

RESEARCH ARTICLE

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Fumarate and nitrate reduction (FNR) dependent activation of the *Escherichia coli* anaerobic ribonucleotide reductase *nrdDG* promoter

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Summary. The *nrdDG* promoter regulates transcriptional expression of the anaerobic ribonucleotide reductase of *Escherichia coli*, an essential enzyme required to supply the building blocks for DNA synthesis. In this work, binding of the pleiotropic FNR (fumarate and nitrate reduction) transcriptional regulator to the *nrdDG* promoter region and the effects of binding on transcription were investigated. Gel retardation analysis with purified FNR* demonstrated FNR interaction at two FNR sites, termed FNR-2 and FNR-1, while studies with altered FNR boxes indicated that the upstream FNR-2 site was essential for anaerobic activation of the *nrdDG* promoter. Although the FNR-1 site was not absolutely required, it allowed maximal expression of this promoter. These results suggest that the two sites have an additive effect in coordinating *nrdDG* expression in response to shifting oxygen concentrations. [*Int Microbiol* 2008; 11(1):49-56]

Key words: *Escherichia coli* · fumarate and nitrate reduction (FNR) · ribonucleotide reductase · gene *nrd*

Introduction

Balanced de novo synthesis of deoxyribonucleotides (dNTPs) is an essential requirement of all DNA-based organisms. This function is supplied by the ubiquitous enzyme ribonucleotide reductase (RNR), whose allosteric regulation and unique chemistry provide the cell with a balanced pool

of all four dNTPs by reducing the corresponding ribonucleotides (NTPs) [21]. Life on Earth has evolved to proliferate under oxic and anoxic environments, an ability due in part to the evolution of three classes of RNRs, enzymes that carry out DNA synthesis and repair [29]. Class I RNRs contain a stable tyrosyl radical and an oxygen-linked diferric center required for radical generation. Since this process requires oxygen, class I enzymes are only functional under oxic conditions. Class II enzymes require *S*-adenosylcobalamin as a radical generator and do not depend on oxygen. Class III RNRs contain a free radical located on a glycyl residue. This radical is generated with the aid of *S*-adenosylmethionine (SAM) together with an iron-sulfur cluster. Thus, class III enzymes are only active under strictly anoxic conditions. It seems reasonable that a strict aerobe should contain a class I enzyme, a strict anaerobe a class III enzyme, and facultative organisms RNRs of class II, but the situation is actually more complex. For example, some facultative microorganisms bear both class I and class III enzymes, but

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not class II [28,29]. In those microorganisms, a mechanism to activate or repress each RNR class in response to changing oxygen concentrations may well be crucial for proper adaptation to the environment.

Escherichia coli is usually a harmless commensal of the mammalian lower gastrointestinal tract. However, there are also many pathogenic strains of *E. coli* that cause intestinal and extra-intestinal infections in humans and animals [3]. This γ -proteobacterium is a facultative anaerobe whose genome includes the genes for two class I enzymes (Ia and Ib, respectively) and one class III RNR. Oxygen availability is one of the most important regulatory signals in *E. coli* and several one- or two-component regulatory systems control the expression of its aerobic and anaerobic metabolism. The *arcA* gene encodes the

pleiotropic transcriptional regulator of the two-component *arc* system (aerobic respiration control). When activated anaerobically by the membrane sensor protein ArcB, ArcA typically represses a number of genes involved in aerobic metabolism [18] and activates genes necessary for anaerobic growth [9,22]. The transcriptional regulator encoded by the *fnr* (fumarate and nitrate reduction) gene is a pleiotropic one-component regulator that activates transcription of genes involved in anaerobic metabolism and represses the synthesis of enzymes required for aerobic growth [13,31].

