Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 6399–6403, July 1995
Microbiology

Genetic and redox determinants of nitric oxide cytotoxicity in a *Salmonella typhimurium* model

(nitrosothiol/peroxynitrite/dipeptide permease/RpoS/superoxide dismutase)

MARY ANN DE GROOTE*, DONALD GRANGER†, YISHENG XU*, GAIL CAMPBELL*, ROBERT PRINCE*, AND FERRIC C. FANG*

*Division of Infectious Diseases, University of Colorado Health Sciences Center, Box B168, 4200 East 9th Avenue, Denver, CO 80262; and †Division of Infectious Diseases, University of Utah School of Medicine, 50 North Medical Drive, Room 4B322, Salt Lake City, UT 84132

Communicated by Irwin Fridovich, Duke University Medical Center, Durham, NC, April 14, 1995

ABSTRACT **Paradoxically, nitric oxide (NO) has been found to exhibit cytotoxic, antiproliferative, or cytoprotective activity under different conditions. We have utilized *Salmonella* mutants deficient in antioxidant defenses or peptide transport to gain insights into NO actions. Comparison of three NO donor compounds reveals distinct and independent cellular responses associated with specific redox forms of NO. The peroxynitrite ($OONO^-$) generator 3-morpholinosydnonimine hydrochloride mediates oxygen-dependent *Salmonella* killing, whereas *S*-nitrosoglutathione (GSNO) causes oxygen-independent cytostasis, and the NO· donor diethylenetriamine–nitric oxide adduct has no antibacterial activity. GSNO has the greatest activity for stationary cells, a characteristic relevant to latent or intracellular pathogens. Moreover, the cytostatic activity of GSNO may best correlate with antiproliferative or antimicrobial effects of NO, which are unassociated with overt cell injury. *dpp* mutants defective in active dipeptide transport are resistant to GSNO, implicating heterolytic NO^+ transfer rather than homolytic NO· release in the mechanism of cytostasis. This transport system may provide a specific pathway for GSNO-mediated signaling in biological systems. The redox state and associated carrier molecules are critical determinants of NO activity.**

Nitric oxide (NO) cytotoxicity has been demonstrated for a rapidly expanding list of helminths, protozoa, yeasts, bacteria, and viruses (reviewed in ref. 1), as well as for tumor cells (2). This property of NO has important implications for understanding mechanisms of antimicrobial defense, antitumor defense, cell injury in inflammatory diseases, and food preservation by nitrites. Potential molecular targets of NO include transition metals, thiols, lipids, and DNA (3). Interaction with reactive oxygen intermediates is generally believed to be required for NO cytotoxicity (4), but the precise mechanisms are incompletely understood. Moreover, it is not presently understood how NO can possess cytotoxic, antiproliferative, or cytoprotective activity under different conditions (5).

Under physiologic conditions, NO may react with thiol-containing molecules such as glutathione (GSH) to form *S*-nitrosothiols (6–8). *S*-Nitrosothiols have been detected in human bronchoalveolar lavage fluid, plasma, platelets, and polymorphonuclear neutrophils (7), with higher concentrations measured in inflammatory states (8). Although conventionally viewed as NO donor compounds that undergo spontaneous homolytic release of NO·, *S*-nitrosothiols are also capable of heterolytic transfer of nitrosonium (NO^+) to other sulfhydryl centers (7). *S*-Nitrosothiols have been recognized to possess antimicrobial activity, including against *Salmonella* (9). These compounds have also been proposed to mediate NO^+ transfer to outer membrane thiols in *Bacillus*, which inhibits spore outgrowth (10). Differences in stability and target responses relative to NO· lend credence to the suggestion that *S*-nitrosothiols are important physiologic redox forms of NO. *S*-Nitrosoglutathione (GSNO) may be of particular biological importance in view of the abundance of GSH in mammalian cells, including macrophages and hepatocytes.

Peroxynitrite ($OONO^-$) is a highly toxic and reactive intermediate, which has been strongly implicated in a number of NO cytotoxicity models (4, 11). Recent observations utilizing tyrosine nitration as a marker of $OONO^-$ indicate that $OONO^-$ is formed *in vivo*, particularly in inflammatory lesions (12). 3-Morpholinosydnonimine hydrochloride (SIN-1), a nonthiol compound, undergoes spontaneous oxidation to produce equimolar quantities of NO· and O_2^- (13), which react nearly instantaneously to produce $OONO^-$ (14). Excess superoxide dismutase (SOD) may limit $OONO^-$ production by scavenging O_2^- (4).

The NO· radical itself has also been implicated in interactions with metalloenzymes, which may contribute to NO cytotoxicity (1). Diethylenetriamine–nitric oxide adduct (DETA/NO) is a long-acting nucleophile adduct developed for the controlled biological release of NO (15), which provides a virtually pure source of the NO· redox form.

