Expression Analysis of the nrdHIEF Operon from Escherichia coli

CONDITIONS THAT TRIGGER THE TRANSCRIPT LEVEL IN VIVO

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SUMMARY

*Escherichia coli* has two aerobic ribonucleotide reductases encoded by the *nrdAB* and *nrdHIEF* operons. While NrdAB is active during aerobiosis, NrdEF is considered a cryptic enzyme with no obvious function. Here, we present evidences that *nrdHIEF* expression might be important under certain circumstances. Therefore, basal transcript levels were dramatically enhanced (25- to 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 owned 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 on *nrdHIEF* regulation indicates strong differences with the regulation of the *nrdAB* operon and that 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.
INTRODUCTION

Ribonucleotide reductases (RRases)\(^1\) 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 in allosteric regulation but also on involvement of auxiliary proteins (recently reviewed in 1). *Escherichia coli* has the coding potential for three different RRases. The NrdAB (class Ia) is active during aerobiosis, 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 glutathione (GSH) (2). *E. 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

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\(^1\) The abbreviations used are: RRase, ribonucleotide reductase; Trx, thioredoxin; Grx, glutaredoxin; GSH, glutathione; RT-PCR, reverse transcription-polymerase chain reaction; SOD, superoxide dismutase; HPI, hydroperoxidase I; H\(_2\)O\(_2\), hydrogen peroxide; PQ, paraquat; tBOOH, t-butyl hydroperoxide; 4NQO, 4-nitroquinoline 1-oxide; MNNG, N-methyl-N’-nitro-N-nitrosoguanidine; ROS, reactive oxygen species.
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 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 reductans 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 normal 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 nrdEF 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 nrdHIEF genes might be required in *E. coli*. 
EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions: All bacterial strains were *E. coli* K-12. UC5710 \([arg56, nad113, araD81, \Delta(uvrB-bio)]\) was considered the parental wild type (14). UC1342 (\(\Delta\text{oxyR::kan}\)), UC1394 (\(\text{oxyR2}\)), UC1333 (\(\Delta\text{sox-8::cat}\)), UC827 (\(\Delta\text{trxA grxA::kan}\)), UC1358 (\(\Delta\text{trxA gshA}\)), UC1363 (\(\Delta\text{trxA gshA OxyR::kan}\)), UC1395 (\(\Delta\text{trxA gshA oxyR2}\)), UC1101 (pKM101), UC498 (\(\text{katG katE::Tn10 pKM101}\)) and UC628 (\((\text{sodA::Mud PR13})25 (\text{sodB-kan})1-\Delta2\) pKM101), have been previously described (10,15,16). UC499 (\(\text{katG17::Tn10}\)), UC4910 (\(\text{ahpCF::kan}\)), UC1049 (\(\text{katG katE::Tn10}\)) and UC4101 (\(\text{katG17::Tn10 ahpCF::kan}\)) were constructed by P1-mediated transduction. Successful transfer of the \(\text{katG17::Tn10}\) mutation was confirmed by assaying catalase HPI activity, as described (17). Successful transfer of the \(\text{ahpCF::kan}\) deletion was confirmed by screening for no DNA amplification with specific primers (18). Strains with the \(\Delta\text{oxyR::kan}\) or \(\Delta\text{sox-8::cat}\) mutant allele do not induce the expression of genes regulated by OxyR or SoxR/S, upon exposure to H\(_2\)O\(_2\) or paraquat, respectively (10). Strains carrying the \(\text{oxyR2}\) allele exhibit constitutive high levels of OxyR-regulated gene expression (10). Strains with the \(\Delta\text{trxA}\) and with either the \(\text{grxA::kan}\) or the \(\text{gshA}\) mutant allele exhibit undetectable levels of Trx1 and of Grx1 or GSH, respectively (15). Double \(\text{katG katE}\) or \(\text{sodA sodB}\) mutants retain <1% of the wild-type catalase or SOD level, respectively (16). Double \(\text{katG ahpCF}\) mutants are known to have increased intracellular levels of peroxides (19,20). Plasmid pKM101 carries the \(\text{mucAB}\) genes, which make bacteria more susceptible to SOS-dependent mutagenesis (21).

