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The NsrR Regulon of *Escherichia coli* K-12 Includes Genes Encoding the Hybrid Cluster Protein and the Periplasmic, Respiratory Nitrite Reductase▽

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Successful pathogens must be able to protect themselves against reactive nitrogen species generated either as part of host defense mechanisms or as products of their own metabolism. The regulatory protein NsrR (a member of the Rrf2 family of transcription factors) plays key roles in this stress response. Microarray analysis revealed that NsrR represses nine operons encoding 20 genes in *Escherichia coli* MG1655, including the *hmpA*, *ytfE*, and *ygbA* genes that were previously shown to be regulated by NsrR. Novel NsrR targets revealed by this study include *hcp-hcr* (which were predicted in a recent bioinformatic study to be NsrR regulated) and the well-studied *nrfA* promoter that directs the expression of the periplasmic respiratory nitrite reductase. Conversely, transcription from the *ydbC* promoter is strongly activated by NsrR. Regulation of the *nrf* operon by NsrR is consistent with the ability of the periplasmic nitrite reductase to reduce nitric oxide and hence protect against reactive nitrogen species. Gel retardation assays were used to show that both FNR and NarL bind to the *hcp* promoter. The expression of *hcp* and the contiguous gene *hcr* is not induced by hydroxylamine. As *hmpA* and *ytfE* encode a nitric oxide reductase and a mechanism to repair iron-sulfur centers damaged by nitric oxide, the demonstration that *hcp-hcr*, *hmpA*, and *ytfE* are the three transcripts most tightly regulated by NsrR highlights the possibility that the hybrid cluster protein, HCP, might also be part of a defense mechanism against reactive nitrogen stress.

The ability of both pathogenic and free-living bacteria to protect themselves against reactive nitrogen species (RNS) generated either as products of their own metabolism or as part of the innate immune response is critical to their survival. A primary source of RNS is the highly reactive nitric oxide (NO), which is generated by phagocytes in response to infection, by the chemical transformation of nitrite formed either in the host or as the product of bacterial nitrate reduction, and by bacteria, either as an intermediate in denitrification or as a by-product of nitrite reduction to ammonia by fermentative bacteria (30).

In *Nitrosomonas europaea*, transcription of the gene encoding a copper nitrite reductase is regulated by a "nitrite-sensitive repressor," NsrR, that is a member of the Rrf2 family of transcription factors (3). The experimental data are consistent with the possibility that the *N. europaea* NsrR senses NO made as a product of nitrite reduction, which may also be the case for an NsrR homologue in *Rhodobacter capsulatus* (9). A recent bioinformatic analysis of Rrf2 family regulators and their predicted binding sites led to the proposal that NsrR homologues in many other bacteria regulate their defense against RNS (40). This predicted role for NsrR has recently been confirmed for *Escherichia coli*, *Bacillus subtilis*, and the obligate human pathogen *Neisseria gonorrhoeae* (4, 33, 34). Four *E. coli* promoters were predicted to be regulated by the NsrR homologue (encoded by the gene formerly designated *yjeB*) in response to RNS (40). These predictions were confirmed experimentally for the *hmpA*, *ytfE*, and *ygbA* promoters (4). The fourth promoter predicted to be regulated by NsrR was P_{hcp}, the promoter of a two-gene operon encoding the hybrid cluster protein, HCP, and its reductase, HCR. In our recent microarray study of the FNR, NarL, and NarP regulons of *E. coli*, members of the NsrR regulon were up-regulated in cultures growing anaerobically in the presence of nitrate and nitrite, presumably as a consequence of NO formation. Genes showing this pattern of expression included *hmpA*, *ytfE*, *ygbA*, and *hcp* (the known and predicted members of the NsrR regulon) and a number of others, raising the possibility that the NsrR regulon might be more extensive than that proposed on the basis of bioinformatic analysis alone (11). The first aim of the current study was therefore to exploit our genome-wide microarrays and a repressor titration approach to obtain a first evaluation of the possible extent of the NsrR regulon.

HCPs are iron-sulfur proteins that contain two iron-sulfur clusters: one is either a conventional [2Fe-2S] or a cubane [4Fe-4S] cluster; the other is a hybrid [4Fe-4S-2O] cluster (2). HCPs are related to the carbon monoxide dehydrogenase protein family (16, 31) and are widely distributed among obligate anaerobes and facultatively anaerobic bacteria. High-resolution crystal structures reveal a potential redox center that can accommodate a small or linear substrate, but their physiological roles remain controversial (31). Two reports implicated HCP in hydroxylamine reduction as part of the nitrate assimilation pathway in *Rhodobacter capsulatus* (9) or as a defense against hydroxylamine toxicity in *E. coli* (46). However, the latter authors were careful not to discount an alternative role

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TABLE 1. Bacterial strains used in this work

