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In Vivo Requirement for Glutaredoxins and Thioredoxins in the Reduction of the Ribonucleotide Reductases of *Escherichia coli*

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ABSTRACT

***Escherichia coli* expresses three types of ribonucleotide reductases (RNRs) that utilize the redox chemistry of cysteine to catalyze the reduction of ribonucleotides. Upon reduction, the cysteines form a disulfide bond and must be reduced. The authors present *in vivo* studies that shed light on the mechanism by which these enzymes are regenerated. The class Ia enzyme, NrdAB, can be reduced by either the thioredoxins 1 and 2 or by glutaredoxin 1. The class Ib enzyme, NrdEF, is reduced *in vivo* by a dedicated glutaredoxin-like protein, NrdH. Despite its similarities to glutaredoxins, this protein is itself reduced by thioredoxin reductase *in vivo*. However, in the absence of thioredoxin reductase and NrdH, glutaredoxin 1 can partially replace NrdH. Despite their similar structures, the NrdEF and NrdAB RNRs differ in their abilities to function under low oxygen conditions. With only traces of oxygen present, NrdAB can allow some growth in the absence of the anaerobic enzyme NrdDG. NrdEF cannot. Furthermore, in anaerobiosis, *E. coli* is dependent for growth on class III RNR, NrdDG, and on having at least one of the two reductive systems, thioredoxin reductase or glutathione reductase. These findings indicate a role for these enzymes either for NrdDG reactivation or some other essential anaerobic process.** *Antioxid. Redox Signal.* 8, 735–742.

INTRODUCTION

Ribonucleotide reductases (RNRs) are enzymes that catalyze the reduction of ribonucleotides to their corresponding deoxyribonucleotides (dNTPs), essential building blocks for DNA synthesis and repair (7, 19). RNRs use radical chemistry to create an activated protein cysteinyl radical (S•) that catalyzes the reduction of nucleotides (37). Appearance of this cysteinyl radical depends on the prior generation of either a tyrosyl or a glycyl radical, depending on the type of RNR being studied. RNRs are divided into three classes (class I, II, and III) based on the mechanism used to create this radical in structurally homologous active sites (6, 31, 36, 37). Class I reductases contain a tyrosyl radical that is generated by a diferric center together with oxygen (20) and, as a result, depend on molecular oxygen for their activity (8, 40). Class I RNRs are further divided into two subclasses (Ia and Ib), based on allosteric regulation and structural differences. Class II reductases also contain a tyrosyl radical, but it is generated by an adenosylcobalamin cofactor (vitamin B12) (2). The formation of the tyrosyl radical does not depend on the presence of oxygen; therefore, reduction of ribonucleotides by class II reductases occurs equally well under both anaerobic and aerobic conditions. Class III enzymes use a glycyl radical instead of the tyrosyl radical. The glycyl radical of this group of enzymes is generated by a complicated activation reaction requiring *S*-adenosylmethionine (SAM) and a [4Fe–4S] iron–sulfur cluster (24). The extreme oxygen sensitivity of this radical limits the function of class III enzymes to bacteria growing in the strict absence of oxygen.

Despite these differences, in all three classes, the cysteinyl radical is directly responsible for ribose reduction by abstracting a hydrogen atom from the ribose ring of the ribonucleotides. Electrons required for this reaction are supplied by different donors: formate for the anaerobic class III enzyme (25), and small thiol-redox proteins such as thioredoxin (Trx)

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and glutaredoxin (Grx) in class I and class II reductases (12, 38). Thioredoxins and glutaredoxins belong to the thioredoxin superfamily. Members of this family are defined by a common "thioredoxin fold" and a conserved active site that contains a cys–x–x–cys sequence (CGPC for thioredoxins and CPYC for glutaredoxins) involved in the redox reactions (12, for review see Ref. 32). While the glutaredoxins and thioredoxins exhibit similar three-dimensional structures and active sites, two different enzymatic systems keep them in a reduced state. Oxidized glutaredoxins are reduced by glutathione and glutathione reductase (Gor), whereas oxidized thioredoxins are reduced by thioredoxin reductase (TrxB). In both cases, electrons are provided by the NADPH pool.

