromised survival when germinating within macrophages. The mechanism underlying bNOS-dependent resistance to macrophage killing relies on NO-mediated activation of bacterial catalase and suppression of the damaging Fenton reaction. Our results demonstrate that pathogenic bacteria use their own NO as a key defense against the immune oxidative burst, thereby establishing bNOS as an essential virulence factor. Thus, bNOS represents an attractive antimicrobial target for treatment of anthrax and other infectious diseases.

anthrax | bacterial NO-synthase | oxidative stress

The spore-producing Gram-positive soil organism, *Bacillus anthracis*, is the causative agent of anthrax, an acute life-threatening infection in humans and domestic animals. Inhalation of *B. anthracis* spores results in a high rate of mortality, because effective treatment must be provided within a very short time after exposure (1). Deliberate dispersal of anthrax spores through the United States Postal Service in 2001 emphasized the importance of developing effective treatments to combat this potential biological scourge.

Although the innate immune response is the first line of defense against *B. anthracis*, its spores survive, germinate, and proliferate in macrophages, eventually bursting them to produce a lethal titer of infectious particles. The mechanism by which *B. anthracis* evades immune attack is not fully understood. Most studies have been focused on major virulence factors found on two plasmids (pXO1 and pXO2) that are responsible for exotoxins, capsule formation, and spore germination (2). These plasmid-borne virulence factors have been the prime candidates for anti-anthrax drug design. However, the ability of pathogens such as *B. anthracis* to survive in phagocytes also depends critically on the state of their oxidative stress defense system. Reactive oxygen species (ROS) play essential roles in innate immunity against many types of microorganisms (3–5). The antibacterial effects of ROS have been largely attributed to DNA and protein damage mediated by the Fenton reaction (6). This process generates hydroxyl radicals that react with DNA bases, sugar moieties, and amino acid side chains, causing various types of lesions (7). We showed that nonpathogenic *B. subtilis* utilizes its own nitric oxide (NO) to gain rapid protection against sudden oxidative damage (8). The mechanism of protection does not rely on transcriptional gene induction but rather on rapid suppression of DNA damage by preventing the Fenton reaction and direct activation of catalase (8). Here, we describe the key role of NO-synthase (bNOS)-derived NO in protecting germinating *B. anthracis* spores from macrophage oxidative attack, establishing the principal role of bacterial NO in pathogenicity.

Results and Discussion

Endogenous NO Protects *B. anthracis* from Oxidative Stress. To investigate the role of endogenous NO in defending *B. anthracis* from oxidative stress, we inactivated the bNOS-encoding gene with a Km cassette. Because the *nos* gene is not transcribed as part of any operon, and because inactivation was done by integration of the Km cassette in the middle of the gene, the mutation should not have any polar effects. The bNOS deletion did not significantly influence the growth rate in rich and sporulation media [supporting information (SI) Fig. 6]. To prove that the *nos* deletion abolished NO production, we took advantage of the highly specific copper-fluorescein-based NO fluorescent probe (CuFL) (9) (Fig. 1*A*). WT, but not Δ*nos*, cells exhibited bright fluorescence upon treatment with CuFL, indicating that bNOS-deficient cells lost their ability to produce NO (Fig. 1*A*). Very low residual fluorescence is probably nonspecific, because no other NO-producing enzymes were found in *B. anthracis*. This result demonstrates that *B. anthracis* NOS generates NO *in vivo* under physiological growth conditions.