The NO donor compounds GSNO, SIN-1, and DETA/NO were used to investigate NO cytotoxicity in the present study, by using the facultative anaerobic bacterium *Salmonella typhimurium* as a target cell. Although each of these compounds generates NO, they differ in critical respects with regard to their redox chemistry. A comparison of the effects of NO donor compounds on isogenic wild-type and mutant *Salmonella* strains reveals discrete oxygen-independent and oxygen-dependent antimicrobial activities associated with different redox forms of NO.

METHODS AND MATERIALS

Media. Luria–Bertani broth (tryptone at 10 mg/ml, yeast extract at 5 mg/ml, and NaCl at 10 mg/ml) was used for routine manipulation and storage of bacteria. M9 minimal medium (Na_2HPO_4 at 7 mg/ml, KH_2PO_4 at 3 mg/ml, NaCl at 0.5 mg/ml, NH_4Cl at 1 mg/ml, thiamine at 5 μg/ml, $MgSO_4$ at 0.12 mg/ml, $CaCl_2$ at 0.015 mg/ml, and glucose at 2 mg/ml) at 37°C was used for NO donor susceptibility studies to avoid potential antagonism by thiol-containing substances in rich medium. EBU medium (16) was used to identify pseudolysogen-free transductants. Agar (1.5%) was added to solid medium. Penicillin (250 μg/ml), kanamycin (50 μg/ml), or tetracycline (15 μg/ml) from Sigma was used for antibiotic selection as indicated.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: GSNO, *S*-nitrosoglutathione; SIN-1, 3-morpholinosydnonimine hydrochloride; SOD, superoxide dismutase; DETA/NO, diethylenetriamine–nitric oxide adduct; GSH, glutathione; GGT, γ-glutamyltranspeptidase.

Bacterial Strains and Plasmids. All studies were performed using wild-type *S. typhimurium* American Type Culture Collection no. 14028s or its isogenic derivatives, except as noted. *S. typhimurium* DLG13 is a spontaneously isolated *rpoS* mutant. *S. typhimurium* SF1005 (*rpoS*) is described in ref. 17, *S. typhimurium* CL2001 (*recBC*) is described in ref. 18, and *S. typhimurium* XF1001 (*katE/katG*) is described in ref. 19. *S. typhimurium* XF1003 (*sodB*), *S. typhimurium* N2-1 (*dppD*), and *S. typhimurium* N2-2 (*dppA*) are described in *Results*. *S. typhimurium* TN3345 (LT2 *galE mutS*::Tn*10*) was used to facilitate P1 transduction from *Escherichia coli* to *S. typhimurium* (20). *S. typhimurium* TT172 is a *leu* auxotrophic LT2 derivative from K. Sanderson (University of Calgary, Calgary, AB Canada). *E. coli* MAD1 is a *ggt* mutant constructed by P1 transduction of *ggt::kan* (21) into wild-type *E. coli* W3110. pUBAD inducibly overexpresses *rpoS* under the control of the P_{BAD} promoter (22).

NO Donor Compounds. GSNO was prepared by adding $NaNO_2$ in 1 M HCl to reduced GSH and then neutralizing with NaOH to pH 7.5. $NaNO_2$ and GSH were obtained from Sigma. SIN-1 was obtained from Casella (Frankfurt), and DETA/NO was obtained from Research Biochemicals (Natick, MA).

Genetic/Biochemical Methods. Routine genetic manipulations were performed by conventional published methods (23, 24). A *S. typhimurium* 14028s MudJ transposon library containing ≈50,000 independent insertions was constructed using *S. typhimurium* TT10288, as described (25). DNA sequencing was performed by a cycle sequencing protocol (Stratagene). The oligonucleotide primer 5′-CCAATGTCCTCCCGGTTTTT-3′ was used to determine host sequences flanking the attL end of MudJ transposon insertions after cloning of transposon junction fragments in pBluescript (Stratagene). SOD activity was detected by the method of Beauchamp and Fridovich (26).

Morphologic Studies. Bacteria were suspended in M9 medium containing 0.5% glutaraldehyde and placed on ice for 1 h. After fixation, cells were washed, resuspended, and stained with safranin O for light microscopic examination.

Microbroth Dilution Susceptibility Assay. Microbroth dilution assays were performed to determine minimal inhibitory and bactericidal concentrations of NO donor compounds, essentially according to the method described in ref. 27. Fifty microliters of a bacterial suspension (7.5×10^5 cells per ml) was added to microtiter plate wells containing 50 μl of M9 medium with 2-fold dilutions of the NO donor at final concentrations ranging from approximately 2 μM to 2 mM. The plates were sealed and incubated at 37°C for 24 h. Stationary-phase bacteria were obtained from overnight cultures; logarithmic-phase bacteria were obtained by diluting overnight cultures 10^3- to 10^4-fold and incubating for several hours until cell densities exceeded 10^7 cells per ml. The minimal inhibitory concentration was recorded as the lowest concentration without visible growth. Three microliters from each well was spotted onto an M9 agar plate and incubated overnight to determine the minimal bactericidal concentration (lowest concentration exhibiting no visible growth, which correlates with ≥99.9% killing).

