Prominent roles of the NorR and Fur regulators in the *Escherichia coli* transcriptional response to reactive nitrogen species

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We examined the genomewide transcriptional responses of *Escherichia coli* treated with nitrosylated glutathione or the nitric oxide (NO)-generator acidified sodium nitrite ($NaNO_2$) during aerobic growth. These assays showed that NorR, a homolog of NO-responsive transcription factors in *Ralstonia eutrophus*, and Fur, the global repressor of ferric ion uptake, are major regulators of the response to reactive nitrogen species. In contrast, SoxR and OxyR, regulators of the *E. coli* defenses against superoxide-generating compounds and hydrogen peroxide, respectively, have minor roles. Moreover, additional regulators of the *E. coli* response to reactive nitrogen species remain to be identified because several of the induced genes were regulated normally in *norR*, *fur*, *soxRS*, and *oxyR* mutant strains. We propose that the *E. coli* transcriptional response to reactive nitrogen species is a composite response mediated by the modification of multiple transcription factors containing iron or redox-active cysteines, some specifically designed to sense NO and its derivatives and others that are collaterally activated by the reactive nitrogen species.

NO gas is generated during the combustion of nitrogenous compounds and the biological decay of organic matter, and as a byproduct of denitrification reactions carried out by microbes. NO is also produced directly by NO synthases in animals, plants, and bacteria (reviewed in ref. 1). At low concentrations, NO functions as a signaling molecule, whereas at high concentrations, NO can be a general poison (reviewed in refs. 2–4). At elevated levels, the soluble gas reacts with heme centers and labile 4Fe–4S clusters and thus inhibits terminal oxidases and aerobic respiration (reviewed in refs. 5 and 6). NO also can react with superoxide (O_2-·) to generate peroxynitrite (OONO-), which can react with other molecules and decompose to the highly reactive hydroxyl (·OH) and nitrogen dioxide (NO_2·) radicals. In addition, NO-derived species can react with small molecule and protein thiols, thereby disrupting protein activity as well as depleting the reduced glutathione pool and generating nitrosylated glutathione (GSNO), which in turn can modify proteins.

Because of the prominent role of reactive nitrogen species in macrophage killing of bacteria, several activities that scavenge or detoxify NO, OONO-, or GSNO or repair damage caused by these compounds have been characterized in *Salmonella enterica* serovar Typhimurium and *Escherichia coli*. For example, it has been proposed that homocysteine reacts with nitrosylating compounds and acts as an NO antagonist because *S. enterica* strains carrying insertions in *metL* encoding aspartokinase II–homoserine dehydrogenase II are hypersensitive to *S*-nitrosothiol (7). The most prominent detoxifying entities that have been identified are the NO dioxygenase, NO denitrosylase, and NO reductase activities associated with the *hmpA*-encoded flavohemoglobin and the NO reductase activity associated with the *norVW*-encoded flavorubedoxin and flavorubedoxin reductase (8–16). Other detoxifying activities include NO reductase activity contributed by *nfrA*-encoded periplasmic cytochrome *c* nitrite reductase (17), and GSNO reductase activity associated with the *adhC*-encoded alcohol-acetaldehyde dehydrogenase (18). The AhpC subunit of the alkylhydroperoxide reductase also has been shown to catalyze the conversion of OONO- to NO_2 (19). Finally, the peptide methionine sulfoxide reductase encoded by *msrA* has been proposed to repair intracellular methionine residues damaged by OONO- (20).

A number of *E. coli* and *S. enterica* transcriptional regulators have been implicated in modulating gene expression in response to reactive nitrogen species. The *E. coli norR* (*ygaA*) gene encodes a homolog of the NO-modulated NorR1 and NorR2 regulators of *Ralstonia eutrophus* (21), and is required for the induction of *norV–lacZ* fusions (15, 16, 22). In *E. coli*, the NO-induction of the *hmpA* gene was reported to be dependent on MetR, a transcriptional regulator of the methionine biosynthetic pathway, under aerobic conditions (23) and on FNR, an oxygen-responsive regulator, under anaerobic conditions (24). In *S. enterica*, the aerobic induction of *hmpA* was reported to depend on Fur, the ferric iron repressor (25).