We previously established the mechanism of endogenous and exogenous NO protection against oxidative stress in *B. subtilis* (8). Because *B. anthracis* and *B. subtilis* are close relatives, NO is likely to protect *B. anthracis* from oxidative stress by the same mechanism. The susceptibility of NOS-deficient *B. anthracis* to peroxide was monitored qualitatively by a modified antimicrobial disk technique. Plates seeded with corresponding bacterial strain were incubated overnight with a filter paper disk saturated with 0.45M H_2O_2 placed on top of brain heart infusion (BHI) agar. Δ*nos* cells formed a clear 5- to 7-mm zone around the H_2O_2 disk (Fig. 1*B*), whereas WT cells grew a complete lawn around the disk, thus demonstrating strong NOS-dependent resistance to hydrogen peroxide. A similar inhibition zone was also observed if the specific NOS inhibitors [*N*-methyl-L-arginine (NMLA) or *N*-nitro-L-arginine methyl ester (LNAME)] were added to the WT strain (Fig. 1*B*). Consistently, we found that the rate of H_2O_2 degradation in crude extracts of WT cells was ≈2-fold greater than in Δ*nos* cells (Fig. 1*C*), suggesting that the antioxidant effect of endogenous NO was at least in part due to stimulation of catalase activity. Indeed, vegetative catalase is the major enzyme that detoxifies hydrogen peroxide in *Bacilli* (10). It is an iron-heme protein and therefore a natural target for NO. Our previous results indicate that NO activates *B. subtilis* catalase directly via an S-nitrosylation mechanism (8).

To prove that NO indeed protected *B. anthracis* from oxidative stress, we examined the effect of a nontoxic single dose of NO

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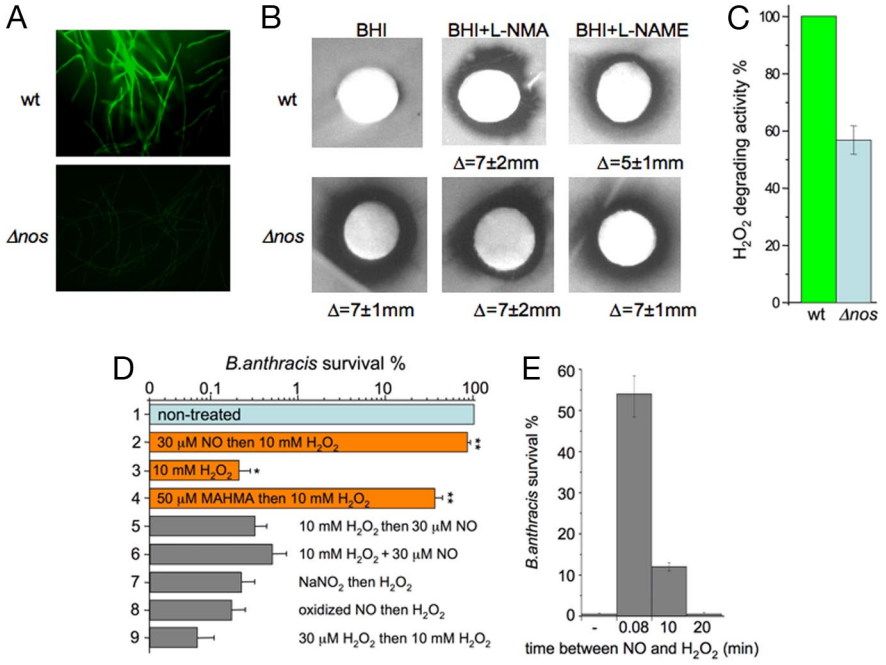

