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Localization of Upstream Sequence Elements Required for Nitrate and Anaerobic Induction of *fdn* (Formate Dehydrogenase-N) Operon Expression in *Escherichia coli* K-12

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Two transcriptional activators, the FNR and NARL proteins, are required for induction of the *fdnGHI* operon, encoding *Escherichia coli* formate dehydrogenase-N. The FNR protein is required for anaerobic expression, while the NARL protein mediates nitrate induction. We used primer extension to locate the transcription initiation site 29 nucleotides upstream of the *fdnG* translation initiation codon. Expression assays with single-copy Φ(*fdnG-lacZ*) gene fusions containing various deletions in the *fdn* 5′-regulatory region delimited three distinct *cis*-acting elements. One site, which is located at approximately −110, was required for nitrate induction. Two other sites share sequence similarity with the FNR protein binding site core consensus. The first site, centered at −42.5, was required for anaerobic induction. We used site-specific mutagenesis to change this putative FNR protein binding site into the CRP protein binding site core consensus. This change caused the *fdn* operon to be expressed aerobically, subject to CRP protein control. On the other hand, converting this putative FNR protein binding site into the FNR protein binding site core consensus resulted in elevated anaerobic induction of the *fdn* operon and also caused weak aerobic expression. The other putative FNR protein binding site, centered at −97.5, was not involved in anaerobic induction. It might play a negative role in *fdn* operon expression during anaerobic growth in the absence of nitrate.

Escherichia coli can use a variety of electron donors and acceptors for respiration. Under anaerobic conditions, nitrate is the preferred electron acceptor. Formate, which is produced from pyruvate during anaerobic growth, is an efficient electron donor for nitrate reduction (for a review, see reference 25). The formate-nitrate respiratory chain consists of formate dehydrogenase-N, quinone, and nitrate reductase. Both enzymes are cytoplasmic membrane-bound enzyme complexes. Formate dehydrogenase-N, which oxidizes formate to carbon dioxide, is encoded by the *fdnGHI* operon at 32 min on the *E. coli* genetic map (4). Nitrate reductase, which reduces nitrate to nitrite, is encoded by the *narGHJI* operon at 27 min on the *E. coli* genetic map (for a review, see reference 25).

Transcription of the *fdnGHI* and *narGHJI* operons is induced during anaerobic growth in the presence of nitrate. Two transcriptional activators, the FNR and NARL proteins, are required for this induction. Anaerobic induction is mediated by the FNR protein (for a review, see reference 23), while nitrate induction is mediated by the NARL protein (4, 24). The FNR protein shares sequence similarity with the cyclic AMP receptor protein (CRP) (for a review, see reference 23), an activator of carbon catabolic operons (for a review, see reference 21). The DNA binding site for the FNR protein has been deduced by sequence comparison and mutational analysis of a number of FNR-dependent promoters (for a review, see reference 23). The core consensus sequence is TTGAT-N_4-ATCAA (where N is any nucleotide). In most cases examined, the FNR protein binding sequence is located approximately 42 bp upstream of the transcription initiation site. The FNR protein binding sequence is quite similar to the core consensus for CRP protein binding, TGTGA-N_6-TCACA. The FNR and CRP proteins may activate transcription initiation by similar mechanisms (for a review, see reference 23).

The NARL protein is also thought to be a DNA binding protein, and it shares substantial sequence similarity with the regulator proteins of bacterial two-component regulatory systems (29). Either of the two sensor proteins, NARX (18, 27) or NARQ (8, 20), monitors the availability of nitrate. In the presence of nitrate, the NARX or NARQ proteins presumably activate the NARL protein by phosphorylation (5, 9). The phosphorylated form of the NARL protein then likely binds to sequences upstream of *fdnGHI*, *narGHJI*, and other operons under its control.

Deletion and mutational analyses of the *narGHJI* operon control region have defined a sequence that is essential for nitrate induction (7, 16). This sequence, TACTCC, is located approximately 200 nucleotides upstream of the transcription initiation site (Fig. 1) (7). In addition, the integration host factor, a sequence-specific DNA bending protein, is required for nitrate induction of *narGHJI* expression (Fig. 1) (19). Presumably, a specifically bent DNA structure brings the NARL protein into the proximity of the transcription initiation site (7, 19).

