Determination of the *Escherichia coli* S-Nitrosogluthathione Response Network Using Integrated Biochemical and Systems Analysis*\(^5\)

Laura R. Jarboe\(^1,2\), Daniel R. Hyduke\(^1\), Linh M. Tran\(^3\), Katherine J. Y. Chou, and James C. Liao\(^4\)

From the Department of Chemical and Biomolecular Engineering, University of California, Los Angeles, California 90095

During infection or denitrification, bacteria encounter reactive nitrogen species. Although the molecular targets of and defensive response against nitric oxide (NO) in *Escherichia coli* are well studied, the response elements specific to S-nitrosothiols are less clear. Previously, we employed an integrated systems biology approach to unravel the *E. coli* NO-response network. Here we use a similar approach to confirm that S-nitrosoglutathione (GSNO) primarily impacts the metabolic and regulatory programs of *E. coli* in minimal medium by reaction with homocysteine and cysteine and subsequent disruption of the methionine biosynthesis pathway. Targeting of homocysteine and cysteine results in altered regulatory activity of MetJ, MetR, and CysB, activation of the stringent response and growth inhibition. Deletion of *metJ* or supplementation with methionine strongly attenuated the effect of GSNO on growth and gene expression. Furthermore, GSNO inhibited the ArcAB two-component system. Consistent with the underlying nitrosative and thiol-oxidative chemistry, growth inhibition and the majority of the regulatory perturbations were dependent upon GSNO internalization by the Dpp dipeptide transporter. Contrastingly, perturbation of NsrR appeared to be a result of the submicromolar levels of NO released from GSNO and did not require GSNO internalization.

*Escherichia coli* is a normal inhabitant of the human digestive tract but is also a causative agent of disease, including cystitis, pyelonephritis, and O157:H7-mediated hemolytic uremic syndrome (1–3). During the course of infection, *E. coli* is exposed to reactive nitrogen species (RNS)\(^4\) produced by the mammalian immune system. The most important RNS are, arguably, nitric oxide (NO) and nitrosothiols (RSNO), such as S-nitrosoglutathione (GSNO) (4–6). Mapping of the regulatory networks that govern the response to these compounds is relevant to understanding infection and in the identification of potential drug targets.

NO and RSNO exhibit distinct chemical reactivities. NO reacts directly with metal centers and free radicals or mediates indirect effects by formation of other RNS in conjunction with oxygen or superoxide (7). Although RSNO can release NO via homolytic cleavage or reaction with copper (I) ions (8–10), its primary biochemical effect is direct reaction with thiol groups through transnitrosation and S-thiolation (11). Additionally, NO can freely diffuse across membranes, whereas, GSNO susceptibility has been shown to be dependent on the Dpp dipeptide ABC transporter in *Salmonella* (12–14). As the first step for studying RNS challenge in *E. coli*, previous investigations often did not distinguish between RSNO and NO-mediated effects (15, 16) and RSNOs were sometimes used as NO donors (17). Such studies set the foundation for further distinction between RSNO and NO. A recent comparison of the *E. coli* response to NO and GSNO during chemostat growth confirmed the expectation that NO and GSNO mediate distinct transcriptional perturbations (18).

*E. coli* possesses a NO-specific response network: the transcription factors (TFs) NorR and NsrR mediate increased expression of defense proteins NO reductase NorV and NO dioxygenase HmpA, respectively, in response to NO (17, 19, 20). Using a systems analysis (21), we have shown that, consistent with the known chemistry, NO primarily affects *E. coli* by reacting with metal groups, including those in the cytochrome oxidases, the Fe-S clusters of IscR, and branched chain amino acid (BCAA) synthase protein dihydroxy acid dehydratase (IlvD). These interactions result in activation of NO defense circuits, respiration inhibition, and BCAA starvation. BCAA starvation in turn leads to metabolic adjustments, including perturbation of biosynthesis regulators, activation of the stringent response, and bacteriostasis.

However, in contrast to the NO response, there are no known *E. coli* TFs that react directly with RSNO or RSNO-consuming defensive proteins. NorR and the ferric iron repressor (Fur) were identified as regulators of the response to GSNO in rich...
medium (15), but whether these TFs react directly with GSNO remains unclear. Other works have suggested that participation of Fur in the GSNO response may be condition-dependent (16). Despite the lack of confirmed protein or TF interactions, GSNO has been shown to react directly with the thiol group of homocysteine (Hcy) in E. coli. Because Hcy is a key metabolite in the methionine (Met) biosynthesis pathway and the co-effect for regulatory activity of MetR, this reactivity affects Met biosynthesis and the regulatory state of MetR (16, 22, 23). Perturbation of Met biosynthesis by GSNO, but not by NO, was recognized as a major difference between the effects of GSNO and NO on E. coli during chemostat growth (18).