Very little is known about RNR transcriptional regulation in response to environmental shifts, especially in those organisms with the ability to express two or more different RNRs. In 2003, Boston and Atlung [4] demonstrated FNR-

Table 1. Strains, plasmids, and bacteriophages used in this study

| | Genotype/characteristics | Source |
|---------------------------|---|------------|
| Strains | | |
| BL21 DE3 | F ⁻ <i>ompT hsdS_B(r_B⁻ m_B⁻) gal dcm</i> (DE3) | Lab. stock |
| DH5 α | <i>recA1 endA1 hsdR17 supE44 thi-1 relA1 Δ(lacZYA-argF)U169 deoR Φ80dlacZM15</i> | Lab. stock |
| MC1061Rif ^R | F ⁻ , <i>araD139, Δ(ara-leu)7697, galE15, galK16, Δ(lac)X74, rpsL(Str^R), hsdR2 (r_K⁻, m_K⁺), mcrA, mcrB1, Rif^R</i> | Lab. stock |
| MC1061pir | F ⁻ , <i>araD139, Δ(ara-leu)7697, galE15, galK16, Δ(lac)X74, rpsL(Str^R), hsdR2 (r_K⁻, m_K⁺), mcrA, mcrB1, lpir</i> | Lab. stock |
| S17-11pir | Tp ^R , Sm ^R , <i>recA, thi, pro, hsdR⁻M⁻RP4:2-Tc:Mu:Km Tn7, λpir</i> | [6] |
| IG40 | MC1061Rif ^R <i>nrdDG-lacZ::Tn5Km-2</i> | This work |
| IG41 | MC1061Rif ^R <i>nrdDFNR-2d-lacZ::Tn5Km-2</i> | This work |
| IG42 | MC1061Rif ^R <i>nrdDFNR-1d-lacZ::Tn5Km-2</i> | This work |
| RZ8480 | <i>Δfnr lacZΔ145 narG::MudI1734</i> | [15] |
| IG34 | IG40 <i>Δfnr</i> | This work |
| ECL963 | ϕ (<i>cyd-lac</i>) <i>bla⁺ cyd⁺ arcA2 zjj::Tn10</i> | [9] |
| IG35 | IG40 <i>arcA2 zjj::Tn10</i> | This work |
| IG36 | IG40 <i>Δfnr arcA2 zjj::Tn10</i> | This work |
| IG37 | IG34 with pGS24 | This work |
| IG38 | IG35 with pMW2 | This work |
| P1 vir Phage | | Lab. stock |
| Plasmids | | |
| pGEM [®] -T easy | A/T cloning vector | Promega |
| pIG72 | pGEM [®] -T easy derivative containing a 479 bp fragment with the <i>nrdDG</i> promoter region | This work |
| pIG73 | pGEM [®] -T easy derivative containing a 479 bp fragment with the <i>nrdDFNR-2d</i> promoter region | This work |
| pIG74 | pGEM [®] -T easy derivative containing a 479 bp fragment with the <i>nrdDFNR-1d</i> promoter region | This work |
| pUJ8 | <i>trp⁻-lacZ</i> promoter probe plasmid vector, <i>lacZ</i> fusions type I | [6] |
| pUTminiTn5Km-2 | Delivery plasmid for mini-Tn5 Km-2 | [6] |
| pMW2 | pBR322 derivative containing the <i>arcA</i> gene | [12] |
| pGS24 | pBR322 derivative with <i>fnr</i> in a 1.64 kb <i>HindIII-BamHI</i> fragment | [25] |
| pGS771 | pGEX-KG derivative with reconstructed <i>fnr</i> * in a 1156 bp <i>NcoI-HindIII</i> fragment | [19] |

mediated oxygen-responsive regulation of the *E. coli nrdDG* operon. Two putative FNR boxes upstream of the +1 transcription start site were also proposed. In this study, we analyzed the in vivo and in vitro interactions of these boxes with the FNR transcriptional regulator, in an attempt to elucidate their roles in *nrdDG* activation.

Materials and methods

Bacterial strains, plasmids, and growth media. The genotypes of the *E. coli* K-12 strains as well as the plasmids and bacteriophages used in this study are listed in Table 1. Cultures were grown routinely at 37°C, either with vigorous shaking in LB broth or on solid medium. When required, antibacterials were added at the following concentrations: ampicillin, 50 µg/ml; kanamycin, 50 µg/ml; chloramphenicol, 34 µg/ml; spectinomycin, 100 µg/ml; tetracycline, 17 µg/ml; rifampycine, 75 µg/ml; and toluidine blue, 260 µg/ml.

