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Nitric Oxide in Chemostat-Cultured *Escherichia coli* Is Sensed by Fnr and Other Global Regulators: Unaltered Methionine Biosynthesis Indicates Lack of S Nitrosation[▽]†

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We previously elucidated the global transcriptional responses of *Escherichia coli* to the nitrosating agent *S*-nitrosoglutathione (GSNO) in both aerobic and anaerobic chemostats, demonstrated the expression of nitric oxide (NO)-protective mechanisms, and obtained evidence of critical thiol nitrosation. The present study was the first to examine the transcriptome of NO-exposed *E. coli* in a chemostat. Using identical conditions, we compared the GSNO stimulon with the stimulon of NO released from two NO donor compounds {3-[2-hydroxy-1-(1-methyl-ethyl)-2-nitrosohydrazino]-1-propanamine (NOC-5) and 3-(2-hydroxy-1-methyl-2-nitrosohydrazino)-*N*-methyl-1-propanamine (NOC-7)} simultaneously and demonstrated that there were marked differences in the transcriptional responses to these distinct nitrosative stresses. Exposure to NO did not induce *met* genes, suggesting that, unlike GSNO, NO does not elicit homocysteine S nitrosation and compensatory increases in methionine biosynthesis. After entry into cells, exogenous methionine provided protection from GSNO-mediated killing but not from NO-mediated killing. Anaerobic exposure to NO led to up-regulation of multiple Fnr-repressed genes and down-regulation of Fnr-activated genes, including *nrfA*, which encodes cytochrome *c* nitrite reductase, providing strong evidence that there is NO inactivation of Fnr. Other global regulators apparently affected by NO were IscR, Fur, SoxR, NsrR, and NorR. We tried to identify components of the NorR regulon by performing a microarray comparison of NO-exposed wild-type and *norR* mutant strains; only *norVW*, encoding the NO-detoxifying flavorubredoxin and its cognate reductase, were unambiguously identified. Mutation of *norV* or *norR* had no effect on *E. coli* survival in mouse macrophages. Thus, GSNO (a nitrosating agent) and NO have distinct cellular effects; NO more effectively interacts with global regulators that mediate adaptive responses to nitrosative stress but does not affect methionine requirements arising from homocysteine nitrosation.

Nitric oxide (NO) is a key component of the host immune response and is encountered by pathogenic bacteria during their lives outside and within hosts. In particular, phagocytic cells of a host produce the antimicrobial radical NO at micromolar concentrations through the activity of inducible NO synthase (20).

Enteric bacteria, such as *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, use two major mechanisms to detoxify NO (Fig. 1) (60), the flavohemoglobin Hmp and the flavorubredoxin NorV. The former enzyme, using an electron from NAD(P)H delivered via the flavin protein domain, catalyzes either an O_2-dependent denitrosylase ("dioxygenase") reaction converting NO to the nitrate ion or an anoxic reductive reaction forming NO^- (63). The flavorubredoxin NorV along with its cognate reductase, NorW, however, catalyzes the reductive detoxification of NO only under microaerobic or anaerobic conditions (25). The synthesis of NorV and NorW is positively regulated at the transcriptional level by the NorR NO-sensing transcription factor (35). The regulation of Hmp synthesis is more complex. First, transcription of the *hmp* gene is repressed anaerobically (61) by the oxygen-responsive regulator, Fnr, but in the presence of NO the DNA-binding activity of Fnr is diminished by formation of dinitrosyl-iron complexes during the reaction of the iron-sulfur cluster with NO (15), so that NO derepresses *hmp* transcription. Second, MetR activates *hmp* transcription. Nitrosation of homocysteine (Hcy) (Fig. 1) forms *S*-nitroso-Hcy and withdraws Hcy, a key intermediate, from the biosynthetic pathway leading to methionine. In the absence of its cofactor, Hcy, MetR binds to the *hmp* promoter and activates transcription (45). Third, *hmp* transcription is repressed by NsrR, an effect reversed by nitrite or NO (6). Fourth, *hmp* is regulated by the ferric uptake regulator (Fur), but this process is unclear. Crawford and Goldberg (13) originally proposed that this iron-responsive protein represses *hmp* transcription in *Salmonella* and that this repression is relieved by NO after inactivation of Fur. Although these proposals have been retracted (14), it is clear that other promoters are indeed controlled by nitrosylation of the Fur iron (17). Furthermore, we recently obtained evidence based on newly constructed *hmp-lacZ* fusions and immunoblotting indicating

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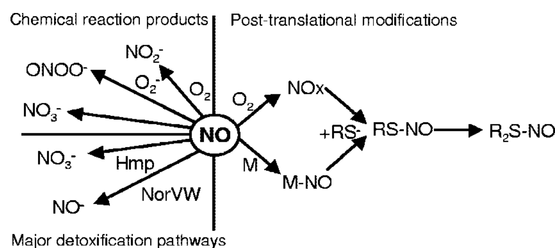

FIG. 1. Targets of NO and its fate. (Top left panel) Unreacted NO appears primarily as nitrite after oxidation or as peroxynitrite after reaction with superoxide. (Lower left panel) The detoxification mechanisms employed by enterobacteria involve primarily the aerobic conversion to the nitrate ion by flavohemoglobin (Hmp) and the one-electron reduction to the nitroxyl anion (NO^-) catalyzed by flavorubredoxin (NorVW). (Right panel) The redox requirements for S nitrosation of thiols (RS^-) are met by transition metals (M) or O_2. Transnitrosation (i.e., transfer of the NO group to R_2S) is directed by nitrosation motifs and/or protein hydrophobic environments.

that Fur is a repressor of *hmp* transcription in both *E. coli* and *Salmonella*, albeit a weak repressor (31).

A powerful approach for investigating bacterial responses to nitrosative stress is to measure the global changes in gene expression that occur upon exposure to this stress. This approach has been used to study the nitrosative stress responses of several bacteria, such *as Bacillus subtilis* (49), *Pseudomonas aeruginosa* (21), and *Mycobacterium tuberculosis* (56). The global transcriptional response of *E. coli* to nitrosative stress has been reported in several recent papers (22, 38, 50), with sometimes conflicting results. Indeed, only three transcriptional units were found in all *E. coli* nitrosative stress microarray studies reported to date; these units are *norVW*, *hmp*, and *nrdH*, the last of which encodes a glutaredoxin-like protein. A probable explanation for the apparent discrepancies is that various reactive nitrogen species (RNS) have been used as mediators of the stress; these RNS include *S*-nitrosoglutathione (GSNO), acidified sodium nitrite, and NO gas, and the contributions of individual stress agents to the patterns of gene expression observed have not been deconvoluted. Nitric oxide and related RNS (NO^+, NO, and NO^-) have unique chemistries (34) that reflect the presence of nitrogen in different oxidation states [N(III), N(II) and N(I), respectively]. Thus, NO per se is not a nitrosating agent, but nitrosation reactions are promoted by the presence of a metal ion or oxygen (Fig. 1). Nitrosating agents (such as GSNO) and NO are often considered interchangeable despite clear evidence that they have quite different effects in biological processes as diverse as caspase activation (7) and the respiratory oscillations of *Saccharomyces cerevisiae* in a chemostat (51).