Disk Diffusion Susceptibility Assay. Susceptibility to NO donor compounds was conveniently measured by a Bauer–Kirby disk diffusion method (28) and found to correlate with the results of microbroth dilution susceptibility assays. Fifteen microliters of 500 mM NO donor compound was added to a 1/4″ paper disk placed over a lawn of 10^6 bacteria spread in a 100-μl volume over M9 minimal agar. The resulting zone of inhibition was measured after overnight incubation at 37°C.

RESULTS

Effects of GSNO on *Salmonella*. *In vitro*, the *S*-nitrosothiol GSNO reversibly inhibits *S. typhimurium* growth. The GSNO precursor compounds GSH and $NaNO_2$ alone did not demonstrate antibacterial activity at neutral pH. Although 500 μM GSNO is cytostatic, even 8-fold higher concentrations are not cytocidal. *S. typhimurium* exposed to subinhibitory GSNO concentrations exhibits cell filamentation (Fig. 1).

Mutant GSNO-resistant strains are readily isolated. One spontaneously isolated GSNO-resistant mutant, *S. typhimurium* DLG13, was initially noted to be catalase deficient. This suggested that DLG13 might harbor a mutation in one of the genes encoding catalase (*katE, katG*) or in the catalase regulatory loci (*rpoS, oxyR*). Expression of *lacZ* transcriptional fusions to *katE* and other *rpoS*-regulated genes was found to be deficient in DLG13 (data not shown) but could be complemented by cloned *rpoS* (29), indicating that DLG13 contains a mutant allele encoding the alternative σ factor RpoS. Cloned *rpoS* but not cloned *katE* was sufficient to restore normal catalase activity to DLG13 (data not shown).

Subsequently, a constructed *rpoS* mutant *S. typhimurium* SF1005 (17) was found to have a level of GSNO resistance identical to that of DLG13 (Fig. 2). Complementation with the cloned *rpoS* gene restores GSNO susceptibility in either *rpoS* mutant strain. Since the RpoS regulon is preferentially expressed by stationary-phase bacteria, it was predicted and confirmed that stationary-phase cells possess heightened susceptibility to GSNO (data not shown). Even 2 mM GSNO is unable to inhibit logarithmically dividing *S. typhimurium*, whereas 500 μM is cytostatic for stationary cells.

SOD and catalase were previously demonstrated to protect eukaryotic cells from NO cytotoxicity, presumably in large part due to the SOD-mediated limitation of $ONOO^-$ production (4). Moreover, NO· activates the *E. coli soxRS* regulon (30), which controls multiple antioxidant defense functions including SOD. Therefore, *Salmonella* mutants deficient in SOD or

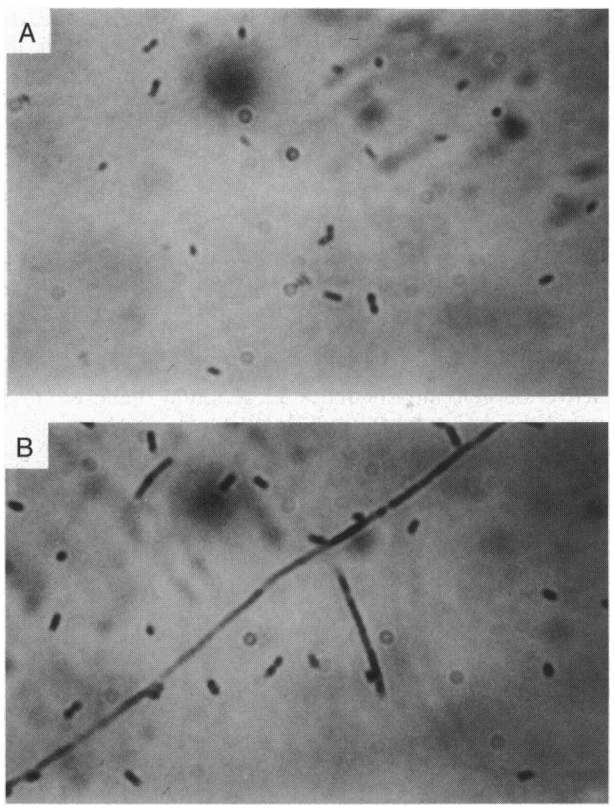

FIG. 1. *Salmonella* morphologic changes induced by GSNO. *S. typhimurium* 14028s were plated onto M9/0.2% glucose/agar and photographed after staining with safranin O. (*A*) Bacteria were not exposed to GSNO. (*B*) Bacteria were obtained from the edge of the zone of inhibition after overnight incubation with a paper disk containing 15 μl of 500 mM GSNO. (×350.)

