

FNR-Mediated Oxygen-Responsive Regulation of the *nrdDG* Operon of *Escherichia coli*

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Transcription of the *nrdDG* operon, which encodes the class III nucleotide reductase, which is only active under anaerobic conditions, was strongly induced after a shift to anaerobiosis. The induction was completely dependent on the transcriptional activator FNR and was independent of the ArcA-ArcB two-component response regulator system. The *nrdD* transcript start site was mapped to a position immediately downstream of two FNR binding sites. Transcription of the other two nucleotide reductase operons, *nrdAB* and *nrdEF*, did not respond to oxygen conditions in a wild-type background, but *nrdAB* expression was increased in the *fnr* mutant under anaerobic conditions.

The *Escherichia coli* genome contains three operons (*nrdAB*, *nrdHIEF*, and *nrdDG*) encoding ribonucleotide reductases, which supply the deoxynucleoside triphosphate (dNTP) substrates for DNA replication. The *nrdAB* operon codes for the primary aerobic reductase, a class Ia tyrosine-cysteine radical enzyme that reduces NDP substrates by using thioredoxin and glutaredoxin (15). The *nrdEF* genes encode an auxiliary weakly expressed class Ib enzyme (15) that, in addition to glutaredoxin, utilizes the *nrdH* gene product as an electron transporter (14). The *nrdD* gene encodes a class III reductase containing an oxygen-sensitive glycy radical (15), similar to that of pyruvate formate lyase. The NrdD reductase is activated by the *nrdG*-encoded activase under anaerobic growth conditions (22) and is irreversibly inactivated by oxygen. The *nrdAB*-encoded reductase is essential under aerobic growth conditions, while the *nrdD* gene is essential under strict anaerobic growth conditions (9) in which the FeIII tyrosyl radical of the class Ia enzyme is inactive.

The *nrdAB* operon is transcribed from two promoters (7), and transcription is activated by Fis (4) and IciA (12) and probably also by DnaA, because mutations in the two DnaA binding sites in the promoter region reduce expression (4). The operon is cell cycle regulated (21), and expression is stimulated by inhibition of the *nrdAB*-encoded nucleotide reductase by hydroxyurea (9, 10) as well as by inactivation of the genes encoding thioredoxin and glutaredoxin (18). Like *nrdAB*, the *nrdHIEF* operon is induced by hydroxyurea (13) and in strains lacking thioredoxin and glutaredoxin (17). In addition, *nrdHIEF* is induced by oxidative stress (17). Very little is known about regulation of expression of the *nrdDG* operon except that the level of enzyme activity is increased under anaerobic growth conditions (9). Here we have constructed single-copy transcriptional *lacZ* fusions of the three *nrd* operons and investigated their expression as a function of aeration and in strains carrying mutations in the two main transcrip-

tional regulators for aerobic/anaerobic shifts, FNR and ArcA. FNR activates transcription of a number of genes coding for enzymes required under anaerobic growth conditions and represses transcription of some genes for aerobic functions (11, 20). Under anaerobic conditions, ArcA, the response regulator of the two-component ArcA-ArcB system, represses many genes for aerobic functions and activates transcription of some other genes (11).

Construction of transcriptional *lacZ* fusions to the *nrd* promoters. The promoter regions of the three *nrd* operons (Fig. 1) were amplified by PCR and cloned in front of the *lacZ* gene of the promoter cloning vector pTAC3953 (6). The fusions were subsequently integrated into the λ attachment site as described previously (2) and transferred to strain LJ24 by P1 transduction to obtain the strains BOS7 to BOS9 illustrated in Table 1, and these strains were used to construct isogenic derivatives carrying the *fnr1* and *arcA1* mutations.