Bacteria were grown in Luria-Bertani (LB) nutrient broth or M9 minimal medium (glucose at 2g/l), as described (10).

Treatments—*E. coli* cells from an overnight culture in LB broth were centrifuged and diluted 100-fold into 50 ml of M9 minimal medium and incubated at 37°C and 150 rpm to reach
an OD$_{600}$ of 0.2. At this stage, the bacteria were further grown in the absence or the presence of hydroxyurea, hydrogen peroxide (H$_2$O$_2$), paraquat (PQ), $t$-butyl hydroperoxide (tBOOH), 4-nitroquinoline 1-oxide (4NQO) or $N$-methyl-$N'$-nitro-$N$-nitrosoguanidine (MNNG), for a fixed time period. The cells were then rapidly cooled to 0°C for total RNA purification.

**Primer** — Primers (listed in Table I) were designed with the Oligo 6.1.1/98 (Molecular Biology Insights Inc., Plymouth, MN) program, in order to obtain the highest specificity and performance in multiplexed PCR reactions. Target genes code for the NrdH-redoxin ($nrdH$), the NrdI stimulatory protein ($nrdI$), and the R1E ($nrdE$) and R2F ($nrdF$) subunits of *E. coli* class Ib RRase. As described previously (10,12), the gapA gene, which codes for D-glyceraldehyde-3-phosphate dehydrogenase, was used as 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 *CYP1A* gene from *Liza aurata*. This external standard has no homology with the *E. coli* genome.

**Multiplex RT-PCR for In Vivo Quantification of $nrdHIEF$ 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 the primer pairs listed in Table I, or using just those for the $nrdH$ and $nrdE$ target genes, the gapA reference gene and the external standard. In this last case, 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 MgCl$_2$, 250 µM 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 (nrdH), 0.09 µM (nrdE), 0.04 µM (gapA) and 0.14 µM (external standard). After incubation at 95°C for 12 min (for activation of DNA polymerase), twenty-six cycles of PCR were performed. Each cycle consisted of 1 min 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 the efficiencies remained constant in the course of the PCR. Reaction products were quantified as described previously (12). Differences among PCR outcomes were normalized by comparing the fluorescent 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 ± standard errors of the means (SEM) from n (e 6) independent multiplexed PCR amplifications. Statistical comparisons were done by a hierarchical ANOVA with SAS software (Statistical Analysis System v. 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 concentration, whether it is the rate of transcription initiation or the rate of transcript turnover.
RESULTS

Quantification of the nrdHIEF Operon Expression—In a prior study (11), expression of nrdEF genes was quantified by a competitive RT-PCR, in which the competitor had an internal deletion of 99 bp 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 differences in fragment label incorporation. When measuring rare species of mRNA, which requires high number of PCR cycles, the control of those problems are of maximal importance, even for relative quantification (e.g. see 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 nrdHIEF expression, and in response to several stress conditions. To achieve this objective, we first confirm that transcription from the nrdHIEF 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 5 min. Thereafter, the expression of all the genes that constitute the nrdHIEF operon was examined by using the primer pair set in Table I. Basal levels of nrdH, nrdI, nrdE and nrdF gene expression were readily detected and increased upon hydroxyurea treatment (data not shown). As 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 is shown in Fig. 1. Maximal expression levels were observed at the initial stages of exponential growth (OD600 = 0.2); then, gene expression decreased rapidly reaching 25-fold lower levels as the culture continued growing from mid-exponential to stationary phase (OD600 = 0.4). Fis and RpoS (also named σS or σ38) 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::767 and rpoS::Tn10 mutants were analyzed in comparison to 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 nrdI 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 (OD600 = 0.2) to 75-fold (OD600 = 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 addition of glucose to the LB nutrient medium (to 2 g/l) 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 SOD 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 HPI and alkyl hydroperoxide reductase activities (UC4101).