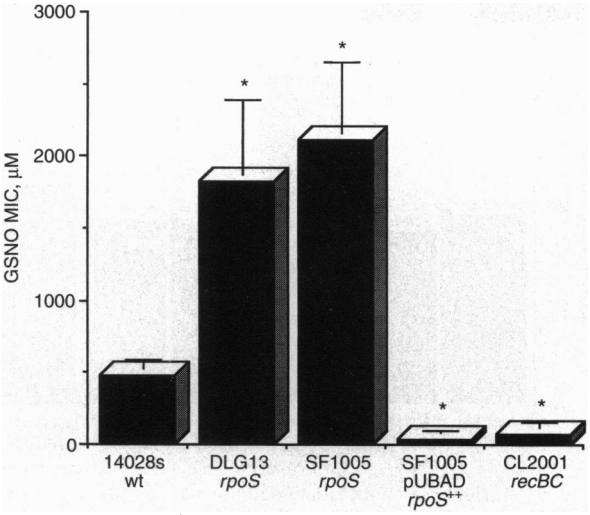

FIG. 2. Influence of *rpoS* and *recBC* on GSNO susceptibility of *Salmonella*. Microdilution minimal inhibitory concentrations (MIC) of GSNO were obtained for *S. typhimurium* strains. All mutant strains are isogenic derivatives of American Type Culture Collection no. 14028s, as described in *Materials and Methods*. pUBAD inducibly overexpresses *rpoS*. *, $P < 0.02$, as determined by Student's *t* test. wt, wild type. In this figure and Figs. 3, 4, and 6, the error bars indicate ± SEM.

catalase production were evaluated for susceptibility to GSNO.

A *sodB S. typhimurium* mutant XF1003 was constructed by transduction of *sodB::kan* from *E. coli* QC774 (31) to *S. typhimurium* TN3345 using P1 and then into *S. typhimurium* 14028s using P22. Appropriate loss of FeSOD activity was demonstrated on a SOD activity gel (26). Unexpectedly, neither the SOD-deficient mutant XF1003 nor catalase-deficient mutant XF1001 (19) demonstrates enhanced susceptibility to GSNO (Fig. 3). In fact, the catalase mutant strain appears to be slightly resistant. Elimination of ambient oxygen in an anaerobic GasPak (Becton Dickinson) similarly has no effect on GSNO susceptibility (Fig. 4). Isogenic *S. typhimurium*

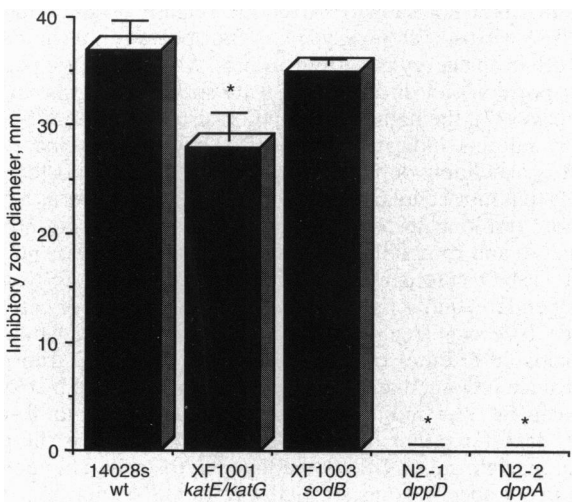

FIG. 3. Influence of antioxidant defense and peptide transport loci on GSNO susceptibility of *Salmonella*. A 24-h disk diffusion assay of GSNO susceptibility (28) was performed using wild-type (wt) and mutant *S. typhimurium*. All mutant strains are isogenic derivatives of American Type Culture Collection no. 14028s, as described in *Materials and Methods*. Susceptibility was measured as the zone diameter surrounding a GSNO-containing disk, placed as in Fig. 1. Zones of inhibition are proportional to GSNO susceptibility. *, $P < 0.01$.

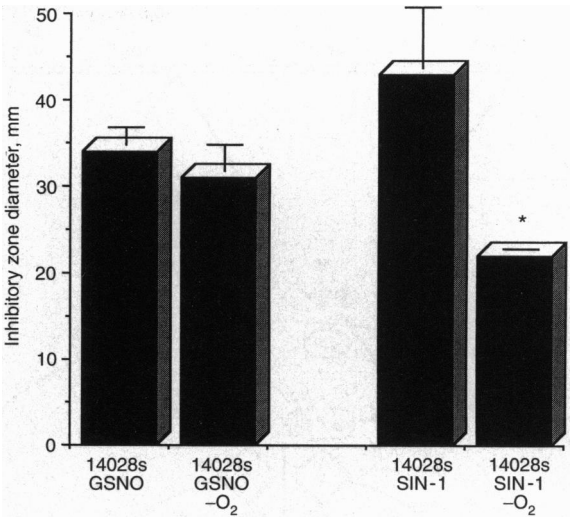

FIG. 4. Susceptibility of *Salmonella* to GSNO and SIN-1 under ambient oxygen and anoxic ($-O_2$) conditions. The disk diffusion assay was performed as in Fig. 3, except that 500 mM SIN-1 was substituted for GSNO. *, $P < 0.01$.