A further shortcoming of most microarray experiments is the difficulty of distinguishing between nitrosative stress per se and the unintentional perturbation of culture behavior arising from the stress. Continuous chemostat culture offers major benefits for postgenomic global studies such as proteomics, transcriptomics, and metabolomics studies (30, 32). The greater biological homogeneity of continuous cultures and the ability to control all relevant growth conditions, such as oxygen levels, pH, and especially growth rate, eliminate the masking effects of secondary stresses and growth rate changes, allowing more precise delineation of the response to an individual stress. In the case of transcriptomics, it has been demonstrated that the reproducibility of analyses between different laboratories is greater when chemostat cultures are used than when identical analyses are performed with batch cultures (58). One criticism of the use of continuous cultures, however, has been the potential selective pressure placed, particularly at low growth rates, on loss-of-function *rpoS* mutations, leading to mutant bacteria overtaking the cultures (53). However, a closer examination of the literature revealed that this phenomenon has not been observed under anaerobic conditions (41) or in the wild-type MG1655 strain used in many array studies (42).

In our previous work we used a combination of chemostat culture in defined growth medium with microarray technology to define the transcriptional response of *E. coli* to GSNO under both aerobic and anaerobic conditions (22). This work revealed important changes in gene expression that had not been observed in previous batch culture-based studies with complex media (50), particularly changes related to the methionine biosynthetic pathway. The chemostat approach allows measurement and control of all key parameters in a culture, including, critically, the growth rate. Thus, even if the addition of GSNO, NO, or some other stressor caused a reduction in the growth rate in a batch culture, the growth rate in a chemostat (i.e., dilution rate) can be constrained by the rate of medium provision, so that the growth rates in the absence and presence of the stressor are equal. The aims of the present work were threefold. First, we set out to explore systematically the effects of NO per se delivered by well-characterized NO-releasing compounds in chemically defined media, in which all components are known and metal speciation, for example, can be predicted. We used as a reference the previous study with GSNO (22). Of particular interest was the possibility that under aerobic conditions NO might exhibit nitrosating activities (Fig. 1), and so, using the same experimental system that was used to study the GSNO stimulon, we compared the NO responses under both aerobic and anaerobic conditions. Previously, such comparisons have been made only using batch cultures and separately under aerobic and anoxic conditions (38, 50). Second, since Fnr has been shown previously to react with NO (15), we sought evidence for global regulation of Fnr-responsive genes under anaerobic conditions. Finally, we looked for additional components of the NorR regulon under anaerobic conditions and assessed the potential protective role of the entire regulon during internalization in murine macrophages.

MATERIALS AND METHODS

Strains and growth conditions. The wild-type *E. coli* strain used in all experiments was MG1655. The mutant strains used were MG1655 derivatives with Tn*5* insertions in desired genes and were purchased from the *E. coli* Genome Project, University of Wisconsin, Madison, unless indicated otherwise. Cultures were grown in defined media, and chemostat cultures were grown as described by Flatley et al. (22) except that under aerobic conditions the concentration of glycerol in the medium was reduced from 54 to 8 mM, so that the aerobic growth yield, measured by determining the optical density at 600 nm, was equal to that of the anaerobic culture. To ensure that prolonged growth in the chemostat did not affect physiological characteristics of the strain or the transcriptome as a result of accumulating *rpoS* mutations, sequencing of the *rpoS* gene region was performed for all chemostat samples using primers described by King et al. (42). All cultures tested were shown to have the wild-type allele.

Viable counts were determined by serial dilution of cultures or macrophage lysates in phosphate-buffered saline (PBS) and plating on nutrient agar (Sigma)

plates supplemented with the relevant antibiotic. Strain RKP3073 (MG1655 *norV*), created via P1 transduction from LMS2710 (29) kindly donated by Ligia Saraiva, and strain FB21836 (*norR* mutant obtained from F. Blattner, University of Wisconsin *E. coli* Genome Project) were plated on nutrient agar supplemented with chloramphenicol (30 μg/ml) and kanamycin (50 μg/ml), respectively. The plates were incubated overnight at 37°C, and the numbers of CFU per ml were expressed as the means of four determinations.

Microarray analysis and real-time PCR. Cell harvesting, RNA isolation, cDNA preparation, and microarray analyses were performed as described previously (22). In brief, cells were harvested directly into RNA Protect (QIAGEN), and total RNA was purified using a QIAGEN RNeasy Mini kit. Equal quantities of RNA from control and GSNO-supplemented cultures were labeled using nucleotide analogues of dCTP containing either the Cy3 or Cy5 fluorescent dye. For each microarray slide, one sample was labeled with Cy3-dCTP, while the other sample contained Cy5-dCTP. Dye swap experiments were performed for each pair to compensate for the different efficiencies of incorporation of the labeled nucleotides. The slides used were *E. coli* K-12 PAN arrays purchased from Ocimum Biosolutions (The Magdalen Centre, Oxford Science Park, Oxford, United Kingdom; previously marketed by MWG Biotech). These slides contain 4,288 gene-specific oligonucleotide probes representing the complete *E. coli* K-12 genome. cDNA synthesis was carried out using 12 μg of RNA primed with 9 μg of $pd(N)_6$ random hexamers (Amersham Biosciences). Reaction mixtures (20 μl) containing 0.5 mM dATP, 0.5 mM dTTP, 0.5 mM dGTP, 0.2 mM dCTP, and 0.11 mM Cy3/Cy5-dCTP were incubated overnight at 37°C with 200 U of Superscript II RNase-H reverse transcriptase (Invitrogen). cDNA was purified using a PCR purification kit (QIAGEN), and equal volumes of cDNAs were combined and evaporated for approximately 45 min in a Thermo Savant SPD121P SpeedVac. cDNA was resuspended in a salt-based hybridization buffer, heated to 95°C for 3 min, and applied to the slides, which were hybridized for 16 to 24 h in a shaking water bath at 42°C. The slides were washed in decreasing salt concentrations, dried by centrifugation, and scanned with an Affymetrix 428 scanner. The average signal intensity and the local background correction were obtained using BioDiscovery Inc. software (Imagene, version 4.0, and GeneSight, version 3.5). The mean values from each channel were $\log_2$ transformed and normalized using the LOWESS method to remove intensity-dependent effects in the $\log_2$ values (ratios). The Cy3/Cy5 fluorescent ratios were calculated from the normalized values. Biological experiments (i.e., chemostat growths) were carried out at least twice, and a dye swap analysis was performed for each experiment, providing a minimum of four technical repeats. Data from independent experiments were combined. Genes differentially regulated ≥2-fold and for which the P value was ≤0.05 (as determined by a t test) were defined as genes that were statistically differentially transcribed. The GEO accession number for the entire series of arrays is GSE5098.