Fig. 1. NO protects *B. anthracis* against oxidative stress. (*A*) bNOS-dependent NO production *in vivo*. Representative fluorescent image of bacteria treated with Cu(II)-based NO-detecting probe (CuFL). *B. anthracis* Sterne (WT) and Δ*nos* cells were grown in LB medium to OD_{600} ≈0.5 followed by CuFL addition. (*B*) bNOS-mediated cytoprotection in *B. anthracis*. WT and Δ*nos* cells were grown aerobically in LB medium to late log phase (OD_{600} ≈0.8–0.9) at 30°C. An aliquot from each culture was diluted with an equal amount of fresh prewarmed LB medium and plated on BHI agar. 3MM disks were saturated with 10 mM H_2O_2 and placed on top of the bacterial lawns. Where indicated, the Brain Heart Infusion (BHI) media contained NOS inhibitors: 1 mM NG-methyl-L-arginine (L-NMA) or 1 mM L-NAME. Δ indicates the increase of the sterile zone. Values shown are the means and SD from four independent experiments. (*C*) Effect of *nos* deletion on catalase activity. Cells in the late exponential growth phase were collected one min after dilution with fresh LB medium and lysed immediately, and H_2O_2 degradation (catalase) activity was measured as described in *Materials and Methods*. Values are the mean ± SE from three experiments. (*D*) Rapid and specific protection against H_2O_2 toxicity by exogenous NO. Reagents were added as indicated to aerobically grown WT (Sterne) cells at OD_{600} ≈0.5 (in LB medium at 37°C). An aqueous solution of NO (bar 2), NO-donor MAHMA NONOate (bar 4), $NaNO_2$ (bar 7), or oxidized NO (bar 8) was added 5 sec before challenging cells with 10 mM H_2O_2 for 30 min. NO was oxidized by bubbling air into an aqueous solution of the gas for 2 h. In additional controls, NO was mixed with H_2O_2 before addition to cells (bar 6), and 10 mM H_2O_2 was added after preincubation (5 sec) with 30 μM H_2O_2 (bar 9). The percentage of surviving cells was determined by colony counting and is shown as the mean ± SD from three experiments. (*E*) Time course of NO-mediated cytoprotection. Culture aliquots were taken after preincubation with NO for the indicated time intervals and challenged with 10 mM H_2O_2 for 30 min. Chloramphenicol (Cm, 200 μg/ml) was added 10 min before NO/H_2O_2 treatment. Data are shown as the mean ± SD from three experiments.

(30 μM aqueous solution) on the survival of bacteria exposed to a lethal dose of hydrogen peroxide (10 mM). Within 5 sec of NO treatment, *B. anthracis* cells became ≈100 times more resistant to H_2O_2 challenge than nontreated bacteria (Fig. 1*D*, bar 2). The addition of NO simultaneously with or after H_2O_2 had no protective effect (Fig. 1*D*, bars 5 and 6), apparently because of rapid NO scavenging by the peroxide-derived radicals (10). Also, no cytoprotection was observed when oxidized NO or nitrite was added instead of NO (Fig. 1*D*, bars 7 and 8). Moreover, pretreatment with the same low concentration of H_2O_2 (30 μM) did not protect bacteria from a subsequent lethal dose (10 mM) of H_2O_2 (Fig. 1*D*, bar 9), indicating that the protective effect of NO is highly specific. These controls and the large molar excess (>300 times) of H_2O_2 rule out the possibility that the protective effect of NO is due to direct reaction with H_2O_2 or its products. A comparable level of protection from H_2O_2 was also achieved with the NO-donors methylamine hexamethylene NONOate (MAHMA NONOate) (bar 4), *S*-nitroso-*N*-acetylpenicillamine (SNAP), and propylamine propylamine NONOate (PAPA NONOate) (data not shown).

Although NO can activate various oxidative stress genes in bacteria to protect cells from reactive oxygen and nitrogen species (11–16), in our experiments, full protection was established within seconds of NO challenge (Fig. 1*E*), suggesting that gene induction is not required. Consistently, we found that inhibition of protein synthesis by the antibiotic chloramphenicol did not compromise NO-mediated cytoprotection (Fig. 1*E*).

Because in all of the experiments the NO-mediated effects were either identical or more potent compared with those observed in *B. subtilis*, we argue that NO protects *B. anthracis* by the same dual mechanism: suppression of the Fenton reaction and activation of catalase (8), with the former being predominant.

NO from *B. anthracis* Is an Essential Virulence Factor. To evaluate the contribution of bNOS to the pathogenicity of *B. anthracis*, the parental Sterne and Δ*nos* mutant strains were compared for virulence after s.c. inoculation of their spores into A/J susceptible mice (17). Δ*nos* spores exhibited a dramatic loss of virulence, with an LD50 increase of approximately three orders of magnitude compared with spores of the parental control strain (Fig. 2*A*). The *nos* gene is monocistronic, which excludes the possibility that the *nos* mutation exerted any polar effect leading to the virulent phenotype *in vivo*. Moreover, in comparison with WT, Δ*nos* mutant cells were much more susceptible to killing by the macrophages (Fig. 2*B*). J774A.1 macrophages were challenged with *B. anthracis* spores at a 1:50 ratio for 40 min, followed by removal of noninternalized bacteria. Infected macrophages were lysed at different time intervals, and the surviving bacteria were plated on Luria–Bertani (LB) agar for colony-forming unit enumeration. To determine whether spores or vegetative cells were responsible for the marked difference in colony-forming units between WT and Δ*nos* strains, each macrophage sample was spread on plates directly or applied to the plates after heat-mediated killing of vegetative cells. The results, expressed