Expression of the *nrd* operons under aerobic and anaerobic conditions. The wild-type strains carrying the three *nrd-lacZ* fusions were grown with full aeration, and part of the cultures were then shifted to anaerobic growth conditions as described in reference 6. Cell mass (optical density at 450 nm [OD₄₅₀]) and β -galactosidase activity were measured throughout the experiment. As expected, expression of the *p_{nrdA}'-lacZ* fusion was very high, and that of the *p_{nrdH}'-lacZ* fusion was very low (Table 2). Expression of both fusions was unaffected by the shift to anaerobiosis (Table 2). During steady-state anaerobic growth, expression of the *p_{nrdA}'-lacZ* and *p_{nrdH}'-lacZ* fusions was reduced to 50% of that observed immediately after the shift (data not shown). In contrast, the level of *nrdD* expression was low during exponential aerobic growth and increased 20-fold upon a shift to anaerobic growth conditions (Fig. 2 and Table 2). The *p_{nrdD}'-lacZ* fusion was also strongly induced in the aerobic culture during the deceleration phase of growth (Fig. 2A), reaching approximately 50% of the anaerobic value (Table 2), while entry into the stationary phase had no effect on expression from the *nrdAB* or *nrdHIEF* promoters (Table 2).

Effect of *fnr* and *arcA* on anaerobic and growth-phase induction of *nrdD*. Introduction of the *arcA1* mutation had no effect on expression of the *nrdD* operon, while introduction of

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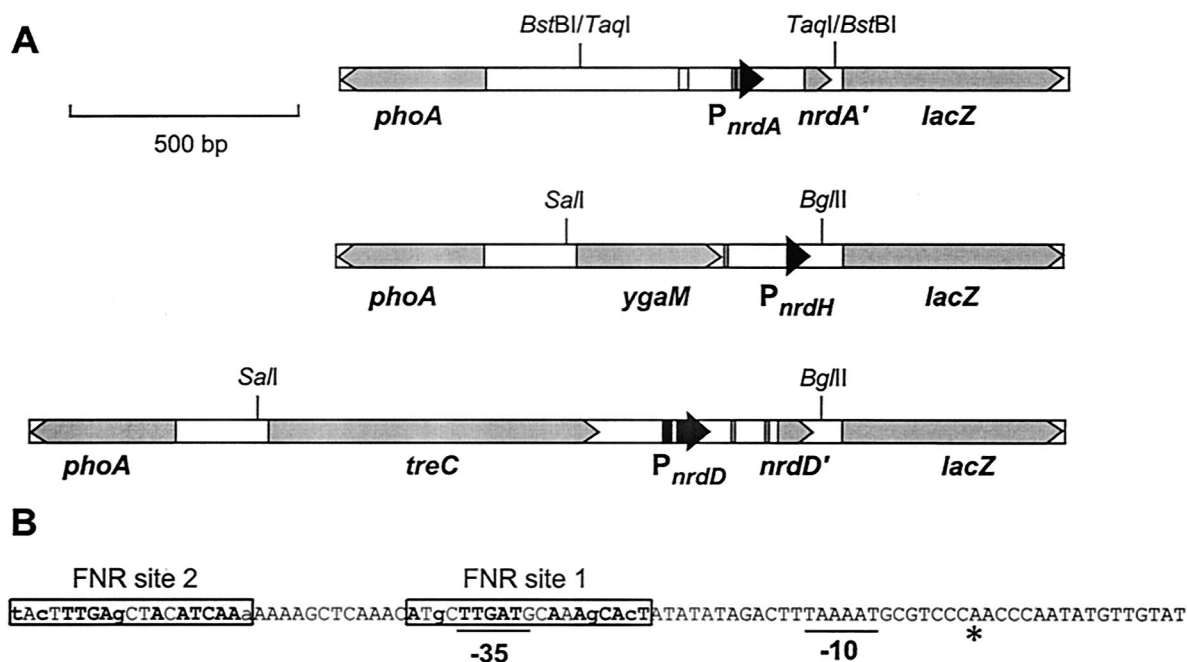