Site-directed mutagenesis. A PCR fragment of 479 bp spanning the 5' end of the *nrdD* gene (184 bp) and the upstream regulatory regions (295 bp) was amplified from *E. coli* MC1061 chromosomal DNA and ligated into the A/T cloning pGEM-T easy vector (Promega), generating plasmid pIG72. The QuickChange Site-Directed Mutagenesis kit (Stratagene) and a complementary set of primers containing the desired mutations were used together with this plasmid to generate plasmids pIG73 (FNR-2d) and pIG74 (FNR-1d). All DNA manipulations were done as described in [24].

Construction of *nrdDG-lacZ* transcriptional fusions and mutant strains. To construct the *nrdDG::lacZ* fusions, DNA fragments of 479 bp containing the 5' end of the *nrdD* gene and the upstream regulatory regions were digested from plasmids pIG72, pIG73, and pIG74, and ligated into the *EcoRI* and *BamHI* sites of plasmid pUJ8 [6], yielding transcriptional fusions between these fragments and the *lacZ* gene. All constructs were confirmed by PCR and DNA sequencing. Fusions were transferred to plasmid pUTminiTn5Km2 [6] by *NotI* digestion and introduced into the *E. coli* conjugative strain S17-1 *λpir*. Biparental conjugation between S17-1 *λpir* and MC1061 Rif^R was used to obtain transposition and insertion of the fusions within the MC1061 chromosome, generating strains IG40, IG41, and IG42.

The *fnr* and *arcA* mutant strains were constructed by introducing the indicated mutation into strain IG40 by P1 transduction [27] followed by selection for the appropriate drug resistance. All *fnr* transductants were tested and proved to be incapable of growing in minimal medium in which glycerol and nitrates were the sole source of carbon and reducing power, respectively, thus confirming the Fnr⁻ phenotype [14]. Similarly, all *arcA* transductants were tested for sensitivity to toluidine blue dye [23].

β-Galactosidase assay. β-Galactosidase activities expressed from *nrdDG-lacZ* fusions were assayed according to the method of Miller [20], using cultures grown in LB broth plus glucose (0.5%), either oxicly or anoxically (in screw-cap tubes filled to the neck with 3.2 mM sodium sulfide) [10]. The quoted specific activities (Miller units) are averages of duplicate samples of at least three independent cultures, variations from the mean being no more than 15%.

Purification of the FNR* protein. FNR*DA154 was overproduced from plasmid pGS771 (Table 1), transformed into the *E. coli* BL21 DE3 strain, and purified from sonicated extracts of aerobic cultures grown in LB plus ampicillin (50 µg/ml) and harvested 3–4 h after induction with 0.5 mM IPTG during the mid-exponential phase of growth. FNR* was purified on a 5-ml GSTrap FF column according to the manufacturer's general procedure

(GE Healthcare). FNR* was further purified and concentrated by ultrafiltration on YM-10 Centriplus (Millipore) equilibrated with Tris-HCl (pH 7.5) and 1 mM DTT. Protein concentration was estimated by Bradford assay [5] using the Coomassie protein assay reagent (Pierce); purity was assessed on a 10% SDS-polyacrylamide gel using a 30:0.8 acrylamide/bisacrylamide ratio (w/v).

Electrophoretic mobility shift assay. FNR* binding to *nrdDG* was assessed by a gel retardation assay, as described by Lazazzera et al. [15]. Briefly, protein was mixed in a 10-µl volume with 0.5 µg of a 479-bp DNA fragment that had been 5'-end DIG-labeled by PCR using labeled primers and which contained the putative FNR-binding sites. The final concentrations of the reaction components were: 0.1 M potassium glutamate (pH 7.5), 10 mM potassium phosphate (pH 7.5), 3 mM Tris-HCl (pH 7.9), 1 mM DTT, 50 µg bovine serum albumin (Roche Diagnostics)/ml, and 5% glycerol. The reactions were incubated at 37°C for 10 min after which they were loaded onto a 5% polyacrylamide gel (30:0.8 acrylamide:bisacrylamide w/v) in 0.5× Tris-borate-EDTA buffer [24], electrophoresed for 30 min at 4°C prior to loading, and then run at 110 V for 2 h.