We have recently applied a systems approach to map the NO response network in E. coli (21). This approach integrated transcriptome analysis, network component analysis (NCA), genetic knockouts, phenotypes, and biochemical experiments to identify response networks and targets. Here, we use a similar approach to identify the GSNO targets and response network in aerobic batch culture, with particular focus on comparison of GSNO- and NO-mediated effects. Consistent with the known chemistry, we have shown that GSNO primarily reacts with thiols and not with the metal groups that are targeted by NO. The GSNO targets include cysteine (Cys) and Hcy. Depletion of Hcy and Cys has many downstream effects, including growth inhibition and altered activity of the CysB, MetJ, and MetR TFs. In addition to metabolic targets, GSNO decreases the activity of the ArcAB two-component system.

**EXPERIMENTAL PROCEDURES**

**Cell Growth**—BW25113 was grown aerobically to mid-log \( A_{600} = 0.4–0.5 \) from an initial \( A_{600} \) of 0.05 at 37 °C in MOPS, 0.2% glucose, as described in Ref. 24 in baffled flasks at 1/10th to 1/5th total volume and shaken at 250 rpm. Amino acid supplementation studies used the concentrations given in Ref. 25. GSNO was made according to Ref. 26 and stored at −80 °C until the day of use and diluted in 0.5 mM diethylenetriamine pentaacetic acid. Diethylammonium (Z)-1-(N,N-diethylamino)diazene-1-ium,1,2-diolate (DeaNO, Cayman Chemical) solutions were made on the day of use with cold Tris/saline buffer, pH > 10.5.

**Cell Harvesting, RNA Purification, and Microarray**—Half of the cell culture was withdrawn for RNA purification as the reference time point; the remainder was harvested 5 min after treatment. Harvesting, RNA purification, array design, hybridization with the Qiagen Array-Ready Oligo Set, and image and data analysis were performed as previously described (21). The MIAME-compliant data have been deposited in the NCBI Gene Expression Omnibus data base (GSE8540) and gene expression ratios are given in supplemental Table S1.

**Network Component Analysis**—NCA is an algorithm that deduces system structure from sparsely connected networks, such as transcriptional regulatory networks (27, 28). Other bipartite network analysis methods have been applied to biological data, such as a probabilistic state-space model (29) and an integration of expression and TF binding data (30). An advantage of NCA is that it can identify changes in transcription factor activity (TFA) when only a subset of a regulon is perturbed; this feature is useful in deconvoluting the output of combinatorial regulation, however, caution should be taken when only a small number of perturbations are considered. NCA decomposed significant gene expression perturbations into TFA ratios and control strengths. The expression and connectivity matrices are provided as supplemental Tables S2 and S3. To determine whether a TF was perturbed in a given experiment, we built null distributions of TFAs and then performed Z tests on individual data points of every TFA. The threshold \( p \) value for significant perturbation was 0.01. The null TFA distributions were constructed by: 1) randomly selecting \( N \) genes from the genome, where \( N \) is the size of the original network; 2) NCA decomposition of the expression data to obtain TFAs of the random network; and 3) repeating steps 1 and 2 100 times.

Furthermore, we wanted to compare the relative response between two significantly perturbed TFAs, which were identified by the above statistical test, and also determine whether the variation in TFA was statistically significant. Because the statistical test described above assumed that the null TFA distribution of each individual data point was normal, the corresponding null distribution of the TFA discrepancy between two data points was also normal. For example, if the TFA \( _{1,t} \) and TFA \( _{2,t} \) of TF \( i \) in experiments \( t_1 \) and \( t_2 \) have null distributions of \( N(\mu_i,\sigma_1) \) and \( N(\mu_i,\sigma_2) \), respectively, the null distribution of TFA \( _{1,t} - TFA_{2,t} \) is \( N(\mu_2 - \mu_1,\sigma_2^2 + \sigma_1^2) \). Therefore, we performed Z tests on the discrepancies between two perturbed TFAs in specific experimental sets, TFA \( _{1,t} - TFA_{2,t} \) based on the new derived null distributions. The variation in TFAs was significant if its \( p \) value was less than 0.01.