FIG. 1. Structure of *nrd-lacZ* fusions. (A) The promoter regions of the *nrdAB*, *nrdDG*, and *nrdHIEF* operons were amplified from chromosomal DNA of strain LJ24 with the primers Nrd5 and Nrd6 (AAGACGGCGTATTTAATCGC and CGAGATACTGATAATCCGGC, respectively), NrdD-F and NrdD-B (TTATGTCTGACCCTGGCAACAAGGAATGCAC and TAAGATCTTTCCGCTGCTTTAGCTGCAC, respectively), and NrdE-1 and NrdE-2 (TTCTGTCTGACGGATCATATGTTAACCAGC and AGGTTAGATCTGAAGATACGGCGCGATG, respectively). The *nrdA* fragment was digested with *TaqI* and cloned into the promoter cloning vector pTAC3953 (6) digested with *BstBI*, while the *nrdD* and *nrdH* promoter fragments were digested with *Sall* and *BglII* and inserted into pTAC3953 digested with the same enzymes. The *lacZ* fusions from the three plasmids were subsequently transferred into the λ attachment site as described in reference 2 to obtain strains BOS7, BOS8, and BOS9. The correct promoter *lacZ* fusion in these strains was verified by sequencing. Restriction sites used in the construction are indicated above the bars showing the relevant segments of the plasmids. Light gray arrows show the position and direction of genes. Gray rectangles represent DnaA boxes with up to one mismatch to the consensus sequence TTATNCACA, while a white rectangle represents the Fis binding site (4), and black rectangles represent FNR binding sites. Black triangles represent promoters. The positions of the promoters for *nrdA* and *nrdH* are according to references 23 and 13, respectively, whereas the position of the *nrdD* promoter is according to our data from Fig. 4. (B) Sequence of the *nrdD* promoter region. The -35 and -10 regions of the promoter sequence are indicated with lines below the sequence, and the transcript start site, determined from Fig. 4, is indicated by an asterisk below the sequence. The FNR binding sites are boxed. Bases fitting the consensus sequence ANANTTGATNNANATCAAT (20) are indicated in boldface, and those not fitting are in lowercase.

the *fnr1* mutation completely eliminated the anaerobic induction of the *nrdD* promoter (Fig. 2B). The differential rate of synthesis of the *p_{nrdD}'-lacZ* fusion increases very shortly after the shift to anaerobiosis in the wild type (Fig. 3). Thus, the kinetics of induction of the *nrdDG* operon are very similar to those observed for other anaerobically induced genes, both the

FNR-regulated *pfl* operon (19) and ArcA-regulated genes (1, 6).

In the absence of the FNR protein, the aerobic exponential expression of *nrdD* was slightly reduced (Table 2) and the stationary-phase induction was virtually absent, indicating that the induction during the deceleration phase of growth is

TABLE 1. *E. coli* K-12 strains used in this study

Strain	Genotype ^a	Source or construction
LJ24	<i>thi-1 leu-6 lacY1 lacI-ZΔ(Mlu) glnV44 tonA21 rpsL rfbD1 rpoS396(Am)</i>	3, 19
TC3266	<i>fnr1 zci::Tn10</i>	6
LB97	<i>arcA1 zjj::Tn10</i>	6
BOS7	<i>attB::p_{nrdA}'-lacZ</i>	This work ^b
BOS8	<i>attB::p_{nrdB}'-lacZ</i>	This work ^b
BOS9	<i>attB::p_{nrdH}'-lacZ</i>	This work ^b
BOS10	<i>arcA1 zjj::Tn10 attB::p_{nrdA}'-lacZ</i>	P1(BOS7) × LB97
BOS11	<i>arcA1 zjj::Tn10 attB::p_{nrdD}'-lacZ</i>	P1(BOS8) × LB97
BOS13	<i>fnr1 zci::Tn10 attB::p_{nrdA}'-lacZ</i>	P1(BOS7) × TC3266
BOS14	<i>fnr1 zci::Tn10 attB::p_{nrdD}'-lacZ</i>	P1(BOS8) × TC3266

^a Genetic symbols are according to reference 5. The genotypes of TC3266 to BOS14 are otherwise like LJ24.