Escherichia coli contains the genetic information for three RNRs, one oxygen-sensitive class III reductase, NrdDG, that is the prototype for all class III RNRs, and two oxygen-dependent class I RNRs, NrdAB (class Ia) and NrdEF (class Ib). NrdAB, encoded by the *nrdAB* operon is the prototype for all class I enzymes and is the only functional enzyme during the aerobic growth of *E. coli* (27). Since NrdEF is not expressed under normal growth conditions, the presence of the genes *nrdEF* on the bacterial chromosome does not allow aerobic growth of *E. coli* when the bacteria are defective for the *nrdAB* genes (15). While transcriptional analysis revealed that *nrdEF* expression is induced in conditions of oxidative stress (23, 39), the physiological role of NrdEF in *E. coli* is still unknown. In *E. coli,* the *nrdEF* genes are transcribed together with two small open reading frames (ORFs), *nrdH* and *nrdI*, located immediately upstream of *nrdE* (16). The *nrdI* gene encodes a protein that stimulates the activity of the class Ib RNR, whereas the *nrdH* gene encodes a novel glutaredoxin-like protein, NrdH (18). However, NrdH presents an unusual structure, as its amino acid sequence is related to glutaredoxins but it lacks the typical motif of glutaredoxins, the GSH-binding site. Furthermore, *in vitro* studies show that NrdH is reduced by thioredoxin reductase but not by glutathione oxidoreductase. This poses two major questions: (a) is NrdH reduced *in vivo* by the thioredoxin reductase TrxB or the glutaredoxin reductase Gor (or both), and (b) is NrdEF specifically reduced by this novel glutaredoxin-like protein or are other thioredoxins and glutaredoxins also able to reduce NrdEF? *E. coli* also contains two thioredoxins, Trx1 and Trx2, encoded by the *trxA* and *trxC* genes and three glutaredoxins, Grx1, Grx2, and Grx3, encoded by the *grxA, grxB,* and *grxC* genes. These proteins are expressed during normal aerobic growth conditions (29). Grx 1, Trx 1, and Trx 2 are each capable of reducing the class Ia ribonucleotide reductase NrdAB to regenerate its activity *in vivo* and *in vitro* (1, 30). *In vitro* studies on the Grx1 and NrdH of *Salmonella enterica* show that each of these proteins is capable of reducing *E. coli* ribonucleotide reductase NrdEF (1, 12, 18, 22), although Grx1 is a considerably less efficient reductant. However, no studies have been done in *E. coli* to determine the physiological reductant of NrdEF *in vivo*.

Another important question regarding *in vivo* function of RNRs concerns the class III enzyme. The NrdD subunit (α) of this enzyme contains the active site for binding of substrates and allosteric effectors and is active as a dimer. The smaller subunit (β), encoded by the *nrdG* gene, is an iron–sulfur protein, also known as "activase," which catalyzes the one-electron transfer from reduced flavodoxin to *S*-adenosylmethionine to generate the stable glycyl radical near the carboxy-terminal portion of the larger subunit (24). The NrdD subunit also contains cysteines in its C-terminus. Electrons required to keep these cysteines in a reduced state can be provided *in vitro* by the NADPH pool via the thioredoxin and thioredoxin reductase system (28). These studies leave open the question, however, of whether the thioredoxin and/or glutaredoxin systems are required *in vivo* for the activation of NrdD and the broader question of whether members of the thioredoxin superfamily are required for anaerobic growth.

As the above summary indicates, a good deal of *in vitro* analysis has been done on the *E. coli* RNRs and the reductants that are capable of maintaining them in the active state. However, very little has been done to determine what actually occurs within the living cell. In the present study, we use a genetic approach to study the *in vivo* requirement for the thioredoxin and glutaredoxin systems for the reduction of each RNR in *E. coli*. Previous *in vivo* studies show that NrdAB can be reduced by either Trx1, Trx2 or Grx1. In this study we show that *in vivo* under normal growth conditions, NrdEF, the class Ib reductase, is specifically reduced by the glutaredoxin-like protein, NrdH. Despite its glutaredoxin-like structure, NrdH appears to be directly reduced by the thioredoxin reductase TrxB but not by the glutathione/glutathione reductase pathway. Further, we find that under certain redox stress conditions, Grx 1 is able to partially replace NrdH in reducing NrdEF.