DNA was electrotransferred to a positively charged nylon membrane and developed by chemiluminescence and colorimetric procedures according to the manufacturer's protocols (Roche Diagnostics).

Results

FNR and ArcA requirements for class III RNR expression. An *nrdDG-lacZ* transcriptional fusion containing 295 bp upstream of the *nrdD* start codon was constructed and inserted as a single copy into the *E. coli* chromosome, as described in Materials and methods, to generate strain IG40 (Table 1). Assay of β-galactosidase activities in this strain showed that LacZ levels were ten-fold higher under anoxic conditions than under oxic conditions, indicating the presence of an oxygen-sensitive regulatory mechanism for this promoter (Fig. 1).

To examine the effects of FNR and ArcA on expression of the *E. coli nrdDG* operon under oxic and anoxic conditions, Δfnr and $\Delta arcA$ mutations were individually transduced into strain IG40 to generate strains IG34 (Fnr⁻), IG35 (ArcA⁻), and IG36 (Fnr⁻/ArcA⁻), respectively. In the *fnr* mutant strain grown in the presence of oxygen, β-galactosidase activities did not differ from those of the wild-type strain. However, when this strain was incubated anoxically, LacZ levels were ten-fold lower than those measured in the *fnr*⁺ strain. To confirm that the *fnr* mutation was responsible for reduced LacZ activities, the mutant strain was complemented with an expression plasmid containing the wild-type *fnr* gene. As determined in β-galactosidase assays, activity in the complemented strain recovered and surpassed that measured in the anoxically grown wild-type strain (Fig. 1). By contrast, β-galactosidase levels in the *arcA* mutant strain were the same as those in the wild-type during either aerobic or anaerobic growth, even when this strain was complemented with an *arcA* containing plasmid, whereas the double mutant