Reactive nitrogen and reactive oxygen species share chemical properties, and two regulators of the *E. coli* responses to oxidative stress can be modified by reactive nitrogen species. The iron–sulfur cluster-containing SoxR protein initially was identified as the sensor of the stress generated by O_2-·-generating compounds, but subsequent studies revealed that SoxR also can be activated by NO (26). Similarly, the OxyR transcription factor, discovered as the primary regulator of the response to hydrogen peroxide (H_2O_2), later was reported to activate target genes on exposure to S-nitrosylated cysteine (27). We have shown that OxyR activation by H_2O_2 is caused by disulfide bond formation between residues Cys-199 and Cys-208 (28, 29). However, others have reported that Cys-199 is modified to *S*-NO, *S*-OH, and *S*-SG and have proposed that these modifications of OxyR elicit different responses at increasing concentrations of GSNO (30). The relative importance the OxyR and SoxR regulons in the response to reactive nitrogen species has not been evaluated on a genomewide level.

To define the global *E. coli* transcriptional response to reactive nitrogen species, we carried out microarray analyses. The recently developed transcriptional profiling technologies allow unbiased, genomewide surveys of an organism's response and have been very successful in characterizing *E. coli* gene expression changes under many different growth and stress conditions (reviewed in ref. 31). We also set out to evaluate the relative contributions of each of the implicated transcriptional regulators by examining the global gene expression patterns as well as the expression of specific genes in mutant strain backgrounds.

Materials and Methods

Strains. The sequences of primers used in this study are given in the supporting information, which is published on the PNAS web

Abbreviation: GSNO, nitrosylated glutathione.

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site. *E. coli* MG1655 was the parental strain used in this study. The MG1655 Δ*oxyR*::*kan* derivative (GSO77) was described (32). The Δ*fur*::*kan* (33) and Δ*soxRS–zjc2205 zjc2204*::Tn*10kan* (34) mutant alleles were moved into MG1655 by P1 transduction to generate GSO82 and GSO83, respectively. The *npt* (kanamycin gene) replacements of *norR* and *metR* were constructed as described (35). PCR fragments obtained by amplifying the kanamycin resistance cassette from pKD4 (36) with primers carrying sequences flanking *norR* and *metR* were purified by using the Gel Extraction kit (Qiagen, Valencia, CA) and transformed into DY330 (35). Recombinants were selected on the basis of kanamycin resistance and confirmed by colony PCR (using one primer homologous to the 5′ flanking sequence of the targeted gene and a second primer homologous to the inserted kanamycin resistance gene). The Δ*norR*::*kan* and Δ*metR*::*kan* mutant alleles then were moved into MG1655 by P1 transduction to generate GSO86 and GSO87, respectively.

Chemicals. Analytical grade H_2O_2, $NaNO_2$, and sodium nitroprusside were purchased from Sigma. Diethylenetriamine (DETA) NONOate was purchased from Cayman Chemical (Ann Arbor, MI). $NaNO_2$ was acidified before use. GSNO was prepared as described (37).

RNA Isolation. For the aerobic samples, cultures were grown at 37°C with shaking in LB-rich medium adjusted to pH 6 with HCl. Overnight cultures were diluted 1:100 and grown to $OD_{600} = 0.1$, 0.4, or 1.0 under the same conditions. For the anaerobic samples, cultures were grown at 37°C with stirring in an anaerobic glove box (Coy Laboratory Products, Grass Lake, MI) in LB medium buffered at pH 6 with 100 mM Mes and supplemented with 0.3% glucose. Overnight cultures were diluted 1:100 and grown to $OD_{600} = 0.4$ under the same conditions. Aliquots (15 or 30 ml) of the cultures at the indicated stages of growth were left untreated or exposed to the indicated concentrations of H_2O_2, acidified $NaNO_2$, or GSNO. After the indicated times, total RNA was isolated and purified by using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions.