Bacterial strain	Relevant genotype	Reference or source
MG1655	Parent strain for microarray experiments, F^-Lac^- prototroph	11
JCB1001	Δ*fnr*, derivative of MG1655	11
JCB1002	Δ*narXL*, derivative of MG1655	11
JCB1003	Δ*narXL* Δ*narP*, derivative of MG1655	11
RK4353	Δ*lacU169 araD139 rpsL gyrA*	41
JCB5000	RK4353 Δ*hcp*	This work
JCB5010	RK4353 Δ*nsrR*	This work
JOEY19	MC1000 *ytfE-lacZ*	4
JOEY61	JOEY19 Δ*nsrR*	4
JOEY103	MC1000 *hcp-lacZ*	This work
JOEY104	JOEY103 Δ*nsrR*	This work
JOEY105	JOEY19 *fnr*::*cat*	This work
JOEY106	JOEY61 *fnr*::*cat*	This work
JOEY126	JOEY103 *fnr*::*cat*	This work
JOEY127	JOEY104 *fnr*::*cat*	This work
RV	Δ*lac*	24

for HCP, noting that, although effective detoxification enzymes typically have very high affinities for their substrates, the K_m for hydroxylamine of *E. coli* HCP is high. The catalytic efficiency of *E. coli* HCP for hydroxylamine reduction increases with increasing pH, with an optimum at the nonphysiological pH of 9 (46). Synthesis of *E. coli* HCP is induced during anaerobic growth, especially in the presence of nitrite (17, 45). Furthermore, HCP from *Salmonella enterica* serovar Typhimurium has been implicated in defense against RNS generated from acidified nitrite (27). This raises the possibility that HCP synthesis might be regulated as part of the response to RNS and, therefore, that it might protect *E. coli* against a reactive species other than hydroxylamine. A second aim of this work was therefore to establish how transcription from P_{hcp} is regulated.

MATERIALS AND METHODS

Bacterial strains, plasmids, and oligonucleotide primers. *Escherichia coli* strains and plasmids used during this study are listed in Tables 1 and 2. The sequences of the oligonucleotide primers are available on request.

Growth conditions. Unless otherwise specified, the strains were grown at 37°C either aerobically in Lennox broth (LB; 10 g/liter tryptone, 5 g/liter yeast extract, 5 g/liter NaCl) or anaerobically in minimal salts (MS) medium [4.5 g/liter KH_2PO_4, 10.5 g/liter K_2HPO_4, 1 g/liter $(NH_4)_2SO_4$, 0.05 g/liter $MgCl_2$, 10 μM ammonium-molybdate, 1 μM sodium-selenate, and 1 ml/liter of *E. coli* sulfur-free salts] (37), supplemented with 5% (vol/vol) LB and, as appropriate, 20 mM $NaNO_3$ or 2.5 mM $NaNO_2$. The carbon and energy source for anaerobic cultures was either 0.4% (wt/vol) glucose, or 0.4% (wt/vol) glycerol and 20 mM sodium fumarate. For the β-galactosidase assays whose results are reported in Table 4, cultures were grown in a minimal medium previously described (4). The source of NO was 0.05 mM spermine NONOate in aerobic cultures or 5 mM nitrite in anaerobic cultures (4).

The bacteria for the microarray experiments were grown anaerobically in the MS medium described above, supplemented with 20 mM trimethylamine-*N*-oxide (11), in the presence or absence of 2.5 mM sodium nitrite. RNA was extracted from four biological replicates, as described previously, from samples of bacteria harvested at the early exponential phase of growth. The pool of RNA used as a control in every experiment was isolated from eight independent cultures of *E. coli* strain MG1655 transformed with pGIT8 (a plasmid with a 1-bp deletion in the NsrR binding site of the *ytfE* promoter; see Table 2) and grown anaerobically in the absence of nitrite.

Whole-genome array analysis. Preparation of mRNA, labeling, hybridization, and analysis of array data were performed as described previously (11), with the following amendments. RNA was extracted from 15-ml samples from four independent cultures of each transformant growing in the presence or absence of nitrite. Each 15-ml sample was mixed with 30 ml of RNAprotect bacterial reagent (QIAGEN Ltd.), and an RNeasy Midikit was used to prepare total RNA according to the manufacturer's instructions (QIAGEN Ltd.). Any contaminating DNA was removed by using a DNase column kit (QIAGEN Ltd.). Total RNA was transcribed to Cy3- and Cy5-labeled cDNA, hybridized onto Corning Ultra GAPI glass slides with the 6,112 70-mer oligonucleotides of the Operon Array-Ready *E. coli* set 1.0 (Operon) as described previously (11). The slides were washed in Advalytix hybridization and wash stations, according to the manufacturer's instructions, and scanned, and the data were analyzed using Genepix and Genespring software as previously described (11).

Identification of NsrR binding sites. The NsrR binding sites at the *hcp*, *hmpA*, *ygbA*, and *ytfE* promoters were used to construct a position weight matrix using Consensus (RSAT tools, http://rsat.scmbb.ulb.ac.be/rsat/). These sites were first identified by Rodionov et al. (40). Bodenmiller and Spiro (4) later defined the NsrR binding site as being 23 bp in length, and these 23-bp binding sites were used to construct the matrix. The *E. coli* genome was searched with this matrix using genomic-scale PATSER (RSAT tools). Only potential NsrR binding sites with a score of >3 within 200 bp of transcription start sites were considered to be significant.