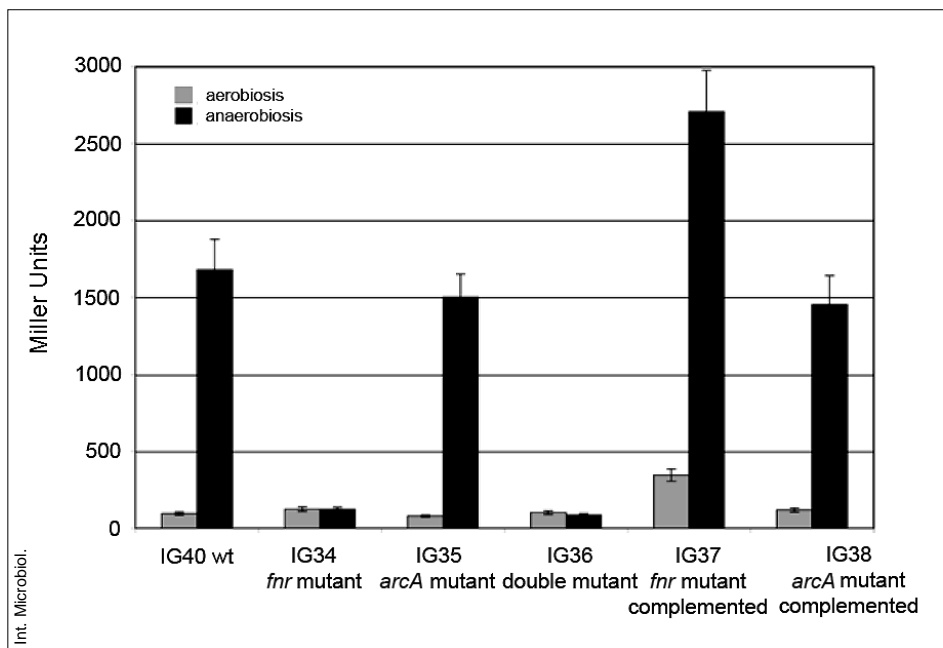


Fig. 1. FNR-mediated regulation of *nrdDG*. β -Galactosidase levels (Miller units) expressed from the *nrdD-lacZ* fusion in the: wild-type strain (wt), *fnr* mutant strain, *arcA* mutant strain, *fnr/arcA* double mutant strain, *fnr* mutant strain complemented with an extrachromosomal *fnr* gene (pGS24), and *arcA* mutant strain complemented with an extrachromosomal *arcA* gene (pMW2) grown under oxic (gray bars) or anoxic (black bars) conditions. Results are expressed as the mean \pm standard error of the mean (SEM) of duplicates from three independent cultures.

strain behaved like the *fnr*⁻ mutant. These results corroborated those of Boston and Atlung [4] and provided further evidence that FNR is indeed responsible for anaerobic activation of *nrdDG* (Fig. 1).

Binding of FNR* to the *nrdDG* promoter. In a preliminary search for specific FNR:DNA binding, gel retardation assays were carried out to investigate whether FNR acts directly on the *nrdDG* promoter region. The same DNA fragment used to construct the *nrdD::lacZ* fusion was end-labeled and used in gel retardation assays against purified FNR* protein (FNR-DA154). The DA154 substitution in this

FNR protein has been shown to enhance dimer stability, thus providing a sufficiently active form of FNR for use in gel retardation analysis under oxic conditions; it has been widely used to simplify the in vitro analysis of FNR–DNA interactions [16,32]. At the lowest protein concentration tested, a mobility shift band was readily detected. As the concentration of FNR* increased, a second retardation band appeared in the gel (Fig. 2), suggesting FNR binding to both putative boxes.

Although ArcA consensus binding sites have been proposed from footprinting studies with several ArcA-controlled promoters [1,7,17,26], a region resembling an ArcA-binding

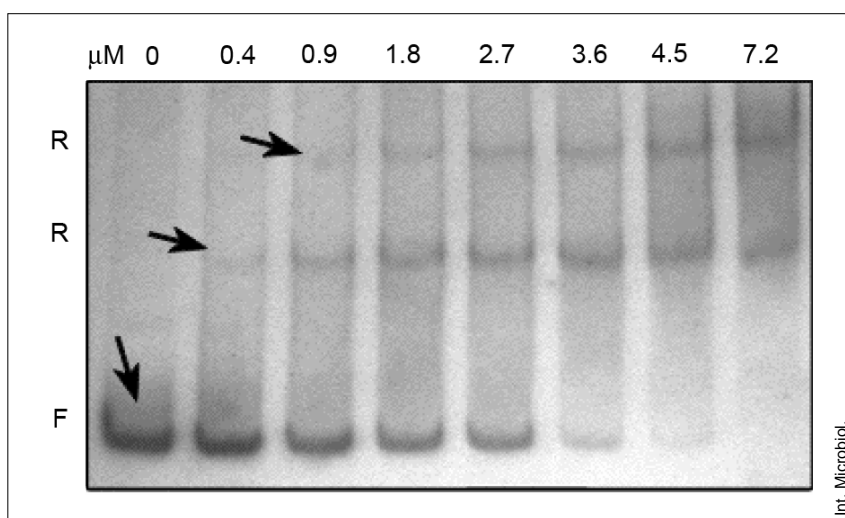


Fig. 2. Electrophoretic mobility shift assay with FNR* and the *nrdDG* promoter region. Arrows indicate positions of the free (F) and retarded (R) DNA. FNR* was added at the concentrations indicated above the lanes.

