Transcriptional Responses of *Escherichia coli* to S-Nitrosoglutathione under Defined Chemostat Conditions Reveal Major Changes in Methionine Biosynthesis*

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Nitric oxide and nitrosating agents exert powerful antimicrobial effects and are central to host defense and signal transduction. Nitric oxide and S-nitrosothiols can be metabolized by bacteria, but only a few enzymes have been shown to be important in responses to such stresses. Glycerol-limited chemostat cultures in defined medium of *Escherichia coli* MG1655 were used to provide bacteria in defined physiological states before applying nitrosative stress by addition of *S*-nitrosoglutathione (GSNO). Exposure to 200 μM GSNO for 5 min was sufficient to elicit an adaptive response as judged by the development of NO-insensitive respiration. Transcriptome profiling experiments were used to investigate the transcriptional basis of the observed adaptation to the presence of GSNO. In aerobic cultures, only 17 genes were significantly up-regulated, including genes known to be involved in NO tolerance, particularly hmp (encoding the NO-consuming flavohemoglobin Hmp) and norV (encoding flavohemoglobin). Significantly, none of the up-regulated genes were members of the Fur regulon. Six genes involved in methionine biosynthesis or regulation were significantly up-regulated; metN, metI, and metR were shown to be important for GSNO tolerance, because mutants in these genes exhibited GSNO growth sensitivity. Furthermore, exogenous methionine abrogated the toxicity of GSNO supporting the hypothesis that GSNO nitrosates homocysteine, thereby withdrawing this intermediate from the methionine biosynthetic pathway. Anaerobically, 10 genes showed significant up-regulation, of which norV, hcp, metR, and metB were also up-regulated aerobically. The data presented here reveal new genes important for nitrosative stress tolerance and demonstrate that methionine biosynthesis is a casualty of nitrosative stress.

Nitric oxide (NO) is recognized to be one of the most important small molecules in biology. It is a lipophilic radical that has the ability to diffuse across biological membranes and through the cytoplasm. At high concentrations NO is viewed as a toxic molecule, capable of reacting with all major classes of biomolecules. Of particular interest is its ability to react with thiols and transition metal centers, often altering the functions of the proteins that contain such groups, including terminal oxidases and other heme proteins that bind dioxygen (1–3). Nitric oxide is also inhibitory to iron-sulfur centers in dehydrogenases such as aconitase (4). These reactions with biomolecules underpin the use of NO as a powerful weapon in the armory of mammalian cells to combat bacterial infection. Because NO is an extremely reactive molecule, it leads to the production of other reactive nitrogen species in biological systems (reviewed in Ref. 5). Peroxynitrite, formed during the oxidative burst of macrophages by the reaction of NO with superoxide is the most highly reactive and potentially cytotoxic of all the reactive nitrogen species (6). Nitrosation is the transfer of an NO group to a nucleophilic center, usually to a sulfur or nitrogen lone pair of electrons (7). Nitrosated compounds such as S-nitrosoglutathione (GSNO), which is an excellent nitrosating agent, are extremely useful laboratory tools for the study of nitrosative stress.

Unsurprisingly, due to the cytotoxic effects of NO and its derivatives, bacterial cells have developed mechanisms for NO detoxification. *Escherichia coli* possesses two prominent NO-detoxifying activities, a nitric-oxide reductase (NorVW) and the flavohemoglobin Hmp. Transcription of the norVW-encoded flavohemoglobin and flavohemoglobin reductase that convert NO to nitrous oxide is activated by NorR, an NO-responsive regulator (8, 9). A second detoxification mechanism is mediated by the flavohemoglobin acting as a nitric-oxide dioxygenase or denitrosylase converting NO to the relatively innocuous nitrate ion (NO$_3^-$) (10–12).

The flavohemoglobins, exemplified by the *E. coli* Hmp, have been extensively examined and reviewed (13, 14). We have elucidated a mechanism for hmp gene regulation (15) that involves the interaction of S-nitrosothiols with homocysteine (Hcy), a cofactor for the regulator of the methionine biosynthetic pathway, MetR. It was proposed that Hcy nitrosation leads to depletion of Hcy and enhanced MetR-mediated transcription of hmp.

Other systems for detoxification of NO, or related agents of nitrosative stress, include the periplasmic cytochrome *c* nitrite reductase, encoded by *nrfA*, which catalyzes the respiratory reduction of nitrite and nitric oxide (16), and a glutathione-dependent formaldehyde dehydrogenase enzyme, encoded by *adhC*, which metabolizes GSNO (17). The importance of reducing oxidized methionine residues by the peptide methionine...
sulfoxide reductase, MsrA, has also been demonstrated in the defense of *E. coli* to peroxynitrite-mediated damage (18).

