Expression Analysis of the nrdHIEF Operon from *Escherichia coli*

**CONDITIONS THAT TRIGGER THE TRANSCRIPT LEVEL IN VIVO**

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*Escherichia coli* has two aerobic ribonucleotide reductases encoded by the *nrdAB* and *nrdHIEF* operons. While NrdAB is active during aerobicis, NrdEF is considered a cryptic enzyme with no obvious function. Here, we present evidence that *nrdHIEF* expression might be important under certain circumstances. Basal transcript levels were dramatically enhanced (25–75-fold), depending on the growth-phase and the growth-medium composition. Likewise, a large increase of >100-fold in *nrdHIEF* mRNA was observed in bacteria lacking Trx1 and Grx1, the two main NrdAB reductants. Moreover, *nrdHIEF* expression was triggered in response to oxidative stress, particularly in mutants missing hydroperoxidase I and alkyl-hydroperoxide reductase activities (69.7-fold) and in cells treated with oxidants (up to 23.4-fold over the enhanced transcript level possessed by cells grown on minimal medium). The mechanism(s) that triggers *nrdHIEF* expression remains unknown, but our findings exclude putative global regulators like RpoS, Fis, cAMP, OxyR, SoxR/S, or RecA. What we have learned about *nrdHIEF* expression indicates strong differences between its regulation and that of the *nrdAB* operon and of genes coding for components of both thioredoxin/glutaredoxin pathways. We propose that *E. coli* might optimize the responses to different stimuli by co-evolving the expression levels for its multiple reductases and electron donors.

Ribonucleotide reductases (RRases)† provide the building blocks for DNA biosynthesis in all living organisms. RRases are grouped into three major classes (class I, II, and III) based on the mechanisms they use for radical generation and on their structural differences. Class I is subdivided further into two subclasses (Ia and Ib) based mainly on allosteric regulation but also on involvement of auxiliary proteins (recently reviewed in Ref. 1). *Escherichia coli* has the coding potential for three different RRases. The NrdAB (class Ia) is active during aerobicis, NrdDG (class III) is strictly anaerobic, and NrdEF (class Ib) is thought to be a cryptic enzyme with no obvious function (1).

Class I enzymes receive the electrons required for the reduction of ribose from small proteins, thioredoxins (Trxs) and glutaredoxins (Grxs), with two redox-active cysteine thiols, which by dithiol-disulfide interchange reduce an acceptor disulfide in the active center of RRase. Trxs and Grxs are kept in a reduced form by NADPH, which reduces the redoxin either via the flavoprotein thioredoxin reductase or via the flavoprotein glutathione reductase and the ubiquitous tripeptide GSH (2). *Escherichia coli* contains two thioredoxins (Trx1 and Trx2), three glutaredoxins (Grx1, Grx2, and Grx3), and a novel redoxin (called NrdH) with thioredoxin-like activity but glutaredoxin-like amino acid sequence (2–5). Grx1 and Trx1 are the two main hydrogen donors of the *E. coli* NrdAB enzyme (3, 4, 6, 7). NrdH is a more specific hydrogen donor for the NrdEF than for the NrdAB enzyme, whereas the opposite is the case for Grx1 (5). Trx1 and Trx2 are hydrogen donors for NrdAB but not for NrdEF (3, 5, 8).

The nrdA and nrdB genes that code for the NrdAB class Ia reductase constitute a tightly regulated transcription unit that does not include the gene for either Trx or Grx. Expression of *nrdAB* genes is cell cycle-regulated and increases when DNA synthesis is inhibited (1). Regulation of *nrdAB* expression in *E. coli* has been shown to be very complex; multiple cis-acting positive regulatory sites identified upstream of the *nrdAB* promoter appear to control, independently but in concert, the cell cycle-dependent transcription and the response to inhibition of DNA synthesis (9). Recent data from our group add further complexity to the *nrdAB* regulation, demonstrating a tight and inverse relation between expression of *nrdAB* and that of genes coding for components of both glutaredoxin and thioredoxin pathways (10). Interestingly, we have observed that induction ratios of *nrdAB* transcription by hydroxyurea (RRase inhibitor) are similar to the increments in *nrdAB* basal expression of mutants lacking both Trx1 and Grx1. Therefore, we have postulated that operation of the NrdAB enzyme in the absence of its two main reductants must lead to disturbances in deoxyribonucleotide production sensed as those caused by hydroxyurea.