Since OxyR can be activated either by challenge with an oxidant such as H$_2$O$_2$ 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 ∆oxyR::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, thus, be attributed to the underexpression in UC1363 of OxyR-regulated genes involved in antioxidant defense, like those coding for catalase HPI and alkyl hydroperoxide reductase (10,18,29). A similar (while opposite) argumentation 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 H_{2}O_{2} or paraquat (a superoxide-generating compound). Since OxyR and SoxR together with SoxS are key regulators of the adaptive responses to H_{2}O_{2} and superoxide radicals, respectively (recently reviewed in 29), strains with mutations that eliminate either OxyR (UC1342) or both SoxR and SoxS (UC1333) were used in conjunction with wild-type bacteria (UC5710). As shown in Fig. 2, induction of *nrdHIEF* mRNA was readily seen in response to both oxidants. Therefore, increments of 23.4-fold and 4.3-fold were quantified shortly (5 min) upon exposure of wild-type cells to 100 µM H_{2}O_{2} or 500 µM PQ concentration, respectively. Inductions were preserved in the *oxyR* and *soxR/S* mutant strains, indicating that neither OxyR (in agreement with the results above) nor
SoxR/S are involved in the oxidative stress stimulation of \textit{nrdHIEF} expression. In fact, as expected from data in Table II, bacteria compromised in the removal of H$_2$O$_2$ (oxyR) or superoxide (soxR/S) exhibited higher (not lower) levels of induction, compared to the otherwise isogenic control strain. Therefore, the increased level of H$_2$O$_2$ induction in the OxyR mutant and that of PQ induction in the SoxR/S mutant is expected for the inability of these bacteria to induce both \textit{katG} and \textit{ahpCF} (18) or \textit{sodA} (30) transcription upon H$_2$O$_2$ or PQ exposure, respectively. Increased level of PQ induction in the OxyR mutant is additionally explained by the spontaneous and SOD conversion of O$_2^{•-}$ to H$_2$O$_2$ (31).

\textit{Gene Expression Induction by Alkyl Hydroperoxide and 4NQO—}AhpC together with AhpF reduce a variety of physiologically relevant alkyl hydroperoxides such as thymine hydroperoxide and linoleic acid hydroperoxide, as well as nonphysiological model alkyl hydroperoxides like tBOOH (32). Encouraged by the high increment in basal level of \textit{nrdHIEF} mRNA caused by the simultaneous deficiency in catalase HPI and alkyl hydroperoxide reductase activity (UC4101 in Table II), we examined the effect of tBOOH treatments on expression of \textit{nrdHIEF} operon (Fig. 3). In this experiment, we used a set of primers (13) that allows to compare the response of \textit{nrdHIEF} genes with that of genes encoding for the main aerobic RRase (\textit{nrdAB} operon), besides that of well-known components of the \textit{E. coli} oxidative stress responses (like \textit{oxyS} and \textit{grxA} genes) (10). While the amount of \textit{nrdAB} transcript remains basically unchanged, the \textit{nrdHIEF} transcript level was readily induced by tBOOH. As shown in Fig. 3, the oxidative-stress responsive genes, \textit{oxyS} and \textit{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 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 study next if this compound increased *nrdHIEF* expression in the absence of RecA protein. No difference between RecA+ and RecA- cells was observed (data not shown), indicating that this effect is not SOS dependent.

In contrast to chemical oxidants, MNNG, 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 these genetic evidences, it is commonly accepted that the *nrdHIEF* operon is underexpressed in bacteria grown at standard aerobic conditions, thus somewhat questioning 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 mid exponential phase (11). Nevertheless, we present the first indication that this low basal level of *nrdHIEF* mRNA can be dramatically enhanced in wild-type bacteria 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 are 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 contained 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 those 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, as it has been postulated that translation of nrdE message might be low because the start codon is TTG (11).

We have been also able to show that 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) (12, this work) that code for both aerobic RRases and for the NrdH reductant. To this respect, its worth to note that (i) NrdH, the specific reductant of the NrdEF enzyme, is also a functional hydrogen donor for the NrdAB reductase (5), and (ii) although the other two known reductants for NrdAB remain in trxA grxA mutant cells, one (Grx3) is highly inefficient (4) and the other (Trx2) has a low level of expression under normal aerobic conditions (3,34).