The structural genes for formate dehydrogenase-N, *fdnGHI*, have been identified and sequenced in our laboratory (3, 4). In this report, we describe experiments to study the regulation of *fdn* operon expression. We used primer extension to locate the *fdn* operon transcription initiation site 29 nucleotides upstream of the *fdnG* translation initiation codon. Through deletion analysis and site-specific mutagenesis, we identified three distinct *cis*-acting sites. A site centered at position −42.5, which shares sequence similarity with the FNR protein binding site core consensus, was required for anaerobic induction. A site located around position −110 was required for nitrate induction. This latter

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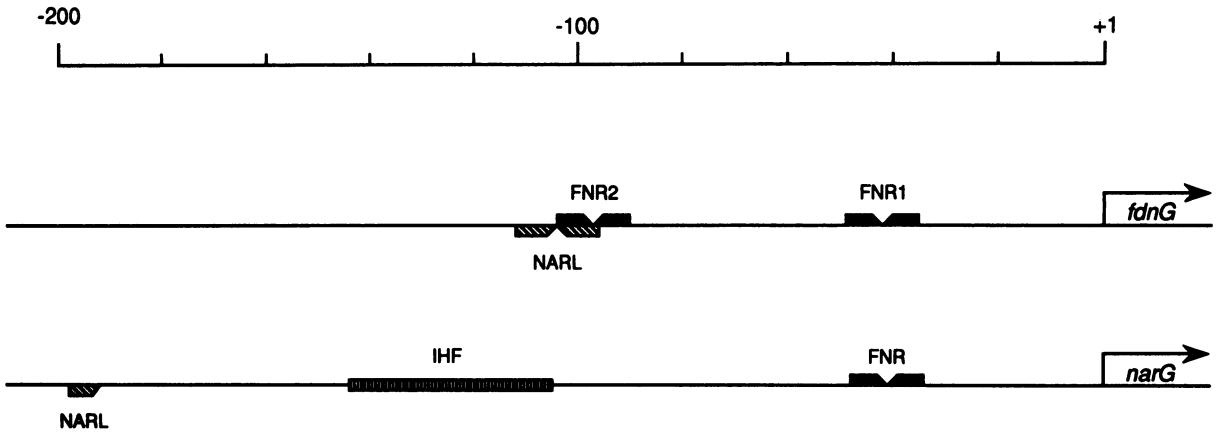

FIG. 1. Schematic representation of the *fdn* and *nar* operon control regions. The transcription initiation sites are indicated by arrows. Protein binding sites are represented as filled boxes. Scale is in base pairs. IHF, integration host factor.

site shares similarity with the NARL protein binding site in the *narGHJI* control region. However, the NARL protein binding site in the *fdn* control region comprised an imperfect dyad symmetry. Finally, a site centered at position −97.5, which also shares sequence similarity with the FNR protein binding site core consensus, might play a negative role in *fdn* operon expression.

MATERIALS AND METHODS

Strains and plasmids. *E. coli* K-12 strains and plasmids used in this study are listed in Table 1. The *narL*::Tn*10*, *fnr*::Tn*10*, and Δ*crp* alleles were transduced into VJS676 derivatives by bacteriophage P1 *kc* (17). pVJS527 contains the fragment from −313 to +2405 of the *fdn* operon and a TGA to TCA change at codon 196 of *fdnG* (3). Using this plasmid, we introduced a *Hin*dIII site at −163 by site-specific mutagenesis. The resulting −163 to +2405 fragment was released with *Hin*dIII and *Bam*HI and inserted into *Hin*dIII-*Bam*HI-digested pGEM7Zf(+) to create pVJS537. Standard methods were used for restriction endonuclease digestion, ligation, and transformation of DNA (6). Restriction enzymes, T4 polynucleotide kinase, and T4 DNA ligase were from New England Biolabs, Inc. (Beverly, Mass.). T4 DNA polymerase, modified T7 DNA polymerase (Sequenase), exonuclease III, and nuclease S1 were from U.S. Biochemical Corp. (Cleveland, Ohio). Avian myeloblastosis virus reverse transcriptase was from Stratagene Cloning Systems, Inc. (La Jolla, Calif.).

Culture media. TYGN contained (per liter) tryptone, 8 g; yeast extract, 5 g; NaCl, 5 g; glucose, 20 mM; $NaNO_3$, 40 mM; Na_2MoO_4, 1 μM; and Na_2SeO_3, 1 μM. Cultures of Φ(*fdnG-lacZ*) strains for β-galactosidase assays were grown in 3-(*N*-morpholino)propanesulfonic acid (MOPS)-buffered minimal medium with either glucose or sorbitol as the sole carbon source (26). The initial pH of this medium was set at 7.8. For *fdn*::Tn*10*Δ*crp*-containing strains, one-half strength MOPS-glucose or MOPS-sorbitol medium was supplemented with tryptone (0.4%), yeast extract (0.125%), and NaCl (0.125%). $NaNO_3$ (40 mM) was added as indicated.