DNA Microarray Analysis. Fabrication of the *E. coli* DNA microarray and procedures for cDNA labeling, hybridization, and array quantification were carried out as detailed (38, 39). Briefly, each RNA sample was used as a template for two cDNA syntheses, each with separate incorporation of Cy3- and Cy5-labeled nucleotides. The reciprocal pairs of differentially labeled, untreated, and treated cDNA samples were hybridized to glass slides printed in duplicate with 4,169 *E. coli* ORFs. Thus, the expression for each gene was measured four times for each RNA sample. The numbers were averaged, and the treated sample/untreated sample ratio was calculated for each ORF. The complete microarray data sets are available in the supporting information.

Primer Extension and Northern Blot Assays. RNA samples were subjected to primer extension analysis as described (40), using avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, FL) and primers specific to the indicated genes. The extension products were separated on 8% sequencing gels. Northern blot analyses were carried out as described (41).

Results and Discussion

Global Responses to GSNO and $NaNO_2$. We examined the transcriptional profile of *E. coli* cells exposed to two commonly used generators of reactive nitrogen species: GSNO, a representative nitrosothiol, and acidified $NaNO_2$, a NO producer. Wild-type cells (MG1655) grown aerobically to exponential phase in LB-rich medium adjusted to pH 6 were left untreated or exposed to GSNO or $NaNO_2$ each at 0.1 and 1 mM. After 5 min, total RNA was isolated from the untreated and treated cultures. Neither concentration resulted in a loss of viability during the time frame of the experiments, and primer extension assays confirmed that the expression of *hmpA*, a gene previously shown to be induced by exposure to reactive nitrogen species (23–25, 42), was elevated in the samples from the GSNO- or $NaNO_2$-treated cells. Fluorescently labeled cDNAs derived from these total RNA samples were used to probe glass slide arrays printed with 4,169 *E. coli* ORFs.

For all four conditions, RNA was isolated from two independent sets of untreated and treated cultures. RNA also was isolated from a separate set of wild-type, Δ*norR*, Δ*fur*, and Δ*oxyR* cells treated with 1 mM GSNO. The complete data sets for all arrays are given in the supporting information. For each of the treatments, only limited numbers of genes were induced ≥5-fold (36, 44, 47, and 49 genes for 0.1 and 1 mM GSNO and 0.1 and 1 mM $NaNO_2$, respectively). The 34 genes whose transcripts showed ≥5-fold accumulation in the samples treated with 1 mM GSNO and the samples treated with 1 mM $NaNO_2$ are listed in Table 1. A similarly small set of 48 genes was found to be induced in a recent microarray analysis of *Mycobacterium tuberculosis* exposed to nontoxic concentrations of NO (43). Only 5 and 7 genes were repressed ≥2-fold by 0.1 mM concentrations of GSNO and $NaNO_2$, respectively, and several hundred genes were repressed ≥2-fold by 1 mM concentrations.

Known Defense Activities Induced by GSNO and $NaNO_2$. As described above, a number of proteins have been implicated in protecting cells against reactive nitrogen species. Among the corresponding genes, three (*norV*, *norW*, and *hmpA*) were induced strongly (>30-fold) by both concentrations of GSNO and $NaNO_2$ (Table 1). These results are consistent with the conclusion (15) that the NO dioxygenase and NO reductase activities encoded by *norV*, *norW*, and *hmpA* play critical roles in protecting *E. coli* cells against the toxic effects of NO. The transcript levels for other postulated defense genes such as *metL*, *nrfA*, *adhC*, *ahpC*, and *msrA* showed no or only minimal (<2-fold) induction under our culture conditions. It may be that basal levels of some of the corresponding proteins suffice to protect against the reactive nitrogen species. Alternatively, these defense activities may not be critical during aerobic growth in rich medium and instead are induced and protective under other culture conditions. Several genes of unknown function (such as *ybaE* and *ychH*) were induced ≥5-fold by both GSNO and $NaNO_2$ (Table 1), suggesting that these genes may be important to the *E. coli* response to reactive nitrogen species. Further characterization of induced genes with unidentified function will likely reveal additional activities that protect against damage caused by reactive nitrogen species.