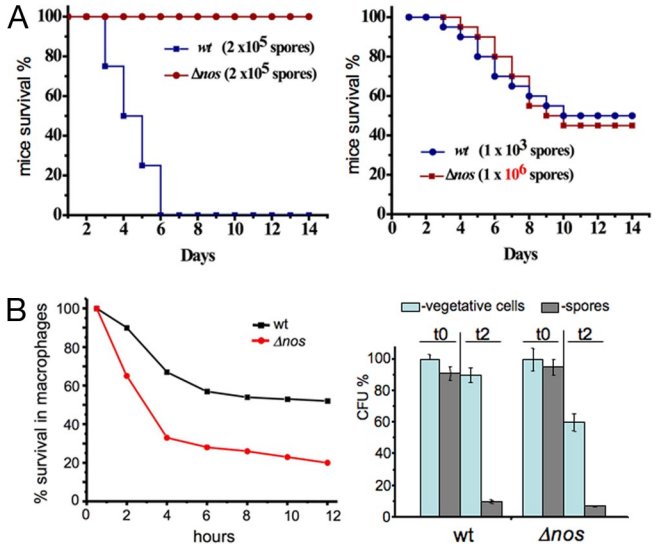

Fig. 2. bNOS is essential for *B. anthracis* virulence in mice and survival in macrophages. (*A*) LD50 of anthrax spores as a function of bNOS activity. The indicated amounts of *B. anthracis* spores (Sterne or Δ*nos*) were inoculated s.c. into 6- to 7-week-old A/J mice ($n = 10$ for each group). Infected animals were monitored and moribund animals were euthanized. Shown are the effect of LD100 (*Left*) and LD50 (*Right*) doses, previously established for this animal model (17) and adjusted to compensate for the compromised virulence of Δ*nos* (*Right*). (*B*) Effect of the *nos* deletion on anthrax spore germination in macrophages. (*Left*) Comparison of WT and Δ*nos B. anthracis* survival in J774A.1 macrophages as a function of time. Viable bacteria within macrophages were recovered and colony-forming units were determined 12 h after infection. Data are the mean of six independent experiments. To follow the spore germination status (*Right*), bacterial colony-forming units from macrophages were determined directly (vegetative cells) or after treatment for 40 min at 65°C (spores) at 40 min (t_0) or 2h (t_2) after phagocytosis. Data are the mean ± SD of three independent experiments ($P < 0.05$).

as a percentage of surviving cells, demonstrate that at 2 h after infection almost all of the spores germinated in macrophages (Fig. 2*B Right*). A control experiment showed that macrophages ingest Δ*nos* and WT spores with the same efficiency. We conclude that germinating bacteria, not spores, account for the increased susceptibility to macrophage-mediated killing of Δ*nos B. anthracis*.

Anthrax NO Production Occurs at the Early Stage of Infection. Considering that macrophages produce a large amount of NO to control infection (18–20) our seemingly paradoxical results can be rationalized if NO production by bacteria and macrophages is separated in time, leading to opposite outcomes. Indeed, it is well established that superoxide generation begins immediately after phagocytosis of bacteria. However, inducible NOS is activated only 8–12 h after infection, when the oxidative burst is largely over (4, 19). In contrast, *B. anthracis* germinating in macrophages would be expected to begin producing its own NO immediately from bNOS to antagonize the antimicrobial action of ROS. To test this hypothesis, we examined the level and source of NO production in infected macrophages at various times (Fig. 3). We first measured NO end products—nitrate and nitrite (NN). NO diffuses freely through cell walls and membranes, and, after escaping from cellular confinement, it is rapidly oxidized in the medium under aerobic conditions to form NN. Therefore, the level of NN in the media reflects NOS activity. We detected significant NN production in macrophages infected by WT, but not Δ*nos*, spores within 2 h after infection (Fig. 3*A*). Consistently, bNOS-specific NO production in infected macrophages was demonstrated by using CuFL (Fig. 3*B*). At 2 h after infection,