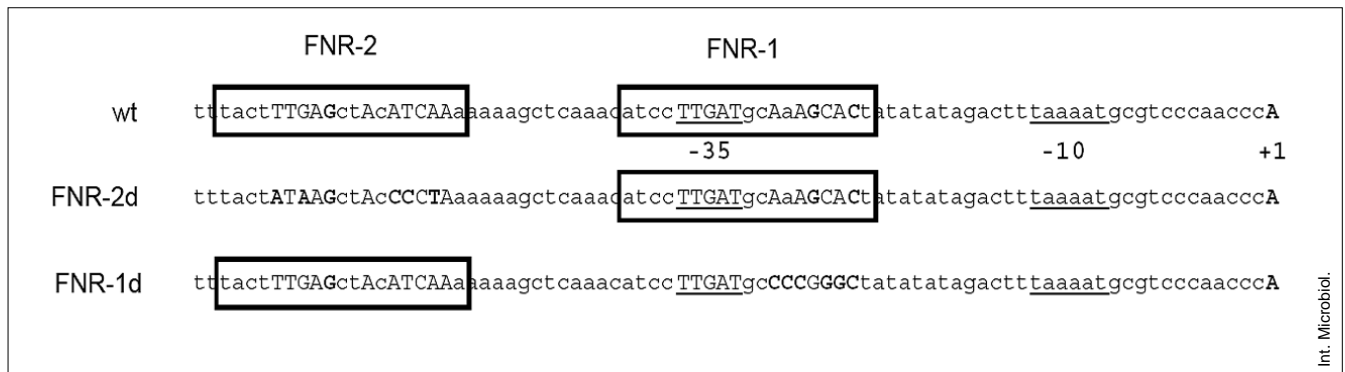


Fig. 3. Sequence of the *nrdDG* promoter regions and the *nrdDFNR-2d* and *nrdDFNR-1d* mutated sequences. The FNR binding sites are boxed in the wild-type sequence, and the -35 and -10 promoter regions are underlined. Bases fitting the consensus FNR sequence TTGATNNANATCAA [11] and the transcription start site [4] are shown in upper-case letters. Mutated bases in the FNR binding sites are indicated in bold.

site within the *nrdDG* promoter region could not be identified. However, the nucleotide sequences described by Boston and Atlung around -35 (FNR-1) and -65 (FNR-2) [4] showed significant similarity with the consensus FNR-binding site, with only one or two mismatches (shown in bold in Fig. 3) in one of the palindromic half-sites, respectively.

The direct involvement of these sequences in FNR recognition and binding in vitro was further demonstrated by independently mutagenizing FNR-2 and FNR-1 (see Materials and methods), yielding two novel DNA fragments each containing a single unaltered FNR site (Fig. 3). These fragments were end-labeled and subsequently probed against FNR* in gel retardation assays. As shown in Fig. 4A, removal of the downstream FNR-1 site (FNR-1d) led to the formation of a single FNR*:*nrdD* complex, while no retardation at all was observed in the fragment lacking the FNR-2 (FNR-2d) site (Fig. 4B). Furthermore, the FNR* concentration resulting in 50% retardation of 1 μ g *nrdD* (K_d) was calculated in all elec-

trophoretic mobility shift assays and found to be similar for the wild-type and the FNR-1d probe ($K_d \sim 4.5 \mu$ M), indicating that the absence of the downstream FNR site did not alter the binding affinity of the FNR-2 site. No shifted bands were generated when FNR* was incubated with a PCR probe lacking the FNR-1 and FNR-2 sites (data not shown). The upstream promoter sequences of the *E. coli nrdAB* and *nrdEF* genes were also probed for FNR:DNA interactions but no retarded bands were detected (data not shown).

In vivo usage of the FNR-2 and FNR-1 sites.