The *nrdE* and *nrdF* genes that code for the NrdEF class Ib reductase form a conserved operon where the promoter is followed by four genes (11). Genes *nrdH*, coding for the NrdH-redoxin, and *nrdI*, coding for a protein with a stimulatory effect on ribonucleotide reduction, are present upstream of *nrdEF* (5). It has been reported that the *E. coli* transcription unit is not expressed in sufficient amounts to support growth under nor-
mal laboratory conditions. Neither inhibitors of DNA replication nor DNA damages induce its expression. Thus far, only hydroxyurea has been shown to stimulate the expression of *ndrEF* genes (11).

We have quantitated the *in vivo* transcription of genes encoding for components of both glutaredoxin and thioredoxin pathways by means of a novel multiplex reverse transcription-polymerase chain reaction (RT-PCR) approach (10, 12). In this protocol, all target genes, a housekeeping gene (also named control gene, reference gene, or internal standard), and one external standard are amplified in the same reaction tube. Specific fluorescent primers are used, and amplification products are analyzed with a DNA sequencer. Putative variations in the expression of the housekeeping gene are controlled by the external standard. Relative expressions of the targets to the reference (or to the external standard) are measured. Lately (13), we have experimentally demonstrated that our methodology fulfills all theoretical requirements for precise quantification of both induction and repression of gene transcription. Because of the PCR amplification step, our method displays a much higher sensitivity than those of current techniques for mRNA quantitation, like Northern blotting or primer extension analyses. Here, we used this sensitive experimental approach to identify those growth conditions and stress circumstances under which expression of *ndrHIEF* genes might be required in *E. coli*.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—All bacterial strains were *E. coli* K-12. UC5710 (argB6, nad113, araD81, lacB-bio) was considered the parental wild type (14). UC1342 (~ΔoxyR::kan), UC1394 (~oxyR2), UC1332 (~ΔoxyR::kan), UC827 (~ΔtrxA gshA::kan), UC13585 (~ΔtrxA gskA), and UC1365 (~ΔtrxA gshA ΔoxyR::kan), UC1385 (~ΔtrxA gshA oxyR2), UC1110 (pKM101), UC498 (katG katE::Tn10 pKM101), and UC628 ((~sodA::Mud PR1325 (sodB-bio-1 Δ2) pKM101) have been previously described (10, 15, 16). UC499 (katG17::Tn10), UC4910 (~ahpCF::kan), UC1049 (katE::Tn10), and UC4101 (katG17::Tn10 ΔahpCF::kan) were constructed by P1-mediated transduction. Successful transfer of the katG17::Tn10 mutation was confirmed by assaying catalase and superoxide dismutase 1 activity, as described (17). Successful transfer of the ~ahpCF::kan deletion was confirmed by screening for no DNA amplification with specific primers (18). Strains with the ΔoxyR::kan or ΔsodB-8::cat mutant allele do not induce the expression of genes regulated by OxyR or SodB upon exposure to H2O2 or paraquat, respectively (10). Strains carrying the oxyR2 allele exhibit constitutive high levels of catalase and superoxide dismutase 1 activity (18). Strains with the ΔoxyR::kan or ΔsodB-8::cat mutant allele exhibit undetectable levels of Trx1 and of Grx1 or GSH, respectively (15). Double katG katE or sodA sodB mutants retain ~1% of the wild-type catalase or superoxide dismutase level, respectively (16). Double katG ahpCF mutants are known to have increased intracellular levels of peroxides (19, 20). Plasmid pKM101 carries the *macAB* genes, which make bacteria more susceptible to SOS-dependent mutagenesis (21). Bacteria were grown in Luria-Bertani (LB) nutrient broth or M9 minimal media more susceptible to SOS-dependent mutagenesis (21). Bacteria (19, 20). Plasmid pKM101 carries the genes for the *NrdH*-redoxin (*ndrH*), the *NrdI* stimulatory protein (*ndrI*), and the *R1E* (*ndrE*) and *R2F* (*ndrF*) subunits of *E. coli* class Ib RRase. As described previously (10, 12), the *gapA* gene, which codes for *N*-glyceraldehyde-3-phosphate dehydrogenase, was used as an internal standard. The internal standard normalizes variations in RNA extraction, reverse transcription, and PCR amplification among samples. To evaluate the extent of putative variations in the expression of the reference gene, an external standard was included, as described (10). The external standard was an *in vitro* synthesized RNA fragment encoded by the *CYP1* gene from *Liza aurata*. This external standard has no homology with the *E. coli* genome.