CL2001 carrying a *recBC* mutation (18) is hypersusceptible to GSNO (Fig. 2).

Two highly GSNO-resistant *S. typhimurium* mutants (N2-1, N2-2) were directly selected by exposing a *S. typhimurium* MudJ transposon library to 2 mM GSNO. P22-mediated transduction of the N2-1 and N2-2 transposon insertions into new wild-type backgrounds demonstrated 100% cotransduction of GSNO resistance with the insertions. Cloning and sequencing of chromosomal regions immediately flanking the transposon insertions demonstrated that the N2-1 insertion lies between nucleotides 5 and 6 of the *S. typhimurium dppD* gene, and the N2-2 insertion is between nucleotides 596 and 597 of the *dppA* gene (Fig. 5*A*). *dppA* and *dppD* are members of an operon encoding dipeptide permease, a transporter of the ABC family responsible for L-dipeptide importation into bacteria (32). *dppA* encodes a periplasmic peptide-binding protein, and *dppD* encodes an ATP-binding subunit of the transmembrane transporter. Since *dppD* is essential for Dpp transporter function, the N2-1 (*dppD*) mutant phenotype was confirmed by introducing this mutation into the *leu* auxotrophic strain *S. typhimurium* TT172; the resulting double mutant showed impaired utilization of the dipeptide L-prolylleucine as a leucine source in minimal medium, in contrast to the *leu* parent strain.

Effects of SIN-1 on *Salmonella*. In contrast to GSNO, SIN-1 is bactericidal with minimal bactericidal concentrations exceeding inhibitory concentrations by less than 2-fold. In further contrast to GSNO, *sodB* mutants have increased susceptibility to SIN-1, while the SIN-1 susceptibility of GSNO-resistant *rpoS, dppA*, and *dppD* mutants is equivalent to that of wild type (Fig. 6). *recBC* is the only mutation thus far identified that confers hypersusceptibility to both GSNO and SIN-1. Multiple attempts to directly obtain SIN-1-resistant mutants have been unsuccessful. Unlike GSNO cytotoxicity, SIN-1 cytotoxicity depends upon ambient O_2 (Fig. 4).

Effects of DETA/NO on *Salmonella*. The long-acting NO· donor compound DETA/NO fails to inhibit or kill any wild-type or mutant *S. typhimurium* strains tested, even at concentrations up to 3 M. Release of NO· from DETA/NO was confirmed by the detection of NO_2^- using the Griess reagent (33).

DISCUSSION

Analysis of the effects of three different NO donors on mutant and wild-type *S. typhimurium* reveals insights into discrete

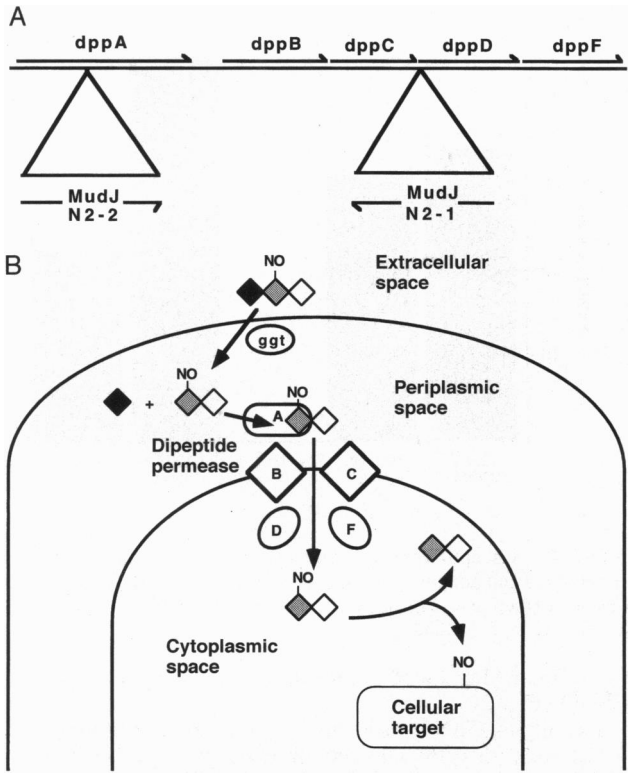

FIG. 5. Role of *dpp* peptide permease loci in GSNO susceptibility. (*A*) *S. typhimurium* mutants N2-1 and N2-2 were obtained by exposing a MudJ transposon library to 2 mM GSNO. Cloning of attL junction fragments indicates that the N2-1 insertion lies at the 5′ end of the *S. typhimurium dppD* gene and that the N2-2 insertion is in the middle of the *dppA* gene. The *dpp* genes make up an operon encoding dipeptide permease, a member of the ABC transporter family specific for importation of L-dipeptides. (*B*) Model of GSNO-mediated cytostasis in *Salmonella*. ◇, Glycine; ◆, cysteine; ◆, glutamate. Periplasmic GGT converts GSNO to glutamate and *S*-nitrosocysteinylglycine, which is actively transported into the cytoplasmic compartment by dipeptide permease (subunits A, B, C, D, and F). Intracellular *S*-nitrosocysteinylglycine is likely to act as a NO^+ donor on an unidentified thiol target, reversibly inhibiting cell division.