NO-releasing agents. The NO-releasing compounds 3-[2-hydroxy-1-(1-methylethyl)-2-nitrosohydrazino]-1-propanamine (NOC-5) and 3-(2-hydroxy-1-methyl-2-nitrosohydrazino)-*N*-methly-1-propanamine (NOC-7), which have half-lives of NO release (at pH 7.4 and 22°C) of 93 and 10 min, respectively, were purchased from Calbiochem. Stock solutions (100 mM) of each compound were prepared with 0.1 M NaOH (in which the compounds are stable), and these stock solutions were added simultaneously to obtain final concentrations of 10 μM. The NO-amine complexes spontaneously released two equivalents of NO under physiological conditions. As described previously (15), use of both compounds ensured continuous release of NO over several tens of minutes. Release of NO after addition of a solution containing both NOC compounds to a defined medium was measured using the apparatus described by Mills et al. (47). NOC-5 and NOC-7 were added simultaneously to chemostat cultures 5 min prior to sampling. NO gas solutions like those used in viability experiments were prepared as described previously (61).

Assay of nitrite levels in defined media. Samples were taken from mid-log-phase cultures of wild-type and *norR* mutant strains grown in batch cultures (25 ml) which were exposed for 5 min to a mixture of NOC-5 and NOC-7 (final concentration of each compound, 10 μM). Samples were centrifuged briefly to pellet the cells, and the supernatants were collected. Nitrite levels in the supernatant were then determined using a Sievers model 280i NO analyzer.

Macrophage culture, phagocytosis, and assay of intracellular *E. coli* viability. Experiments with J774.2 mouse macrophages were performed essentially as described previously (75), except that the macrophages used for infection were cultured in Dulbecco's modified Eagle's media (DMEM) (D5796; Sigma) supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. Where indicated below, macrophages were activated with gamma interferon (1,000 U/ml) for 24 h prior to infection. Bacteria grown in L broth were resuspended in 1 ml DMEM and diluted to obtain an inoculum containing approximately 2×10^7 CFU in 500 μl DMEM (multiplicity of infection, 100). Suspensions of bacteria were declumped by vortexing them twice for 20 s and then used to infect J774.2 cells seeded at a density of 2×10^5 cells per well. Trays were incubated on ice for 1 h in order to allow binding but not internalization of bacteria. The bacterial suspensions were then removed by aspiration, and wells were washed twice with PBS. To examine binding of bacteria to macrophages, cells were fixed for microscopy, whereas to quantify bacterial internalization, 1 ml of prewarmed DMEM was added to each well and the trays were then incubated at 37°C for 0, 15, 30, 45, and 60 min. At the appropriate time, infected macrophages were washed twice in PBS, and cells were fixed for microscopy using 2% paraformaldehyde in PBS. Internalization was scored microscopically as described previously (75). One hundred macrophages were sampled per condition.

A gentamicin exclusion assay was used to monitor bacterial survival within macrophages (74). Bacteria and macrophages were incubated on ice for 30 min; the supernatant was then removed, and the wells were washed twice using PBS. Prewarmed DMEM (0.5 ml) was added to each well and incubated at 37°C as described above for 1.5 h (period of maximum internalization). The supernatant was then removed, and the wells were washed twice with PBS. Extracellular bacteria were killed by incubation with 1 ml of gentamicin at a concentration of 150 μg/ml (the minimum bactericidal concentration) for 30 min at 37°C. The cells were then washed twice with PBS, and 0.5 ml of fresh DMEM was added to each well. The numbers of surviving intracellular bacteria were estimated by determining viable counts after lysis of cells with 1% saponin, using a standard dilution technique before addition of gentamicin and 0.5, 2, 5, 18, and 24 h after addition of gentamicin. Dilutions were plated on nutrient agar supplemented with the relevant antibiotics. All results were confirmed in six independent experiments.

RESULTS

Treatment of cultures with NO. NO is oxidized by molecular oxygen to NO_2, which, when generated in an aqueous solution, reacts with NO to form nitrite (NO_2^-); the lifetime of NO in solution and the appearance of nitrite (itself a mediator of nitrosative stress) are expected to vary with culture aeration. Furthermore, since *E. coli* cells consume NO avidly with or without oxygen, we avoided bolus additions of NO and instead utilized NOC compounds, which release NO with experimentally useful halftimes. To ascertain the concentration of NO in solution in growth medium in the presence or absence of oxygen, NOC-5 and NOC-7 were added simultaneously to medium samples (final concentration of each compound, 10 μM) without bacteria, and the NO content was determined using a WPI NO electrode. Under anaerobic conditions, the concentration of NO reached in the medium (approximately 5 μM) was slightly greater than the concentration reached under aerobic conditions (approximately 4 μM) over 5 min (results not shown). It should be noted that these values were steady-state values measured in the media and reflected both NO release from NOC compounds and nonbiological removal. This period of exposure was used for all microarray experiments. Previous results with *E. coli* cultures (22) showed that the maximal response to GSNO is complete in this interval.

Anaerobic exposure to NO. In this paper, we describe a direct comparison of the anaerobic and aerobic transcriptomes of *E. coli* under NO stress conditions. For anaerobic growth, the culture was sparged with nitrogen, and the glycerol-limited medium was supplemented with 50 mM fumarate to serve as a terminal electron acceptor, as described previously (22). The level of oxygen in the culture was below the detection limit of the oxygen electrode. Tables 1 and 2 show genes that had altered expression ratios in response to simultaneous exposure to NOC-5 and NOC-7 (final concentration of each compound, 10 μM) under anaerobic conditions. A full data set is presented in the supplemental material. Sixty-one genes were up-

TABLE 1. Genes up-regulated by NO anaerobically via known or presumed NO-sensing mechanisms[a]

Regulon[b]	Gene	Fold regulation	*P* value	Gene product or function
NorR	*norV*	180	1.9×10^{-4}	Flavorubredoxin
	norW	85	2.519×10^{-5}	Flavorubredoxin oxidoreductase
NsrR	*hmp*	27	6.8×10^{-4}	Flavohemoglobin
	hcp	8.5	0.003	Hydroxylamine reductase
	hcr	5.9	0.002	Hcp reductase
	ytfE	19	7.6×10^{-4}	Possible role in protection and repair of Fe-S clusters
	ygbA	2.2	0.013	Unknown function
SoxR	*soxS*	5.0	0.011	Regulatory protein of SoxRS regulon
Fur	*bfd*	5.7	0.004	Complexes with Bfr for iron storage and mobility
	exbB	4.1	0.009	Forms complex which transduces energy from the inner membrane to the outer membrane for ferrous iron uptake systems
	exbD	4.3	0.003	Forms complex which transduces energy from the inner membrane to the outer membrane for ferrous iron uptake systems
	tonB	2.8	0.011	Forms complex which transduces energy from the inner membrane to the outer membrane for ferrous iron uptake systems
	feoA	3.3	0.059	Ferrous iron uptake system component
	feoB	3.9	0.004	Ferrous iron uptake system component
	fepB	2.6	0.010	Ferrienterobactin uptake system component
	fepG	2.2	0.055	Ferrienterobactin uptake system component
	nrdH	2.2	0.043	Hydrogen donor for ribonucleotide reductase system
IscR	*iscR*	6.4	0.007	Regulator of *isc* operon
	iscS	4.9	0.032	Mobilization of S from L-cysteine
	iscU	3.7	0.022	Scaffold for Fe-S assembly
	iscA	3.1	0.024	Scaffold for Fe-S assembly
	sufA	2.2	0.030	Scaffold for Fe-S assembly
Fnr	*cydA*	3.9	0.043	Cytochrome *d* terminal oxidase subunit I
	cydB	3.2	0.078	Cytochrome *d* terminal oxidase subunit II
	ndh	5.1	0.001	Respiratory NADH dehydrogenase II
	yfiD	3.4	0.014	Glycine radical cofactor