We also show that class Ia (NrdAB) and class Ib (NrdEF) enzymes have different requirements for oxygen since NrdAB is able to function in a highly limited oxygen context, whereas NrdEF is not. This finding may reflect the differing physiological roles of these two enzymes.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Strains were grown routinely in NZ medium at 37°C (10). Microaerophilic growth was achieved by incubation of the plates in an anaerobic jar containing a BBL GasPack Anaerobic system (Becton Dickinson, Cockeysville, MD). Anaerobic growth was achieved by incubation of the plates in an anaerobic jar containing a Pack Anaero (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan). Plates were routinely preincubated in anaerobic conditions for 24–48 h prior to growth experiments, and returned promptly to anaerobic conditions after streaking of the strains onto the plates.

Antibiotic selection was maintained for all markers on plasmids at the following concentrations: ampicillin, 200 μg/ml; chloramphenicol, 30 μg/ml; kanamycin, 40 μg/ml; spectinomycin, 100 μg/ml. Antibiotic selection for chromosomal markers was done at the following concentrations: chloramphenicol, 10 μg/ml; kanamycin, 40 μg/ml. Induction of *nrdAB*, *nrdEF*, *nrdHIEF*, or *nrdDG* from the *trc* promoter of the pDSW204 plasmid was accomplished by addition of 0–100 μ*M* isopropyl β-D-thiogalactopyranoside (IPTG). Induction and repression of the arabinose promoter of the

TABLE 1. STRAINS AND PLASMIDS USED IN THIS WORK

Strains/plasmids	*Relevant genotype*	*Reference*
Plasmids		
pDSW204	*ori* pBR, amp^R, IPTG inducible	41
pBAD18-cm	*ori* pBR, cm^R, arabinose inducible	11
pBAD18	*ori* pBR, amp^R, arabinose inducible	11
pBAD33	*ori* pACYC, cm^R, arabinose inducible	11
pBAD43	*ori* pSC101, spc^R, arabinose inducible	11
pSMG7	pDSW204-*nrdAB*	27
pSMG8	pDSW204-*nrdEF*	This work
pSMG9	pDSW204-*nrdHIEF*	This work
pSMG10	pDSW204-*nrdDG*	This work
pDR1024	pBAD18-*trxB*	33
pBAD18cm-*gor*	pBAD18cm-*gor*	D. Ritz
pBAD33-*trxB*	pBAD33-*trxB*	F. Aslund
pBAD43-*gor*	pBAD43-*gor*	This work
pFA18	pBAD33-*nrdH*	F. Aslund
pSMG13	pDSW204kan-*nrdEF*	This work
Strains		
DHB4	Δ(*ara–leu*)7697 *araD139* Δ *lacX74 galE galK rpsL phoR* Δ (*phoA*)*Pvu*II Δ *malF3 thi*	4
MJF45	DHB4 Δ*nrdAB*::Cm^R	This work
MJF48	DHB4 Δ*nrdAB*	This work
MJF7	DHB4 Δ*nrdEF*::Km^R	This work
MJF8	DHB4 Δ*nrdDG*::Cm^R	This work
MJF16	DHB4 Δ*nrdDG*	This work
MJF46	DHB4 Δ*nrdAB::Cm*^{R} Δ*nrdEF*	This work
MJF18	DHB4 Δ*nrdDG* Δ*nrdEF*::Km^R	This work
MJF61	DHB4 Δ*nrdAB* Δ*nrdDG*::Cm^R / pSMG7	This work
MJF62	DHB4 Δ*nrdAB* / pSMG7	This work
MJF250	DHB4 Δ*nrdAB* / pSMG8	This work
MJF63	DHB4 Δ*nrdAB* / pSMG9	This work
MJF266	DHB4 Δ*nrdAB* / pSMG10	This work
MJF269	DHB4 Δ*nrdAB* Δ*nrdDG*::Cm^R / pSMG10	This work
MJF249	DHB4 Δ*nrdAB* / pFA18	This work
MJF255	DHB4 Δ*nrdAB* / pSMG8 pFA18	This work
MJF13	DHB4 Δ*nrdEF*::Km^R	This work
MJF23	DHB4 Δ*nrdEF*	This work
MJF49	DHB4 Δ*nrdAB* Δ*nrdEF*	This work
SMG58	DHB4 Δ*trxB*::Cm^R	This work
SMG59	DHB4 Δ*trxB*::Km^R	This work
SMG60	DHB4 Δ*gor*::Cm^R	This work
SMG63	DHB4 Δ*trxB* Δ*gor*::Km^R / pDR1024	This work
SMG65	DHB4 Δ*trxB* Δ*gor* / pDR1024	This work
MJF217	DHB4 Δ*nrdAB* Δ*nrdEF* Δ*gor*::Km^R / pBAD18cm-*gor*	This work
MJF218	DHB4 Δ*nrdAB* Δ*nrdEF* Δ*trx*B::Km^R / pBAD33-*trxB*	This work
MJF265	DHB4 Δ*nrdAB* Δ*trxB*::Km^R / pSMG9 pBAD33-*trxB*	This work
MJF295	DHB4 Δ*nrdAB* Δ*gor*::Km^R / pSMG9 pBAD43-*gor*	This work
MJF308	DHB4 Δ*nrdAB* Δ*trxB* Δ*gor*::Km^R / pSMG9 pBAD33-*trxB*	This work
MJF309	DHB4 Δ*nrdAB* Δ*trxB grxA*:: Km^R / pSMG9 pBAD33-*trxB*	This work
MJF310	DHB4 Δ*nrdAB*::CmR Δ*trxB* / pSMG13	This work