^b The *lacZ* fusions from the three plasmids in Fig. 1 were inserted into the λ attachment site as described previously (2) and transferred by P1 transduction to strain LJ24 by selection for the kanamycin resistance gene linked to the fusions.

TABLE 2. Oxygen and growth-phase regulation of transcription of the *nrd* operons

Strain	Promoter- <i>lacZ</i> fusion	Regulatory genotype	β -Galactosidase sp act ($U \cdot ml^{-1} \cdot OD_{450}^{-1}$) ^a			
			Exponential growth phase		Stationary phase	
			Aerobic	Anaerobic	Aerobic	Anaerobic
BOS8	<i>nrdD</i>	Wild type	1.5	30	16	50
BOS11	<i>nrdD</i>	<i>arcA1</i>	1.5	30	20	50
BOS14	<i>nrdD</i>	<i>fnr1</i>	1.1	1	4	4
BOS7	<i>nrdA</i>	Wild type	370	350	320	200
BOS10	<i>nrdA</i>	<i>arcA1</i>	380	360	330	220
BOS13	<i>nrdA</i>	<i>fnr1</i>	380	320	330	800
BOS9	<i>nrdH</i>	Wild type	0.2	0.2	0.2	0.2

^a The strains were grown and β -galactosidase activity was determined as described in the legend to Fig. 2. The values for aerobic exponential growth are the average of specific β -galactosidase activity in at least six samples taken from the cultures at a cell mass represented by an OD_{450} of between 0.1 and 0.8. The values for anaerobic exponential growth are the slopes from differential plots of β -galactosidase versus OD_{450} (see Fig. 3 for an example), where the slopes are based on at least six points at OD_{450} s of between 0.1 and 0.8. The specific β -galactosidase activity values for the stationary phase are the average of the two to three samples taken after cessation of growth. All values are the average of at least two experiments, which all showed less than 25% variation between experiments.

caused by oxygen limitation. A slight residual stationary-phase induction was, however, observed in the *fnr* mutant. This induction occurred at a much later stage (higher cell density) under both aerobic (Fig. 2A) and anaerobic (Fig. 2B) growth conditions. In the wild type, there was also an approximately twofold stationary-phase induction under anaerobic conditions

(Table 2). This stationary-phase induction is probably not due to RpoS, the stationary-phase sigma factor, because the promoter does not contain any of the RpoS-dependent promoter signatures (Fig. 1B) and the induction occurs very late in the growth cycle relative to the time at which maximal RpoS levels are reached in this strain under these growth conditions (T. Atlung, unpublished data).

Localization of the *nrdD* promoter. Analysis of the sequence between the *treC* and *nrdD* genes revealed two good homologues to FNR binding sites (20) located upstream of two putative -10 promoter sequences (Fig. 1B). To determine which of these is actually the promoter, we mapped the transcript start site by primer extension using the *nrdDG2* primer, complementary to sequences 160 residues downstream of FNR site 1. We detected only one transcript start site in RNA from anaerobically grown wild-type cells, while no specific primer extension products were detected with RNA from the *fnr* mutant (Fig. 4). The same result was obtained with a primer located closer to the promoter (data not shown). Thus the *nrdDG* operon is transcribed from a single promoter that has two FNR