[a] Genes showing a greater-than-twofold alteration in the mRNA level and having a *P* value of <0.05 are included. References for the proposed regulation are indicated in the text. Functional annotations were obtained from the EchoBase online database (48; http://www.e.coli-york.org).
[b] Regulators shown are those proposed to be responsible for the major regulation of the genes.

regulated (Table 1; see Table S1 in the supplemental material), while 59 genes were down-regulated (Table 2; see Table S2 in the supplemental material). Twenty-seven of the up-regulated genes are members of known regulons (Table 1). The transcription factors that appeared to be primarily affected by NO in this study are NorR, NsrR, SoxR, Fur, IscR, and Fnr. The *norV* and *norW* genes (under the transcriptional control of NorR) and *hmp* (exhibiting multiple regulatory mechanisms [60, 63] but shown in Table 1 as a gene that is regulated primarily by NsrR [6]) were the most highly induced genes (180-, 85-, and 27-fold, respectively).

Of the 120 genes that displayed altered expression in this study, 32 are known to require Fnr for transcriptional regulation, as demonstrated in other array studies (10, 39, 68); these genes are the up-regulated genes *cydA*, *cydB*, *ndh*, *yfiD*, and *hmp* and 27 diverse down-regulated genes (Table 2). The Fnr regulon is, therefore, the largest single group of genes found in this study. The [4Fe-4S] cluster of the Fnr protein is required for DNA binding and is sensitive to NO exposure (15). Therefore, induction of genes that are Fnr repressed is anticipated during anaerobic NO exposure. Interestingly, the *nrfAB* genes that encode the cytochrome c_{552} nitrite reductase were down-regulated (Table 2). This appears to be paradoxical, given the claim (59) that this enzyme may play a role in the anaerobic detoxification of NO. Similarly, genes of the *nar*, *nir*, *nap*, and *frd* operons, all of which are positively regulated by Fnr, were down-regulated in response to NO (Table 2).

NsrR, a recently discovered regulator of *hmp*, *ygbA*, *ytfE*, and *hcp-hcr* (6), is thought to sense the presence of NO via an unknown mechanism. Thus, in the presence of NO, NsrR-regulated genes (*hmp*, *hcp*, *hcr*, *ytfE*, and *ygbA*) are expected to be induced, as observed here under anaerobic conditions (Table 1). The genes encoding Hmp and YtfE were also induced under aerobic conditions, but *ygbA*, *hcp*, and *hcr* were not induced (Table 3).

As noted above for Fnr, iron-sulfur clusters (5) are targets for NO; therefore, the induction of members of the Isc operon, which encodes the machinery for Fe-S assembly and is itself regulated via the labile Fe-S cluster within the IscR regulator protein (69), is not unexpected. Additionally, it has been demonstrated recently (27) that in the apo form, IscR is an activator of *sufA* expression, explaining the induction found here.

Many of the genes that exhibited altered transcriptional levels in response to NO are not known to be regulated by any of the proven NO-responsive regulators (see Table S1 in the supplemental material). Under anaerobic conditions, some of

TABLE 2. Genes down-regulated by NO anaerobically via known or presumed NO-sensing mechanisms[a]

Regulon[b]	Gene	Fold regulation	*P* value	Gene product or function
Fnr	*ackA*	−3.8	0.004	Acetate kinase
	ansB	−2.1	0.010	Asparaginase II
	arcA	−2.1	0.005	Response regulator of ArcAB two-component system
	caiF	−3.1	0.007	Transcriptional activation of *cai* operon
	dcuC	−2.5	0.009	Anaerobic C_4-dicarboxylate transport
	dcuR	−3.9	0.037	Anaerobic C_4-dicarboxylate transport; regulator
	dmsA	−2.0	0.030	Dimethyl sulfoxide reductase subunit A
	dmsB	−2.4	0.016	Dimethyl sulfoxide reductase subunit B
	fliJ	−2.7	0.025	Flagellar biosynthesis
	frdB	−2.8	0.037	Fumarate reductase iron-sulfur protein subunit
	frdC	−2.6	0.001	Fumarate reductase membrane anchor polypeptide
	frdD	−2.2	0.048	Fumarate reductase membrane anchor polypeptide
	glpB	−2.1	0.023	Glycerol-3-phosphate dehydrogenase subunit
	napD	−3.1	0.005	Essential for NapAB activity
	napF	−3.7	3.24×10^{-04}	Involved in electron transfer from ubiquinol to NapAB
	napH	−2.2	0.008	Involved in electron transfer from ubiquinol to NapAB
	narG	−2.8	0.008	Nitrate reductase I (NRA), alpha subunit
	nirC	−2.2	0.025	Nitrite uptake transporter; membrane protein
	nrfA	−4.9	0.005	Nitrite reduction; tetraheme cytochrome c_{552}
	nrfB	−2.9	0.012	Nitrite reduction; pentaheme cytochrome *c*
	pykA	−2.1	0.010	Pyruvate kinase A(II)
	ycbJ	−2.3	0.028	Unknown function
	ydhV	−3.7	0.000	Unknown function
	ydjX	−2.3	0.001	Unknown function
	ydjY	−7.3	0.001	Unknown function
	ydjZ	−3.5	0.001	Unknown function
	yecH	−3.8	0.000	Unknown function
Fur	*ftn*	−4.6	0.028	Ferritin; negatively regulated by *ryhB* RNA

[a] Genes showing a greater-than-twofold decrease in the mRNA level and having a *P* value of <0.05 are included. References for the proposed regulation are indicated in the text. The annotations are from the source described in Table 1, footnote *a*.
[b] Regulators shown are those proposed to be responsible for the major regulation of the genes.

the most intriguing of these genes include *copA*, which encodes a copper-transporting ATPase (57, 64), exhibited 4.9-fold induction by NO, and was also induced twofold by GSNO in our previous study (22). Also highly up-regulated were the xanthosine transporter gene *xapB* and *phoR*, which encodes the signal sensor of the *pho* regulon two-component system.

TABLE 3. Genes up-regulated by NO aerobically[a]

Regulon	Gene	Fold regulation	*P* value	Gene product or function
NorR	*norV*	50	8.4×10^{-06}	Flavorubredoxin
	norW	7.5	2.6×10^{-05}	Flavorubredoxin oxidoreductase
NsrR	*hmp*	3.2	7.4×10^{-04}	Flavohemoglobin
	ytfE	2.2	0.007	Unknown function
IscR	*iscR*	2.3	0.022	Regulator of *isc* operon
	iscU	2.1	0.017	Scaffold for Fe-S assembly
Unknown	*bglF*	3.2	0.046	β-Glucoside phosphotransferase
	cydA	2.4	0.016	Cytochrome *d* terminal oxidase subunit I
	cydB	2.5	0.010	Cytochrome *d* terminal oxidase subunit II
	ybcH	2.7	0.035	Unknown function
	ydiT	3.1	0.049	Unknown function

[a] Genes showing a greater-than-twofold increase in the mRNA level and having a *P* value of <0.05 are included. The regulators proposed to be responsible for the NO-mediated induction of the genes are indicated by bold type. References for the proposed regulation are indicated in the text. The annotations are from the source described in Table 1, footnote *a*.