Major Contributions of the NorR and Fur Transcription Factors. The expression of the two most strongly regulated genes, *norV* and *norW*, has been shown to be regulated by NorR by assays of *lacZ* operon fusions (15, 16, 22). NorR regulation of these two genes was confirmed by microarray analysis of total RNA isolated from Δ*norR* mutant with and without exposure to 1 mM GSNO; induction of *norV* and *norW* was abolished in the Δ*norR* mutant strain. The induction of other genes, such as *ybiJ*, was also eliminated, suggesting that these genes may be additional NorR targets. Primer extension assays of *norV* mRNA levels in strains carrying mutations in all of the transcription factors implicated in the response to reactive nitrogen species under aerobic conditions showed that only the NorR regulator is required for the GSNO and $NaNO_2$ induction of *norV* (Fig. 1).

Four of the genes induced ≥5-fold by 1 mM GSNO and $NaNO_2$ (*fes*, *nrdH*, *sufA*, and *fhuF*) and 13 of the genes induced ≥5-fold by the 0.1 mM concentrations are members of the Fur regulon (44). In microarrays of Δ*fur* mutants treated with 1 mM

Table 1. Genes showing ≥5-fold induction by both 1 mM GSNO and 1 mM $NaNO_2$

Gene	b no.	GSNO		$NaNO_2$		Function
		0.1 mM	1 mM	0.1 mM	1 mM	
norV	b2710	173	85	75	78	Flavorubredoxin
norW	b2711	338	98	169	52	Flavorubredoxin reductase
hmpA	b2552	70	37	53	31	Flavohemoglobin
ytfE	b4209	38	29	21	50	Homology to NO-dependent regulators
soxS	b4062	2	27	2	36	Regulator of *soxRS* regulon
yftA	b2597	3	19	2	22	Homology to σ^{54} modulators
fes	b0585	11	15	14	17	Enterochelin esterase
yhjX	b3547	2	14	3	9	Homology to oxalate/formate antiporters
nrdH	b2673	17	14	18	10	Glutaredoxin-like protein
nrdI	b2674	18	8	19	5	Probably involved in ribonucleotide reductase function
ybaE	b0445	1	14	1	9	Putative ABC-type transporter
ychH	b1205	1	11	1	9	Putative membrane protein
hofF	b3327	6	11	7	11	Putative general secretory pathway protein
ygbA	b2732	5	10	4	8	Conserved hypothetical protein
tdcB	b3117	2	10	2	7	Threonine dehydratase
uspA	b1895	1	9	1	6	Universal stress protein A
hycI	b2717	15	8	14	6	Hydrogenase 3 maturation protease
ybhG	b0795	2	8	2	7	Homology to HlyD-family secretion proteins
sufA	b1684	6	7	7	7	Homology to iron-binding protein IscA
yadG	b0127	7	7	6	10	Putative ABC-type transporter
fhuF	b4367	9	7	9	10	Ferric iron reductase protein
yqeB	b2875	1	6	1	5	Homology to xanthine and CO dehydrogenases maturation factors
yhaK	b3106	1	6	1	25	Homology to pirin-related proteins
yjjM	b4357	1	6	1	9	Homology to transcriptional regulators
yfiD	b2579	1	6	1	6	Homology to formate acetyltransferases
ybaR	b0484	2	6	4	7	Homology to copper-transporting P-type ATPase
asnA	b3744	4	6	4	5	Asparagine synthetase A
deoC	b4381	1	6	1	5	Deoxyribose-phosphate aldolase
ilvC	b3774	2	6	2	8	Ketol-acid reductoisomerase
ybiJ	b0802	2	6	2	44	Conserved hypothetical protein
ascF	b2715	2	6	1	6	Phosphotransferase system (PTS) system enzyme IIABC
ycgT	b1200	2	5	1	5	Homology to dihydroxyacetone kinase
adhE	b1241	2	5	2	6	Aldehyde-alcohol dehydrogenase
ycfR	b1112	2	5	2	17	Conserved hypothetical protein

Columns show average of induction ratios for two independent RNA samples isolated from MG1655 cells left untreated or exposed to 0.1 or 1 mM concentrations of GSNO or $NaNO_2$ for 5 min. For all induction ratios ≥2, the *P* values are <0.05. The *norVW* and *nrdHI* genes, which are contained within operons, are grouped together. For most genes within operons, all genes show induction, though the induction ratios decrease for the downstream genes. Descriptions of function are taken from http://genolist.pasteur.fr/Colibri and/or www.ncbi.nlm.nih.gov/BLAST.