In addition to specific NO defense mechanisms, there are two known regulators in *E. coli* that are thought to respond primarily to redox stresses but which can also be activated by NO and reactive nitrogen species. These are the SoxRS and OxyR regulons, both of which mediate the expression of a number of genes involved in oxidative and nitrosative stress responses. Activation of SoxR by NO occurs through the nitrosylation of the Fe-S centers, forming mixed dinitrosyl-iron-dithiol complexes (19). The OxyR regulon is thought to respond most strongly to the presence of hydrogen peroxide (20) as well as superoxide-generating agents and nitrosothiols (21).

Although the genes regulated by SoxRS and OxyR have been postulated to play a role in NO detoxification, to date there is no evidence demonstrating their specific importance.

Recently, the genome-wide response of *E. coli* to GSNO and acidic sodium nitrite in aerobic batch cultures has been reported (22). This revealed that NorR and Fur are major regulators of the nitrosative defense system under the conditions tested. In the present report, we describe a different approach, namely the use of a chemically defined medium in continuous culture to profile, under both aerobic and anaerobic conditions, the genome-wide transcriptional responses to GSNO of *E. coli*. The use of chemostat cultures for microarray studies has recently been reported for the analysis of the response to changes in growth-limiting nutrients in *E. coli* (23) and *Saccharomyces cerevisiae* (24). Under these conditions, microorganisms are grown under defined and controlled conditions allowing changes in gene expression to be attributed solely to the specified environmental changes and not, for example, to accompanying changes in growth rate. Here transcriptional profiling of chemostat cultures exposed to GSNO confirms the role of genes previously known to be involved in the nitrosative stress response, but also reveals genes required for methionine biosynthesis to be critical for GSNO tolerance under both aerobic and anaerobic conditions. Under conditions, in a medium with adequate supplies of chelated metal ions, Fur is not involved in responses to nitrosative stress.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth—** *E. coli* strain MG1655 was grown in a New Brunswick Scientific Bioflow III fermenter under continuous cell-limited fed-batch conditions. Cells were grown in a medium containing 54 mM glycerochrome as the sole and limiting source of energy and carbon (25). The medium also contained 23 mM KH₂PO₄, 7.3 mM KH₂PO₃, 18.7 mM NH₄Cl, 69 μM CaCl₂, 15 mM K₂SO₄, 1 mM MgCl₂ and the following trace elements in 134 μM EDTA: 31 μM FeCl₃·6H₂O, 18.1 μM CuCl₂·2H₂O, 340 μM CoNO₃·6H₂O, 1.6 μM H₂BO₃ (all final concentrations) in distilled water. The pH of the medium was maintained automatically at 7.0 by the addition of sterile 1 N NaOH or HCl and monitored using a Broady James Fermpen (F-635-B200-DH). To test for glycerochrome limitation, culture supernatant was subjected to an enzymatic assay as described by Garland and Randle (26). The working volume was 1 liter. The maximum specific growth rate aerobically (determined in batch cultures) in this defined medium was 0.6 h⁻¹, whereas anaerobically it was about 0.3 h⁻¹. Despite these invariable differences, we elected to maintain a constant dilution rate, D, of 0.2 h⁻¹ for both aerobic and anaerobic conditions to eliminate any growth rate-dependent changes in gene expression. For aerobic growth, the airflow rate was 1 liter min⁻¹, and the dissolved oxygen tension was maintained at 40% air saturation by measuring oxygen dissolved in the culture using a Broady James D140 OxyProbe® electrode and automated adjustment of stirring rate. For anaerobic growth, nitrogen was sparged through the chemostat medium prior to inoculation and throughout the course of the experiment at a rate of 0.2 liter min⁻¹. No dissolved oxygen was detectable using the OxyProbe. Sodium fumarate was added to anaerobic medium at a final concentration of 50 mM to act as a terminal electron acceptor (27). Small anaerobic cultures were grown in glass universal bottles filled to the brim and closed with metal screw caps with rubber liners. Where indicated, chloramphenicol was added before harvest to a final concentration of 200 μg ml⁻¹ and GSNO to a final concentration of 200 μM.