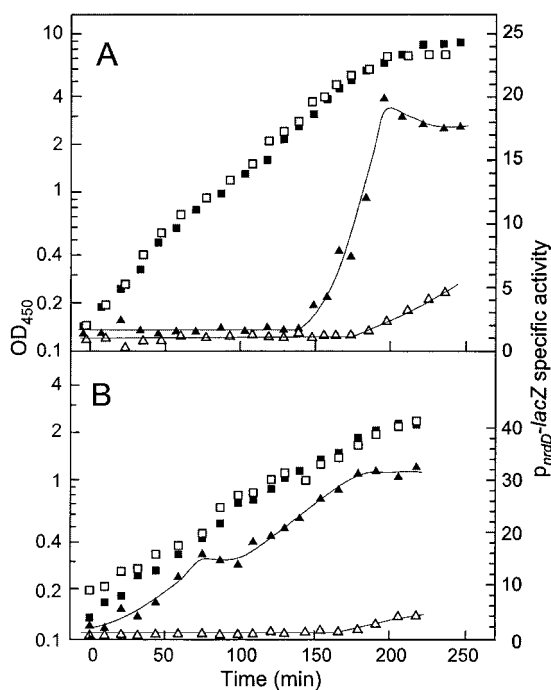


FIG. 2. p_{nrdD}' -*lacZ* expression under aerobic (A) and anaerobic (B) growth conditions. Strains BOS8 (*fnr*⁺) and BOS14 (*fnr1*) were grown with good aeration at 37°C in AB minimal medium (8) supplemented with 1% Casamino Acids (Difco), 0.2% glucose, and 1 μ g of thiamine per ml. At the time corresponding to $t = 0$ min, part of the cultures were shifted to anaerobic growth conditions obtained as previously described (6), while the other part of the cultures remained under aerobic conditions. Samples were taken for determination of cell mass and β -galactosidase activity as described previously (6). Solid symbols indicate BOS8 (*fnr*⁺), and open symbols indicate BOS14 (*fnr1*). Squares indicate OD_{450} , and triangles indicate β -galactosidase specific activity (units per milliliter $\times OD_{450}$).

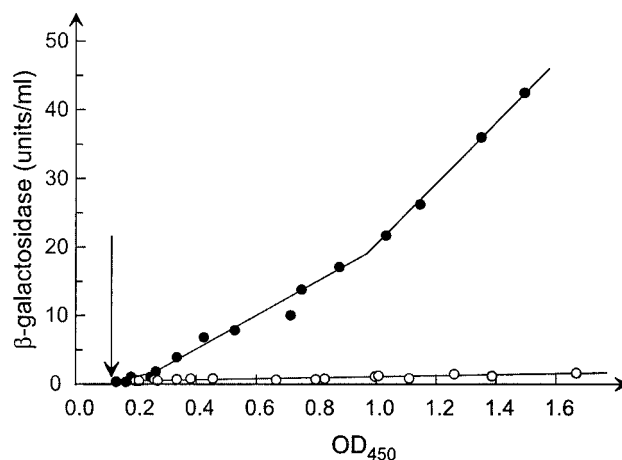


FIG. 3. Differential plot of p_{nrdD}' -*lacZ* expression during a shift to anaerobiosis. The strains BOS8 (*fnr*⁺) and BOS14 (*fnr1*) were grown as described in the legend to Fig. 2 and shifted to anaerobic growth conditions at the OD_{450} indicated by the arrow. Solid circles represent BOS8 (*fnr*⁺), and open symbols represent BOS14 (*fnr1*).

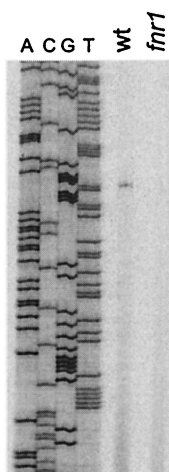


FIG. 4. Primer extension analysis of the *nrdD* transcript start site. The wild-type (wt) strain BOS8 (*fnr*⁺) and mutant strain BOS14 (*fnr1*) were grown under anaerobic conditions as described in the legend to Fig. 2, and RNA was prepared with the MasterPure RNA purification kit from EPI-CENTRE. Primer extension analysis (16) was carried out with 5 μ g of RNA and the ³²P end-labeled primer nrdDG2 (GGTTATCCACAGAA ATTGGGAAAGG). The primer extension products were analyzed by electrophoresis on an 8% acrylamide gel containing 8 M urea, alongside sequencing reactions performed with the same end-labeled primer and the *nrdD* promoter PCR fragment as a template.

sites: one centered at position -65 relative to the transcript start site and the second located on top of the -35 sequence of the promoter. It is therefore very likely that transcription from the *nrdD* promoter is stimulated directly by FNR upon its activation by a shift to anaerobiosis.