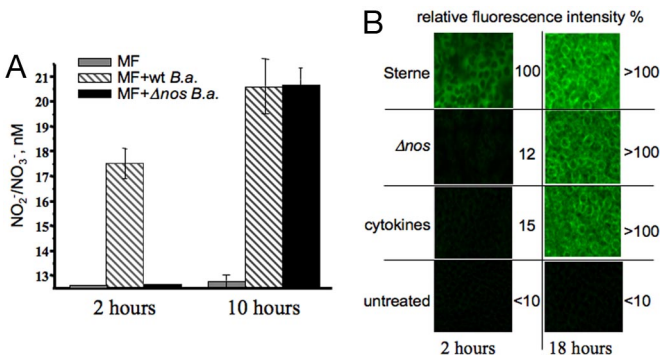

Fig. 3. NO production in *B. anthracis* infected macrophages. (*A*) NN accumulation in clarified supernatants of J774A.1 macrophages 2 and 10 h after infection with Sterne or Δ*nos* spores or noninfected control macrophages (MF). Experimental conditions are as in Fig. 2*B*. Values are the mean ± SD from four experiments. (*B*) Direct monitoring of NO production in J774A.1 infected or cytokines-treated macrophages (IL1β, 1 nM; TNFα, 50 ng/ml; and IFN, 250 μg/ml) with Cu(II)-based NO-detecting probe (CuFL) at 2 and 18 h after infection. The relative intensity of the fluorescent signal was calculated by IPLab Scientific image processing software.

germinating WT (Sterne) spores caused a bright fluorescence signal indicative of a high level of NO. However, the NO level remained at a basal level after infection by Δ*nos* spores or treatment with cytokines. Notably, at 18 h after infection, macrophages challenged with either WT or Δ*nos* spores or cytokines generated the large amount of their own NO (Fig. 3*B*). Taken together, these results show that the only major source of NO at an early stage of infection (≈2 h) is that produced by ingested bacteria, not by macrophages.

Model for the NO-Mediated Defense System in *B. anthracis*. To determine whether bacterial NO indeed protects *B. anthracis* from macrophage-inflicted oxidative damage, we monitored the state of bacterial genomic DNA in macrophages infected with either WT or Δ*nos* spores. Formation of double-strand breaks in DNA after peroxide exposure is the major cause of bacterial death (21, 22). As mentioned earlier, this global damage to bacterial DNA is a result of the Fenton reaction (7), which can be suppressed by NO (8). The integrity of DNA can be analyzed by quantitative PCR (23). Accordingly, treatment of exponentially growing bacteria by H_2O_2 caused a significant decrease in the yield of the PCR product (Fig. 4, lanes 5 and 6). We next isolated bacterial DNA at 2 h after infection and analyzed it by the same quantitative PCR method. DNA obtained from *nos* mutant cells was damaged to a much greater extent that that

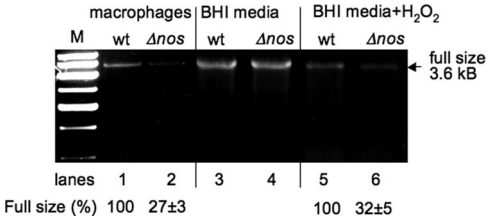

Fig. 4. *B. anthracis* NO protects bacteria from the macrophage-inflicted oxidative damage. bNOS-dependent bacterial DNA protection during macrophage infection. Chromosomal damage was monitored by qPCR. A representative agarose gel shows a 3.6-kb PCR fragment amplified from the Sterne or Δ*nos* chromosome. Genomic DNA was isolated from J774A.1 macrophages at 2 h after infection. Control DNA was isolated from bacteria grown exponentially in BHI media without (lanes 3 and 4) or with (lanes 5 and 6) 10 mM H_2O_2 treatment. M, 1 kb DNA marker. % indicates the fraction of the full size PCR products. Values are the mean ± SD from three experiments.