**GSNO Chemostat Experiments and RNA Isolation—** Cells were grown as above to steady state, defined as constant optical density (OD₆₀₀), after ~5 culture volumes had passed through the vessel. At steady state, GSNO (synthesized according to the method of Hart (28)) was added to the chemostat culture and simultaneously to the nutrient feed at a final concentration of 200 μM unless otherwise stated. Samples were taken immediately prior to the addition of GSNO and after a period of 5-min exposure to GSNO for subsequent analysis using microarrays. Cells were harvested directly into RNA Protect (Qiagen) to stabilize RNA, and total RNA was purified using Qiagen's RNeasy Mini kit as recommended by the suppliers. The integrity of the RNA was determined by electrophoresis on a 1.25% agarose gel run in 1 × MOPS, and the concentration and purity of the nucleic acid were measured at 260 and 280 nm on a Beckman DU® 650 spectrophotometer (29).

**Preparation of Labeled cDNA and Hybridization—** Equal quantities of RNA from control and GSNO-supplemented cultures were labeled using nucleotide analogues of dCTP containing either Cy3 or Cy5 fluorescent dyes. For each microarray slide, one sample was labeled with Cy3-dCTP while the other incorporated Cy5-dCTP. Dye-swap experiments were performed for each pair to compensate for different efficiencies of incorporation of the labeled nucleotides. The slides used were E. coli MG1655 arrays purchased from MWG Biotech. These slides contain 4,288 gene-specific oligonucleotide probes representing the complete *E. coli* (K12) genome. cDNA synthesis was carried out using 12 μg of RNA, primed with 9 μg of pd(N)₆ random hexamers (Amersham Biosciences). Reactions mix (20 μl) containing 0.5 mM dATP, dTTP, and dGTP, 0.2 mM dCTP, and 0.11 mM Cy3/Cy5-dCTP were incubated overnight at 37 °C with 200 units of Superscript II RNase-H Reverse Transcriptase (Invitrogen). Following synthesis, cDNA was purified using a PCR purification kit (Qiagen) to remove unincorporated dNTPs, fluorescent dyes, and primers. Equal volumes of cDNA were combined and evaporated for ~45 min in a Thermo Savant SPD121P Speed Vac®. For hybridization to the microarray slide, cDNA was resuspended in an appropriate volume of salt-based hybridization buffer (provided by MWG). Prior to addition to the slides, cDNA samples were heated to 90 °C for 3 min. Slides were placed in MWG hybridization chambers and incubated for 16–24 h in a shaking water bath at 42 °C.

**Washing and Scanning of Slides—** Following incubation, the slides were washed in decreasing salt concentrations, as recommended by MWG Biotech, ranging from 2 × SSC, including 1% SDS, to 0.5 × SSC at 37 °C, with gentle agitation. Slides were dried by centrifugation at 1200 rpm for 5 min and subsequently scanned on an Affymetrix 440 scanner.

**Data Analysis—** The average signal intensity and local background correction were obtained using a commercially available software package from BioDiscovery, Inc. (Imagem, version 4.0 and GeneSight, version 3.5). The mean values from each channel were log2 transformed and normalized using the LOWESS method to remove intensity-dependent bias (30). The mean ratios were calculated from the normalized values. Biological experiments, i.e. chemostat growths, were carried out twice, and dye-swap analysis was performed on each experiment providing four technical repeats, two from each biological experiment. Data from the independent experiments were combined. Genes differentially regulated ≥2-fold and displaying a p value of ≤0.05 (using t test) were defined as being statistically, differentially transcribed. The GEO accession numbers are GSE2095 (aerobic data) and GSE2129 (anaerobic data) at www.ncbi.nlm.nih.gov/geo.

**Real Time-PCR—** RNA was extracted as described above. cDNA synthesis was carried out using 4 μg of starting material, primed with 9 μg of pd(N)₆ random hexamers (Amersham Biosciences). Reaction mixes (20 μl) containing 0.5 mM dATP, dTTP, dGTP, and dCTP were incubated for 2 h at 42 °C with 200 units of Superscript II RNase-H Reverse Transcriptase (Invitrogen). Following synthesis, cDNA was purified using a PCR purification kit (Qiagen) to remove unincorporated dNTPs and primers.

Gene-specific primers were designed to amplify 50–150 nucleotide fragments of target genes using PRIMER3 software (30). Each reaction contained 1 μl of cDNA sample, 25 μl of reaction mix, 2.5 μl of each of the two primers, and 5 μl of cDNA sample. PCR amplification was carried out in an ABI 7700 thermocycler (PE Applied Biosystems) with the following thermal cycling conditions: 50 °C for 2 min; 95 °C for 10 min; 40 cycles of 95 °C for 15 s; 60 °C for 1 min. The data were analyzed using the Sequence Detector System software (PE Applied Biosystems). A
standard curve was established using genomic DNA for each gene studied to confirm that the primers amplified at the same rate and to validate the experiment. The relative levels of expression of genes of interest, compared with untreated controls, were calculated following the protocol for the Standard Curve Method in the User Bulletin #2 (ABI Prism 7700 Sequence Detection System, Subject: Relative Quantification of Gene Expression) supplied by Applied Biosystems.