pBAD plasmids was accomplished by addition of 0.2% arabinose or glucose, respectively.

Plasmid and strain construction

The *nrdEF, nrdHIEF,* and *nrdDG* coding sequences were amplified by PCR using DHB4 chromosomal DNA as template. We used primers that introduce an *Eco*RI and a *Hin*dIII restriction site, respectively. The product was digested with *Eco*RI and *Hin*dIII and ligated into the same sites of the pDSW204 (41) to create plasmids pSMG8, pSMG9, and pSMG10. The DNA fragment containing the kanamycin-resistance gene and its promoter region was extracted from the pBAD18kan (11) by digestion with *Bsa*I and *Ahd*I. This prod-

uct was ligated into the same sites of pSMG8 to create plasmid pSMG13.

The *gor* coding sequences was amplified by PCR using DHB4 chromosomal DNA as template. We used primers that introduce a *Nhe*I and a *Hin*dIII restriction site, respectively. The product was digested with *Nhe*I and *Hin*dIII and ligated into the same sites of the pBAD43 (11) to create plasmid pBAD43-*gor.*

The DNA sequences of all constructs were verified by sequencing by the Micro Sequences Core Facility at the Department of Microbiology and Molecular Genetics, Harvard Medical School. Strains were constructed by P1 transduction. Standard techniques were used for cloning and analysis of DNA, PCR, electroporation, transformation, and P1 transduction (21, 34).