Construction of reporter fusions. The 400-nucleotide *hcp* promoter region between nucleotides 912971 and 913339 in *E. coli* genomic DNA was amplified by PCR from genomic DNA using primers hcp-prom1 and hcp-prom2, which create unique EcoRI and HindIII restriction sites at the 5′ and 3′ ends, respectively, of the amplified fragment. Plasmid pNF383 was constructed by ligating the EcoRI/HindIII-digested PCR fragment into pAA182 that had been digested with the same enzymes (Table 2). The *hcp-lacZ* fusion was crossed onto λRS45 by homologous recombination, and the resulting phage was used to isolate monolysogens (confirmed by PCR). The construction of the *ytfE-lacZ* fusion has been described previously (4).

Construction of *hcp* mutants. The *hcp* gene in strain RK4353 was first replaced by a chloramphenicol resistance cassette (*cat*) to give the strain JCB4999 (Table 1) (14). The *hcp*::*cat* mutation was transferred to other strains by bacteriophage P1 transduction. Purified transductants were checked by PCR, transformed with pCP20, and grown at 30°C to isolate derivatives, such as JCB5000, that had lost the *cat* cassette.

TABLE 2. Plasmids used in this work

Plasmid	Description	Reference or source
pAA182	11.2-kb Amp^r promoter-probe vector with ColE1 origin of replication, carrying the *lac* operon without the *lac* promoter (see Fig. 2)	24
pCP20		14
pGIT1	205-bp *ytfE* promoter fragment in pSTBlue-1	4
pGIT8	205-bp *ytfE* promoter fragment with ΔA deletion in pSTBlue	4
pNF383	*hcp* regulatory region (383 bp) cloned into pAA182 to create the *hcp-lacZ* fusion	This work
p*nrf53*	*nrfA* promoter cloned into the promoter-probe vector pRW50	5
pKD3	Amp^r and Cm^r marker used as a PCR template to replace a gene of interest with a *cat* cassette	14
pKD46	Amp^r marker with temperature-sensitive origin of replication (active at 30°C), expressing λ *red* β, γ, and *exo* genes (the products of which enable homologous recombination) under control of the *parCB* promoter	14
pRW50	Broad-host-range *lacZ* fusion vector for cloning promoters on EcoRI-HindIII fragments containing the RK2 origin of replication and encoding tetracycline resistance	29

Gel retardation assays. The *hcp* promoter fragment from pNF383 was gel purified, end labeled with [γ-^{32}P]ATP, and incubated at 37°C for 30 min with different concentrations of purified FNR and NarL proteins, and protein-DNA complexes were separated by electrophoresis on a 6% nondenaturing polyacrylamide gel containing 2% glycerol for 3 to 4 h, following the protocol described previously (6). Gels were analyzed using a Bio-Rad molecular imager FX and Quantity One software (Bio-Rad).

Chemical and biochemical assays. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (43). For β-galactosidase assays, strains were grown and assayed as described by Jayaraman et al. (24) or according to Miller (32). Protein concentrations were determined by the Lowry method.

Effects of hydroxylamine and other RNS on growth. Test tube cultures of each test strain were grown without aeration in 10 ml of MS medium supplemented with 0.8% (vol/vol) glycerol, 20 mM sodium fumarate, 2.5% (vol/vol) LB, and various concentrations of hydroxylamine in the range of 0.1 to 1.0 mM. The inoculum was 60 μl of an exponential-phase aerobic culture in LB. The tubes were incubated at 37°C, and the density of each culture was determined at intervals. When relevant, the β-galactosidase activity of each culture was assayed.