| Substrate respired | Control chemostat cells | GSNO-treated cells |
|--------------------|-------------------------|--------------------|
|                    | Respiration rate         | Inhibition of respiration by NO |
|                    | nmoles O$_2$/min         | 45 µM NO  | 90 µM NO |
| Endogenous         |                         | min       |
| Glucose            | 33.6 (± 11)             | ND*       | ND       |
| Glycerol           | 87.6 (± 25)             | 0.67 (± 0.06) | 5.0 (± 0.9) |
|                   | 82.3 (± 12)             | 1.1 (± 0.3) | 6.4 (± 0.5) |
|                   | 15.9 (± 4.1)            | ND       | ND       |
|                   | 76.7 (± 9.5)            | 0 (± 0)   | 2.1 (± 1) |
|                   | 32.5 (± 4.1)            | 0 (± 0)   | 0.13 (± 0.2) |

* ND, not determined.

To assess the impact of nitrosative stress on respiratory metabolism of chemostat-grown E. coli cells and, in particular, to determine whether short (5 min) exposure to GSNO was sufficient to elicit an adaptive response, O$_2$ uptake rates of cells harvested from the chemostat were measured (Table I). Expressed with respect to total cell protein, respiration rates were diminished in GSNO-treated cultures. With endogenous respirable substrates (i.e., measured in washed cell suspensions from glycerol-limited chemostats, not further supplemented with an energy source), respiration rates in GSNO-treated cells were about one-half of control values. Addition of glucose (final concentration, 100 µM) to the polarographic chamber markedly stimulated respiration rates for the treated and untreated cultures (Table I). However, although glycerol-supported respiration was increased for GSNO-treated bacteria, it did not reach the levels achieved with the untreated samples (Table I).

When NO solutions were added to respiring bacterial suspensions, a transient inhibition of O$_2$ uptake was observed. The period of cessation of O$_2$ uptake was dependent on both the NO concentration and the prevailing O$_2$ tension in the chamber, as described before (32), with inhibition increasing at lower O$_2$ tensions (results not shown). The respiratory response of chemostat-grown E. coli MG1655 to NO was investigated by adding NO, to a final concentration of 45 µM, when the oxygen tension had reached 90 µM, and then making a second addition of NO, to a final concentration of 90 µM, when the O$_2$ tension reached 30 µM (Table I). Bacteria from control chemostats exhibited a NO dose-dependent inhibition with either respirable substrate (glucose or glycerol), increasing to 5- to 6-min duration at 90 µM NO. In contrast, cells from GSNO-treated chemostats were completely insensitive to 45 µM NO and exhibited only brief periods of inhibition when exposed to 90 µM NO. This indicates that exposure to GSNO for 5 min is sufficient to elicit an adaptive response.

Similar experiments were attempted to demonstrate the sensitivity to nitrosative stress of fumarate reductase, which will be the predominant terminal reductase (27) under these anoxic growth conditions. However, the standard assay employing fumarate-dependent benzyl viologen oxidation was unsuitable, because addition of solutions of NO gas to the reaction resulted in immediate, fumarate-independent viologen oxidation. This may result from rapid metabolization of the added NO accompanied by enzymic consumption (by flavo-bredoxin or, flavohemoglobin) of reductant in the extract. We demonstrated, however, the inactivation of fumarate reductase by GSNO. Fumarate reductase activity decreased from control values (mean of two determinations, 15 µmol min$^{-1}$ (mg protein)$^{-1}$) by 37 and 57% after 5 and 30 min, respectively, exposure to 200 µM GSNO. However, when chloramphenicol was added immediately prior to GSNO, the inactivation of fumarate reductase was much greater, namely 66 and 79% after 5 and 30 min, respectively, exposure to 200 µM GSNO. These data suggest that exposure to GSNO for 5 min is also sufficient to elicit an adaptive response and partial reductase protection under anoxic conditions.