**Multiple RT-PCR for *in Vitro* Quantification of *ndrHIEF* Transcription**—RNA purification and cDNA synthesis were as described (10). At least two independent RNA preparations were isolated for each experimental condition, each RNA sample being retrotranscribed on three separated occasions. PCR amplification of cDNA was carried out using all of the primer pairs listed in Table I or using just those for the *ndrH* and *ndrE* target genes, the *gapA* reference gene, and the internal standard. The multiplex PCR amplification was performed in a mixture (25 μl final volume) containing MPCR buffer 3 (Maxim Biotech Inc., San Francisco, CA) supplemented with 1 mM MgCl2, a 250 μM concentration of each dNTP, 1 μl of cDNA solution, 1.25 units of AmpliTaq Gold DNA polymerase, and primers at the following concentrations: 0.07 μM (*ndrH*), 0.09 μM (*ndrE*), 0.04 μM (*gapA*), and 0.14 μM (external standard). After incubation at 95 °C for 12 min (for activation of DNA polymerase), 26 cycles of PCR were performed. Each cycle consisted of 1 min of denaturation at 94 °C and 45 s of annealing and enzymatic primer extension at 70 °C. These multiplex PCR conditions were optimized as detailed (13) to ensure that the amplifications were in the exponential phase and that the efficiencies remained constant in the course of the PCR. Reaction products were analyzed as described previously (12). Differences among PCR outcomes were normalized by comparing the fluorescence intensity of each band to that resulting from *gapA* amplification (internal standard). The levels of *gapA* in reference to the external standard remained essentially equal among the strains and experimental conditions investigated in this work. Consequently, changes detected with reference to the housekeeping *gapA* gene were accurately attributed to variations in the expression levels of the target genes under analysis. Samples for comparison of different experimental conditions or different bacterial strains were handled in parallel. Data are the means ± S.E. from *n* ≥ 6 independent multiplexed PCR amplifications. Statistical comparisons were done by a hierarchical analysis of variance with SAS software (Statistical Analysis System, version 6.03). Data presented here as relative expression ratios do not provide any indication of the mechanism that contributes to the dynamic control of a particular mRNA expression, whether it is the rate of transcription initiation or the rate of transcript turnover.

**RESULTS**

**Quantification of the *ndrHIEF* Operon Expression**—In a prior study (11), expression of *ndrEF* genes was quantified by a competitive RT-PCR, in which the competitor had an internal deletion of 99 base pairs and the reaction products were resolved in ethidium bromide-agarose gels and analyzed by densitometry. Several points at which errors can occur by using such a methodology include differences in amplification efficiencies because of the great size difference between the competitor and the target, possible heteroduplex formation, and the need to compensate for differences in fragment label incorporation. When measuring rare species of mRNA, which requires a high number of PCR cycles, the control of those problems is of maximal importance, even for relative quantification (e.g. see Refs. 22–24). In this work, by taking advantage of the high sensitivity, accuracy, and reproducibility of our multiplex RT-PCR procedure (13), we monitored variations in basal levels of *ndrHIEF* expression and in response to several stress conditions. To achieve this objective, we first confirmed that transcription from the *ndrHIEF* promoter normally takes place in wild-type cells and increases upon hydroxyurea exposure (11). UC5710 cells were, thus, exposed to increasing concentrations of hydroxyurea (varying from 1 to 30 mM) for 30 min. Thereafter, the expression of the genes that constituted the *ndrHIEF* operon was examined by using the primer pair set in Table I. Basal levels of *ndrH*, *ndrI*, *ndrE*, and *ndrF* gene expression were readily detected and increased upon hydroxyurea treatment (data not shown). As an average, an induction level of 12.4 ± 2.5-fold (relative to untreated bacteria) was observed at the minimal dose assayed of 1 mM. It is worth noting that in the prior study by Jordan et al. (11), such an
induction level was observed under much more acute treatment conditions (bacteria grown in the presence of 50 mM hydroxyurea). Since we do not detect differences in the number of times that expression of each nrd gene was increased by hydroxyurea, the multiplex PCR was simplified in further experiments by amplifying just two (nrdH and nrdE) of the four genes included in the nrdHIEF transcription unit (in addition to the internal and external standards).