RESULTS

Microarray analysis of the *E. coli* NsrR regulon. The *nsrR* gene of *E. coli* MG1655 is located immediately upstream of, and is cotranscribed with, the *rnr* gene encoding RNase R (10). The expression of this transcription unit is cold-shock inducible (10), which may well involve secondary structures in the mRNA in the vicinity of the *nsrR* coding region. Deletion of the *nsrR* gene might therefore perturb *rnr* expression by a simple consequence of polarity or by disruption of mRNA structural elements involved in posttranscriptional regulation. The consequence would likely be secondary effects on global RNA stability; hence, a comparison of the transcriptomes of an *nsrR* mutant and its parent would be difficult to interpret. To avoid such potential artifacts, we exploited the observation that regulation by NsrR is extremely sensitive to repressor titration by NsrR binding sites provided on a multicopy plasmid (4). Transformation of an *nsrR*$^+$ strain with plasmid pGIT1, which carries the *ytfE* promoter in multicopy, titrates out NsrR to phenocopy an NsrR$^-$ mutation. As a control, pGIT8 carries the same promoter fragment, but a 1-bp deletion mutation in the NsrR binding site eliminates repressor titration in vivo (4). Strain MG1655 transformed with either pGIT1 or pGIT8 was grown anaerobically under the carefully controlled conditions described previously, so that growth rates were almost identical (11), and RNA was isolated from early-exponential-phase cultures grown in the presence or absence of nitrite. For each growth condition, RNA was isolated from four independent cultures, and to validate comparisons between different plasmids and growth conditions, a pool of RNA from the control strain (MG1655 transformed with pGIT8 and grown in the absence of nitrite) was included in every microarray to provide a common reference. Nine transcripts encoding 20 genes were either more abundant in cultures in which NsrR was titrated out by the *ytfE* promoter than in the control cultures or more abundant in cultures grown in the presence than in the absence of nitrite (Table 3). Operons observed to be differentially expressed include not only all four of the transcripts, *hmpA*, *ytfE*, *ygbA*, and *hcp-hcr*, known or predicted to be NsrR-regulated (4, 40), but also other transcripts predicted to be regulated by nitrite or RNS generated from nitrite, for example, nitric oxide (11). Particularly interesting is the demonstration that genes encoding the periplasmic nitrate and nitrite reductases, Nap and Nrf, but not the cytoplasmic nitrate and nitrite reductases, NarGHI and NirBD, are also part of the NsrR regulon. At least in the case of Nrf, there is a documented role in the defense against RNS, since Nrf is both a nitrite and an NO reductase (36). Also repressed by NsrR was the promoter of the *yeaR-yoaG* genes of unknown function, previously implicated in the metabolism of RNS (Table 3) (see Table 5 of reference 11).

A further 22 transcripts fulfilled the statistical criteria to be considered to be activated by NsrR (Table 3). In the case of the *ydbC* promoter, the differences in transcript levels between the two transformants were at least as great as those for the NsrR-repressed genes, making P_{ydbE} a prime candidate for direct activation by NsrR. At least one other Rrf2 family transcription factor, IscR, can also function both as a repressor and as a transcription activator (19, 47). The microarray data for the other promoters that are apparently activated by NsrR must be evaluated cautiously, however, because few of these transcripts encode proteins known or suspected to be involved in the response to RNS, and most of the differences were much smaller (typically two- to threefold apparent activation) than the 30-fold repression of *ytfE*, the 14-fold repression of *hmpA*, and the up-to-15-fold repression of *hcp*. It is likely that most of these other apparent transcription activation effects of NsrR are indirect consequences of the NsrR titration. Nevertheless, whether the effect is direct or secondary, it is interesting that the genes encoding the cytochrome *bo* oxidase, but not the cytochrome *bd* oxidase, appear to be modestly activated by NsrR. Recent microarray analysis and real-time quantitative PCR data revealed that the *cydAB* mRNA was the transcript most strongly induced as part of the nitrosative stress response when *Staphylococcus aureus* was exposed to *S*-nitroso *N*-acetyl DL-penicillamine (39).

In summary, the microarray data established that NsrR plays a central role in *E. coli* defense against RNS and implicated additional genes of unknown function in this response. The prediction that P_{hcp} is part of the NsrR regulon (40) was confirmed. Subsequent experiments were designed to confirm these and other key results from the microarray analysis.

Binding of FNR and NarL at the *hcp* promoter. We recently reported microarray data for *E. coli* strain MG1655 that showed that transcription from P_{hcp} is induced during anaerobic growth (11). Anaerobic induction was totally dependent upon a functional FNR protein, partially dependent on the nitrate-sensing two-component regulatory system NarXL, and independent of the alternative nitrate response protein NarP. To determine whether the effects of FNR and NarL on *hcp* transcription are direct or indirect (for example, due to inactivation of NsrR by nitrite or nitric oxide generated during anaerobic growth), the *hcp* promoter fragment from pNF383 was incubated with different concentrations of purified FNR and NarL proteins. Gel retardation assays showed that both proteins formed multiple complexes; therefore, the effects of FNR and NarL on transcription are likely to be direct (Fig. 1). This correlates with the previously noted presence of FNR and NarL binding sites in the promoter located so that FNR would function, unusually, as a class 1 activator and NarL as a class 2 activator (8, 17).

Regulation of *hcp* transcription in response to nitric oxide and NsrR but not by hydroxylamine. There was a striking

TABLE 3. Genes differentially regulated in strains carrying pGIT1 and pGIT8[a]