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2 M. Coopamah and R. K. Poole, unpublished data.
Global Transcriptional Responses to GSNO in an Aerobic Chemostat Culture—The data described above suggested that *E. coli* is able to mount an adaptive response within 5 min of being exposed to 200 μM GSNO. To determine how the transcriptome changes to mediate this rapid adaptation, total RNA was isolated from aerobic chemostat cultures before the addition of GSNO and 5 min after addition of the stress reagent. The corresponding fluorescently labeled cDNAs were synthesized and were used to probe arrays. The entire chemostat run was replicated and, in each case, two arrays were prepared in which the sample-dye pairings were reversed. Under these conditions, a surprisingly small set of genes exhibited increased transcript levels within 5 min of GSNO treatment. It should be noted that the high cell densities achieved when cultures were impregnated with 100 mM GSNO was sufficient to induce an adaptive respiratory response (see above) and increasing the GSNO concentration to 1 mM elicited a response similar to that observed with 200 μM GSNO (data not shown). 17 genes (0.5% of the total) were up-regulated in response to GSNO (9, 33, 34). Unlike the recent data and those of Storz’s group (22) clearly show that these genes are also up-regulated aerobically. To probe the anoxic responses of *E. coli* to GSNO stress under rigidly controlled growth conditions, the chemostat experiments were repeated in nitrogen-sparged cultures in which no oxygen was detectable polarographically throughout growth. Under anaerobic conditions, *E. coli* is capable of both fermentative and respiratory modes of energy conservation. Therefore, for comparison with aerobic growth on glycerol, a non-fermentable carbon source, cells were cultivated anaerobically by supplementing the glycerol medium with fumarate as terminal electron acceptor. Anoxic, fumarate-supported, glycerol-limited growth gave cell yields considerably lower (A_{600} 0.4) than in aerobic glycerol-limited cultures at the set dilution rate.

Ten genes were up-regulated >2-fold after exposure to GSNO under anaerobic conditions (Table IV). Most of these genes, including the *met* genes (*metB, metF, and metR*) were also induced under aerobic conditions (indicated in Table IV).

### Table II

| Gene name<sup>a</sup> | -Fold regulation | p value | Gene product/function<sup>b</sup> |
|-----------------------|-----------------|---------|---------------------------------|
| **Aerobic**            |                 |         |                                 |
| codB                  | -2.21           | 0.043   | Cytosine deaminase              |
| ydcC                  | -2.12           | 0.025   | Function unknown                |
| yeaD                  | -2.11           | 0.037   | Function unknown                |
| nac                   | -2.1            | 0.039   | Nitrogen assimilation control   |
| **Anaerobic**          |                 |         |                                 |
| bcp                   | -2.21           | 0.046   | Thioredoxin-dependent thiol peroxidase |
| rpmB                  | -2.05           | 0.048   | 50 S ribosomal subunit protein L28 |
| rpmG                  | -2.41           | 0.03    | 50 S ribosomal subunit protein L33 |

<sup>a</sup> Gene names were taken from bmb.med.miami.edu/EcoGene/EcoWeb/

<sup>b</sup> Gene product description was obtained from bmb.med.miami.edu/EcoGene/EcoWeb/

Transcriptional Profile of Anaerobic Continuous Cultures to GSNO—The flavorubredoxin and its associated oxido-reductase have been previously considered to be most significant in NO detoxification under anaerobic conditions (8), yet the current data and those of Storz’s group (22) clearly show that these genes are also up-regulated aerobically. To probe the anoxic responses of *E. coli* to GSNO stress under rigidly controlled growth conditions, the chemostat experiments were repeated in nitrogen-sparged cultures in which no oxygen was detectable polarographically throughout growth. Under anaerobic conditions, *E. coli* is capable of both fermentative and respiratory modes of energy conservation. Therefore, for comparison with aerobic growth on glycerol, a non-fermentable carbon source, cells were cultivated anaerobically by supplementing the glycerol medium with fumarate as terminal electron acceptor. Anoxic, fumarate-supported, glycerol-limited growth gave cell yields considerably lower (A_{600} 0.4) than in aerobic glycerol-limited cultures at the set dilution rate.

Aside from genes encoding known NO-detoxifying enzymes (*hmp* and *norV*) and those (*met*) involved in maintaining a flux of intermediates in the methionine biosynthetic pathway in response to Hcy reaction with GSNO, the two most highly up-regulated genes were *hcpl*, encoding hydroxylamine reductase (the “hybrid cluster protein”) (36) and *yhaO*, encoding a hypothetical transport protein, possibly involved in amino acid transport. Both of these were previously shown to be up-regulated in response to nitrosative stress in batch cultures in broth medium (22). In this work, regulation of these two genes was confirmed by real-time RT-PCR using RNA prepared from a third aerobic chemostat run in which samples were withdrawn from the culture at 5-min intervals after simultaneous addition of GSNO to both culture vessel and medium reservoir. The results (Fig. 1) clearly show dramatic up-regulation of both genes, reaching maximal transcript accumulation (*yhaO* 50-fold; *hcpl* 30-fold) only 5 min after GSNO treatment and declining thereafter.