Effects of Growth Conditions on Basal Levels of nrdHIEF Expression—The expression profiles of nrdH and nrdE genes throughout the growth curve of wild-type UC5710 cells in rich LB medium are shown in Fig. 1. Maximal expression levels were observed at the initial stages of exponential growth (A_{600} \approx 0.2); then gene expression decreased rapidly, reaching 25-fold lower levels as the culture continued growing from midexponential to stationary phase (A_{600} \approx 4). Fis and RpoS (also named σ{^32} or σ{^{32S}}) are two regulatory proteins with acute growth phase-dependent expression in LB medium (25, 26). To determine the hypothetical influence of Fis and RpoS on the nrdH and nrdE transcript levels during the course of cell growth in LB, both fis::Tn7 and rpoS::Tn10 mutants were analyzed in comparison with otherwise isogenic wild-type cells. Data obtained (not shown) indicate that neither the presence of Fis nor the presence of RpoS affects the expression of nrdH and nrdE genes in E. coli.

Notable variations in gene expression were observed also with respect to the composition of the growth medium (Fig. 1). Thus, bacteria growing on M9 minimal medium with glucose had from 2-fold (A_{600} = 0.2) to 75-fold (A_{600} = 0.7) higher levels of nrdH and nrdE transcripts than did bacteria growing on rich LB medium alone. Since carbon sources control intracellular cAMP levels (27), we examined next the effects on nrdH and nrdE expression, of the addition of glucose to the LB nutrient medium (to 2 g/liter), and of replacement of glucose by lactose as the sole carbon source in the M9 minimal medium. No dependence on carbon source was observed (data not shown).

As indicated above, -fold variations were identical for both nrdH and nrdE genes; nrdH data will henceforth be considered representative of the entire nrdHIEF transcription unit.

Effects of Deficiencies in Thioredoxin and Glutaredoxin/GSH Pathways or in Antioxidant Enzymes on Basal Levels of nrdHIEF Expression—In addition to the classic function of acting as reductants for RRase, both the Trx and Grx/GSH pathways are required to maintain the low thiol-disulfide redox potential of the bacterial cytoplasm (20, 28). On the other hand, catalase, alkyl hydroperoxide reductase, and superoxide dismutase activities maintain the steady-state concentrations of peroxides and superoxide beneath their respective toxicity thresholds (29). The influence of missing various components of these systems on basal levels of nrdHIEF expression is studied in Table II. Significant increments in the steady-state levels of nrdHIEF transcript were detected in mutants compromised either in both the Trx- and Grx/GSH-reducing pathways or in the removal of endogenous oxidants. Of particular note is the large up-regulation (>30-fold) quantified in bacteria lacking Trx1 in combination with either Grx1 (UC827) or GSH (UC1358) or missing both catalase hydroperoxidase I and alkyl hydroperoxide reductase activities (UC4101).