Oxidative stress is an unavoidable by-product of aerobic life. Oxidative stress is caused by exposure to H2O2, superoxide anion and hydroxyl radical, which in turn damage proteins, nucleic acids, and cell membranes producing detrimental molecules like alkyl hydroperoxides (29,32). In this paper, we present evidences that nrdHIEF expression is triggered in E. coli when
confronting oxidative stress. Imbalances in the intracellular pro-oxidant/antioxidant ratio were produced either by exposing the cells to different chemical oxidants (H\textsubscript{2}O\textsubscript{2}, PQ, tBOOH and 4NQO) or by loss of major antioxidant defenses (catalase, SOD and alkyl hydroperoxide reductase activities). Under both situations large increments in \textit{nrdHIEF} transcript levels were quantified. Of particular note is the vast up-regulation observed in bacteria missing both catalase HPI and alkyl hydroperoxide reductase (69.7-fold) and, in general, in bacteria exposed to the oxidants. In this latter situation the increments (up to 23.4-fold) were observed shortly (5 min) upon oxidant exposure and over the already enhanced basal levels of \textit{nrdHIEF} mRNA displayed by cells grown on minimal medium.

What might an enhanced \textit{nrdHIEF} expression do in the \textit{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 \textit{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 ROS escaping from antioxidant defenses, can inflict many oxidative damages on DNA (42).

The mechanism by which \textit{nrdHIEF} expression is triggered under oxidative stress conditions remains elusive, but data reported indicate that the presence of ROS must be sensed by regulators that are distinct from both OxyR and SoxR/S. Contrarily to what we have learned in this work about \textit{nrdHIEF} expression, the expression of \textit{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 (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 next coming experiments. Nevertheless, quantifications at the protein level will be also necessary in order to unravel the relationships between mRNA production and protein synthesis.

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FIGURE LEGENDS

Fig. 1. Effect of growth phase and growth medium on basal levels of gene expression. Wild-type cells (UC5710) from an overnight culture in LB broth were centrifuged and diluted 100-fold into 50 ml of fresh LB broth or M9 minimal medium. Bacteria were then incubated at 37°C with shaking at 150 rpm. Samples were collected at regular time intervals and frozen with liquid nitrogen. Culture growth was monitored by measuring OD600. Growth rate was 28 min on the rich LB medium and 48 min on M9 minimal medium. The mean values of the fluorescence signal of the nrdH (left) or nrdE (right) target sequence relative to that of gapA (internal standard) were plotted as a function of the OD600 value. Bacteria grown in LB broth are indicated by the line and bacteria grown in M9 minimal medium by lightly shaded bars (OD600 of 0.2 and 0.7). Error bars represent SEM.

Fig. 2. Induction of gene expression by H2O2 and PQ. UC5710 (wild type), UC1342 (oxyR::kan) and UC1333 (sox-8::cat) cells were treated (see Experimental Procedures) for 5 min with H2O2 or PQ at the indicated concentrations. The mean values of the fluorescence signal of the nrdH target sequence (considered representative of the entire nrdHIEF transcription unit) relative to that of gapA (internal standard) were plotted for untreated and H2O2 or PQ treated bacteria. Error bars represent SEM. To facilitate the comparison among different bacterial strains, data were divided by that of untreated UC5710. These fold increments are given in parentheses. Only statistically significant increments are indicated.

Fig. 3. Induction of gene expression by tBOOH and 4NQO. UC5710 (wild type) cells were exposed (see Experimental Procedures) to increasing concentrations of tBOOH or 4NQO (varying from 3 to 300 µM) for 5 min. The set of primers (see ref. 13) used in these experiments
Regulation of E. coli NrdHIEF Expression

considers the nrdE and nrdA sequences as representative of the entire nrdHIEF and nrdAB transcription units, respectively. The mean values of the fluorescence signal of each target sequence were referred to that of gapA (internal standard). To facilitate the comparison among different genes, data from treated bacteria were divided by those from the corresponding untreated samples. These fold increments were plotted as a function of the log values of tBOOH or 4NQO concentrations. Significant increments are indicated by filled-in symbols.
TABLE I

PCR primer characteristics

Forward primers (marked with an asterisk) were labeled with 4,7,2',4', 5',7',-hexachloro-6-carboxyfluorescein. The housekeeping gapA gene was used as internal standard.