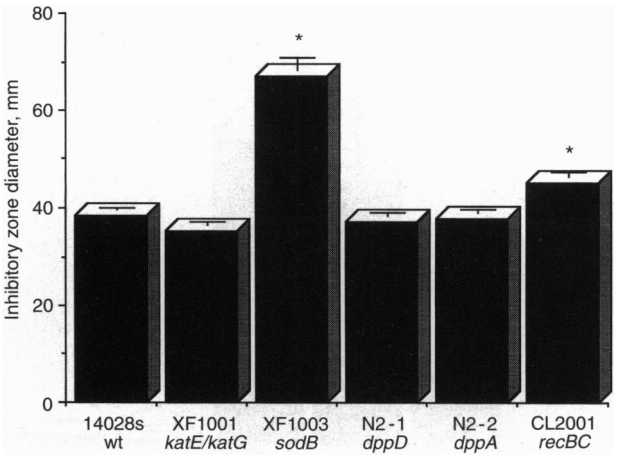

FIG. 6. Influence of antioxidant defense and peptide transport loci on SIN-1 susceptibility of *Salmonella*. A disk diffusion assay (28) was performed as in Fig. 4. Zones of inhibition are proportional to SIN-1 susceptibility. *, $P < 0.01$.

cytotoxic or antiproliferative mechanisms associated with different redox states of NO.

The *S*-nitrosothiol GSNO is reversibly cytostatic for *S. typhimurium*. *S. typhimurium* exposed to GSNO exhibits cell filamentation (Fig. 1), suggesting that GSNO disrupts cell division. *S. typhimurium* carrying a mutation in the gene encoding the alternative σ factor RpoS is relatively resistant to GSNO. The GSNO resistance of *rpoS* mutant *S. typhimurium* is unexpected, since *rpoS* mutants are generally hypersusceptible to environmental stresses (17). RpoS regulates the stationary-phase expression of >30 genes (reviewed in ref. 34), including genes involved in cell division, and one or more of these gene products may interact with GSNO to result in cytostasis.

The greater activity of GSNO for stationary target cells is a unique feature among antimicrobial effector molecules. Since intracellular bacteria appear to behave physiologically as if they were in stationary phase (35, 36), this property may be particularly relevant to host defenses against intracellular or latent microbes. Our *in vitro* observations indicate that GSNO might interfere with the transition from stationary phase to logarithmic growth. Although *rpoS* mutant *Salmonella* has increased resistance to GSNO, it is hypersusceptible to other environmental stresses and attenuated for virulence (17). This underscores the importance of the multiple antimicrobial mechanisms employed by phagocytes, including production of reactive oxygen intermediates, nutrient limitation, phagosome acidification, and production of antimicrobial peptides.

Our investigations of the influence of cellular antioxidant defense mechanisms (SOD, catalase) or ambient oxygen availability indicate that GSNO cytotoxicity for *Salmonella* is oxygen independent and does not involve $OONO^-$. The hypersusceptibility of *recBC* mutant *S. typhimurium* suggests that the mechanism of GSNO cytotoxicity may involve DNA damage.

The surprising discovery that transporter mutations confer high-level GSNO resistance indicates that GSNO is not simply an extracellular donor of NO·, which would be anticipated to freely enter target cells without requiring active uptake. Cellular importation of GSH is dependent upon periplasmic γ-glutamyltranspeptidase (GGT), which converts GSH to glutamate and cysteinylglycine. A proposed model for GSNO entry wherein GGT converts GSNO to glutamate and *S*-nitrosocysteinylglycine is depicted in Fig. 5*B*. In support of this model, a *ggt* mutant derivative of *E. coli* W3110 (21) was found to be GSNO-resistant compared with the isogenic wild-type parent (data not shown). After the release of glutamate by GGT, *S*-nitrosocysteinylglycine is transported into the bacterial cell in an energy-requiring process. Although other peptide transporters with different substrate specificities exist in *Salmonella* (37), the high-level GSNO resistance of the N2-1 and N2-2 mutants indicates that *S*-nitrosocysteinylglycine transport is absolutely dependent upon the Dpp transporter. It is likely that intracellular *S*-nitrosocysteinylglycine acts as a NO^+ donor, resulting in *S*-nitrosylation of a cytoplasmic protein target(s) and reversible inhibition of cell division. It is notable that GSNO cytotoxicity requires active uptake, is oxygen-independent, and is uninfluenced by cellular SOD or catalase. Since NO· can freely permeate membranes and would be anticipated to enter cells in the absence of GSNO transport, and since NO·-mediated cytotoxicity is potentiated by oxygen radicals (4), our findings suggest that heterolytic thiol-to-thiol NO^+ transfer rather than homolytic NO· release is the principal mechanism of GSNO-mediated cytostasis. The identification of a specific transport pathway for GSNO may represent an additional pathway for NO-mediated signal transduction, a possibility that can be explored in other biological systems since both GGT (21) and the ABC family of transporters (38) are found in organisms ranging from bacteria to humans.