Gene	Product	Transcript expression ratio in[c]: pGIT8 $Ø_2$ vs pGIT1 $Ø_2$	pGIT8 $Ø_2$ vs pGIT8 with NO_2^-	pGIT8 $Ø_2$ vs pGIT1 with NO_2^-	Operon structure	Coregulation by[d]: FNR	NarL	NarP	NsrR site[e]	Reference
NsrR repressed										
hcr	NADH oxidoreductase	12.20	4.47	13.09	*hcp-hcr*	A[f,g]	A[g]		+6 wrt mapped TSS	17
hcp	Hybrid cluster protein	15.65	5.28	9.93						
yccM	Predicted 4Fe-4S membrane protein	4.89	3.19	4.18	*yccM*	A[f]			+69 wrt predicted TSS	
uspF	Nucleotide binding protein	2.09	1.63	1.78	*uspF*					
yeaR	Hypothetical protein	2.57	3.57	3.12	*yeaR-yoaG*	R	A	A	−20 wrt predicted TSS	
ccmG	Cytochrome *c* biogenesis system	2.75	2.17	2.64	*napFDAGHBC-ccmABCDEFGH*	A[f,g]	R[g]	A[g]	−30 wrt mapped TSS	13
ccmF	Cytochrome *c* biogenesis system	2.08	1.73	2.34						
napB	Periplasmic nitrate reductase	2.07	3.49	3.75						
napH	Periplasmic nitrate reductase	2.13	3.39	3.86						
napD	Periplasmic nitrate reductase	2.02	3.49	2.80						
napF	Periplasmic nitrate reductase	2.41	3.99	3.00						
hmpA[b]	Flavohemoglobin	13.07	8.50	14.44	*hmpA*	R[f,g]	A		+1 wrt mapped TSS	12
ygbA[b]	Hypothetical protein	4.58	3.20	3.86	*ygbA*	R[f]			−7 wrt mapped TSS	
nrfA	Periplasmic nitrite reductase	5.28	7.37	6.66	*nrfABCDEFG*	A[f,g]	R[g]	A[g]	−63 wrt mapped TSS	6
nrfB	Periplasmic nitrite reductase	3.54	4.31	4.67						
nrfC	Periplasmic nitrite reductase	4.51	5.30	6.19						
nrfD	Periplasmic nitrite reductase	3.68	3.98	3.99						
nrfE	Periplasmic nitrite reductase	4.72	5.15	6.44						
nrfF	Periplasmic nitrite reductase	2.65	3.29	2.47						
ytfE[b]	RNS-induced conserved protein	31.91	7.84	33.18	*ytfE*	R[g]	A		−12 wrt mapped TSS	25, 26
NsrR activated										
insB_1	IS*1* protein InsB	0.40	1.29	0.60						
yafE	Predicted *S*-adenosylmethionine-dependent methyltransferase	0.36	0.36	0.35	*yafDE*					
yafU	Predicted inner membrane protein	0.23	0.44	0.16	*yafU*					
ykfJ	Hypothetical protein	0.43	0.52	0.68	*ykfJ[prfH]*					
mmuP	Predicted ABC transporter	0.37	0.57	0.27	*mmuPM*	A				
insB_2	IS*1* protein InsB	0.41	1.46	0.63						
yagA	Hypothetical protein	0.23	0.56	0.18	*yagA*					
insB_3	IS*1* protein InsB	0.40	1.07	0.59						
ykgF	Predicted amino acid dehydrogenase	0.40	0.42	0.49	*ykgEFG*	R	A			
betB	Betaine aldehyde dehydrogenase	0.47	0.70	0.54	*betIBA*	R	A			
cyoE	Cytochrome *bo* terminal oxidase	0.44	0.94	0.47	*cyoABCDE*	R[f]	A			
cyoC	Cytochrome *bo* terminal oxidase	0.36	0.96	0.51						
cyoB	Cytochrome *bo* terminal oxidase	0.29	0.67	0.38						
cyoA	Cytochrome *bo* terminal oxidase	0.26	0.79	0.35						
ompF	Outer membrane porin	0.43	0.63	0.40	*ompF*					
insB_4	IS*1* protein InsB	0.35	1.18	0.54						
dadA	D-Amino acid dehydrogenase	0.49	0.76	0.49	*dadAX*					
ydbC	Putative oxidoreductase	0.03	0.03	0.01	*ydbC*					
insB_5	IS*1* protein InsB	0.40	1.25	0.61						
ygeF	Hypothetical protein	0.43	0.94	0.49	*ygeF*					
insB_6	IS*1* protein InsB	0.38	1.30	0.61						
yiiL	L-Rhamnose mutarotase	0.17	0.23	0.16	*rhaBAD-yiiL*	A				
yjbB	Putative α-helix protein	0.35	0.42	0.23	*yjbB*		A			
yjiV	Hypothetical protein	0.30	0.26	0.45	*yjiU*					

[a] This table lists genes that display at least a twofold difference in transcript abundance between anaerobically grown cultures of *E. coli* strain MG1655 harboring pGIT1, a multicopy plasmid with a copy of the *ytfE* promoter to which NsrR binds, and pGIT8, the same plasmid with a mutation which abolishes NsrR binding. Also shown are the ratios of transcript abundance in cultures carrying either pGIT8 or pGIT1 grown in the presence of 5 mM nitrite compared to those in cultures carrying pGIT8 in the absence of nitrite.

[b] Gene previously shown to be regulated by NsrR (4).

[c] $Ø_2$, anaerobic growth conditions.

[d] Observed differential regulation in strains carrying *fnr*, *narXL*, and *narXLP* in Constantinidou et al. (11). A, activation; R, repression.