**Real-time RT-PCR**—Real-time PCR was performed in a Cepheid SmartCycler with QuantiTect RT-PCR SYBR mixture (Qiagen), as previously described (21). Transcript abundance was normalized relative to chaA, which is not perturbed in any of the related microarray data. The RT-PCR primers are listed in supplemental Table S4.

**Gene Deletion**—Deletion mutants were generated using the method described by Datsenko and Wanner (31). Deletions that could potentially disrupt expression of co-transcribed genes were designed to be non-polar.

**NO Concentration Measurements**—Extracellular NO concentration was measured using a microchip NO electrode (ISO-NOPMC; World Precision Instruments). Experiments were performed aerobically at 37 °C in a total volume of 10 ml.

**GSNO Reactivity Assays**—Metabolites and GSNO, both 500 \( \mu M \), were incubated in MOPS/glucose medium at 37 °C in the dark in a total volume of 1 ml. GSNO concentration was monitored by measuring \( A_{335} \).

**RESULTS**

**GSNO and NO Inhibit Growth through Distinct Mechanisms**—GSNO and NO both transiently inhibit E. coli growth, and E. coli has an adaptive response to both compounds: after adaptation to an initial dose, a second dose does not impact growth (Fig. 1, A and B (21)). However, whereas adaptation to GSNO (Fig. 1A) or the nitrating agent sodium nitroprusside (32) increased resistance to NO, adaptation to NO did not increase GSNO growth resistance (Fig. 1B). This indicates that whereas the GSNO response network includes NO-defensive elements, the NO response network does not include GSNO-defensive
elements and therefore GSNO and NO have distinct modes of causing growth inhibition.

In biological conditions, GSNO can release NO (8–10), but the amount of NO released from 500 \( \mu M \) GSNO was much lower than that from 5 \( \mu M \) DEA-NO (Fig. 1C). The amount of NO released from 5 \( \mu M \) DEA-NO is insufficient for growth inhibition in our condition (21) and therefore, the amount of NO released from 500 \( \mu M \) GSNO is insufficient to account for the observed growth inhibition. Whereas NO is a small uncharged molecule that can freely cross membranes, GSNO is a larger charged molecule that requires transport. In Salmonella typhimurium GSNO is internalized via the Dpp transporter system (12). To verify that growth inhibition by GSNO required GSNO internalization, as opposed to extracellular evolution of NO, we verified that a \( \Delta dppABCDEF \) E. coli has increased resistance to GSNO (data not shown). Whereas concentrations of up to 1000 \( \mu M \) GSNO did not inhibit growth of the \( \Delta dpp \) mutant, the NO sensitivity of this strain was similar to the WT (data not shown). These results provide further evidence for distinct NO and GSNO inhibitory mechanisms.

**NCA Identifies Potential Regulators of the GSNO Response**— To identify GSNO-sensitive TFs, we performed NCA on transcriptome data of the GSNO response, as measured 5 min after addition of 100 or 500 \( \mu M \) GSNO (supplemental Table S1). To verify that NCA attributed transcriptional perturbations to the appropriate regulator and that the regulators contribute to mediation of the transcriptional response, we repeated the 500 \( \mu M \) GSNO exposure, transcriptomic analysis, and NCA for individual TF deletion mutants. Additionally, previously reported transcriptome data from treatment with 100 \( \mu M \) GSNO in rich medium batch cultures (15) and 200 \( \mu M \) GSNO in minimal medium chemostat cultures (16) were subjected to NCA to assess the effect of growth conditions on the GSNO response.

NCA is a mathematical approach that employs biologically relevant constraints to identify perturbed regulators in transcriptome data by accounting for regulator activity and separating the effects of multiple regulators (27, 28). Many TFs have both an active and an inactive state, where only the active state is able to affect transcript abundance. TFA is often dictated by properties that are not reflected in expression of the gene encoding the TF, such as oxidation state or phosphorylation. NCA accounts for both TFA and overlapping regulatory elements to identify regulators with significantly altered TFA relative to a random network (33). The initial connectivity data used in NCA comes from existing databases (34, 35). Additionally, we have included a virtual “stringent factor” (SF) TF, which includes both the direct and indirect effects of the stringent response; the stringent factor regulon was defined by analyzing the transcriptomic response to serine starvation (21). Although MetR has been reported to regulate \( hmpA \) (22), RT-PCR showed that \( hmpA \) abundance in a \( \Delta metR \) mutant was equiva-
To further investigate our results, we measured the transcript abundance of two regions of norV by RT-PCR. The two regions flank the microarray probe and are separated by ~600 bp (Fig. 2B). Expression of the region near the transcriptional start increased nearly 10-fold in response to 500 μM GSNO but the region downstream from the microarray probe was unperturbed, consistent with the microarray measurement. Contrastingly, the two regions were both increased ~100-fold in the NO response (Fig. 2B). Other researchers have reported unexpected trends in norV expression: primer extension assays based near the transcriptional start demonstrated oscillatory expression patterns (15) and comparison of protein and transcript abundance in the NO response was inconsistent (38). Investigation of the GSNO response during chemostat growth in minimal medium showed that whereas norV expression was increased in aerobic and anaerobic conditions, norW expression was increased only during anaerobic, and not aerobic, growth (16). Together, these data suggest that there may be as yet unidentified elements contributing to the regulation of norV.