In contrast, the cytocidal activity of SIN-1 reflects the synergistic cytotoxicity of NO· and O_2^-, presumably mediated by $OONO^-$. The O_2 dependence of SIN-1 cytotoxicity results from a requirement of O_2 for generation of NO· and O_2^-.

Residual SIN-1 cytotoxicity in the anaerobic environment most likely reflects residual O_2 present in the GasPak system during the first few hours of incubation. DNA damage has been attributed to NO (15), and the increased susceptibility of the *recBC* mutant to both GSNO and SIN-1 suggests that DNA damage is a common feature of damage resulting from NO· release by either compound. It is possible that increased susceptibility to DNA damage mediated by reactive nitrogen intermediates *in vivo* may play a role in the attenuated macrophage survival and mouse virulence exhibited by *recBC S. typhimurium* (18). However, DNA damage is unlikely to represent the primary mechanism of GSNO cytotoxicity in *S. typhimurium*, since NO-mediated DNA mutagenicity requires actively dividing cells (39), whereas stationary-phase cells demonstrate maximal susceptibility to GSNO.

Interestingly, DETA/NO failed to exhibit bacteriostatic or bactericidal activity in this *Salmonella* model. This suggests that NO· alone may not be cytotoxic for *S. typhimurium* or that NO· delivery from DETA/NO is quantitatively insufficient to manifest this activity. In support of the former possibility, NO· has recently been shown to be incapable of inactivating *E. coli* aconitase (40) in the absence of oxygen, while incubation with $OONO^-$ results in rapid inactivation. Moreover, other investigators have recently reported a lack of antimicrobial activity exhibited by 1 mM aqueous NO for *E. coli* under conditions in which SIN-1 and $OONO^-$ are bactericidal (41).

In summary, analysis of *Salmonella* mutants reveals discrete oxygen-independent and -dependent antimicrobial activities associated with different redox states of NO. NO donated by SIN-1 has a cytocidal effect attributable to $OONO^-$ formation. GSNO has a cytostatic effect resulting from NO^+ transfer (*S*-nitrosylation) to an intracellular target. DETA/NO has no effect on *Salmonella*, suggesting that NO· itself may not be cytotoxic.

Although redox forms of NO such as $OONO^-$ can clearly produce cell damage or death (4, 13), this mechanism cannot readily explain reversible NO-mediated cytostatic or antimicrobial effects in numerous situations that do not appear to be associated with overt cell injury (1, 42). The oxygen-independent cytotoxic activity associated with GSNO in the present study may better account for many cytostatic and antiproliferative activities associated with NO in a variety of biological systems. The existence of GSNO has been demonstrated *in vivo* (8), underscoring the potential biological relevance of these observations. The identification of a specific transpeptidase and peptide permease required for GSNO cytostasis has broad implications for understanding signal transduction pathways involving *S*-nitrosothiols.

The authors are grateful to J. Roth, N. Buchmeier, K. Sanderson, S. Maloy, D. Touati, and H. Suzuki for generous gifts of bacterial strains. This work was supported in part by grants from the National Institutes of Health (AI32463) and the U.S. Department of Agriculture (9401954).