The transcript level of the *soxS* gene, encoding the superoxide-sensitive response regulator SoxS, increased fivefold anaerobically in response to NO (Table 1). Transcription of *soxS* is activated upon nitrosylation of the [2Fe-2S] cluster of its regulator protein, SoxR (19). However, none of the genes known to be activated by SoxS were up-regulated under these conditions. This may have been due to the short time of exposure before sampling (5 min), but similar results were reported for previous anaerobic batch culture studies, despite the greater exposure time that was used (20 min) (38). It seems probable that the levels of SoxS achieved are not adequate to reach a threshold level for induction of the SoxS-activated genes. The SoxRS system provides protection against NO-mediated macrophage killing (54).

Of the 61 genes up-regulated anaerobically, 9 are implicated in iron homeostasis (Table 1) (44). The addition of NO therefore appears to mimic the effects of iron limitation, inducing genes involved in the import of both ferric and ferrous iron, as well as the ExbD-TonB-TonD complex, which transduces energy to the outer membrane for iron import (2). In addition, the *bfd* gene is induced, which may play a role in releasing the iron stored in bacterioferritin. Whether there is any physiological advantage to this response is not clear as, paradoxically, a *fur* mutant strain in which all genes of the regulon are constitutively expressed shows increased sensitivity to nitrosative stress (1, 50). The *nrdH* transcript was also induced in this study under anaerobic conditions, presumably due to the relief

of Fur-mediated repression (44). The *ftn* gene encoding ferritin, which is indirectly and positively activated by Fur, was down-regulated upon anaerobic NO exposure (Table 2).

In marked contrast to our previous studies with GSNO (22), *met* genes were not up-regulated either anaerobically or aerobically, a result that we attribute to a lack of Hcy nitrosation (see below).

Aerobic exposure to NO. For comparison of the transcriptional profiles under aerobic and anoxic conditions, identical chemostat experiments were performed, except that for aerobic growth the glycerol-limited medium lacked fumarate and was sparged with air; the dissolved oxygen tension was maintained automatically at 40% of air saturation by using an oxygen electrode immersed in the culture with feedback control of the stirrer speed as described previously (22). Cells that were in the aerobic steady state, in which the cell density was equal to the cell density attained anaerobically, and were exposed to a mixture of NOC-5 and NOC-7 exhibited significant changes in the transcriptional levels of only 11 genes after 5 min of treatment (Table 3); this number is strikingly less than the number observed under anaerobic conditions. The relatively small number of genes showing changes in expression under aerobic conditions may have been due in small part to the slightly lower NO levels in aerated media but more likely was due to the instability under aerobic conditions of regulators such as Fnr and other Fe-S-containing transcriptional factors that control many of the genes observed (see Discussion).

For the genes that were significantly up-regulated, the transcripts that were increased the most were again the transcripts of *norVW* and *hmp* (Table 3), encoding the two well-studied detoxifying mechanisms in *E. coli*. The aerobic induction of *norVW* appears to be paradoxical as there is no biochemical evidence for an aerobic activity of the NorV protein; however, induction of this protein aerobically by GSNO and NO has been reported previously (22, 35, 50). Under both anaerobic and aerobic conditions, the extent of up-regulation of the major NO-detoxifying proteins (Hmp and NorVW) was markedly greater with 10 μM NO (this study) than with GSNO, even at a concentration of 200 μM, presumably reflecting the preeminent role of NO as an antimicrobial agent in macrophages and other environments and the reactivity of Fnr, SoxR, Fur, and perhaps NsrR with NO itself. Figure 2 shows the anaerobic and aerobic profiles observed both for GSNO stress (22) and in the present study with NO. Common to the two data sets is up-regulation of genes of the NorR and NsrR regulons implicated in NO detoxification, together with *copA*, *yhgG*, and *nrdH*. In addition, the NO stimulon includes large numbers of genes in the Fnr, Fur, and IscR regulons, as well as *soxS*. In contrast, the GSNO stimulon is characterized by six *met* genes, *mdtC*, and *yhaONM*, in which only the presence of the *met* genes can be rationalized with our present knowledge.

E. coli possesses two alternative terminal oxidases (cytochromes *bd* and *bo*′), and the respiration supported by each oxidase is sensitive to inhibition by NO (73). The preferential induction (Table 3) of the genes encoding only the cytochrome *bd* terminal oxidase subunits, *cydAB*, is therefore surprising, but it may be related to the complements of redox centers in the oxidases (see Discussion). For the transcripts that were down-regulated in response to NO, no large changes were observed. The only significant change based on the criteria used in this study

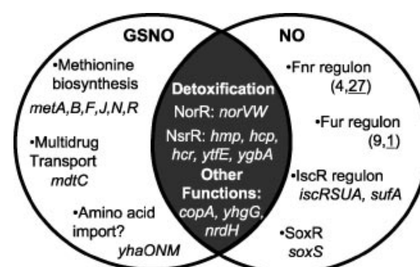

FIG. 2. Simplified comparative overview of GSNO- and NO-mediated responses. Transcript levels altered in response to NO or GSNO or both are shown in the form of a Venn diagram. Numbers in parentheses are numbers of genes that are up-regulated (not underlined) or down-regulated (underlined).

(>2-fold change; $P < 0.05$) was the change in *ygeR*, which encodes a putative lipoprotein belonging to the M37 family.

Real-time PCR confirmed increases in several transcript levels. Quantitative real-time PCR was used to confirm the up-regulation of selected genes in the microarray data set. Several genes up-regulated in this study have been shown repeatedly to be regulated by NO. These genes were not reexamined here and include *hmp* and *soxS*, both of which are up-regulated both aerobically and anaerobically by NO in solution (55, 61). In the case of *norV*, NO is a powerful inducer; the maximal effect is seen anoxically (25, 35), but a significant effect is also observed aerobically (24). Therefore, these genes were not reexamined by real-time PCR in this study. For anaerobic cultures, up-regulation by NO of *ndh*, *hcp*, *hcr*, and *ytfE* was confirmed by the following increases (the values obtained in the anaerobic arrays are indicated in parentheses): 78-fold (5.1-fold), 140-fold (8.5-fold), 87-fold (5.9-fold), and 130-fold (20-fold), respectively. Aerobically, induction of *cydA*, *iscR*, *iscU*, and *ytfE* was confirmed by the following increases (the values obtained in the aerobic arrays are indicated in parentheses): 2.5-fold (2.4-fold), 2.4-fold (2.3-fold), 9.1-fold (2.1-fold), and 2.5-fold (2.2-fold), respectively. Although the changes obtained by quantitative real-time PCR exceeded those measured in the arrays, these data validate the microarray approach for the identification of genes up-regulated by NO (see Discussion).