Exogenous Methionine Abrogates GSNO Sensitivity—It has been shown that Hcy, an intermediate in methionine biosynthesis, is sensitive to nitrosation by compounds such as GSNO and SNP in *vitro* and that the intracellular depletion of Hcy is implicated in up-regulation of *hmp* (15). To test whether shortfalls in methionine, caused by GSNO-mediated nitrosation reactions, contribute to the enhanced transcription of *met* genes (Tables III and IV) and the hypersensitivity to GSNO of *met* mutants, cultures of strain MG1655 were supplemented with exogenous methionine. We assayed the ability of cells to retain viability on exposure to GSNO (up to 3 mM) for 45 min in defined medium, having previously demonstrated that high concentrations of the nitrosating agent are required to cause loss in viability. Addition of GSNO to final concentrations of 0.3, 1, or 3 mM caused progressive decreases in cell viability to about 70% of control values (Fig. 3). However, inclusion in the medium of 3 mM methionine prevented killing by 0.3 and 1 mM GSNO. Only at very high GSNO concentrations (3 mM), was a higher methionine concentration needed to prevent killing. In the rich
Levels in the control mRNAs were arbitrarily set to 1. referenced to levels of the transcript in control cultures lacking GSNO. internal reference controls. The normalized values were subsequently reported, but the present report reveals important new aspects of regulating under anaerobic conditions.

The advent of microarray technology has given laboratories the tools required to analyze the genome-wide transcriptional response of bacteria to a variety of different conditions. Here the responses of steady-state chemostat-cultured E. coli to the nitrosating agent, GSNO, under both aerobic and anaerobic growth conditions are reported. The transcription of a surprisingly small number of genes was found to respond to exposure to GSNO with only 17 genes up-regulated and four down-regulated under aerobic conditions, whereas 10 genes were up-regulated and 3 down-regulated under anaerobic conditions.

The defense mechanisms of E. coli against NO and NO-releasing compounds have been extensively examined and reported, but the present report reveals important new aspects of nitrosative stress responses. Thus, a recent genome-wide profile of the response of aerobically grown E. coli to GSNO and the NO-releaser acidified sodium nitrite demonstrated that the sensing of NO was mediated by the modification of a number of transcription factors, including NorR and Fur (22). The mechanism of NO sensing by NorR has not been elucidated, but sequence analysis shows the protein to have two cysteines in a CXXC motif characteristic of redox-active cysteines (22). Fur acts as a transcriptional repressor when bound to ferrous ion, sterically hindering RNA polymerase at “iron boxes” within the promoter regions of genes involved in iron uptake (37). Inactivation of Fur by NO and related species has been shown to involve the formation of a Fur-bound iron-nitrosyl complex, which disrupts the DNA-binding capabilities of Fur and results in the expression of genes required for iron acquisition (38) and the reconstitution of iron proteins damaged by NO. Four genes reported in a previous study (22) to be induced ≥5-fold by 1 mM GSNO and NaNO₂ (fes, nrdH, sufA, and fhuF), and 13 of the genes induced ≥5-fold by 0.1 mM GSNO and NaNO₂ were members of the Fur regulon. However, in a fur mutant, the induction ratios of these genes was reduced to <2-fold suggesting that elevated expression of these genes was due to relief from Fur repression. Interestingly, one of these genes, fhuF, was also shown by D’Autreux et al. (38) to be up-regulated by an NO-releasing agent, again suggesting inactivation of Fur, and effectively mimicking a lack of Fe²⁺, the corepressor with Fur. That iron status influences whether NO (and its congeners) derepresses Fur-regulated genes is predicted from the recent work of Storz’s group, who used LB-rich medium (22), and also in the recent work from Helmann’s laboratory, who examined the response of Bacillus subtilis to NO (39), in contrast to the lack of Fur involvement in the present work, where we used defined media, might be explained by LB broth being iron-limited. Indeed, the poor bioavailability of iron in media lacking chelators is well documented (40). In our experiments, the choice of growth medium appears to negate the effect of GSNO on Fur. The defined medium employed contains 31 μM Fe(III), and the additional presence of 134 μM EDTA ensures metal ion solubility. Evidence that these cultures are not iron-limited is as follows: (i) exhaustiv extraction of defined medium is necessary to iron-lmit chemostat growth (41), and (ii) aerobic growth yield is limited on glycerol concentration in the medium (results not shown) all of which is consumed during growth, i.e. growth of the cultures is glycerol-, not iron-, limited. A possible explana-