GSNO, the induction ratios of the Fur-repressed genes were all reduced to <2-fold, indicating that GSNO induction of these genes occurs via the Fur regulator. The primer extension assays also showed that the mRNA levels for *fes* as well as *ydiE*, another known Fur target gene, in untreated Δ*fur* mutants were similar to the levels in wild-type cells treated with 1 mM H_2O_2, GSNO, or $NaNO_2$ (Fig. 1). These results suggest that elevated expression of these genes in response to H_2O_2, GSNO, or $NaNO_2$ is due to relief from Fur repression.

To further assess the roles of the NorR and Fur transcription factors in protecting cells against deleterious effects of reactive nitrogen species, we also examined the growth of wild-type and mutant strains treated with 3 mM GSNO or 2 mM $NaNO_2$ (shown in supporting information). The Δ*norR* mutants recovered quickly from the exposure to either GSNO or $NaNO_2$ and did not show a substantial growth delay compared to the parental strain. We suggest this lack of sensitivity may be due to some redundancy between the *norVW*-encoded NO reductase and other enzymes such as the *hmpA*-encoded NO reductase. The Δ*fur* mutant exhibited severe growth delays on both treatments. This finding that Δ*fur* mutants, in which Fur target genes are constitutively derepressed, were hypersensitive to GSNO and $NaNO_2$ is consistent with a prominent Fur role in the *E. coli* response to reactive nitrogen species, but seems contradictory to the observation that Fur target genes are derepressed on GSNO treatment. However, Fur is an extremely abundant protein of >10,000 molecules per cell (45) and may perform functions other than its role as a transcriptional repressor. In addition, the benefits of temporary induction of Fur target genes may not be observed on constitutive derepression of these genes.

Minor Contributions of the SoxR and OxyR Transcription Factors. The expression of *soxS*, whose transcription is controlled directly by SoxR was induced ≥5-fold by both GSNO and $NaNO_2$. However, with the exceptions of *fpr* (induced 3.5- and 3.0-fold by GSNO and $NaNO_2$, respectively) and *sodA* (induced 2.7- and 2.8-fold), none of the other known members of the SoxRS regulon were induced >2-fold. The induction of *sodA* is through SoxRS because the primer extension assays in Fig. 1 showed that *sodA* induction by H_2O_2, GSNO, or $NaNO_2$ was reduced in the Δ*soxRS* mutant strain, consistent with previous findings (26).

The OxyR transcription factor has been described as a sensor of nitrosative stress in *E. coli* (27, 30). However, the only OxyR target genes, *sufA* and *fhuF*, showing ≥5-fold induction by GSNO or $NaNO_2$, are known to be regulated by both Fur and OxyR. Although one other target gene, *grxA*, was induced 4-fold,

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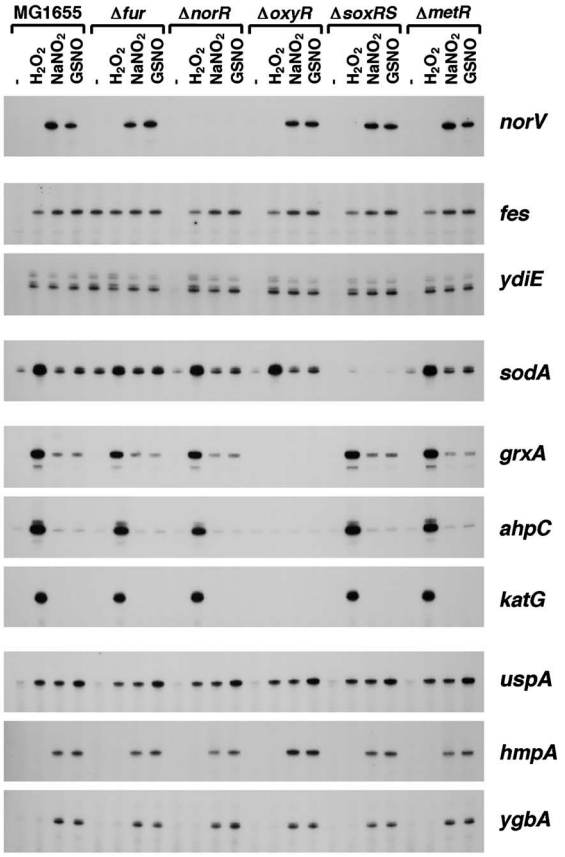