NCA of deletion mutants verified CysB, MetJ, NsrR, and FlhDC as regulators responding to GSNO; the GSNO-induced change in TFA ratios became insignificant for the deletion mutants relative to the control experiment and were significantly different from the GSNO-treated WT (Fig. 3, p < 0.01). ΔmetR mutants require Met supplementation, but MetR TFA was not perturbed by GSNO in the Met-supplemented WT (Fig. 3). Thus, we were unable to separate the effects of Met supplementation and metR deletion and participation of MetR in the GSNO response was not confirmed. Interestingly, Met supplementation drastically reduced the impact of GSNO on the transcriptional network of E. coli (Fig. 3), consistent with the report that Met supplementation provides growth protection from GSNO (16).

The GSNO transport-deficient Δdpp strain allowed us to confirm that most of the regulatory perturbations require GSNO internalization. Fewer than 10% of the transcriptional perturbations caused by 100 μM GSNO in the WT were observed in the Δdpp strain (supplemental Table S1) and perturbation of CysB, MetR, and the SF TFA was significantly reduced relative to the WT (Fig. 3, p < 0.01). Contrastingly, perturbation of NsrR was comparable in the WT and Δdpp mutant (Fig. 3). This indicates that CysB, MetR, and SF, and possibly MetJ, FlhDC, and TTA, require GSNO internalization for TFA perturbation but NsrR does not. Because 100 μM GSNO delivers submicromolar levels of NO, this result illus-
GSNO Response Network Mapping in E. coli

FIGURE 3. NCA identifies GSNO-responsive TFs. All TFs that were significantly perturbed in the WT 5 min after the addition of 100 μM GSNO relative to the control sample (p < 0.01), which was treated only with 0.5 mM diethylenetriamine pentaacetic acid, as well as NorR and Fur, are shown. The rich medium and chemostat GSNO and DeaNO response data sets were previously reported (15, 16, 21). *, TFs that are significantly different (p < 0.01) from the control experiment; this comparison was performed only for the WT 100 and 500 μM GSNO and TF deletion mutant experiments. †, indicates TFs that are significantly different (p < 0.01) from the WT 100 μM GSNO response; this comparison was only performed for the Δdpp 100 μM GSNO to identify TFA perturbations that are dependent on GSNO internalization. ‡, indicates TFs that are significantly different (p < 0.01) from the WT 500 μM response; this comparison was only performed for the TF deletion mutants to confirm the NCA results and Met-supplemented WT 500 μM GSNO to highlight effects that are reduced by Met supplementation.

trates the exquisite sensitivity of NsrR and suggests that GSNO perturbs the other TFs through transnitrosation or S-thiolation.

GSNO Induces the Stringent Response by Targeting Hcy and Cys—The stringent response is mediated by ppGpp(p) in response to stress conditions that require resource conservation (39), such as amino acid depletion. We have previously shown that by damaging the Fe-S cluster of BCAA biosynthesis enzyme IlvD, NO causes BCAA starvation and induces the stringent response. Accordingly, derepression of the Fe-S repair system by deletion of the \( \text{iscR} \) TF increased the growth resistance to NO (21). However, neither BCAA supplementation nor derepression of the \( \text{isc} \) system increased the resistance to GSNO (Fig. 4A, data not shown).

Although GSNO does not appear to cause BCAA depletion, it does perturb the TFA of four regulators associated with amino acid biosynthesis: MetJ, MetR, CysB, and SF. The TFAs of these regulators are dependent upon the abundance of Met/Cys pathway intermediates (a pathway diagram is shown in Fig. 4A). Met biosynthesis repressor MetJ requires binding of Met derivative S-adenosyl-L-methionine (AdoMet) for activity (40). CysB is a dual regulator that requires binding of Cys precursor acetylserine for activity (41). Contrastingly, binding of Hcy to the MetR dual regulator increases the regulatory effect on some promoters and decreases the effect at others (42). GSNO is known to react with the thiol groups of Cys and Hcy (22, 23, 43), and this reaction with Hcy is believed to be the cause of MetR perturbation in response to GSNO (22). Cystathionine and Met, which contain non-exposed sulfur groups, as well as Met precursor homoserine were not GSNO-reactive (Fig. 4B, homoserine and cystathionine not shown). Therefore, we have concluded that Cys and Hcy are the only direct GSNO targets in this pathway.