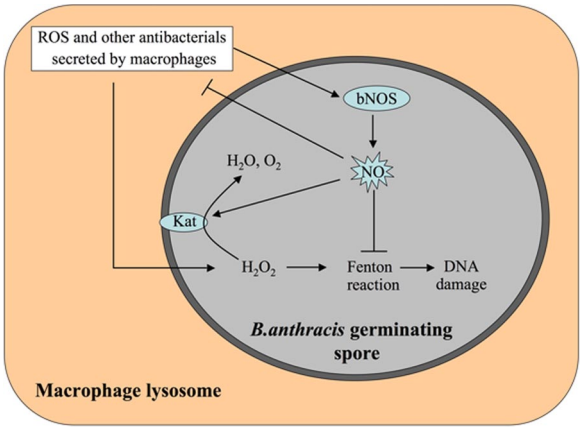

Fig. 5. The proposed mechanism of the NO-mediated defense system in *B. anthracis*. Upon germination, bNOS, which has been accumulated in the spore during the sporulation phase (32, 33), generates NO that instantly protects the pathogen from H_2O_2 toxicity by a dual mechanism. NO interrupts the production of damaging hydroxyl radicals from the Fenton reaction and directly activates catalase (Kat) (8), which has already been shown to be a part of the exosporium (34).

from the parental strain (Fig. 4). The yield of full-length PCR fragment decreased ≈4-fold. No DNA damage was detected in either mutant or WT vegetative cells in the absence of macrophages (Fig. 4, lanes 3 and 4). These results demonstrate that bNOS-derived NO protects the *B. anthracis* chromosome from the macrophage-induced Fenton reaction at an early stage of infection.

A large body of evidence implicates ROS in microorganism killing by stimulated phagocytes (3, 6, 18, 24). Our *in vitro* studies indicate that NO directly and rapidly activates a latent oxidative stress defense system in *B. anthracis*, which does not require additional protein synthesis (Fig. 1*E*) and thus must be available immediately upon spore germination. Taking together, the results presented in this report lead us to propose a novel antioxidant defense mechanism used by NOS-containing pathogenic bacteria such as anthrax (Fig. 5). In this mechanism, endogenous NO rapidly protects pathogens against immunological oxidative stress by suppressing the harmful Fenton reaction and directly activating catalase (8). Although the superoxide anion generated in the phagosome can react with NO to form toxic peroxynitrite ($ONOO^-$), the inability of O_2^- to pass through the bacteria cell wall and membrane makes this event highly unlikely. Instead, H_2O_2, a product of spontaneous or enzymatic O_2^- dismutation, readily enters bacterial cells, where it is the major cytotoxic species because it fuels the damaging Fenton reaction inside bacteria. Therefore, the rapid NO preconditioning that occurs via a dual mechanism—the inhibition of the Fenton reaction and the activation of catalase (8)—renders bacteria resistant to immune oxidative attack (Fig. 5). The primary sequence and high-resolution structure of bNOS reveal several unique features that distinguish it from mammalian NOS counterparts (25, 26), suggesting that bNOS-specific small molecular inhibitors can be designed. Indeed, some potent bNOS inhibitors are described in ref. 27. Because bNOS represents an important virulence factor, its selective inhibition offers a previously uncharacterized and specific approach to the development of antimicrobial therapy.

Materials and Methods

Chemicals and Reagents. CuFL was prepared as described in ref. 28. Kanamycin (Km), erythromycin (Em), lysozyme, H_2O_2, MAHMA NONOate, PAPA NONOate, and SNAP were purchased from Sigma. Restriction enzymes and T4 ligase were purchased from New England BioLabs. PCR was carried out with Ex TaqDNA polymerase (TaKaRa). NO solution was prepared in an airtight device by bubbling NO gas (Aldrich) that had been purified from higher oxides by passing it through a 1 M solution of KOH into water until the concentration of dissolved NO reached ≈300 μM. Milli-Q-grade water was deaerated by boiling and then cooling under argon (Praxair). Immediately before reactions were performed, the NO concentration was measured with an iso-NO Mark II electrode (WPI Instruments).