NO does not elicit up-regulation of genes for the methionine biosynthetic pathway. In our previous studies (22) we demonstrated that the methionine requirement of GSNO-stressed cells was increased, which was attributed to the nitrosation of homocysteine and withdrawal of this compound as a key intermediate in the biosynthetic pathway (45). Addition of exogenous methionine was shown to protect the viability of cultures exposed to GSNO. Figure 2 shows that *met* genes were among the genes that were most highly up-regulated by GSNO, but they were not observed in any array experiment involving cells exposed to the NOC compounds. To corroborate the discrete effects of GSNO and NO, the protective effect of methionine from NO-mediated killing was investigated. While exogenous methionine protects cells from the lethal effects of GSNO, it offers no protection against NO per se (Fig. 3). We considered the possibility that addition of methionine to the medium might act exogenously by reaction with GSNO. Therefore, we used a *metN* mutant strain, which can import only L-methio-

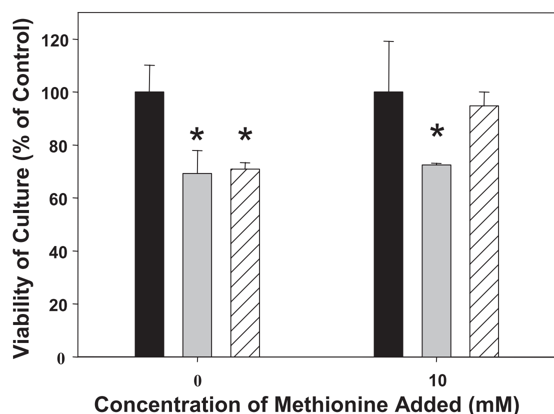

FIG. 3. Exogenous methionine protects *E. coli* from the effects of GSNO but not from the effects of NO. Cultures were grown aerobically to the mid-log phase in defined medium and exposed to 2 mM GSNO for 45 min (striped bars) or to 200 μM NO for 5 min (shaded bars) before viable counts were determined. Experiments were repeated in the presence of 10 mM methionine. An asterisk indicates a significant difference from the unexposed control (solid bars) (P value $<$ 0.05; $n = 3$).

nine (46), and showed that D-methionine offered no protection against GSNO, while a mixture of D-methionine and L-methionine did protect against GSNO, indicating that import of methionine into the cell is required for the protective effects of this compound (data not shown). Thus, depletion of intracellular methionine pools via nitrosation of homocysteine (22, 45) is not a major consequence of exposure to NO per se.

Role of NorR. As NorVW is induced aerobically (22, 25, 35, 50) despite an apparent lack of activity under such conditions (23), it seems possible that there are other genes induced by NorR that play a role in the response to NO. Attempts to identify other members of the NorR regulon by promoter sequence searching for consensus binding sites have not identified any candidates (77). We therefore repeated the previous anaerobic chemostat experiment using a *norR* null mutant strain. The RNA harvested from the cultures exposed to NO was used to produce cDNA which was hybridized in competition with exposed wild-type sample RNA, providing a direct transcriptional profile comparison between wild-type and *norR* mutant strains exposed to NO.

The transcripts whose abundance was reduced most in the mutant were the *norV* (54-fold) and *norW* (32-fold) transcripts, as expected (not shown). However, two genes having unknown functions, *yjiH* and *yjiG*, were also down-regulated relative to the wild-type strain, albeit to a much lesser extent (4.5- and 3.5-fold, respectively). We also observed lower levels of the *rpoN* transcript, perhaps due to the decreased demand for the σ^{54} subunit, which is usually recruited by NorR to promote transcription of *norVW*.

The gene whose transcript level increased the most (~7-fold) in the mutant was *nrfA*; this gene encodes the cytochrome *c* nitrite reductase NrfA, which may represent a further NO detoxification mechanism (59), particularly under anaerobic conditions in the absence of NorV. We hypothesized that *nrfA* induction may be due to an increase in the level of nitrite formed from the NO that accumulates in the absence of NorVW activity. To test this hypothesis, small anaerobic cultures of wild-type and *norR* strains were grown to the mid-log phase and exposed to the mixture of NOC compounds used in chemostats. Samples of the growth media were taken before exposure and after 5 min of exposure, and the levels of nitrite were determined using an NO analyzer. In supernatants from cultures of the wild-type strain not exposed to the NOC compounds, around 2.5 μM nitrite was detected (not shown). Based on the specified maximum levels of nitrogen impurities present in the AnalaR-grade medium components, and assuming that all of the nitrogen impurities were nitrite (or nitrate that could be reduced to nitrite), the calculated nitrite level might have been as high as 25 μM, fully accounting for the nitrite levels measured. These levels were not increased significantly by addition to wild-type cultures of NOC compounds, suggesting that NO is detoxified by reduction, presumably ultimately to N_2O by NorVW activity. In cultures of the *norR* mutant, detectable nitrite levels were also within the range that could be attributed to contaminating medium levels (approximately 5 μM); however, in cultures treated with NOC compounds, the nitrite levels were significantly elevated. The theoretical concentration of nitrite that accumulated in such cultures, if the NOC compounds quantitatively released NO that was wholly converted to nitrite, was 40 μM; the measured values (approximately 60 μM) included background nitrite levels in the medium. Thus, nitrite was present at much higher levels in the *norR* mutant culture and might have contributed to *nrfA* induction.

Interaction of J774.2 mouse macrophages, the wild-type *E. coli* strain, and *norV* and *norR* mutants. We previously demonstrated that mutation of the flavohemoglobin Hmp decreases survival of *Salmonella enterica* (75) and *E. coli* (T. M. Stevanin, R. C. Read, and R. K. Poole, unpublished data) in human macrophages. To analyze the roles of the *norVW* genes and other genes of the NorR regulon in macrophages, we compared the levels of intracellular survival of the wild-type and *norR* and *norV* mutant strains using the mouse macrophage cell line J774.2. Thus, any uncharacterized members of the NorR regulon, not only *norV*, were included in this analysis. All strains grew similarly, and the levels of binding to macrophages of the wild-type strain and the *norV* and *norR* mutants were found to be equivalent (1.31 ± 0.31, 1.24 ± 0.16, and 1.31 ± 0.17 bacteria per macrophage, respectively; the values are means ± standard deviations) (results not shown). The rates and extents of internalization of the three strains were also indistinguishable, and all strains exhibited optimum internalization (~95%) after 1 h of incubation at 37°C. The gentamicin exclusion assay was used to investigate the relative levels of survival of all strains in J774.2 mouse macrophages that had been stimulated using gamma interferon for 24 h prior to infection. Six independent replicate intracellular survival experiments were performed; the results (data not shown) showed that, compared with the survival ability of the wild-type strain, the survival abilities of the *norV* and *norR* mutants were not impaired.