Because depletion of metabolites downstream from Cys and Hcy could contribute to the observed growth inhibition, we tested whether supplementation with individual Met/Cys pathway components provided growth protection from 500 μM GSNO (Fig. 4A). In addition to the previously reported protective effect of Met and Hcy supplementation (16, 22), we found that Cys, homoserine, and cystathionine were growth protective but Cys precursors serine and acetylserine and Met derivative AdoMet were not. Because it is unclear if a transport mechanism exists for homoserine derivative...
GSNO Response Network Mapping in E. coli

mutant was 10-fold higher than the WT (Fig. 4D). This increased expression of the Met biosynthesis pathway may be the source of the increased GSNO resistance of the ΔmetJ mutant.

GSNO Induces HmpA Expression through NO Release—When hmpA-deficient cells were treated with 500 μM GSNO, the concentration of NO was increased relative to the WT (Fig. 5A), verifying that HmpA contributes to consumption of the NO released by GSNO. Whereas activation of the GSNO response network increased the NO growth resistance of the WT (Fig. 1A), this effect was not observed in the ΔhmpA mutant (Fig. 5B), suggesting that HmpA is the underlying element that links the GSNO and NO response networks. Elevated expression of HmpA in the ΔnsrR mutant increased the consumption of and resistance to NO (21), but the growth resistance to 500 μM GSNO was not increased (Fig. 5C), demonstrating that HmpA does not provide protection from GSNO-mediated depletion of the Cys/Met pathway and the resulting growth inhibition.

ArcAB Is a Differential RNS Sensor—We have previously reported that the ArcAB system is activated by NO, and that this activation is likely due to inhibition of cytochrome oxidase activity by NO (21) and alteration of the redox state of the cell. The phosphorylation state of ArcB, the transmembrane protein component of the Arc (aerobic respiration control) two-component system, is determined by the state of Cys residues in the linker region (reviewed in Ref. 45). When the Cys thiol residues are reduced, ArcB is in the active (kinase) state; formation of disulfide bonds between the Cys residues results in the non-active state. When active, ArcB phosphorylates, and therefore activates ArcA. We hypothesize that targeting of the Cys residues in the ArcB linker region could decrease ArcB activity, resulting in decreased ArcA TFA.

Because ArcAB activity is normally low during aerobic growth (46), a decrease in activity will be small and potentially difficult to measure with DNA microarrays. NCA, which is based upon transcriptome data, did not identify a change in ArcA TFA in the 100 or 500 μM GSNO transcriptome data (data not shown). To increase measurement sensitivity, we used RT-PCR to measure the transcript abundance of cydA, which encodes the cytochrome bd terminal oxidase subunit I and is known to be activated by ArcA and FNR (47).
target, demonstrating that in addition to depleting Cys and Hcy pools, GSNO also interacts with protein thiol groups.

**DISCUSSION**

We have employed a systems approach to map the key GSNO response network in *E. coli*. NCA of the transcriptomic response to GSNO in minimal medium indicated that a number of transcription factors (CysB, MetJ, MetR, NsrR, SF, FlhDC, and TTA) were perturbed by GSNO (Fig. 3). RT-PCR indicated that GSNO also perturbs ArcA (Fig. 6) and NorR (Fig. 2B) in our condition. However, RT-PCR results indicate that currently unidentified regulatory elements might be truncating *norVW* transcription, possibly in response to oxygen availability, and masking the effect of NorR. Using a GSNO transport-deficient strain, we verified that the majority of the regulators were responding to events mediated by direct GSNO chemistry (transnitrosation or S-thiolation). Phenotypic analysis of amino acid supplements, as well as regulator and *hmpA* deletions, contributed to construction of a model of the crucial components of the interactions of GSNO with *E. coli* (Fig. 7). In this model, GSNO targets the thiol groups of Met biosynthesis intermediates (Hcy and Cys), resulting in amino acid depletion and growth inhibition, whereas the NO-defensive TFs NsrR and NorR are activated by small amounts of NO released from GSNO.