RESULTS

In aerobiosis, NrdEF, when produced, is able to substitute for NrdAB

Previous studies (9) showed that NrdAB is an essential enzyme for *E. coli* grown in aerobiosis. To study the ability of the different RNRs to substitute for NrdAB, we first constructed a Δ*nrdAB* deletion strain by replacing the *nrdAB* operon with a chloramphenicol-resistance marker. Since NrdAB is essential for aerobic growth of *E. coli*, the strain construction was achieved under anaerobic conditions where the class III RNR (NrdDG) is active and allows growth. The resulting strain (MJF48) grows anaerobically but not aerobically or microaerophilically (See Materials and Methods for conditions used for these different growth experiments) (Table 2). Thus, in an otherwise wild-type background, neither chromosomal expression of NrdDG nor NrdEF can substitute for the deleted NrdAB under aerobic or microaerophilic conditions (Table 2). That chromosomally-encoded NrdEF could not substitute for NrdAB is not surprising as the *nrdEF* genes are normally not expressed (23). That NrdDG could not substitute for NrdAB can be explained by the fact that it has an oxygen-sensitive radical in its active site and is inactivated by oxygen *in vitro*. In addition, the *nrdDG* operon is under the control of FNR, the global transcriptional regulator of anaerobiosis, and, therefore, is also not expressed in aerobiosis (3). In order to determine whether these explanations account for the failure of the NrdDG and NrdEF enzymes to replace NrdAB, we constructed two plasmids expressing either the *nrdDG* or the *nrdHIEF* operons under the control of an IPTG inducible promoter. Both of these plasmids express their respective proteins as indicated by the ability of p*nrdDG* to complement a *ΔnrdDG* mutant strain (data not shown) and of p*nrdHIEF* to complement a *nrdAB* deletion strain as shown by results later in this section. The resulting plasmids, pSMG10 and pSMG9, respectively, were introduced into the MJF48 strain under strict anaerobic conditions. The Δ*nrdAB* strain carrying the complementing plasmid expressing *nrdDG* (MJF266) is not able to grow in aerobiosis even in the presence of IPTG. This result shows that the NrdDG reductase when expressed aerobically is not able to function, probably because of its sensitivity to oxygen. In contrast, the Δ*nrdAB* strain transformed with the plasmid carrying *nrdHIEF* (MJF63) is able to grow aerobically in the presence of IPTG. This result shows that, when expressed, the normally cryptic NrdEF enzyme can function aerobically. This result also confirms the proposal that the normal low level of expression of the nrdHIEF chromosomal copy is not sufficient to support growth in the absence of the main reductase NrdAB (Table 2) (14).

TABLE 2. GROWTH IN AEROBIOSIS, MICROAEROBIOSIS, AND ANAEROBIOSIS

Strain (genotype)	*Aerobiosis*	*Microaerobiosis (Gas-Pack anaero, BBL)*	*Anaerobiosis (Pack-Anaero Mitsubishi)*
DHB4	+++	+++	+++
DHB4 Δ*nrdDG*	+++	+++	++ (flat colonies)
DHB4 Δ*nrdEF*	+++	+++	+++
DHB4 Δ*nrdAB*	—	—	+++
DHB4 Δ*nrdAB* Δ*nrdDG* /pDSW204-*nrdAB*	++ (IPTG)	++ (IPTG)	+ (flat colonies)
DHB4 Δ*nrdAB* Δ*nrdDG* /pDSW204-*nrdDG*	—	—	++ (IPTG)
DHB4 Δ*nrdEF* Δ*nrdDG*	+++	+++	++ (flat colonies)
DHB4 Δ*nrdAB* /pDSW204-*nrdAB*	++ (IPTG)	++ (IPTG)	+++
DHB4 Δ*nrdAB* /pDSW204-*nrdEF*	—	—	+++
DHB4 Δ*nrdAB* /pDSW204-*nrdHIEF*	++ (IPTG)	++ (IPTG)	+++
DHB4 Δ*nrdAB* /pDSW204-*nrdDG*	—	—	++ (IPTG)

Which thiol-redox enzymes reduce the NrdEF reductase in vivo in E. coli?

NrdH and Grx1 are each capable of reducing the class Ib ribonucleotide reductase NrdEF of *E. coli in vitro* (Grx1 less efficiently) (1, 12, 18, 22). However, no *in vivo* studies have been done to determine the physiological reductant of NrdEF in *E. coli* (18). In order to address this question, we compared the ability of NrdEF to restore growth to a *ΔnrdAB* strain in the absence of *nrdH*. We cloned the *nrdEF* genes (without *nrdH*) into a plasmid where they were expressed from an IPTG-inducible promoter. The resulting plasmid (pSMG8) was introduced under anaerobic conditions into the Δ*nrdAB* strain (MJF250). In contrast to the Δ*nrdAB* strain carrying the pSMG9 plasmid that expresses the entire *nrdHIEF* operon, MJF250, expressing only NrdEF in the presence of IPTG, does not grow in aerobiosis. However, when two compatible plasmids, one expressing NrdEF (pSMG8) and the other NrdH (pBAD33–*nrdH*), are introduced into an Δ*nrdAB* strain (MJF255), the cells grow only in the presence of the two inducers, IPTG and arabinose. This result suggests that NrdH is essential for NrdEF reduction in *E. coli*.