Animals. A/J mice were purchased from The Jackson Laboratory. Six-week-old female mice, each weighing 18 to 22 g, were used in all experiments. This strain of mice is susceptible to spores of the *B. anthracis* Sterne strain (17).

Bacterial Strains, Plasmids, and Mammalian Cell Lines. *B. anthracis* Sterne 34F2 ($pXO1^+pXO2^-$) was used as a parent strain. Plasmids were constructed by using standard methods and amplified in *E. coli* TG1 [*supE* Δ (*hsdM-mcrB*)5 ($r_k^-m_kMcrB$) *thi*Δ(*lac-proAB*) *F′* (*tra*Δ36 *proAB*+*lacI*^{q} *lacZ*Δ*M*15)]. Plasmids for *B. anthracis* transformation were first isolated from *E. coli* GM 2163 [*F*$^-$ *ara-14 leuB6 fhuA31 lacY1 tsx78 glnV44 galK2 galT22 mcrA dcm-6 hisG4 rfbD1 rpsL136 dam13*::*Tn9 xylA5 mtl-1 thi-1 mcrB1 hsdR2*] in unmethylated form. All PCR fragments were amplified from *B. anthracis* Sterne 34F2 chromosomal DNA, using *ExTaq* DNA polymerase (TaKaRa). Oligonucleotide primers (IDT) used for PCR of the Sterne *nos* (JGI ''Locus Tag'' BAS5299) are shown in *SI Materials*.

To construct pKS1::baNOS, two ≈500-bp fragments upstream and downstream of *B. anthracis nos* were amplified by PCR and cloned into pKS1 (29). The resulting plasmid (pKS1::baNOS) carries the kanamycin (Km) resistance gene flanked with these fragments. Sterne strain cells were transformed with pKS1::baNOS, Km^R Em^S colonies were selected, and double cross-over recombination events were confirmed by PCR. Preparation of electroporation-competent *B. anthracis* cells was carried out as follows: A 50-ml culture of *B. anthracis* was grown in BHIG [BHI (Difco) and 0.5% glycerol] at 37°C to OD_{600} ≈0.6. The cells were harvested by centrifugation at 4°C, and all subsequent steps were performed on ice. The cells were washed three times with an equal volume of 10% glycerol plus 1 mM Hepes (pH 7.0), resuspended in 2.5 ml of the same glycerol-Hepes solution, and kept on ice. Up to 5 μl of plasmid DNA (0.1–4 μg in water) was added to a 100-μl aliquot of cells, which was pulsed (2.5 kV, 25 μF, 200 Ω) in a 0.2-cm gap cuvette. The cells were resuspended in 1 ml of BHIG and incubated for 1.5 h at 30°C with aeration. Recovered cells were spread on LB medium or BHI agar plates, containing Km and Em. Colonies were visible after 20–24 h.

Bacteria were grown aerobically at 30 or 37°C in Luria–Bertani (LB) or brain heart infusion (BHI) media (Difco). The antibiotics Km and Em were used at final concentrations of 50 μg·ml^{-1} and 300 μg·ml^{-1} for *E. coli* or 100 μg·ml^{-1} and 3 μg·ml^{-1} for *B. anthracis*, respectively.

The macrophage cell line J774.A1 (TIB-67) was obtained from American Type Culture Collection and was maintained in DMEM containing 10% FBS (Gemini Bio-Products) and an antibiotic-antimycotic mixture (penicillin–streptomycin–fungizone) (Invitrogen) at 37°C with 5% CO_2.