1. DeGroote, M. A. & Fang, F. C. (1995) *Clin. Infect. Dis.*, in press.
2. Hibbs, J. B. J., Taintor, R. R. & Vavrin, Z. (1987) *Science* **235,** 473–476.
3. Nathan, C. (1992) *FASEB J.* **6,** 3051–3064.
4. Lipton, S. A., Choi, Y. B., Pan, Z. H., Lei, S. Z., Chen, H. S. V., Sucher, N. J., Loscalzo, J., Singel, D. J. & Stamler, J. S. (1993) *Nature (London)* **364,** 626–632.
5. Choi, D. W. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 9741–9743.
6. Stamler, J. S., Simon, D. I., Osborne, J. A., Mullins, M. E., Jaraki, O., Michel, T., Singel, D. J. & Loscalzo, J. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 444–448.
7. Stamler, J. S. (1994) *Cell* **78,** 931–936.
8. Gaston, B., Reilly, J., Drazen, J. M., Fackler, J., Ramdev, P., Arnelle, D., Mullins, M. E., Sugarbaker, D. J., Chee, C., Singel, D. J., Loscalzo, J. & Stamler, J. S. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 10957–10961.
9. Incze, K., Farkas, J., Mihalys, V. & Zukal, E. (1974) *Appl. Microbiol.* **27,** 202–205.
10. Morris, S. L. & Hansen, J. N. (1981) *J. Bacteriol.* **148,** 465–471.
11. Radi, R., Beckman, J. S., Bush, K. M. & Freeman, B. A. (1991) *J. Biol. Chem.* **266,** 4244–4250.
12. Haddad, I. Y., Pataki, G., Hu, P., Galliani, C., Beckman, J. S. & Matalon, S. (1994) *J. Clin. Invest.* **94,** 2407–2413.
13. Feelisch, M. (1989) *J. Cardiovasc. Pharmacol.* **14,** Suppl. 11, S13–22.
14. Huie, R. E. & Padmaja, S. (1993) *Free Radical. Res. Commun.* **18,** 195–199.
15. Wink, D. A., Kasprzak, K. S., Maragos, C. M., Elespuru, R. K., Misra, M., Dunams, T. M., Cebula, T. A., Koch, W. H., Andrews, A. W., Allen, J. S. & Keefer, L. K. (1991) *Science* **254,** 1001–1003.
16. Bochner, B. R. (1984) *BioTechniques* **2,** 234–240.
17. Fang, F. C., Libby, S. J., Buchmeier, N. A., Loewen, P. C., Switala, J., Harwood, J. & Guiney, D. G. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 11978–11982.
18. Buchmeier, N., Lipps, C. J., So, M. H. Y. & Heffron, F. (1993) *Mol. Microbiol.* **7,** 933–936.
19. Buchmeier, N. A., Libby, S. J., Xu, Y., Loewen, P. C., Switala, J., Guiney, D. G. & Fang, F. C. (1995) *J. Clin. Invest.*, **95,** 1047–1053.
20. Rayssiguier, C., Thaler, D. S. & Radman, M. (1989) *Nature (London)* **342,** 396–401.
21. Suzuki, H., Kumagai, H. & Tochikura, T. (1987) *J. Bacteriol.* **169,** 3926–3931.
22. Volkert, M. R., Hajec, L. I., Matijasevic, Z., Fang, F. C. & Prince, R. (1994) *J. Bacteriol.* **176,** 7638–7645.
23. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
24. Gerhardt, P., Murray, R. G. E., Wood, W. A. & Krieg, N. R. (1994) *Methods for General and Molecular Bacteriology* (Am. Soc. for Microbiol., Washington, DC).
25. Hughes, K. & Roth, J. R. (1988) *Genetics* **119,** 9–12.
26. Beauchamp, C. & Fridovich, I. (1971) *Anal. Biochem.* **44,** 276–277.
27. Reimer, L. G., Stratton, C. W. & Reller, L. B. (1981) *Antimicrob. Agents Chemother.* **19,** 1050–1055.
28. Bauer, A. W., Kirby, W. M. M., Sherris, J. C. & Turck, M. (1966) *Am. J. Clin. Pathol.* **45,** 493–496.
29. Prince, R. W., Xu, Y., Libby, S. J. & Fang, F. C. (1994) *Biochim. Biophys. Acta* **1219,** 198–200.
30. Nunoshiba, T., deRojas-Walker, T., Wishnok, J. S., Tannenbaum, S. R. & Demple, B. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 9993–9997.
31. Carlioz, A. & Touati, D. (1986) *EMBO J.* **5,** 623–630.
32. Abouhamad, W. N., Manson, M., Gibson, M. M. & Higgins, C. F. (1991) *Mol. Microbiol.* **5,** 1035–1047.
33. Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S. & Tannenbaum, S. R. (1982) *Anal. Biochem.* **126,** 131–138.
34. Loewen, P. C. & Hengge-Aronis, R. (1994) *Annu. Rev. Microbiol.* **48,** 53–80.
35. Fierer, J., Eckmann, L., Fang, F., Pfeifer, C., Finlay, B. B. & Guiney, D. (1993) *Infect. Immun.* **61,** 5231–5236.
36. Guiney, D. G., Libby, S., Fang, F. C., Krause, M. & Fierer, J. (1995) *Trends Microbiol.*, in press.
37. Higgins, C. F., Hiles, I. D., Salmoned, G. P. C., Gill, D. R., Downie, J. A., Evans, I. J., Holland, I. B., Gray, L., Buckel, S. D., Bell, A. W. & Hermodson, M. A. (1986) *Nature (London)* **323,** 448–450.
38. Higgins, C. F. (1992) *Annu. Rev. Cell Biol.* **8,** 67–113.
39. Arroyo, P. K., Hatch-Pigott, V., Mower, H. F. & Cooney, R. V. (1992) *Mutat. Res.* **281,** 193–202.
40. Hausladen, A. & Fridovich, I. (1994) *J. Biol. Chem.* **269,** 29405–29408.
41. Brunelli, L., Crow, J. P. & Beckman, J. S. (1995) *Arch. Biochem. Biophys.* **316,** 327–334.
42. Mannick, J. B., Asano, K., Izumi, K., Kieff, E. & Stamler, J. S. (1994) *Cell* **79,** 1137–1146.