We then asked whether glutathione and glutathione reductase (Gor) and/or thioredoxin reductase (TrxB) is responsible for NrdH recycling *in vivo*. A deletion of *trxB* was introduced into the MJF63 strain (*ΔnrdAB*/pDSW204–*nrdHIEF*) containing a pBAD33–trxB complementing plasmid. The resulting strain, *ΔnrdAB trxB::*Km /pBAD33–*trxB* pDSW204–*nrdHIEF* (MJF265) does grow on rich medium with glucose and IPTG, where the *trxB* gene is shut off and *nrdHIEF* is turned on, although it forms smaller colonies than the *trxB*$^+$ parent. This result was surprising, as *in vitro* studies suggested that only TrxB and not Gor could reduce NrdH.

To determine whether Gor might be involved in the survival of cells missing TrxB, a deletion of *gor* was also introduced into strain MJF265 (*ΔnrdAB* Δ*trxB*/pBAD33–*trxB* pDSW204–*nrdHIEF*). The resulting strain (MJF308) does not grow at all, indicating that when *trxB* is absent, *gor* plays a role in NrdEF reduction. To determine whether Gor is directly or indirectly (through glutaredoxin) responsible for NrdEF reduction, we introduced a *grxA* deletion into strain MJF265. The resulting strain (MJF309) also did not grow in the presence of glucose. We considered two explanations for these results: (a) NrdEF can be reduced by either NrdH or GrxA. Ordinarily, GrxA is produced in only low quantities in the cell, insufficient for reduction of NrdEF. However, in a *trxB*$^-$ background, GrxA is overproduced (29) and may suffices for NrdEF reduction, or (b) GrxA can reduce NrdH. In order to distinguish between these explanations, we constructed the MJF310 strain (*ΔnrdAB* Δ*trxB*/pDSW204kan–*nrdEF*). This construction had to be done under anaerobiosis as we could only be certain that the anaerobic reductase, NrdDG, would be functioning. Therefore, if GrxA is acting to directly reduce NrdEF, the strain should grow. The resulting strain (MJF310) is able to grow in aerobiosis in the presence of glucose. Thus, in this particular strain, NrdEF reduction no longer requires NrdH and the enzyme is apparently reduced by GrxA. Western blot analysis revealed that expression of *grxA* is derepressed in the MJF310 strain (data not shown).

In strict anaerobiosis the main RNR is NrdDG, and NrdAB is poorly active

In *E. coli*, the class III ribonucleotide reductase, NrdDG, is believed to be the only enzyme capable of providing cells with dNTPs in anaerobiosis. To test this hypothesis, we constructed, under aerobic growth conditions, a Δ*nrdDG* strain, replacing the *nrdDG* operon with a chloramphenicol-resistance marker. The resulting strain MJF8 grows in aerobiosis and microaerobiosis (Table 2). Surprisingly, in conditions which are generally considered to be strict anaerobiosis, the *ΔnrdDG* strain (MJF8) is still able to form isolated colonies. However, colony morphology differs from the wild-type DHB4 in these conditions. MJF8 colonies are flat, very thin, spread out to give a larger diameter than the DHB4 on the surface of the agar, perhaps indicating that the cells are looking for traces of oxygen dissolved in the growth medium. Furthermore, examination of the cells under the microscope shows extensive instances of cell filamentation. One explanation for the remaining growth of the MJF8 strain is that the cells are relying on the activity of either NrdEF or NrdAB. To test this explanation we constructed strains that, in addition to the *ΔnrdDG* mutation, carried null mutations of either *nrdAB* or *nrdEF*. Deletion of *nrdEF* in the *ΔnrdDG* strain does not reduce the growth of MJF16 strain in strict anaerobiosis, indicating that NrdEF is not responsible for the growth observed in strict anaerobiosis. The double *ΔnrdDG ΔnrdAB* mutant strain was constructed by transduction of the *ΔnrdDG::Cm* allele into the *ΔnrdAB* strain (MJF61) carrying a complementing plasmid expressing *nrdAB* under IPTG control. Deletion of *nrdAB* in the *ΔnrdDG* strain abolishes growth in strict anaerobiosis. Growth in this strain is restored in anaerobiosis either with expression of *nrdAB* or *nrdDG* in *trans* from a complementing plasmid (pSMG7 or pSMG10). Expression of *nrdHIEF* in trans from a plasmid (pSMG9) does not restore anaerobic growth. This result shows that NrdAB is responsible for the remaining growth observed in strict anaerobiosis in the *ΔnrdDG* strain, and, thus, is able to function even in the presence of highly restricted amounts of oxygen. These results suggest that although both NrdAB and NrdEF belong to the same group of RNRs (class I), their catalytic properties, and, therefore, their functions, differ in response to differing oxygen content in the cells.