Defined, complex, and indicator media for routine genetic manipulations were used as described previously (6, 17). Ampicillin, streptomycin, and tetracycline were used at 200, 15, and 20 μg/ml, respectively. Agar and dehydrated media were from Difco Laboratories (Detroit, Mich.). Other components were from Sigma Chemical Co. (St. Louis, Mo.).

Culture conditions. Cultures were grown at 37°C. Culture densities were monitored with a Klett-Summerson photoelectric colorimeter (Klett Manufacturing Co., New York,

TABLE 1. Strains, plasmids, and phage

Strain, phage, or plasmid	Genotype, phenotype, or characteristic	Source or reference
E. coli strains[a]		
JM83	*ara*Δ(*lac-proAB*)*X111 rpsL* Φ80d Δ(*lacZ*)*M15*	32
LS854	Δ*crp-45 rpsL136 his-85 metE70 trpA9605 trpR55*	CGSC[b]
RK4353	*araD139* Δ(*argF-lac*)*U169 flhD5301 gyrA219 non-9 rpsL150 ptsF25 relA1 deoC1*	24
RK5278	As RK4353 but *narL215*::Tn*10*	24
VJS533	As JM83 but *recA56*	26
VJS632	Prototroph	26
VJS676	As VJS632 but Δ(*argF-lac*)*U619*	Laboratory collection
VJS1741	As RK4353 but *fnr-271*::Tn*10*	Laboratory collection
Phage λRS45	*att*$^+$ *int*$^+$ *imm*^{21} *'lacZ lacY*$^+$ *lacA*$^+$	22
Plasmids		
pGEM-7Zf+	Ap^r, T7 Φ10 promoter	Promega
pRS414	Ap^r *'lacZ lacY*$^+$ *lacA*$^+$	22
pVJS523	Ap^r, *Eco*RI-*Bam*HI, −313 to +2405 of *fdnG* in pRS414	2
pVJS527	Ap^r, *Eco*RI-*Bam*HI, −313 to +2405 of *fdnG* in pGEM-7Zf+	This work
pVJS537	Ap^r, *Hin*dIII-*Bam*HI, −163 to +2405 of *fdnG* in pGEM-7Zf+	This work
pVJS538	As pVJS527 but *Bam*HI at *fdnG* codon 33	This work

[a] All strains are F$^-$ and λ$^-$.
[b] Coli Genetic Stock Center, courtesy of B. J. Bachmann.

N.Y.) equipped with a number 66 (red) filter. Anaerobic and aerated cultures for β-galactosidase assays were grown in screw-cap tubes and baffled flasks, respectively, as described previously (26).

Primer extension analysis. *E. coli* VJS533 containing pVJS523 was grown in 15 ml of TYGN broth under anaerobic conditions to late exponential phase (approximately 60 Klett units). Total RNA was prepared by the method of Gilman (11). To determine the 5′ end of *fdn* mRNA, a synthetic oligonucleotide complementary to nucleotides 30 to 59 of the *fdnG* coding region was end labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase and was coprecipitated with 20 μg of RNA. The primer extension reaction with avian myeloblastosis virus reverse transcriptase was performed by the method of Kingston (14). The sequence ladder corresponding to the mRNA sequence was generated by using the pVJS523 DNA template and the same oligonucleotide as a primer.

Unidirectional deletions. Unidirectional deletions were constructed by the method of Henikoff (13). pVJS537 was cleaved at the *Kpn*I site, which is in the polylinker of the pGEM7Z(+) vector, and at the introduced *Hin*dIII site, which is at −163 of the *fdn* operon. Exonuclease III does not efficiently attack a 3′ protrusion, so digestion proceeded from the 5′-protruding *Hin*dIII site into the *fdn* control region. The extent of exonuclease III digestion was varied by stopping the reaction at different times. The remaining undigested single-stranded DNA was removed by S1 nuclease. Klenow polymerase was added to blunt the ends, and then DNA ligase was added to recircularize the molecules. Plasmids with different deletion endpoints were identified by restriction enzyme mapping and DNA sequencing.

Site-specific mutagenesis. Oligonucleotide-mediated site-specific mutagenesis was carried out by the procedure of Kunkel et al. (15). We engineered an in-frame *Bam*HI site at codon 33 of *fdnG* to form plasmid pVJS538, which was used to make site-specific mutations in the *fdn* control region. All of the mutagenized templates were sequenced from the polylinker region of the vector to the *Bam*HI site at codon 33 of *fdnG* in order to confirm the mutational alterations and to ensure that no spurious changes were introduced.