[e] NsrR sites located by position weight matrix search of the *E. coli* MG1655 genome using a matrix generated from the NsrR binding sites at the *hcp*, *hmpA*, *ygbA*, and *ytfE* promoters. Predicted transcription start sites (TSS) are taken from RegulonDB (http://regulondb.ccg.unam.mx/index.html). wrt, with respect to.

[f] An FNR binding site is located in the promoter region of this gene.

[g] Regulation has been independently documented.

discrepancy between the apparent NarP dependence of *hcp* transcription revealed in preliminary experiments with a multicopy reporter plasmid, pNF353 (17), and the microarray data, in which RNA expressed from a single chromosomal copy of the *hcp-hcr* operon was analyzed (11). As multicopy plasmids titrate out the effects of NsrR (4) (Table 3), a single-copy chromosomal *hcp*::*lacZ* fusion was constructed to investigate the role of NsrR at this promoter. The previously characterized

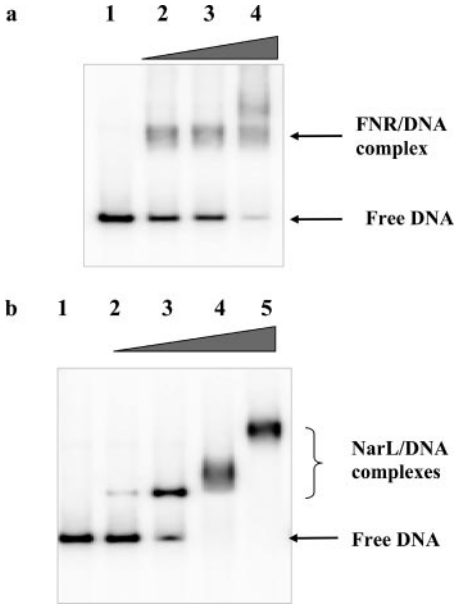

FIG. 1. Binding of FNR and NarL to the *hcp* promoter. The *hcp* regulatory region was prepared by digesting plasmid pNF383 with EcoRI and HindIII. The *hcp* regulatory region was labeled with radioactive [γ-^{32}P]ATP and mixed with FNR or NarL at a range of concentrations, indicated by the gray triangles. The reaction mixtures were incubated at 37°C for 30 min and run on polyacrylamide electrophoresis image gels for 3 to 4 h. The gels were fixed and dried, and the phosphorscreen image was developed. Free DNA and complexes of DNA with FNR and NarL are denoted. (a) Gel retardation assay with FNR. Lane 1, no FNR; lane 2, 0.25 μM FNR; lane 3, 0.5 μM FNR; lane 4, 1.0 μM FNR. (b) Gel retardation assays with NarL. Lane 1, no NarL; lane 2, 0.4 μM NarL; lane 3, 0.8 μM NarL; lane 4, 1.6 μM NarL; lane 5, 3.2 μM NarL.

ytfE::*lacZ* fusion was used as a control (Table 4). During aerobic growth, the very low level of transcription from the *hcp* promoter was induced 2.5-fold by spermine NONOate in the parental strain but only 1.6-fold in an *fnr* mutant. The *hcp* gene was constitutively expressed at a higher level in the *nsrR* mutant, and this constitutive level was lower in the *fnr nsrR* double mutant.

During anaerobic growth, the background level of *hcp* transcription was sixfold higher than during aerobic growth and was induced a further 21-fold by nitrite. Increased transcription during anaerobic growth was strongly dependent upon FNR (Table 4). The highest expression level, 6,180 units, was detected during anaerobic growth of the *nsrR* mutant in the presence of nitrite, but the activity was slightly lower, 4,880 units, in the absence of nitrite, presumably reflecting the smaller effect of NarL and NarP under these conditions. These data clearly indicate that the *hcp* promoter is activated by FNR and repressed by NsrR. In the case of the *ytfE* promoter, repression by NsrR is the dominant regulatory mechanism, though there is also some evidence for anaerobic repression by FNR, as reported previously (25). The effect of the *fnr* mutation may well be indirect in this case, since FNR probably controls the expression of genes that are involved in the reduction of nitrite to NO. In the absence of both FNR and NsrR, *ytfE* promoter activity was significantly higher in anaerobic cultures, but the underlying mechanism is not known.

No evidence was obtained for the induction of *hcp* transcription by hydroxylamine when the promoter activity was assayed using the multicopy plasmid pNF383 in strain RK4353 or in a *pcnB* derivative in which the plasmid copy number is decreased to about one (data not shown). Furthermore, growth of both the parental strain and the *hcp* mutant, JCB5000, was totally inhibited by 1 mM hydroxylamine, unaffected by 0.2 mM hydroxylamine, and partially inhibited by 0.3 or 0.5 mM hydroxylamine; there were no significant differences in hydroxylamine toxicity between the two strains.