TABLE III

| Gene name<sup>a</sup> | -Fold regulation | p value | Gene name<sup>b</sup> |
|----------------------|------------------|---------|--------------------------|
| norV                 | 6.80             | 0.0017  | Flavorubredoxin           |
| metB                 | 6.21             | 0.011   | Cystathionine γ-synthase   |
| metF                 | 6.18             | 0.055   | 5,10-Methyltetrahydrofolate reductase |
| hcp                  | 5.89             | 0.002   | Hydroxylamine reductase (Hybrid-cluster protein) |
| metR                 | 5.18             | 0.0024  | HTH-type transcriptional regulator |
| yhaO                 | 4.89             | 0.0098  | Hypothetical transport protein |
| metA                 | 3.83             | 0.020   | Homoserine O-succinyltransferase |
| yeaJ                 | 3.83             | 0.0079  | Hypothetical protein |
| ybdL                 | 3.66             | 0.0025  | Hypothetical aminotransferase |
| metN                 | 2.91             | 0.032   | ni-methionine transporter, ATP-binding protein |
| metJ                 | 2.76             | 0.036   | Methionine regulon repressor |
| yebY                 | 2.26             | 0.015   | Hypothetical protein |
| alsK                 | 2.30             | 0.043   | Allose kinase |
| mdtC                 | 2.25             | 0.024   | Multidrug transporter |
| hmp                  | 2.14             | 0.025   | Flavohemoglobin |
| yhaN                 | 2.10             | 0.029   | Hypothetical protein |
| yhaM                 | 2.03             | 0.0218  | Hypothetical protein |

<sup>a</sup> Gene names were taken from bmb.med.miami.edu/EcoGene/EcoWeb.  
<sup>b</sup> Gene product description was obtained from bmb.med.miami.edu/EcoGene/EcoWeb.  

FIG. 1. Real-Time PCR expression of hcp and yhaO in GSNO-treated chemostat cultures. Real time quantitative RT-PCR analysis of changes in expression was performed at various times points following chemostat exposure to 200 μM GSNO. Mean values are plotted for three replicate assays; bars show standard deviation but in most cases are within the size of the data points. mRNA levels were normalized to trxB mRNA for hcp and to reca for yhaO, which served as internal reference controls. The normalized values were subsequently referenced to levels of the transcript in control cultures lacking GSNO. Levels in the control mRNAs were arbitrarily set to 1.

The involvement of Fur in the work of Storz’s group, who used LB-rich medium (22), and also in the recent work from Helmann’s laboratory, who examined the response of Bacillus subtilis to NO (39), in contrast to the lack of Fur involvement in the present work, where we used defined media, might be explained by LB broth being iron-limited. Indeed, the poor bioavailability of iron in media lacking chelators is well documented (40). In our experiments, the choice of growth medium appears to negate the effect of GSNO on Fur. The defined medium employed contains 31 μM Fe(III), and the additional presence of 134 μM EDTA ensures metal ion solubility. Evidence that these cultures are not iron-limited is as follows: (i) exhaustiv extraction of defined medium is necessary to iron-lmit chemostat growth (41), and (ii) aerobic growth yield is limited on glycerol concentration in the medium (results not shown) all of which is consumed during growth, i.e. growth of the cultures is glycerol-, not iron-, limited. A possible explana-
The flavohemoglobin, Hmp, has a prominent role in NO detoxification (reviewed by Wu et al. (43)). The Hmp protein acts as an NO oxygenase (10, 11) or denitrosylase (44) thus protectingaconitase activity (45) and respiration (32). Regulation of hmp expression is partly mediated by the oxygen-sensitive transcription factor, FNR. Under anaerobic conditions, FNR acquires $[4\text{Fe}-4\text{S}]^{2+}$ clusters, which promote protein dimerization and site-specific DNA binding to repress hmp transcription. In the presence of NO (or oxygen), the FNR iron-sulfur clusters are modified, and repression of hmp expression is lifted (46). Under the conditions used here, hmp was up-regulated only under aerobic conditions suggesting that the NorVW NO reductase activity is sufficient to detoxify the effects of GSNO under anaerobic conditions. Furthermore, although FNR has been shown to sense NO, there is no evidence that, under anoxic conditions, GSNO can generate NO sufficient for reaction with FNR.

Intracellular levels of Hcy, an intermediate in the biosynthesis of methionine, influence hmp expression via MetR, a member of the LysR family of DNA-binding proteins (47) that regulates methionine biosynthesis in response to the intracellular Hcy pool (15). The transcriptional profiles obtained are consistent with GSNO-mediated depletion of the Hcy pool thereby minimizing the expression of hmp.