Since OxyR can be activated either by challenge with an oxidant such as H₂O₂ or directly by a change in the cellular thiol-disulfide status caused by the inactivation of the two Trx- and Grx/GSH-reductive pathways (20, 28), it seemed possible that the transcriptional induction of nrdHIEF that we report in Table II was due to activation of OxyR. This possibility was investigated by studying the effects of oxyR mutant alleles on basal levels of gene expression in UC1358. As shown in Table II, expression of nrdHIEF genes was modulated by mutations in the oxyR regulatory locus, but it followed a pattern opposite to that exhibited by most OxyR-regulated genes (10, 29). Therefore, the high basal level of nrdHIEF message in UC1358 (32.7-fold) was further elevated (not diminished) in its ΔoxyR::kan null mutant derivative (UC1363), where a 54.4-fold increase in the amount of nrdHIEF transcript was quantified. On the contrary, a decrease (not an increase) in the steady-state level of UC1358 was observed in the strain (UC1395) that carries the oxyR2 constitutive mutation. These results indicated that OxyR is not directly involved in the nrdHIEF up-regulation reported in Table II.

The higher increment in basal level of nrdHIEF expression caused by the ΔoxyR::kan null allele might therefore be attributed to the underexpression in UC1363 of OxyR-regulated genes involved in antioxidant defense, like those coding for catalase hydroperoxidase I and alkyl hydroperoxide reductase (10, 18, 29). A similar (while opposite) argument might explain the lower increment quantified in bacteria with the oxyR2 constitutive mutation. Likewise, the difference between UC827 and UC1358 with respect to nrdHIEF expression can be explained by differences in the expression levels of OxyR-regulated genes. The strain (UC1358) with higher levels of antioxidant defenses (10) displayed the lower increment in nrdHIEF/gapA ratio (Table II).

Gene Expression Induction by Hydrogen Peroxide and Superoxide—To gain further information for the regulation of nrdHIEF expression under oxidative stress conditions, wild-type bacteria were exposed to increasing concentrations of H₂O₂ or paraquat (a superoxide-generating compound). Since OxyR and SoxS together with SoxS are key regulators of the adaptive responses to H₂O₂ and superoxide radicals, respec-
Basal levels of gene expression in bacteria defective in Trx and Grx/GSH pathways or in antioxidant enzymes

Cells were grown in LB broth to reach an OD_{600} of 0.7. Data are the mean ± S.E. of the fluorescent signal of the nrd target sequence relative to that of gapA (internal standard). Boldface type indicates statistically significant increments relative to wild type UC5710.

| Strain          | Relevant phenotype | nrdHIEF/gapA ratio | Variation |
|-----------------|--------------------|--------------------|-----------|
| UC5710          | Wild type          | 0.013 ± 0.001      | 1.0       |
| UC1342          | OxyR               | 0.020 ± 0.002      | 1.5       |
| UC1394          | OxyR               | 0.012 ± 0.001      | 0.9       |
| UC1101          | Wild type/pKM101   | 0.020 ± 0.002      | 1.5       |
| Deficient in Trx and Grx/GSH pathways |                |                    |           |
| UC827           | Trx1 - Grx1−       | 1.342 ± 0.112      | 103.2     |
| UC1358          | Trx1 - Gh−         | 0.425 ± 0.023      | 32.7      |
| UC1363          | Trx1 - Gh− OxyR−   | 0.707 ± 0.103      | 54.4      |
| UC1395          | Trx1 - Gh− OxyR−   | 0.154 ± 0.011      | 11.8      |
| Deficient in peroxide/superoxide scavenging enzymes |                |                    |           |
| UC499           | KatG−              | 0.015 ± 0.001      | 1.2       |
| UC4910          | AhpCF−             | 0.009 ± 0.001      | 0.7       |
| UC1049          | KatG − KatE−       | 0.031 ± 0.002      | 2.4       |
| UC4101          | KatG− AhpCF−       | 0.906 ± 0.057      | 69.7      |
| UC498           | KatG− KatE− /pKM101| 0.225 ± 0.003      | 1.9       |
| UC628           | SodA− SodB− /pKM101| 0.076 ± 0.004      | 5.8       |