NsrR: sixth protein to regulate transcription factor at P_{nrfA}. Transcription initiation at the *nrfA* promoter is activated by FNR in the absence of oxygen and induced further by NarL and NarP in response to low concentrations of nitrate or to nitrite (35, 44). FNR-dependent transcription is also repressed by two nucleoid-associated factors, Fis and IHF, which bind to multiple sites within the promoter (6). Thus, P_{nrfA} is a complex promoter with at least five proteins directly controlling transcription initiation. However, there is a residual response to nitrite even in a *narL narP* double mutant (38), and the possibility that a sixth factor might regulate transcription initiation at P_{nrfA} was apparent from detailed experimental analysis of this promoter (5–7, 11). Two approaches were used to confirm that NsrR is this sixth factor that regulates P_{nrfA}.

Plasmid pnrf53, which includes DNA from 209 bases upstream of the transcription start and a further 131 bases downstream cloned into the low-copy *lacZ* expression vector pRW50 to generate a P_{nrfA}::*lacZ* transcriptional fusion (44), was transformed into strain RK4353 and its *nsrR* null mutant,

TABLE 4. Regulation of *hcp* transcription by FNR, NsrR, and sources of nitric oxide[a]

Promoter	Background	β-Galactosidase activity under the indicated growth conditions			
		Aerobic	Aerobic with NONO	Anaerobic	Anaerobic with nitrite
ytfE	Wild type	27 ± 1.2	943 ± 53	34 ± 3	1,200 ± 195
	nsrR	4,450 ± 317	5,155 ± 188	9,580 ± 553	8,615 ± 477
	fnr	37 ± 5	936 ± 30	239 ± 26	1,740 ± 115
	nsrR fnr	6,140 ± 150	4,460 ± 200	17,600 ± 1,050	19,900 ± 650
hcp	Wild type	8 ± 1	20 ± 2	49 ± 6	1,064 ± 34
	nsrR	147 ± 5	152 ± 4	4,880 ± 364	6,180 ± 786
	fnr	8 ± 2	13 ± 2	9 ± 2	34 ± 2
	nsrR fnr	114 ± 3	109 ± 5	165 ± 5	161 ± 3

[a] Cultures were grown and β-galactosidase assayed as previously described (4). Data are expressed in Miller units ± standard deviations (32). NONO, spermine NONOate.

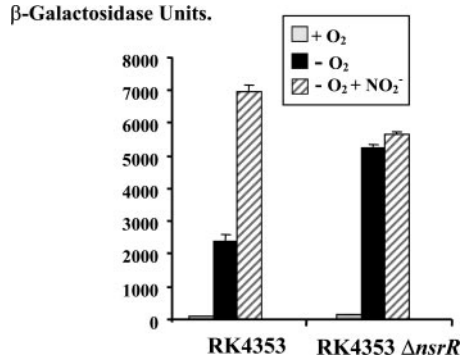

FIG. 2. NsrR represses anaerobic expression from the *nrf* promoter. The figure shows the β-galactosidase activities of RK4353 and its Δ*nsrR* derivative strain JCB5010, carrying pRW50 containing the pnrf53 promoter fragment. Bacteria were grown aerobically and anaerobically in MS medium and nitrite was added to a final concentration of 2.5 mM where indicated. The β-galactosidase activities are expressed as nmol of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed min^{-1} (mg dry cell mass)$^{-1}$. Each level of activity shown is the average of three independent determinations ± standard deviation.

strain JCB5010. Purified transformants were grown either aerobically or anaerobically in the presence or absence of nitrite, and the β-galactosidase activities were determined. Anaerobic expression from P_{nrfA} was increased 2.2-fold by the disruption of *nsrR* (Fig. 2), consistent with the observation from microarray data that NsrR is a weak repressor of P_{nrfA}. Nitrite induction was not observed in the *nsrR* null strain, indicating that NsrR might also play a role in the response of P_{nrfA} to nitrite, possibly indirectly due to the formation of NO from nitrite.

In view of the ability of multicopy plasmids to titrate out the repressor activity of NsrR and the possibility that a mutation in *nsrR* might have secondary polar effects on the downstream *rnr* gene encoding RNase R (10), the repressor titration experiments were repeated with the Lac$^-$ strain RV, doubly transformed with pnrf53 and either pGIT1 or pGIT8. Bacteria were grown anaerobically with fumarate, trimethylamine-*N*-oxide, and either glucose or glycerol as the main carbon source, and the β-galactosidase activities were determined. The ability of the NsrR binding site on the multicopy plasmid pGIT1, but not on pGIT8, to titrate out the NsrR repressor and hence derepress P_{nrfA} transcription was measured during anaerobic growth in the absence of nitrate and nitrite. The data from independent duplicate cultures were completely consistent with NsrR repression of P_{nrfA} [1,200 and 1,270 nmol of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed · min^{-1} · (mg bacterial dry mass)$^{-1}$ for the pGIT1 transformant compared with 180 and 230 units for the pGIT8 transformant]. However, the double transformants were unable to grow anaerobically with glycerol rather than glucose as the primary carbon source. Even with glucose, the growth of the double transformants was extremely slow compared with the growth of bacteria transformed with one of the pGIT plasmids alone and was variable between experiments.