General Methods. *B. anthracis* overnight cultures, grown in liquid brain heart infusion (BHI) media, were diluted 1:100 in fresh BHI and grown at 37°C with aeration to OD_{600} ≈1.0, unless indicated otherwise. To determine H_2O_2 resistance, *B. anthracis* cells were swabbed on BHI agar plate, and 3MM disks were applied on the plate surface. Ten milliliters of 450 mM H_2O_2 was added on each disk, and plates were incubated at 37°C overnight. To prepare bacterial cell extracts, *B. anthracis* cells were harvested, dissolved in lysis buffer [20 mM Tris·HCl (pH 7.9) and 150 mM NaCl] containing 125 μg/ml lysozyme (Sigma), incubated for 5 min at 37°C, sonicated, and clarified by centrifugation. The protein concentration was determined by using a BioRad protein assay kit. Nitrite was measured in clarified infected macrophage cell culture supernatants, using the fluorometric nitrite assay kit (Cayman). Macrophages were lysed, and bacteria colony-forming units were counted on LB agar plates. One-half milliliter of a 5×10^5 macrophage cell suspension was loaded in each well of a 24-well plate and incubated at 37°C with 5% CO_2 for 24 h. Spores were added to the macrophages (50:1) and incubated for 40 min Gentamicine (2 mg/ml) was then added to eliminate noningested spores and vegetative bacteria. After 20 min, the antibiotic media was replaced by fresh media and incubation was continued. At each subsequent hour, macrophages were lysed with 0.05% sodium desoxyholate and plated either directly or after heat-treatment on LB agar to determine the colony-forming units (vegetative cells or non-germinated spore). DNA manipulations and plasmid DNA isolation were performed by using standard procedures (30). *B. anthracis* spores were prepared in Difco sporulation medium as described in ref. 31.

Detection and Measurement of Bacterial NO. To quantify nitrate/nitrite (NN), cell culture supernatants were clarified by centrifugation and then filtered through YM-3 microcon (Millipore). NN were measured in the flow-through fraction with a fluorometric and calorimetric nitrate/nitrite assay kit (Cayman). NO production *in vivo* was detected by the NO-specific intracellular fluorescent CuFL probe, as described in ref. 9. CuFL was prepared immediately before use by mixing FL and $CuCl_2$ in a 1:1 ratio and then added to the growing bacterial cultures or macrophages to a final concentration of 10 μM. One hour later, fluorescent and visible images of bacteria treated with the NO-detecting probe were taken with a digital camera attached to an Axio microscope (Zeiss MicroImaging). The percentage of fluorescent bacteria was calculated by IPLab Scientific image processing software.

Catalase Activity Assay. Degradation of H_2O_2 was monitored in real time as a decrease in absorbance at 240 nm (10). Aliquots of extracts to be monitored or of pure catalase were mixed with 50 mM phosphate buffer (pH 7.0) and placed into a 1-ml quartz cuvette. H_2O_2 solution (40 mM) was added, and the kinetics of its degradation were recorded. Total H_2O_2 degrading activity was measured as the decrease of H_2O_2 concentration per milligram of total protein per second. OD_{240} was converted to the concentration of H_2O_2 according to the calibration curve (10 mM H_2O_2 = 0.36 OD_{240}).

Measurement of DNA Damage. Macrophages infected with WT or Δ*nos* spores were lysed and centrifuged to collect bacteria. Total genomic DNA was isolated from bacteria pellets according the GenElute bacterial genomic DNA kit protocol for Gram-positive bacteria (Sigma). DNA was extracted with phenol/chloroform and quantified by using a PicoGreen dsDNA quantitation reagent (Molecular Probes) and lambda phage DNA as a standard. An ≈3.6-kb fragment of AE017225 contig (JGI) was used for qPCR. Primer sequences were as follows: 5′-CTCAGCTGGTTAGAGCGCACGCCTG-3′ (forward) and 5′-CACCCCTTCTCCCGAAGTTACGGGGTC-3′ (reverse). PCR was performed by using Phusion DNA polymerase (Finnenzymes). The 50-μl PCR mixture contained 0.05–0.5 ng of genomic DNA as a template, 1.5 μM primers, 200 μM dNTPs (Fermentas), 5× Phusion GC PCR buffer, and 0.5 μl of DNA polymerase. DNA was subjected to 30 cycles of PCR (98°C for 30 s, 58°C for 30 s, 72°C for 9 min). PCR products were separated by electrophoresis in an 0.8% agarose gel, stained with ethidium bromide, scanned, and quantified with an AlphaImager (Imgen Technologies).

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