Cells require either thioredoxin reductase, TrxB, *or glutathione reductase,* Gor, *for anaerobic growth*

In vitro studies show that formate can act as the electron donor for reduction of the ribonucleotide by the *E. coli* class III RNR, NrdDG. If this were the case *in vivo*, it would represent a different pathway for the passage of electrons than that seen for the NrdAB and NrdEF reductases where the thioredoxin and glutaredoxin pathways provide electrons. However, recent *in vitro* studies suggest that thiol groups located in NrdD C-terminus also require reduction by the thioredoxin pathway in order for the NrdDG enzyme to be active. According to Fontecave and co-workers (28), reduced thioredoxin 1 (Trx1) is directly responsible for reduction of cysteines located in NrdD and oxidized Trx1 is in turn reduced by thiore-

doxin reductase (TrxB). To ask whether the thioredoxin and/or the glutaredoxin pathways are implicated *in vivo* in the activity of the NrdDG RNR, we followed the anaerobic growth of a Δ*nrdAB* Δ*nrdEF* strain carrying deletions of either the *trxB* or *gor* genes. We transduced the *ΔtrxB::Km* or the *Δgor::Km* allele into the DHB4 strain carrying complementing plasmids expressing either *trxB* or *gor*, respectively, under control of an arabinose inducible promoter. The resulting strains Δ*nrdAB* Δ*nrdEF* *ΔtrxB::Km*/pBAD33–trxB (MJF218) and Δ*nrdAB* Δ*nrdEF* *Δgor::Km* / pBAD18cm–gor (MJF217) do not exhibit any growth defect in anaerobiosis in the presence of glucose. These results suggest that neither *trxB* nor *gor* is absolutely required for growth or for NrdG reduction *in vivo*. However, the thiol–redox enzymes that belong to the thioredoxin superfamily can often efficiently act on the same substrates (see introduction) (26). Thus, the absence of growth defects in anaerobiosis of mutations in *trxB* and *gor* could be due to overlapping specificities of the two reductive pathways in reduction of NrdG as well.

We then asked whether elimination of both the TrxB and Gor reductive pathways in the same strain would cause defects in anaerobic growth. We constructed a *ΔtrxB Δgor* double mutant carrying a complementing plasmid expressing *trxB* under the control of the arabinose promoter (pBAD18–*trxB*). The resulting strain (SMG65) did not grow under strict anaerobic conditions in the presence of glucose unless DTT was added to the growth medium. This result suggests either that NrdDG activity is dependent on the presence of at least one of the two reductive pathways, that TrxB and Gor are required for the function of another protein(s) which is essential in anaerobiosis, or that both NrdDG and another essential protein are reduced by these pathways. These findings conflict with previous results suggesting that a *trxB⁻ gor⁻* strain was able to grow although extremely slowly (30). We have compared the two *trxB⁻ gor⁻* strains side by side and the original strain always grew a little while the other did not. We cannot explain the difference between the two strains, although ours contains a complete deletion of the *gor* gene, while the earlier strain has a transposon inserted in it which leaves approximately 115 amino acids of the original protein present. This region includes the active site and part of the glutathione binding site. Further analysis of multiple mutants of the two reductive pathways [e.g., in analogy to studies with the *trxA,trxC,grxA* triple mutants studied previously, (27); Gon *et al.*, unpublished observations] may resolve this question, as they did in the case of NrdAB.