Fig. 1. Primer extension assays of *norV*, *fes*, *ydiE*, *grxA*, *ahpC*, *katG*, *sodA*, *uspA*, *hmpA*, and *ygbA* expression in wild-type and isogenic regulatory mutants. The parent MG1655 and Δ*fur*, Δ*norR*, Δ*oxyR*, Δ*soxRS*, and Δ*metR* mutant strains were grown to $OD_{600} = 0.4–0.6$ in LB medium, pH 6. Cultures were split, and aliquots were left untreated or treated with 1 mM H_2O_2, $NaNO_2$, or GSNO. After 5 min, total RNA was isolated from each untreated and treated culture. All assays were carried out by using 5 μg of the same RNA preparation.

no other known target was elevated >2-fold. To further explore the OxyR role in the responses to GSNO or $NaNO_2$ and to compare these to the OxyR-mediated response to H_2O_2, we also assayed the expression of three target genes by primer extension (Fig. 1). All three genes were strongly induced by H_2O_2, but showed differences in induction by GSNO and $NaNO_2$; *grxA* was induced moderately by both compounds via OxyR and *ahpC* showed slight induction, whereas there was no detectable induction of *katG*. The *katG* gene was shown to be activated by nitrosylated OxyR *in vitro* (30), and a plasmid-borne *katG–lacZ* fusion was reported to be induced by 1 mM *S*-nitrosocysteine *in vivo* (27). However, in our measures of *katG* mRNA levels produced from the normal location in a wild-type strain, we do not detect any induction by reactive nitrogen species under any of the conditions that we have tried, including exposure to 0.1, 1, and 5 mM GSNO, $NaNO_2$, sodium nitrosoprusside, or diethylenetriamine (DETA) NONOate over a 90-min time course (data not shown). Given that we see strongly elevated mRNA levels for other genes previously reported to be NO-regulated in the identical total RNA sample, the lack of *katG* induction cannot be attributed to a use of inactive NO generators. The Δ*soxRS* and Δ*oxyR* mutants also both showed wild-type sensitivity to 3 mM GSNO and 2 mM $NaNO_2$.

These results indicate that SoxR and OxyR do not serve as primary regulators of the *E. coli* response to reactive nitrogen species during aerobic growth. Although the redox-active Cys-199 residue in OxyR probably can undergo modification to *S*-NO, this form of the protein is at best capable of limited activation of a subset of promoters. Thus, we propose that the *E. coli* response to reactive nitrogen species is brought about by the modification of some transcription factors, such as NorR, that control the expression of NO detoxifying activities and are dedicated to sensing reactive nitrogen species, and other redox-active transcription factors such as Fur, SoxR, and OxyR, where the reaction with reactive nitrogen species is incidental. In this context, the *E. coli* NorR protein should be favored as a model for examining effects of reactive nitrogen species on transcription factor activity.

Additional Transcriptional Regulators of the Responses to GSNO and $NaNO_2$. The microarray analysis revealed that the induction of several of the genes was unaffected in the Δ*fur*, Δ*norR*, and Δ*oxyR* mutant strains. We further examined expression of three of these genes by primer extension assays (Fig. 1). These experiments showed that *uspA*, *hmpA*, and *ygbA* genes had wild-type induction by GSNO and $NaNO_2$ in the Δ*fur*, Δ*norR*, Δ*oxyR*, Δ*soxRS*, and Δ*metR* backgrounds. Thus, additional regulators must exist whose activities are modulated, directly or indirectly, by reactive nitrogen species. Our finding that induction of *hmpA* by GSNO under aerobic conditions did not require MetR contradicts a previous study of an *hmpA–lacZ* fusion (23), but possibly can be explained by differences in strain backgrounds or growth conditions.