The protective effect of Met and Hcy supplementation against growth inhibition by GSNO has been previously recognized (16, 22, 23). Here we have shown that supplementation with Cys, homoserine, or cystathionine, or increased expression of the Met biosynthesis enzymes via deletion of *metJ* also increased GSNO resistance and greatly reduced the impact of GSNO on the transcriptome.

CysB, which has not been previously linked to the GSNO response, activates sulfur metabolism genes and the GSNO-responsive TFA decrease is reflected as decreased expression of these genes. Production of the CysB cofactor acetylserine by serine acetyltransferase (*CysE*) is inhibited by Cys (48). Because targeting by GSNO alters Cys abundance, it is not surprising that acetylserine, and CysB TFA, are affected in turn. However, it is unclear why CysB TFA is decreased and not increased; if Cys depletion reduces inhibition of CysE, acetylserine production should increase, increasing acetylserine abundance and CysB TFA. This unexpected result warrants further investigation.

Although other TFs, namely FlhDC and TTA, were responsive to GSNO, they did not appear to play a significant role in the GSNO response. Recent crystallographic analysis on master motility regulator FlhDC identified a tertiary fold in FlhC with a zinc ion ligated to four Cys residues (49). Whereas it is possible that targeting of these Cys residues by GSNO could cause the observed decrease of FlhDC TFA, we were unable to detect a motility decrease in the presence of GSNO (data not shown).
The TTA transcriptional attenuator was significantly activated in the WT by 100 μM GSNO, but not by 500 μM. It is possible that GSNO-mediated depletion of Cys and Met has an indirect effect on TTA biosynthesis, but it is unclear why this perturbation is not observed in the 500 μM WT response.

A key issue in analyzing regulatory responses to chemical perturbations, such as GSNO, is the sensitivity of the observed response to the experimental conditions. Because \textit{E. coli} is exposed to RNS in a variety of conditions, it is useful to identify the condition-dependent response components. Therefore, we compared the NCA results for our own minimal medium batch cultures to two other published aerobic GSNO-response transcriptome data sets: rich medium batch cultures (15) and minimal medium chemostat cultures (16). NsrR and NorR are the only regulators that are similarly perturbed in all three conditions (Fig. 3). However, given that (i) \textit{hmpA} deletion does not strongly influence the growth response to GSNO (Fig. 5B), (ii) \textit{norVW} transcription is incomplete (Fig. 2B) or erratic (15), and

![Diagram](https://example.com/diagram.png)
GSNO Response Network Mapping in E. coli

(iii) deletion of NsrR or NorR (15) does not alter the phenotypic response to GSNO, it is likely that these regulators do not strongly contribute to GSNO defense. Instead, they may be responding to the small amounts of NO that are released from GSNO in biological conditions. The lack of CysB, MetR, and MetJ perturbations in rich medium is likely due to a buffering effect of the abundant Met biosynthesis metabolites in rich medium, similar to our Met supplementation results.

In addition to the effect of environmental factors on the response network, it is important to consider the specific biochemical effects of different RNS. The marked differences in the response of E. coli to GSNO and NO are consistent with the differences in the underlying biochemistry. The crucial interactions between NO and E. coli involve the direct reaction of NO with metal centers (21, 50, 51). Contrastingly, most of the GSNO-mediated regulatory perturbations appear to result from transnitrosation or S-thiolation of the accessible sulfurs of Cys and Hcy and required GSNO internalization. An important factor to account for when examining the biological effects of GSNO, is whether the observations arise from direct GSNO chemistry, NO evolved from GSNO, or a combination. The exquisite sensitivity of NsrR to NO was illustrated by its strong activation by low levels of NO released from GSNO, even when GSNO uptake was reduced by deleting Dpp (Fig. 3). Although NsrR was activated in our condition, it did not appear to play a significant role in GSNO defense: deletion of nssR or hmpA did visibly alter GSNO sensitivity (Fig. 5). Contrastingly, Gilberthorpe et al. (52) recently reported that deletion of nssR in Salmonella increased GSNO resistance and deletion of hmpA decreased GSNO resistance. However, Gilberthorpe et al. (52) used rich medium and roughly 10-fold more GSNO (3 mM) than the concentrations used in our study. Although GSNO releases NO slowly in biological conditions, 3 mM GSNO will yield a GSNO concentration of NO donor 2-fold, there was no growth inhibition and no perturbation of MetJ ((21), MetJ data not shown). This further emphasizes the condition-dependent interactions of RNS with E. coli. RNS, particularly NO, are known to elicit a number of effects in mammalian systems that are dependent on RNS concentration and exposure time (53).