Since modification of the putative FNR boxes affected FNR binding to the *nrdD* promoter sequence, we examined whether such modifications also altered in vivo transcription. Accordingly, chromosomal *lacZ* fusions to the defective promoter sequences were constructed as described in Materials and methods, generating strains IG41 (*nrdDFNR-2d::lacZ*) and IG42 (*nrdDFNR-1d::lacZ*). The two strains were assayed

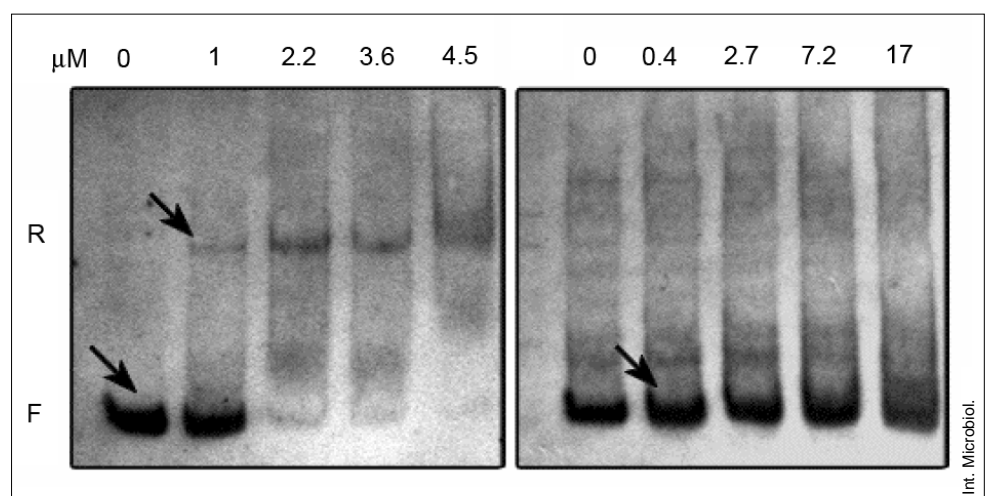


Fig. 4. Electrophoretic mobility shift assays with FNR* and the *nrdDFNR-1d* (A), and *nrdDFNR-2d* (B) promoter regions. Arrows indicate positions of the free (F) and retarded (R) DNA. FNR* was added at the concentrations indicated above the lanes.

for β -galactosidase activity under oxic and anoxic growth conditions. β -Galactosidase levels in strain IG41 were similar under the two conditions (46 ± 5 and 50 ± 5 Miller units, respectively), suggesting that this strain was unable to activate β -galactosidase expression when grown anoxically and thus behaved like the FNR mutant strain. By contrast, the lack of the downstream FNR-1 site did not impair β -galactosidase expression in strain IG42 when incubated in an anoxic environment, although expression was two-fold lower than in the wild-type fusion (760 ± 10 and 1676 ± 12 Miller units for IG42 and IG40, respectively). These results corroborated those of the electrophoretic mobility shift assays.

Discussion

Very few studies in the literature have examined the genetic regulation of *nrd* genes, and most of them focused on the genes encoding the aerobic classes of enzymes (*nrdAB* and *nrdEF*). Consequently, the mechanism driving the expression of the anaerobically controlled enzyme is poorly understood. However, since three different RNR classes are found within the *E. coli* genome, the presence of a mechanism in which expression of the *nrdDG* promoter is switched on and off in response to shifting oxygen concentrations can be assumed. Boston and Atlung [4] showed that regulation of the *E. coli* anaerobic enzyme is FNR-dependent, either through direct interaction of the protein with the promoter sequence or by means of a more complex pathway. Results obtained with the transcriptional fusions between the *E. coli nrdDG* promoter and the *lacZ* gene described here corroborated Boston and Atlung's findings, while complementation studies of an *fnr* mutant strain provided further evidence for FNR-dependent activation under anaerobic growth. It also seems clear from our experiments that ArcA is not involved in *nrdDG* regulation and that the *nrdAB* and *nrdEF* promoters are not FNR-dependent (data not shown). However, although Boston and Atlung identified two putative FNR recognition sequences within the *nrdDG* promoter, they did not provide direct evidence of the involvement of those sequences in the anaerobic activation of the *E. coli* class III enzyme. The electrophoretic mobility shift assays carried out in this work demonstrate a direct interaction between FNR* and the *nrdDG* promoter at specific FNR boxes (see Fig. 3). The fact that two shifted bands appeared in the gel also indicated involvement of both FNR sites in FNR-dependent regulation. Furthermore, in the presence of a DNA fragment lacking the downstream FNR-1 site, FNR* rendered a single shifted band, whereas no retardation was observed at the tested protein concentrations in the absence of FNR-2. A requirement for the FNR-2 site to

obtain anaerobic transcription from the *nrdDG* promoter was also found when transcriptional fusions with these defective promoter sequences were analyzed. Similarly, the lower β -galactosidase levels resulting from the fusion lacking FNR-1 supported the involvement of the FNR-1 site.