DISCUSSION

The exploitation of the ability of a multicopy plasmid to titrate out NsrR and hence relieve NsrR repression enabled us to demonstrate that NsrR is a global regulator of the response to RNS in *E. coli* (4). This approach is similar to the ferric uptake regulator titration assay (42), with the inclusion of a microarray analysis to identify the genes derepressed by NsrR titration. Some of the small increases in transcript abundance detected when NsrR is active were almost certainly (but it was not independently confirmed) due to secondary effects, but the primary effects of NsrR as a repressor of operons involved in the relief of reactive nitrogen stress were largely confirmed by independent evidence. Previous studies have reported that *hmpA* transcription is induced during NO- or *S*-nitrosoglutathione-induced stress (18, 26), and NO also induces synthesis of the di-iron protein YtfE (4, 25, 26). We recently predicted that there is a previously undocumented nitrite-responsive transcription factor that regulates the expression of the *nrf* operon encoding the periplasmic nitrate reductase, as well as genes of unknown function that include *ytfE* and *yeaR-yoaG* (11). All of these transcripts were more abundant in the transformant in which the pool of NsrR was depleted than in the control transformant, validating our repressor titration approach and indicating that NsrR is the additional transcription factor.

One interesting result from the microarray analysis was the observation that the complete periplasmic pathway for nitrate reduction to ammonia is regulated by NsrR. Pathogenic bacteria must be able to defend themselves from RNS originating from four sources: products of their own metabolism; products of other bacteria that share their ecological niche (for example, NO generated by lactic acid bacteria in the gastrointestinal tract); NO generated as part of host defense mechanisms; and products of nonspecific chemical reactions. Most of the above threats originate outside enteric bacteria, so it is appropriate that they can be neutralized by the NO reductase activity previously documented for the periplasmic nitrite reductase Nrf (36). If so, how does *E. coli* protect itself against RNS that enter or are made in the cytoplasm?

A major protection mechanism against NO in the cytoplasm is provided by flavorubredoxin and its reductase, NorVW, which are synthesized in response to NO activation of the transcription activator NorR (15, 20, 22). The three operons most strongly up-regulated in response to NsrR titration are *hmpA*, *ytfE*, and *hcp-hcr* (Table 3). Both Hmp and YtfE are clearly established as cytoplasmic components of the RNS response (25, 26, 28), suggesting that the same might be true for HCP. Microarray analysis of the *E. coli* FNR, NarXL, and NarQP regulons and supporting transcription fusion data revealed that *hcp* expression is regulated in parallel with the cytoplasmic NADH-dependent nitrite reductase Nir (11). We inferred that this implied a function for HCP in detoxifying a product generated when the NarXL two-component system is activated, possibly an RNS generated as a side product of nitrite reduction to ammonia by Nir. The *hcr* product has been shown to be an NADH-dependent HCP reductase that presumably functions to provide electrons for the reduction of the HCP substrate (45), which was tentatively identified to be hydroxylamine (46). However, there is a hydroxylamine reductase activity associated with the cytoplasmic NADH-dependent nitrite reductase NirBD that is at least as effective as that of HCP (23). While it is possible that HCP is a back-up mechanism to provide protection against hydroxylamine toxicity, con-

ditions under which its role is significant remain to be revealed, so other physiological roles for HCP must be considered. Even when it is expressed from a single chromosomal copy of the *hcp* gene, HCP accumulates as an abundant protein (45). Possibly HCP simply binds hydroxylamine stoichiometrically to prevent it from inhibiting bacterial metabolism until it can be reduced by Nir to ammonia (23). A precedent for such a detoxification mechanism is cytochrome *c′*, which protects pathogenic neisseria by binding nitric oxide (21, 43). A further possibility meriting consideration is that HCP repairs NO- or hydroxylamine-induced damage to iron-sulfur centers, for example, the Fe-S centers of NirB.

Recently an entirely different role was proposed for HCP, namely, that it functions as a peroxidase (1). This suggestion was based upon observations that HCP is oxidized by hydrogen peroxide, that an *hcp* mutant is more sensitive to hydrogen peroxide than its parent, and that *hcp* transcription is regulated by OxyR. However, other links between RNS and the OxyR regulon have been reported (18), and the phenotype of the *hcp* mutant was rather weak. We therefore suggest that the physiological substrate reduced by HCP remains to be determined but is more likely a reactive nitrogen compound (other than hydroxylamine) than a reactive oxygen species.

The microarray analysis confirmed our previous suggestion that transcription of the two-gene operon *yeaR-yoaG* is subject to NsrR repression. This operon is therefore another candidate for encoding proteins that protect *E. coli* against RNS, especially in anaerobic environments where FNR might be inactivated by severe NO damage (11, 12, 18). Future studies must focus on the biochemical functions of YeaR, YoaG, YtfE, and yet again, HCP.

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