| Primer   | Fragment size (bp) | Sequence                                      |
|----------|-------------------|-----------------------------------------------|
| External standard* | 93               | 5'-TCCTTCAACCAGACCGTTTCTCA-3'                 |
| External standard |                   | 5'-CCGCTTTCCCAAGCCAAAAACCATC-3'               |
| nrdH*    | 105               | 5'-GTTGCGTGCTAGGGCTTTCGTAGT-3'                |
| nrdH     |                   | 5'-TGTTGGCGCTGGATGCAGACGGTTAAT-3'             |
| nrdI*    | 112               | 5'-GCCAGCTCGTCTACTTCTCCAGCAGC-3'              |
| nrdI     |                   | 5'-TCTACCTGAATCCGTTCCGCCGTCATT-3'             |
| nrdE*    | 130               | 5'-CGTCCGAAACAGTGTGACCTTCAGTACGCCC-3'         |
| nrdE     |                   | 5'-GTCGCTGGGTAACACGCGTAAATGACAAA-3'          |
| nrdF*    | 136               | 5'-GGCTACGAAACCCTTTTCCGCAGAA-3'              |
| nrdF     |                   | 5'-CAACCGCTTTCCCATCAACATAGAGG-3'             |
| gapA*    | 143               | 5'-CGTTCTGGGCTACACCAGAGATGACG-3'             |
| gapA     |                   | 5'-AACCGGTTTCGTGCTACCAGGA-3'                 |
**Regulation of E. coli NrdHIEF Expression**

**TABLE II**

*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 ± SEM of the fluorescent signal of the *nrd* target sequence relative to that of *gapA* (internal standard). Boldface type indicates statistical significant increments relative to wild type UC5710.

| Strain       | Relevant phenotype | nrdHIEF/gapA ratio | Fold variation |
|--------------|--------------------|--------------------|----------------|
| UC5710       | Wild type          | 0.013 ± 0.001      | 1.0            |
| UC1342       | OxyR$^-$           | 0.020 ± 0.002      | 1.5            |
| UC1394       | OxyR$^c$           | 0.012 ± 0.001      | 0.9            |
| UC1101       | Wild type/pKM101   | 0.020 ± 0.002      | 1.5            |

**Deficient in Trx and Grx/GSH pathways**

| Strain       | Relevant phenotype | nrdHIEF/gapA ratio | Fold variation |
|--------------|--------------------|--------------------|----------------|
| UC827        | Trx1$^-$ Grx1$^-$  | 1.342 ± 0.112      | 103.2          |
| UC1358       | Trx1$^-$ Gsh$^-$   | 0.425 ± 0.023      | 32.7           |
| UC1363       | Trx1$^-$ Gsh$^-$ OxyR$^-$ | 0.707 ± 0.103 | 54.4          |
| UC1395       | Trx1$^-$ Gsh$^-$ OxyR$^c$ | 0.154 ± 0.011 | 11.8          |

**Deficient in peroxide/superoxide scavenging enzymes**

| Strain       | Relevant phenotype | nrdHIEF/gapA ratio | Fold variation |
|--------------|--------------------|--------------------|----------------|
| 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 | 0.025 ± 0.003 | 1.9            |
| UC628        | SodA$^-$ SodB$^-$/pKM101 | 0.076 ± 0.004 | 5.8            |
Fig. 1

OD$_{600}$

nrdH/gapA ratio

M9 medium

LB broth

nrdE/gapA ratio

M9 medium

LB broth
**Fig. 2**

Bar graph showing the nrdHIEF/gapA ratio under different conditions:

- **Untreated**
  - WT: (3.6)
  - OxyR-: (2.3)
  - SoxR/S-: (4.3)

- **H₂O₂**
  - 10 µM: (23.4)
  - 100 µM: (40.7)
  - 100 µM: (21.2)

- **PQ**
  - 100 µM: (14.4)
  - 100 µM: (8.8)
  - 500 µM: (4.3)

Legend:
- **WT**
- **OxyR-**
- **SoxR/S-**
Fold increase relative to untreated bacteria

**tBOOH**

- **nrdHIEF**
- **nrdAB**
- **oxyS**
- **grxA**

**4NQO**

- **nrdHIEF**
- **nrdAB**
- **oxyS**
- **grxA**

**Fig. 3**
Expression Analysis of the nrdHIEF Operon from Escherichia coli: conditions that trigger the transcript level in vivo
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