Gene Expression Induction by Alkyl Hydroperoxide and 4NQO—AhpC together with AhpF reduces a variety of physiologically relevant alkyl hydroperoxides such as thymine hydroperoxide and linoleic acid hydroperoxide as well as non-physiological model alkyl hydroperoxides like tBOOH (32). Encouraged by the high increment in basal level of nrdHIEF mRNA caused by the simultaneous deficiency in catalase hydroperoxidase I and alkyl hydroperoxide reductase activity (UC4101 in Table II), we examined the effect of tBOOH treatments on expression of nrdHIEF operon (Fig. 3). In this experiment, we used a set of primers (13) that allows us to compare the response of nrdHIEF genes with that of genes encoding for the main aerobic RRase (nrdAB operon) besides that of well known components of the E. coli oxidative stress responses (like oxyS and grxA genes) (10). While the amount of nrdAB transcript remains basically unchanged, the nrdHIEF transcript level was readily induced by tBOOH. As shown in Fig. 3, the oxidative stress-responsive genes, oxyS and grxA, gave also a clear positive response to tBOOH treatments.

4NQO is a widely studied model mutagen and carcinogen, which derives its activity from the induction of both bulky adducts (for that, it is often referred to as UV radiation-like).
and oxidative damages in cellular DNA (33). As in the case of tBOOH, treatments with 4NQO elevated the *nrdHIEF* and the *oxyS* and *grxA* transcript levels without affecting the *nrdAB* expression. Since 4NQO is known to trigger the SOS response (33), we next studied whether this compound increased *nrdHIEF* expression in the absence of RecA protein. No difference between RecA<sup>+</sup> and RecA<sup>-</sup> cells was observed (data not shown), indicating that this effect is not SOS-dependent.

In contrast to chemical oxidants, *N*-methyl-*N*-9-nitro-*N*-nitroso-guanidine, which is a strong monofunctional alkylating agent that methylates cellular DNA, resulting in multiple types of primary lesions (33), did not affect *nrdHIEF* expression in wild-type *E. coli* (data not shown).

**DISCUSSION**

Previous studies have reported that suppression by the *nrdHIEF* genes of the inviability of *E. coli* strains defective in either the NrdAB reductase or three of its reductants (Grx1, Trx1, and Trx2) requires a second gene copy placed in either the chromosome or a cloning plasmid (11, 34). Based on this genetic evidence, it is commonly accepted that the *nrdHIEF* operon is underexpressed in bacteria grown at standard aerobic conditions, thus somewhat calling into question its physiological significance. The main goal of this study was the accurate quantification by multiplex RT-PCR of variations in *nrdHIEF* transcript levels in order to elucidate the growth conditions and stress circumstances under which expression of *nrdHIEF* genes might become important to *E. coli*.

Results presented in this work confirm that transcription of the *nrdHIEF* operon normally takes place in *E. coli* cells grown in LB medium to midexponential phase (11). Nevertheless, we present the first indication that this low basal level of *nrdHIEF* mRNA can be dramatically enhanced in wild-type cells as a function of the growth phase and the composition of the growth medium: a pronounced increase (from 25- to 75-fold) in *nrdHIEF* transcript could be monitored at the initial stages of exponential growth in LB and in cells cultured in M9 minimal medium. Of note is the additional observation of no significant differences among the relative transcript levels for the genes of the *nrdHIEF* operon, indicating for the first time that the expression of the genes encoding the NrdEF ribonucleotide reductase is tightly co-regulated with that of genes encoding accessory proteins, like its specific NrdH-redoxin hydrogen donor.

We hypothesize that the strong growth phase- and growth medium-dependent regulation of *nrdHIEF* transcription might have a functional significance for wild-type cells. It is well known that the physiology of a bacterial cell shifts between the phases of a culture and with the quality of the growth medium and that many of these changes are realized at the level of gene expression (35). Therefore, while the rich medium contains preformed building blocks of macromolecule synthesis, in the minimal medium the carbon backbone of the glucose molecule
is rearranged through biosynthetic pathways to generate each of the building blocks de novo. The higher expression of *nrdHIEF* genes in minimal medium might thus be indicative of the need to generate the building blocks for DNA biosynthesis de novo from glucose. Accordingly, a recent single experiment that used DNA arrays of the entire set of *E. coli* genes to discover genomic expression patterns has revealed that these genes with a pivotal role in central metabolism tend to be expressed at higher levels in minimal medium than in rich LB medium (36). Here, we verified this tendency for the expression of the *nrdHIEF* operon. Many genes having a growth phase- and growth medium-dependent regulation are under global regulatory mechanisms like those mediated by RpoS, Fis, or the intracellular levels of cAMP (35, 36). Our data indicate, however, that these global regulators are not responsible for the *nrdHIEF* expression pattern, thus making a difference with the *nrdAB* operon, which is known to be under the positive regulation of Fis protein (37).