Effect of *fnr* and *arcA* on expression of *nrdA*. We also tested the effect of mutations in the two transcriptional regulators on expression of the *nrdAB* promoter, because we had observed a small but reproducible decrease in the levels of β -galactosidase activity in the wild type in the stationary phase under anaerobic growth conditions (Table 2). Expression of *nrdA* was independent of ArcA (Table 2), but we found a significant increase in the anaerobic stationary-phase expression of *nrdA* in the *fnr* mutant. No FNR sites are found close to the *nrdA* promoter, but there is a site showing reasonable homology for one half-site overlapping the promoter-proximal IciA binding site, and since IciA stimulates transcription (12), FNR could repress transcription by interfering with IciA binding. It has previously been found that *nrdAB*-encoded nucleotide reductase levels were increased in an *nrdD*-null mutant under microaerophilic conditions (9). We therefore favor the alternative hypothesis that the low level of NrdD ribonucleotide reductase activity in the *fnr* mutant causes the increased expression of *nrdA* by the same unknown induction mechanism as inhibition of NrdAB reductase activity by hydroxyurea, *nrdA* inactivation by mutation, or the absence of thioredoxin and glutaredoxin.

Conclusions. Expression from the *nrdD* promoter exhibits FNR-dependent and ArcA-independent induction by anaerobiosis and by oxygen limitation in the stationary phase. The promoter is most probably directly activated by binding of FNR to the two FNR binding sites found in positions similar to those found for other FNR-regulated promoters. Expression of the *nrdA* promoter is independent of oxygen availability and

growth phase, except for prolonged growth under anaerobic conditions or entry into the stationary phase during anaerobic growth, where expression is reduced in the wild type but increased in the *fnr* mutant, probably in response to the sum of activity of the nucleotide reductases.