Oscillatory Expression of *norV*. To determine whether the SoxRS and OxyR regulators play more important roles at different time points or under other conditions, we also assayed the dose dependence (Fig. 2*A*) and time course of induction (Fig. 2*B*) for representative target genes. The expression profiles showed differences that are likely to have consequences for the *E. coli* response to reactive nitrogen species. The *norV*, *ydiE*, *soxS*, *grxA*, and *hmpA* genes all were maximally induced by 1 mM GSNO and $NaNO_2$, whereas *ygbA* was induced equally by 0.1 and 1 mM concentrations. The *norV*, *ydiE*, and *grxA* transcript levels also were slightly induced by 0.1 mM $NaNO_2$, and *hmpA* and *ygbA* even showed induction by 0.01 mM concentrations of both GSNO and $NaNO_2$ indicating exquisite sensitivity to these reactive nitrogen species. The elevated levels of the *ygbA* mRNA persisted for >90 min. In contrast, the expression of *ydiE*, *soxS*, and *grxA*, targets of Fur, SoxRS, and OxyR, respectively, was maximal at 5 min and then returned to pretreatment levels within 15 min. Surprisingly, the two genes encoding known NO-detoxification activities, *norV* and *hmpA*, showed two peaks of induction, one peak at 5 min and another at 90 min.

The apparent oscillation in *norV* mRNA levels was examined in more detail by monitoring *norV* expression for 3 h in cells exposed to 1 mM $NaNO_2$ at different stages of aerobic growth (Fig. 3). Multiple peaks of induction were observed for all three aerobic cultures. Interestingly, however, the period between the peaks varied among the cultures; the period for the early exponential phase culture was ≈45 min, the period for the mid-exponential phase culture was ≈75 min, and the period for the early stationary phase culture was ≈90 min. Simultaneous measurements of the OD_{600} and pH of the cultures showed that all cultures continued to grow and that the pH of the cultures did not change significantly (shown in supporting information), and Northern blots probed for a control RNA confirmed that equal amounts of total RNA were isolated (Fig. 3). For anaerobically grown cells treated with 1 mM $NaNO_2$ at mid-exponential phase, *norV* mRNA levels remained high for the duration of the experiment and no oscillation was observed, suggesting that oxygen is required for the undulating expression pattern.

Various groups have constructed synthetic genetic circuits that display oscillatory behavior (46–48). In one case, the oscillator