The discrepancy between the presented data and those of Storz’s group (22) is therefore that the broth medium used in the latter work is sufficiently poor in iron to reveal Fur-mediated, GSNO-sensitive repression of genes involved in iron acquisition. Although we did not find Fur to be a significant regulator of GSNO-induced genes in defined medium, the NorR-regulated norV and norW transcripts were strongly up-regulated. The NorR protein is an NO-responsive transcription factor that interacts with $\sigma^{34}$-RNA polymerase to activate target gene expression (9). The norV gene encodes a flavorubredoxin with an NO-binding di-iron center, and norW encodes an NAD(P)H:flavorubredoxin oxidoreductase (42). Anaerobically, we found that norV induction was greater than norW; indeed norV was the most highly up-regulated gene in GSNO-treated cultures (21-fold compared with 2.8-fold for norW). Aerobically, only norV was strongly up-regulated (6.8-fold). Because norV and norW are co-transcribed from a single promoter, these differences may reflect differential stability of the mRNA transcripts from these genes. The up-regulation of norV and, to a lesser extent, hmp may be sufficient to maintain NO concentrations below that required to induce adhC, encoding the glutathione-dependent formaldehyde dehydrogenase enzyme that detoxifies GSNO and S-nitrosylated proteins (17).
enhancing MetR binding to the metA and metB promoters, to enhance the synthesis of Hcy from homoserine, and to the hmp promoter region, to detoxify NO (Fig. 4). The observed increase in metF expression could ensure that there is sufficient N\(^5\)-methylenetetrahydrofolate available to direct some Hcy toward methionine biosynthesis to maintain growth (Fig. 4). The metE and metR genes are divergently transcribed from overlapping promoters. Transcription of metE requires MetR and Hcy (48). Thus, the lack of enhanced metE expression in the transcript profile of GSNO-treated cultures is consistent with nitrosylation of the Hcy pool. Expression of metR is controlled by the MetJ repressor, which is functional as a complex with S-adenosylmethionine, generated by the product of the metK gene (49). Depletion of the Hcy pool is likely to lead to lower levels of methionine, which in turn will lead to lower levels of S-adenosylmethionine resulting in relief of MetJ-mediated repression of metR, metJ, and metK (48). This hypothesis is consistent with the enhanced metR, metJ, and metK transcription under aerobic conditions (metK transcription was induced >2-fold aerobically with a p value of 0.11).

We have demonstrated the importance of methionine in protecting E. coli against GSNO using a variety of met mutants. We propose that the differences observed in the sensitivity experiments of the met mutants are directly due to the products that these genes encode. metN and metF are reported to encode components of an ABC-type methionine transporter (a putative ATPase and membrane-spanning region, respectively). In contrast, metA and metP encode members of the methionine biosynthetic pathway (homoserine trans-succinylase and \(N^\beta,N^\gamma\)-methylene tetrahydrofolate reductase, respectively) (50). Therefore, the presence of exogenous methionine in these experiments bypasses the requirement of metA and metF, because methionine can be transported into the cell; however, metN and metF mutants are unable to transport methionine, resulting in the apparent decrease in viability observed for these strains. In addition, MetR is required for the regulation of hmp (15).

The hybrid cluster protein, encoded by hcp, was up-regulated under both aerobic and anaerobic conditions. HCP (previously known as the prisamine protein) has been suggested to play a role in nitrogen respiration, because it is optimally expressed under anaerobic conditions in the presence of either nitrate or nitrite (36). In addition, hcp expression was not detected under aerobic growth or anaerobically using fumarate as an electron acceptor; however, it was induced using rich media or minimal media enhanced with nitrate or nitrite as the terminal electron acceptor (36). Although very little is known about the role of HCP in E. coli, it has been shown to have a hydroxylamine reductase activity (51). Sequence analysis of the hcp promoter region has suggested that the regulation of hcp may be under the control of the fumarate and nitrate reduction response regulator (FNR) (36) and also the nitrate response regulatory proteins, NarL or NarP. The data presented here clearly show that hcp expression is enhanced in both anaerobic and aerobic conditions, and in the presence of fumarate as the terminal acceptor, suggesting that there is more to learn about the regulation of hcp expression and the role of the protein in the defense against nitrosative stress.

During revision of this report, Justino et al. (52) published an analysis of genes up-regulated by NO (50 \(\mu\)M final concentration, achieved by adding an NO-saturated aqueous solution). As in Ref. 22, growth was in batch culture, but using minimal salts medium. In addition to the flavohemoglobin gene, a large number of genes were described as up-regulated, including ytfE and yidZ of poorly defined function, and genes encoding iron-sulfur cluster assembly systems, DNA repair enzymes, and stress response regulators. The consequences of the "visible effects on the growth curve" caused by NO could not be evaluated in this work.

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