DISCUSSION

This study was the first direct comparison of the aerobic and anaerobic NO stimulons of *E. coli* under well-defined growth conditions. A mixture of the NO-releasing compounds NOC-5 and NOC-7 elicited markedly different changes in gene expression under aerobic and anaerobic conditions, and the most notable difference involved the far more numerous changes in

gene expression observed under anaerobic conditions. To allow direct comparison with our previous experiments involving GSNO (22), an exposure time of 5 min was chosen. The facile diffusion of NO to intracellular targets, compared with the multistep access of a nitrosating species from GSNO (involving GSNO hydrolysis in the periplasm, followed by transport of *S*-nitrosocysteinylglycine via the dipeptide permease system) (18), suggests that the effects of NO should not be slower than those of GSNO. To maximize the fidelity of comparisons between the current data sets and our previous work (22), standardized procedures were also used for the growth vessel, growth medium, RNA labeling, hybridization, microarray processing, data acquisition, and data normalization, as advocated by Bammler et al. (3). Since more than one-half of the variability in gene expression measurements between laboratories and platforms is attributable to the microarray platform itself (3), differences in expression ratios between the values reported here and the values obtained in other laboratories are not unexpected; however, the MIAME (Minimum Information About a Microarray Experiment)-compliant data reported here are reproducible and provide reliable descriptions of the genes up-regulated in response to NO for comparison with previous data obtained with GSNO.

The major effects of NO that we observed were effects on the global regulator Fur and the more specific NO-sensitive regulators NorR and NsrR, in agreement with previous array-based transcriptional studies of nitrosative stress (38, 50), as well as the predictive studies of Rodionov and colleagues (66). The present data also provide the strongest evidence to date of the importance in vivo of the interaction of the Fnr protein and NO (15), as patterns of gene expression consistent with NO-mediated deactivation of Fnr were clearly observed under anaerobic conditions.

Perhaps most significantly, this work revealed important differences in the responses of *E. coli* to different forms of nitrosative stress. To our knowledge, such a comparative analysis has not been attempted previously; the chemostat is ideally suited to maintaining the constant and controlled growth conditions necessary to reveal subtle changes in physiology and gene expression. Previous directly comparable experiments using the nitrosating agent GSNO (22) led to the induction of genes involved in methionine biosynthesis via nitrosation of homocysteine, a key intermediate in the pathway (78). In striking contrast, no *met* genes were induced by exposure to NO in this study, and methionine added exogenously had no protective effect in NO-stressed cells, showing the specific effects of related but chemically and biologically distinct species. It appears that while the NO-specific response regulators in *E. coli*, NorR and NsrR, respond to both exogenous GSNO and NO, perhaps due to the small amounts of NO released upon cleavage or decomposition of GSNO, the secondary effects of the two stresses are remarkably different.

A further clear distinction between the present data for the NO stimulon and our previous description of the GSNO responses (22) is the involvement of Fur. Fur acts as a transcriptional repressor when it is bound to ferrous ion, sterically hindering RNA polymerase at "iron boxes" in the promoter regions of genes involved in iron uptake (2). When *E. coli* was challenged with GSNO under conditions identical to those used in this work, no evidence for modification of Fur by nitrosative species was obtained (22), despite recognition that Fur is inactivated by NO (17). Reaction of Fur with NO involves 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 (17) and the reconstitution of iron proteins damaged by NO. We assume that NO is reactive with Fur under our conditions (as shown by the gene expression patterns in Table 1) but that GSNO or its transnitrosation products are not reactive with Fur. Interestingly, however, a previous study of the GSNO stimulon of *E. coli* did reveal Fur-regulated genes; in this study quite different growth conditions were used—the cultures in complex medium were exposed to GSNO or acidified nitrite for 5 min. We suggest that the broth medium used in the previous study (50) was a poor source of bioavailable iron and revealed Fur-mediated, GSNO-sensitive repression of genes involved in iron acquisition. In the defined, iron-replete medium used in this study, GSNO did not effectively react with Fur (22), whereas the NO generated here increased Fur repression of gene expression.

The genes whose transcription was altered in response to both GSNO and NO in these studies include *hmp*, *norVW*, *hcp*, *nrdH*, and *copA* (Fig. 2), all of which have also been described in the previous batch culture studies mentioned above. *nrdH* is the first gene of the ribonucleotide reductase operon, but its function in response to nitrosative stress is unclear. The induction of *copA* is particularly interesting, as a requirement for the major cellular copper efflux pump (64) is not easily understood in this context. However, it is interesting that the CopA paralogue in *Neisseria gonorrhoeae* is coregulated with a gene encoding a GSNO reductase, AdhC (40). Both genes are regulated by the MerR-like regulator NmlR, which is a zinc-dependent transcriptional repressor that changes to an activator upon loss of the Zn atom (in a classical MerR-like mechanism) in the face of disulfide stress (40). Strikingly, an *nmlR* mutant strain of *N. gonorrhoeae* is hypersensitive to NO (71). The *copA* gene of *E. coli* is regulated by the related, MerR-like regulator CueR, which acts in a similar manner, using Cu as its metal cofactor. This raises the possibility of a mechanism in which exposure to NO leads to an increase in the level of free copper, due to liberation from Cu-containing proteins, triggering the induction of *copA*.

A comparison of the patterns of gene expression in anaerobic and aerobic conditions, while equal growth rates and biomass densities were maintained in the chemostats, highlighted significant differences. The most notable difference is the far higher number of genes that are up-regulated more than twofold anaerobically than aerobically (compare Table 1 and Table S1 in the supplemental material with Table 3). While the slightly higher steady-state levels of NO observed extracellularly under anaerobic conditions may be a factor, it seems probable that intracellular NO levels or the sensitivity of transcriptional regulatory mechanisms to sense NO is more important. The first possible explanation is that Hmp, whose NO detoxification function requires O_2 (23, 47), is more effective in NO removal than NorVW is anoxically. However, wild-type cells grown anoxically and challenged with NO had NO consumption rates around 47 nmol NO/min/10^8 cells (25), an activity that was lost after mutation of *norVW*, whereas aerobically grown cells also challenged with NO exhibited rates

attributable to Hmp that were only marginally higher (26). Thus, after pretreatment with NO, aerobically and anaerobically grown *E. coli* cells appear to be equally effective in detoxifying NO. The fact that the number of genes found to be up-regulated anaerobically by the same regulator (NsrR) is greater anaerobically (five genes) (Table 1) than aerobically (two genes) (Table 3) might suggest that the NsrR NO sensor is more NO responsive in the absence of O_2. The expression of *hmp* and other NsrR-regulated genes in the absence of RNS under aerobic conditions is similar to the expression of *hmp* and other NsrR-regulated genes in the absence of RNS under anoxic conditions (6). The levels of expression are also similar after RNS treatment, but the aerobic and anaerobic data presented previously (6) are not directly comparable, since aerobic cultures treated with NO were shown alongside anaerobic cultures treated with nitrite. Thus, an O_2-sensitive, NO-sensing metal center, perhaps an Fe-S cluster, as proposed for the NsrR protein of *Bacillus subtilis* (52), might explain the present data. Similarly, the large number of genes down-regulated anaerobically (Table 2) but not aerobically presumably reflects the oxygen sensitivity of Fnr (12).