DISCUSSION

NrdEF is reduced only by NrdH under normal growth conditions

In the present study, we show that the only thioredoxin-like enzyme able to efficiently reduce NrdEF *in vivo* under normal growth conditions is NrdH, indicating that the physiological reductant of NrdEF is NrdH. NrdH shares a considerable degree of similarity with glutaredoxins and glutaredoxin-like proteins (17, 35). Yet, NrdH is not a member of the subclass of glutaredoxins since it lacks the specific sequence responsible for glutathione binding (GSH-binding site) found in other glutaredoxins (5). Also, the active site sequence (C–M/V–Gln–C) of NrdH differs from the typical C–P–Y–C of glutaredoxins and from the conserved C–G–P–C motif found in the thioredoxins. We show that *in vivo*, NrdH is reduced by thioredoxin reductase and not by the glutathione/ glutathione reductase pathway. These findings are consistent with *in vitro* studies.

However, in a mutant strain lacking thioredoxin reductase, the NrdEF ribonucleotide reductase can be reduced by Grx 1. This reduction occurs, even though in other strain backgrounds where the *trxB* gene is intact, the presence of Grx 1 is not sufficient for NrdEF reduction. This apparent paradox is explained by the following facts: (a) glutaredoxin 1 is normally produced in very low amounts; (b) glutaredoxin 1 is a much less efficient reductant of NrdEF than NrdH; and (c) in strains that are defective in thiol–redox pathways such as the thioredoxin pathway, other redox components (such as Grx 1) are derepressed. In other words, in a *trxB* knockout strain, overexpression of Grx 1 occurs, raising its concentration high enough so that despite its weak activity towards NrdEF, the reduction is sufficient to allow growth. Since a strain deleted for *nrdAB*, and expressing only NrdEF, is unable to grow, it appears that the deficiency in deoxyribonucleotide synthesis does not generate any signal that triggers significant derepression of Grx 1. The ability of the weakly active Grx 1 to reduce NrdEF, when overexpressed, is reminiscent of the ability of the weakly active Grx 3 to reduce NrdAB, when the latter is overexpressed (27).

NrdAB and NrdEF class I ribonucleotide reductases behave differently

In this article, we studied the activity of each *E. coli* RNR under different levels of oxygen. We show that one major difference between these class Ia and class Ib enzymes relates to the amount of oxygen required for the formation of the tyrosyl radical. Under our anaerobic growth conditions, NrdAB is still partially active and is able to provide deoxyribonucleotides for DNA replication, partially replacing the deleted anaerobic enzyme, NrdDG, and allowing slow growth. However, NrdHIEF is not able to function in a comparable fashion under these conditions, and cannot substitute for NrdDG.

It is generally thought that Class I type RNRs require oxygen for the generation of their tyrosyl radical (8). If this is the case, then our finding that NrdAB can still function at highly limited oxygen concentration suggests that NrdAB may be more efficient at generating its radical than NrdEF or may have a higher affinity for O_2 than NrdEF. Whether this difference has any relationship to its possible function in oxidative stress (23) is still unclear. An enhanced ribonucleotide reductase activity during oxidative stress may be advantageous since reactive oxygen species cause DNA damage (13). If this is the main reason for the presence of the NrdHIEF operon in the bacteria, then one could speculate that the highly oxidative conditions would not require a ribonucleotide reductase with high affinity for oxygen in order for the formation of the free radical.

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ABBREVIATIONS

NTP, deoxyribonucleotide triphosphate; Grx, glutaredoxin; RNR, ribonucleotide reductase; SAM, *S*-adenosylmethionine; Trx, thioredoxin.

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