In 1996, Garriga et al. [10] demonstrated that the *E. coli* anaerobic enzyme is essential for growth under strict anaerobiosis, but that an *nrdDG* null-mutant can also proliferate under microaerophilic conditions by overexpressing the *nrdA* gene (aerobic class Ia). Our gel retardation experiments revealed that the two FNR sites displayed a differential affinity towards the FNR protein, with FNR-2 site having the highest binding capacity. Thus, FNR-1 and FNR-2 should be involved in fine tuning the shift from aerobic to anaerobic growth, and the following model accounting for these results can be proposed:

Maximal transcription from the *nrdDG* promoter requires the binding of two FNR dimers, with one dimer bound at each FNR site. Comparison of the half-maximal expression values ($pO_{0.5}$) for FNR- and ArcA-regulated genes suggested that the transition point from aerobic to anaerobic metabolism is found within the range of 1–5 millibars of oxygen, such that there is a coordinated substitution of the aerobic pathways by the anaerobic pathways [2]. The FNR content in *E. coli* cells remains almost constant throughout aerobic and anaerobic growth. However, the proportion of the active reduced form must increase gradually with decreasing pO_2 [8]. As the pO_2 decreases (microaerophilia), the amount of FNR able to bind DNA increases proportionally, yielding amounts of active FNR in the cell that are sufficient to allow binding to the high-affinity FNR-2 site (which also displays greater similarity to the FNR consensus sequence), but not to the FNR-1 site. Accordingly, expression from the *nrdDG* promoter is not maximal; instead, by overexpressing *nrdA* the cell is able to proliferate. The lower β -galactosidase levels detected in our FNR-1d fusions provided evidence for this scenario.

By contrast, oxygen depletion (strict anaerobiosis) renders class Ia RNR non-functional, and the anaerobic enzyme becomes the only source of reduced ribonucleotides. At this point, the amount of active FNR is maximal such that the protein binds to both FNR sites. Since FNR-1 is centered over the putative -35 sequence, it is not clear how occupancy of this site contributes to the higher levels of expression obtained with the wild-type fusion. In most FNR-dependent promoters, FNR acts as an activator by binding to a single FNR site centered near position -41 (class II promoters, which overlap the -35 sequence) or near positions -61 or -71 (class I promoters). FNR activation of both the class I and class II promoters is achieved through direct contacts

between FNR and the RNA polymerase σ subunit (RNAP σ) [30]. However, when a second FNR site is located in an upstream position, binding of FNR to both sites down-regulates rather than enhances expression from the promoter. Green and co-workers have shown that this down-regulation is due to specific interactions between the two in-tandem-bound FNR molecules [2]. In this respect, the *nrdDG* promoter may be considered as constituting a mixture of class I and class II FNR-dependent promoters, and its unique architecture might allow simultaneous contacts of RNAP σ with the two in-tandem FNR proteins, resulting in FNR-dependent activation rather than repression. Note that equivalent positions in FNR-2 and FNR-1 are 31 bp apart (three helical turns), which indicates binding on the same face of the helix. In addition, the closely spaced FNR sites might also allow protein-protein contacts between FNR dimers, such that cooperative binding and activation cannot be excluded. A similar situation is found in the *dcuC* promoter in *E. coli*, which contains two FNR activating regions centered over positions -34 and -65 [33].

Although there is no doubt about the additive effect of FNR-1 in the FNR-mediated activation of the *nrdDG* promoter, the specific activating mechanism remains unclear. To our knowledge, ours is the first direct evidence for the involvement of two FNR sites in the up-regulation of an FNR-dependent promoter. Further studies aimed at understanding the mechanisms driving this regulation are under way.

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