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REFERENCES

1. Atlung, T., and L. Brøndsted. 1994. Role of the transcriptional activator AppY in regulation of the *cyx appA* operon of *Escherichia coli* by anaerobiosis, phosphate starvation, and growth phase. *J. Bacteriol.* **176**:5414–5422.
2. Atlung, T., A. Nielsen, L. J. Rasmussen, L. J. Nellemann, and F. Holm. 1991. A versatile method for integration of genes and gene fusions into the λ attachment site of *Escherichia coli*. *Gene* **107**:11–17.
3. Atlung, T., H. V. Nielsen, and F. G. Hansen. 2002. Characterisation of the allelic variation in the *rpoS* gene in 13 K12 and six other nonpathogenic *Escherichia coli* strains. *Mol. Gen. Genomics* **266**:873–881.
4. Augustin, L. B., B. A. Jacobson, and J. A. Fuchs. 1994. *Escherichia coli* Fis and DnaA proteins bind specifically to the *nrd* promoter region and affect expression of an *nrd-lac* fusion. *J. Bacteriol.* **176**:378–387.
5. Bachmann, B. J. 1990. Linkage map of *Escherichia coli* K-12, edition 8. *Microbiol. Rev.* **54**:130–197.
6. Brøndsted, L., and T. Atlung. 1994. Anaerobic regulation of the hydrogenase 1 (*hya*) operon of *Escherichia coli*. *J. Bacteriol.* **176**:5423–5428.
7. Casado, C., M. Llagostera, and J. Barbe. 1991. Expression of *nrdA* and *nrdB* genes of *Escherichia coli* is decreased under anaerobiosis. *FEMS Microbiol. Lett.* **83**:153–157.
8. Clark, D. J., and O. Maaløe. 1967. DNA replication and the division cycle in *Escherichia coli*. *J. Mol. Biol.* **23**:99–112.
9. Garriga, X., R. Eliasson, E. Torrents, A. Jordan, J. Barbe, I. Gibert, and P. Reichard. 1996. *nrdD* and *nrdG* genes are essential for strict anaerobic growth of *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **229**:189–192.
10. Gibert, I., S. Calero, and J. Barbe. 1990. Measurement of in vivo expression of *nrdA* and *nrdB* genes of *Escherichia coli* by using *lacZ* gene fusions. *Mol. Gen. Genet.* **220**:400–408.
11. Gunsalus, R. P., and S. J. Park. 1994. Aerobic-anaerobic gene regulation in *Escherichia coli*—control by the ArcAB and Fnr regulons. *Res. Microbiol.* **145**:437–450.
12. Han, J. S., H. S. Kwon, J. B. Yim, and D. S. Hwang. 1998. Effect of IciA protein on the expression of the *nrd* gene encoding ribonucleoside diphosphate reductase in *E. coli*. *Mol. Gen. Genet.* **259**:610–614.
13. Jordan, A., E. Aragall, I. Gibert, and J. Barbe. 1996. Promoter identification and expression analysis of *Salmonella typhimurium* and *Escherichia coli* *nrdEF* operons encoding one of two class I ribonucleotide reductases present in both bacteria. *Mol. Microbiol.* **19**:777–790.
14. Jordan, A., F. Aslund, E. Pontis, P. Reichard, and A. Holmgren. 1997. Characterization of *Escherichia coli* NrdH—a glutaredoxin-like protein with a thioredoxin-like activity profile. *J. Biol. Chem.* **272**:18044–18050.
15. Jordan, A., and P. Reichard. 1998. Ribonucleotide reductases. *Annu. Rev. Biochem.* **67**:71–98.
16. Meleforts, O., and A. von Gabain. 1988. Site-specific endonucleolytic cleavages and the regulation of stability of *E. coli ompA* mRNA. *Cell* **52**:893–901.
17. Monje-Casas, F., J. Jurado, M. J. Prieto-Alamo, A. Holmgren, and C. Pueyo. 2001. Expression analysis of the *nrdHIEF* operon from *Escherichia coli*—conditions that trigger the transcript level in vivo. *J. Biol. Chem.* **276**:18031–18037.
18. Prieto-Alamo, M. J., J. Jurado, R. Gallardo-Madueno, F. Monje-Casas, A. Holmgren, and C. Pueyo. 2000. Transcriptional regulation of glutaredoxin and thioredoxin pathways and related enzymes in response to oxidative stress. *J. Biol. Chem.* **275**:13398–13405.
19. Rasmussen, L. J., P. L. Møller, and T. Atlung. 1991. Carbon metabolism regulates expression of the *pfl* (pyruvate formate-lyase) gene in *Escherichia coli*. *J. Bacteriol.* **173**:6390–6397.
20. Spiro, S., and J. R. Guest. 1990. FNR and its role in oxygen-regulated gene expression in *Escherichia coli*. *FEMS Microbiol. Rev.* **75**:399–428.
21. Sun, L., and J. A. Fuchs. 1992. *Escherichia coli* ribonucleotide reductase expression is cell-cycle regulated. *Mol. Biol. Cell* **3**:1095–1105.
22. Tamarit, J., E. Mulliez, C. Meier, A. Trautwein, and M. Fontecave. 1999. The anaerobic ribonucleotide reductase from *Escherichia coli*—the small protein is an activating enzyme containing a [4Fe-4S]²⁺ center. *J. Biol. Chem.* **274**:31291–31296.
23. Tuggle, C. K., and J. A. Fuchs. 1986. Regulation of the operon encoding ribonucleotide reductase in *Escherichia coli*—evidence for both positive and negative control. *EMBO J.* **5**:1077–1085.