A further feature of the anaerobic-aerobic comparisons is the significant differences in the amounts of regulation observed. For example, *hmp* is up-regulated by NO only 3.2-fold aerobically (Table 3) but is up-regulated by NO 27-fold anaerobically (Table 1). One explanation might be the action of FNR in anaerobically repressing basal levels of *hmp* (15), as well as *norVW* and *ygbA*. Thus, under these conditions, the changes observed when NO is added reflect not only the aerobic relief from FNR repression but also the positive regulation by NsrR. Interestingly, *hmp* is not as highly up-regulated by NO, either anaerobically or aerobically, as *norVW* is. Nevertheless, the preeminent role of *hmp* in the resistance of *Salmonella* to nitrosative stress in macrophages is clear (75), and we must assume that the expression levels in vivo are not mimicked in highly aerated laboratory cultures.

Of the 61 genes up-regulated aerobically, 8 were also induced anaerobically; these genes were *norVW*, *hmp*, *cydAB*, *iscR*, *iscU*, and *ytfE*. The roles of the two detoxifying systems (*hmp* and *norVW*) and the iron-sulfur cluster synthesis system (*iscR* and *iscU*) seem clear, but the roles of *cydAB* and *ytfE* are less clear. The induction of cytochrome *bd* but not cytochrome *bo′* is interesting given that both oxidases are sensitive to NO (73). Recently, however, Mason et al. (43) have shown that NO inhibition of respiration involves both competitive (at the heme) and noncompetitive (at the copper) binding to cytochrome *c* oxidase. Significantly, cytochrome *bd* contains no copper (36), whereas the cytochrome *bo′* quinol oxidase possesses a heme-Cu_B active site like that of cytochrome *c* oxidase, and the copper atom is capable of binding NO (9). Cytochrome *bd* is widely considered to confer tolerance to a number of apparently unrelated cellular stresses, including metal ions, cyanide, and reductants (62). The anaerobic up-regulation of *cydAB* might be related to the reaction of NO with Fnr, which is involved in complex transcriptional control of the operon (11).

Some evidence for inactivation of Fnr upon NO exposure was obtained in a previous microarray study (38). Batch culture exposure to NO resulted in the down-regulation of Fnr-activated genes (*narG*, *pyrD*, *yjiH*, *aroP*, and *rmuC*) and the up-regulation of FNR-repressed genes (*ndh*, *hmp*, *gpmA*, and *lpdA*). A recent review (72) points out that earlier data do not support regulation by Fnr of *nrfA* in response to *NO*. However, the anaerobic induction in the present work of a large number of Fnr-repressed genes and the down-regulation of a large number of Fnr-activated genes, including the classically Fnr-activated *nrf*, *nir*, *nar*, *frd*, and *dms* genes, is strong evidence that Fnr senses NO under anaerobic conditions.

YtfE has been observed in most microarray studies of nitrosative stress, and Justino and colleagues (38) demonstrated that the growth of a strain lacking the gene encoding YtfE exhibits increased sensitivity to NO. YtfE appears to play some role in the protection or repair of Fe-S clusters during NO stress (37). The *ytfE* gene is up-regulated in concert with other genes of the NsrR regulon in *E. coli* growing in the human urinary tract, an environment that is deduced to be nitrate rich, judging from the induction of genes involved in nitrate respiration (67).

Notable among the genes down-regulated by NO anaerobically were five genes involved in arginine biosynthesis (see Table S2 in the supplemental material). Whether these changes are reflected in changes in intracellular arginine pools after NO treatment is unknown. It is intriguing that nitrite, the stable end product of NO, is an inhibitor of arginase (33). This enzyme is employed by certain pathogens, notably *Helicobacter pylori*, to inhibit NO production from NO synthase (28), which utilizes arginine as a substrate. Taken together, these findings may indicate that changes in *arg* gene expression are implicated in bacterial adaptation to the presence of host cell arginine.

Our analysis of the transcriptional responses of the *norR* mutant strain had two purposes: (i) to identify genes regulated by NorR and (ii) to observe any secondary anaerobic protective mechanisms that might be observed more clearly in the absence of NorVW. The paucity of alterations in gene expression after mutation of *norVW* seems to indicate that compensatory changes in gene expression do not occur and perhaps that Hmp or other pathways for NO detoxification (possibly NrfA) suffice. In the wild-type strain, 4.9-fold down-regulation of *nrfA* was observed upon exposure to NO. However, a comparison of the exposed wild-type and mutant RNA profiles revealed a ~7-fold-higher level of the *nrfA* transcript in the *norR* mutant; the increase may have been due to the nitrite that accumulated in cultures of the *norR* mutant. We speculate that nitrite might form under these anoxic conditions via metal ion-dependent oxidation of NO to NO^+, which during reaction with H_2O yields NO_2^- (34, 76). NorVW presumably detoxifies NO to nitrous oxide so rapidly that it prevents the formation of nitrite in the wild type, but in the mutant formation of nitrite induces *nrfA* transcription (8, 16).

The higher levels of the *yjiH* and *yjiG* transcripts may well be explained by the recent findings of Giel et al. (27), who discovered that the *yjiH-yjiG-iadA-yjiE* operon may be under the transcriptional control of IscR, which is thought to repress the transcription of the genes in its Fe-S-bound state. Up-regulation of all four operon members was observed in the *norR* mutant strain when it was compared to the wild-type strain in response to NO (4.5-, 3.5-, 2.1-, and 1.7-fold up-regulation, respectively). This indicates that although the IscR regulon is induced in the wild type in response to NO, it is induced to a

greater extent in the absence of NorR. This is presumably due to the lower levels of anaerobic NO detoxification in the mutant, which leads to greater exposure of the IscR protein to NO or else leads to greater loss of Fe-S clusters generally, which is sensed by IscR.

Only two NO defense mechanisms (Hmp and NorVW) have consistently been shown to be significant in enterobacteria. Indeed, a comparison of the mRNA microarray profiles of chemostat-grown cultures of *hmp* and *norR* mutant strains exposed to NO with the profile of NO-exposed wild-type cells revealed no obvious candidates for further defense mechanisms (S. Pullan and R. K. Poole, unpublished). However, genes with unknown functions were up-regulated; in the *hmp* null background, the levels of *yedN* and *yddK* transcripts were increased compared to the wild-type levels, while in the *norR* null background the levels of the *yciQ* and *yfkB* transcripts were increased.

Compared with the wild-type strain, *norV* and *norR* mutants were not impaired in terms of the ability to survive within J774.2 cells, indicating that the flavorubredoxin NorV and the transcriptional regulator NorR are not required for NO detoxification within mouse macrophages. This is consistent with the recent findings of Bang et al. (4), who demonstrated that mutants lacking *hmp* were attenuated in a mouse virulence model, while mutants lacking *norV* were not attenuated. Our data also demonstrate that no other NorR-regulated gene contributes significantly to intracellular survival. This work and recent studies with other bacterial pathogens, including *Yersinia pestis* (70) and *Staphylococcus aureus* (65), reaffirm the key role of Hmp as the major NO detoxification and defense mechanism in bacteria.

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