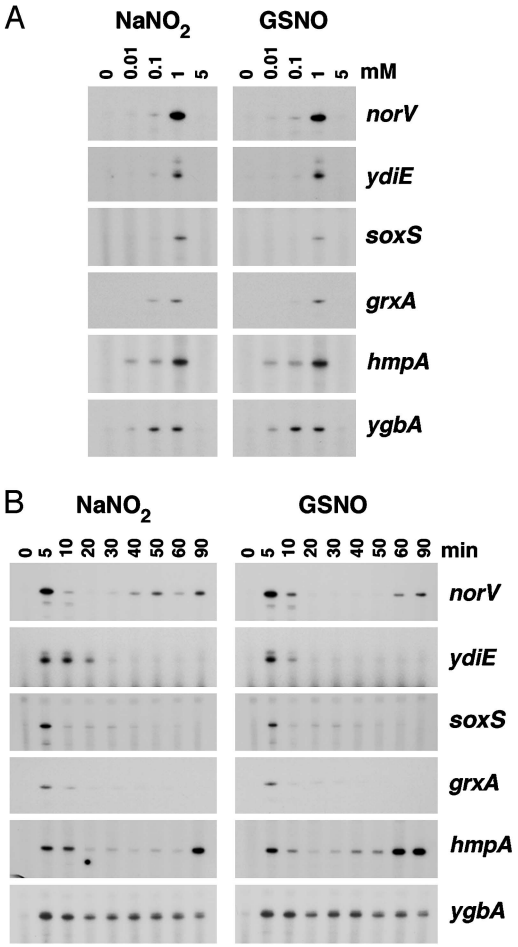

Fig. 2. Primer extension assays of *norV*, *ydiE*, *soxS*, *grxA*, *hmpA*, and *ygbA* expression in wild-type cells treated with 0.01, 0.1, 1, and 5 mM GSNO or $NaNO_2$ for 5 min (*A*) or with 1 mM GSNO or $NaNO_2$ for 5, 10, 20, 30, 40, 50, 60, or 90 min (*B*). MG1655 cells were grown to $OD_{600} = 0.4$ in LB medium, pH 6. Aliquots of the cultures were left untreated or treated with the indicated concentrations of GSNO or $NaNO_2$ and harvested after the indicated times. Total RNA was isolated from 15 ml of each untreated and treated culture, and 5 μg of each RNA sample was used in each primer extension reaction by using primers to the indicated genes.

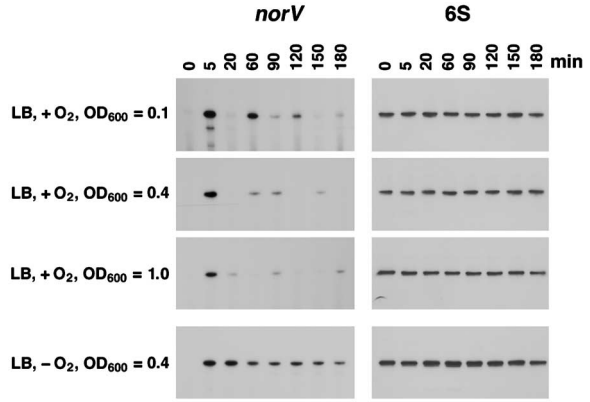

Fig. 3. Primer extension assays of *norV* expression in aerobic and anaerobic wild-type cells treated with 1 mM $NaNO_2$. MG1655 cells were grown aerobically to $OD_{600} = 0.1$, 0.4, and 1.0 or anaerobically to $OD_{600} = 0.4$ and treated with 1 mM $NaNO_2$ for 0, 5, 20, 60, 90, 120, 150, or 180 min. Total RNA was isolated from 15–30 ml of each untreated and treated culture; 5 μg of each RNA sample was used in each primer extension reaction using the *norV*-specific primer, and 1 μg of each RNA sample was used in Northern analysis for the 6S RNA.

consisted of three genes encoding repressors linked in a daisy chain; in the second case, oscillatory expression was achieved by the differential degradation and synthesis of an inhibitory protein; in the third case, the oscillator was composed of an activator and repressor that cross-regulated each other's expression. Given that only a limited number of natural genetic circuits have been found to exhibit oscillatory behavior, it is noteworthy that some human fibroblast genes induced by the NO-donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) also show biphasic expression (49). Further characterization of the regulatory mechanisms underlying the oscillations in *norV* mRNA levels should provide insights into the metabolism of reactive nitrogen species.

Regulation by Multiple Redox-Active Transcription Factors. Our studies of the global transcription response of *E. coli* cells treated with GSNO and $NaNO_2$ showed that the *E. coli* response to reactive nitrogen species during aerobic growth in rich media is a composite response in which NorR and Fur have major roles, SoxR and OxyR have minor roles, and additional regulators remain to be identified. It is interesting to note that NorR, Fur, SoxR, and OxyR all appear to contain redox-active cysteines and, in some cases, iron. The mechanism of NorR activation remains to be elucidated, but the *E. coli* NorR protein has seven cysteines, three of which are conserved in *R. eutropha* NorR1 and NorR2 and two of which are not conserved but are found in a CXXC motif characteristic of redox-active cysteines. For the *E. coli* Fur repressor whose activity is regulated by cellular iron levels, NO was found to react with the bound iron to form an iron–Fur–NO complex and thereby abolish the DNA binding activity of this repressor (50). NO also has been shown to react directly with the 2Fe–2S centers of the *E. coli* SoxR dimer to generate a dinitrosyl–iron–dithiol form of SoxR (51), and as described above, GSNO can react with the redox-active C199 residue in OxyR. We suggest that NorR, and perhaps the unidentified regulators, were evolved to sense reactive nitrogen species, whereas Fur, SoxR, and OxyR were evolved to sense iron and reactive oxygen species and are collaterally activated by reactive nitrogen species.

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