ArcA is part of the two-component global regulatory system that mediates the response to decreased respiratory throughput. Activation of ArcA by NO was somewhat expected because NO is a known inhibitor of respiration, and the ArcAB system is activated by a downshift in respiration. However, the negative impact of GSNO on ArcA activity is less obvious. Although GSNO, like NO, can strongly perturb the cellular redox state, it is not known to significantly inhibit cytochrome bo oxidase. Instead, GSNO interferes with the cellular redox state by oxidizing thiols. This result highlights the importance of thiol-oxidative chemistry in the GSNO response and demonstrates that in addition to targeting the thiol groups of metabolites, GSNO also targets protein thiol groups.

Acknowledgments—We thank Jon M. Fukuto for insightful discussion, Alice Lee for assistance with experimental analysis, and Katy Kao, Eileen Fung, and Wilson Wong for assistance in generating mutant strains.

REFERENCES

1. Hooton, T. M., and Stamm, W. E. (1997) Infect. Dis. Clin. N. Am. 11, 551–581
2. Talan, D. A., Stamm, W. E., Hooton, T. M., Moran, G. J., Burke, T., Iravani, A., Reuning-Scherer, J., and Church, D. A. (2000) J. Am. Med. Assoc. 283, 1583–1590
3. Tauxe, R. V., Griffin, P. M., Ostroff, S. M., and Wachsmuth, I. K. (1991) Lab. Med. 22, 55–56
4. Fang, F. C. (2004) Nat. Rev. Microbiol. 2, 820–832
5. Gao, A. J., Buerk, D. G., and Ischiropoulos, H. (1997) J. Biol. Chem. 272, 2841–2845
6. Inoue, K., Akaite, T., Miyamoto, Y., Okamoto, T., Sawa, T., Otagiri, M., Suzuki, S., Yoshimura, T., and Maeda, H. (1999) J. Biol. Chem. 274, 27069–27075
7. Wink, D. A., and Mitchell, J. B. (1998) Free Radical Biol. Med. 25, 434–456
8. Stamler, J. S. (1999) Coron. Artery Dis. 10, 273–276
9. Singh, R. J., Hogg, N., Joseph, J., and Kalyanaraman, B. (1996) J. Biol. Chem. 271, 18596–18603
10. Dicks, A. P., Swift, H. R., Williams, D. L. H., Butler, A. R., AlSadoni, H. H., and Cox, B. G. (1996) J. Chem. Soc. Perkin Trans. 2, 481–487
11. Hogg, N. (2002) Annu. Rev. Pharmacol. Toxicol. 42, 585–600
12. Degroote, M. A., Granger, D., Xu, Y. S., Campbell, G., Prince, R., and Fang, F. C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6399–6403
13. Abouhamad, W. N., Manson, M., Gibson, M. M., and Higgins, C. F. (1991) Mol. Microbiol. 5, 1035–1047
14. Olson, E. R., Dunyk, D. S., Jurss, L. M., and Poorman, R. A. (1991) J. Bacteriol. 173, 234–244
15. Mukhopadhyay, P., Zheng, M., Bedzyk, L. A., LaRossa, R. A., and Storz, G. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 745–750
16. Flatley, J., Barrett, J., Pullan, S. T., Hughes, M. N., Green, J., and Poole, R. K. (2005) J. Biol. Chem. 280, 10065–10072
17. Bodenmiller, D. M., and Spiro, S. (2006) J. Bacteriol. 188, 874–881
18. Pullan, S. T., Gidley, M. D., Jones, R. A., Barrett, J., Stevanin, T. A., Read, R. C., Green, J., and Poole, R. K. (2007) J. Bacteriol. 189, 1845–1855
19. Rodionov, D. A., Dubchak, I. L., Arkin, A. P., Alm, E. J., and Gelfand, M. S. (2005) PLoS Comput. Biol. 1, 415–431
20. D’Autreaux, B., Tucker, N. P., Dixon, R., and Sprio, S. (2005) Nature 437, 769–772
21. Hyduke, D. R., Jarboe, L. R., Tran, L. M., Chou, K. J., and Liao, J. C. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 8484–8489

22. Membrillo-Hernandez, J., Coopamah, M. D., Channa, A., Hughes, M. N., and Poole, R. K. (1998) Mol. Microbiol. 29, 1101–1112

23. DeGroot, M. A., Testerman, T., Xu, Y. S., Stauffer, G., and Fang, F. C. (1996) Science 272, 414–417

24. Wanner, B. L. (1994) in Methods in Molecular Genetics (Adolph, K. W., ed) Vol. 3, pp. 291–310, Academic Press, Orlando, FL