The striking up-regulation of the *nrdHIEF* operon in wild-type cells under certain growth conditions raises an intriguing question: why is the inactivation of the *nrdAB* operon lethal to *E. coli* cells in the presence of oxygen? The straight answer to this question is that this up-regulation is conceivably insufficient to complement the lack of the first *NrdAB* RRase or three of its reductants (Grx1, Trx1, and Trx2) unless a second extra copy of the *nrdHIEF* operon is placed on the bacterial chromosome. In this context, quantification at the protein level would be of maximal interest, since it has been postulated that translation of *nrdE* message might be low because the start codon is TTG (11).

We have also been able to show that the basal level of *nrdHIEF* mRNA is dramatically increased (>100-fold) in bacteria (UC827) simultaneously lacking Trx1 and Grx1, the two main reductants of the *NrdAB* reductase. We speculate that this enormous increment in *nrdHIEF* expression might be physiologically relevant for the viability of UC827 (38). This *trxA grxA* double mutant would maintain the balanced supply of deoxyribonucleotides required for DNA synthesis by triggering the transcription of the operons (*nrdAB* and *nrdHIEF*) (Ref. 12; this work) that code for both aerobic RRases and for the *NrdH* reductant. In this respect, it is worth noting that while DNA-damaging agents that induce the SOS response produce in general an overexpression of *nrdAB* genes, these agents have no effect on *nrdHIEF* expression (Ref. 11; this work).

In short, this report strongly suggests that *nrdHIEF* expression might be important under specific physiological circumstances. Findings presented here open numerous ways for future studies. One challenge will be the construction of a mutant lacking the entire *nrdHIEF* transcription unit in order to elucidate further compensations among the expression of both aerobic RRases and their reductants under either normal or stressed conditions. The multiplex RT-PCR approach will be of relevance in these coming experiments. Nevertheless, quantifications at the protein level will also be necessary in order to unravel the relationships between mRNA production and protein synthesis.

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What might an enhanced *nrdHIEF* expression do in the *E. coli* oxidative stress response? An answer to this question could be to increase the free radical scavenging capacity of cells by increasing the *NrdH* protein level. In this respect, it is worth noting that Trx is a highly efficient antioxidant with a role in protecting *E. coli* against oxidative stress (39–41); *NrdH* with a redox potential of −248.5 mV is as potent a reductant as Trx (5). Furthermore, an enhanced ribonucleotide reduction capacity should be advantageous under oxidative stress conditions, since reactive oxygen species escaping from antioxidant defenses can inflict much oxidative damage on DNA (42).

The mechanism by which *nrdHIEF* expression is triggered under oxidative stress conditions remains elusive, but data reported indicate that the presence of reactive oxygen species must be sensed by regulators that are distinct from both OxyR and SoxRS. Contrary to what we have learned in this work about *nrdHIEF* expression, the expression of the *nrdAB* operon that codes for the main class I reductase was not induced by oxidative stress, in agreement with previous results (10). Interestingly, however, genes that code for two (Grx1 and Trx2) out of the five known *NrdAB* reductants, together with those that code for the enzymes (glutathione reductase and thioredoxin reductase) that regenerate their reduced forms, are part of the OxyR regulon. These findings suggest that *E. coli* might optimize the responses to different stress situations by co-evolving the expression levels for multiple RRases and reductants. In this respect, it is worth noting that while DNA-damaging agents that induce the SOS response produce in general an overexpression of *nrdAB* genes, these agents have no effect on *nrdHIEF* expression (Ref. 11; this work).
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