25. Maloy, S. R. (1989) Experimental Techniques in Bacterial Genetics, Jones and Bartlett, Boston

26. Gladwin, M. T., Wang, X. D., Reiter, C. D., Yang, B. K., Vivas, E. X., Bonaventura, C., and Schechter, A. N. (2002) J. Biol. Chem. 277, 27818–27828

27. Liao, J. C., Boscolo, R., Yang, Y. L., Tran, L. M., Sabatti, C., and Roychowdhury, V. P. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 15522–15527

28. Tran, L. M., Brynildsen, M. P., Kao, K. C., Suen, J. K., and Liao, J. C. (2005) Metab. Eng. 7, 128–141

29. Sanguinetti, G., Lawrence, N. D., and Rattray, M. (2006) Bioinformatics 22, 2775–2781

30. Gao, F., Foat, B. C., and Bussemaker, H. J. (2004) BMC Bioinformatics 5, 31

31. Datsenko, K. A., and Wanner, B. L. (2000) Proc. Natl. Acad. Sci. U. S. A. 100, 6640–6645

32. Stevanin, T. M., Ioannidis, N., Mills, C. E., Kim, S. O., Hughes, M. N., and Poole, R. K. (2000) J. Biol. Chem. 275, 35868–35875

33. Galbraith, S. J., Tran, L. M., and Liao, J. C. (2006) Bioinformatics 22, 1886–1894

34. Salgado, H., Gama-Castro, S., Martinez-Antonio, A., Diaz-Peredo, E., Sanchez-Solano, F., Peralta-Gil, M., Garcia-Alonso, D., Jimenez-Jacinto, V., Santos-Zavaleta, A., Bonavides-Martinez, C., and Collado-Vides, J. (2004) Nucleic Acids Res. 32, D303–D306

35. Keseler, I. M., Collado-Vides, J., Gama-Castro, S., Ingraham, J., Paley, S., Paulsen, I. T., Peralta-Gil, M., and Karp, P. D. (2005) Nucleic Acids Res. 33, D334–D337

36. Hutchings, M. I., Mandhana, N., and Spiro, S. (2002) J. Bacteriol. 184, 4640–4643

37. Gardner, A. M., Gessner, C. R., and Gardner, P. R. (2003) J. Biol. Chem. 278, 10081–10086

38. da Costa, P. N., Teixeira, M., and Saraiva, L. M. (2003) FEMS Microbiol. Lett. 218, 385–393

39. Magnusson, L. U., Farewell, A., and Nystrom, T. (2005) Trends Microbiol. 13, 236–242

40. Shoeman, R., Redfield, B., Coleman, T., Greene, R. C., Smith, A. A., Brot, N., and Weissbach, H. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 3601–3605

41. Kredich, N. M. (1992) Mol. Microbiol. 6, 2747–2753

42. Urbanowski, M. L., and Stauffer, G. V. (1989) J. Bacteriol. 171, 3277–3281

43. Tsikas, D., Sandmann, J., Rossa, S., Gutzki, F. M., and Frolich, J. C. (1999) Anal. Biochem. 270, 231–241

44. Lee, L. W., Ravel, J. M., and Shive, W. (1966) J. Biol. Chem. 241, 5479–5480

45. Malpica, R., Sandoval, G. R., Rodriguez, C., Franco, B., and Georgellis, D. (2006) Antioxid. Redox Signal. 8, 781–795

46. Kredich, N. M. (1992) Mol. Microbiol. 6, 2747–2753

47. Cotter, P. A., and Gunsalus, R. P. (1996) J. Bacteriol. 178, 1094–1098

48. Cotter, P. A., and Gunsalus, R. P. (1992) FEMS Microbiol. Lett. 91, 31–36

49. Wasser, I. M., de Vries, S., Moenne-Loccoz, P., Schroder, I., and Karlin, K. D. (2002) Chem. Rev. 102, 1201–1234

50. Draper, J. C., and Bouton, C. (1996) Bioessays 18, 549–556

51. Gilberthorpe, N. J., Lee, M. E., Stevanin, T. M., Read, R. C., and Poole, R. (2007) Microbiology 153, 1756–1771

52. Mancardi, D., Ridnour, L. A., Thomas, D. D., Katori, T., Tocchetti, C. G., Espey, M. G., Miranda, K. M., Paolocci, N., and Wink, D. A. (2004) Curr. Mol. Med. 4, 723–740