Construction of Φ(*fdnG-lacZ*) gene fusions. Gene fusions between *fdnG* and *lacZ* were constructed on plasmid pRS414, which is a vector for gene (translational) fusion (22). The *Eco*RI-*Bam*HI (codon 33) fragments containing the different mutations were cloned into plasmid pRS414. Confoundingly, these Φ(*fdnG-lacZ*) gene fusions at codon 33 had very low β-galactosidase activity. To remedy this problem, we subsequently introduced a *Bam*HI fragment containing *fdnG* codons 33 to 793 into each of the fusion constructs. These longer gene fusion constructs expressed full levels of β-galactosidase activity. The *Eco*RI-*Bam*HI (codon 793) fragments containing different control region deletions from pVJS537 were cloned into pRS414 individually.

All of the gene fusion constructs were crossed to λRS45 as described by Simons et al. (22). Single-copy lysogens of strain VJS676 were isolated and verified by genetic tests as described previously (28). Each fusion consists of the first 793 codons of *fdnG* fused to codon 9 of *lacZ*. Thus, expression of hybrid β-galactosidase requires transcription and translation of the *fdnG* sequence. All the fusions carry a serine codon (TCA) at position 196 of *fdnG* instead of a selenocysteine codon (TGA) (2, 3).

β-Galactosidase assay. Assays were carried out at room temperature (approximately 21°C). Cell pellets were suspended in 4 ml of Z buffer (17) and stored on ice. β-Galactosidase activity was measured in $CHCl_3$-sodium dodecyl sulfate-permeabilized cells by monitoring the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside. Activities are expressed in terms of cell density (A_{600}) by using the formula of Miller (17). Each culture was assayed in duplicate. Reported values were averaged from at least two independent experiments.

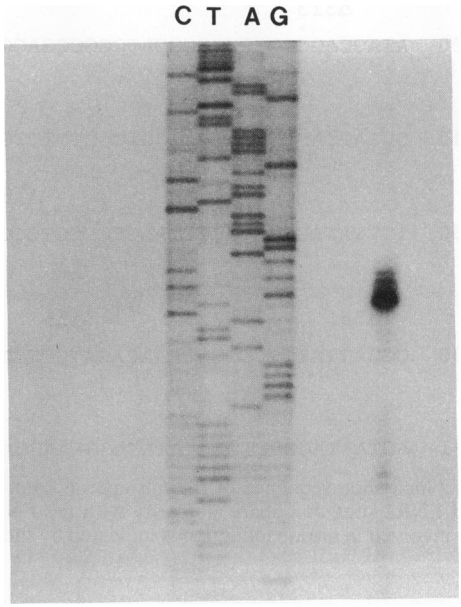

FIG. 2. Primer extention of *fdn* operon mRNA. mRNA was isolated and subjected to primer extension as described in the text. The lanes marked C, T, A, and G show the corresponding dideoxynucleotide chain termination sequencing reactions. The primer extension reaction is shown in the lane to the right of the sequencing ladder.

RESULTS

Control region of the *fdn* operon. A primer extension experiment was performed to identify the 5′ end of the *fdn* operon mRNA. We isolated RNA from a strain carrying pVJS523, a Φ(*fdnG-lacZ*) plasmid. The culture was grown anaerobically in the presence of nitrate. Using a primer complementary to nucleotides 30 to 59 of the *fdnG* coding region, we observed a single major cDNA (extended from the primer) which ended at the position of a T in the sequence ladder (Fig. 2). This T corresponded to an A in the *fdnG* coding strand. No upstream endpoints were observed. Thus, this A is the likely transcriptional initiation site, designated position +1. It is 29 nucleotides upstream of the *fdnG* translation initiation site (Fig. 3).

Upstream from the transcriptional initiation site, two potential −10 sites share similarity with the consensus sequence of prokaryotic promoters (12). Further upstream, two sites share sequence similarity with the FNR protein binding site consensus. Sites FNR1 and FNR2 are centered at positions −42.5 and −97.5, respectively.

Three gene (translational) fusions were constructed to monitor regulation by the *fdn* control region. They contained ~1,400, 313, and 163 bp of DNA upstream of the transcriptional initiation site, respectively. β-Galactosidase activities were measured in fusion-containing strains grown under aerobic and anaerobic conditions without or with nitrate.

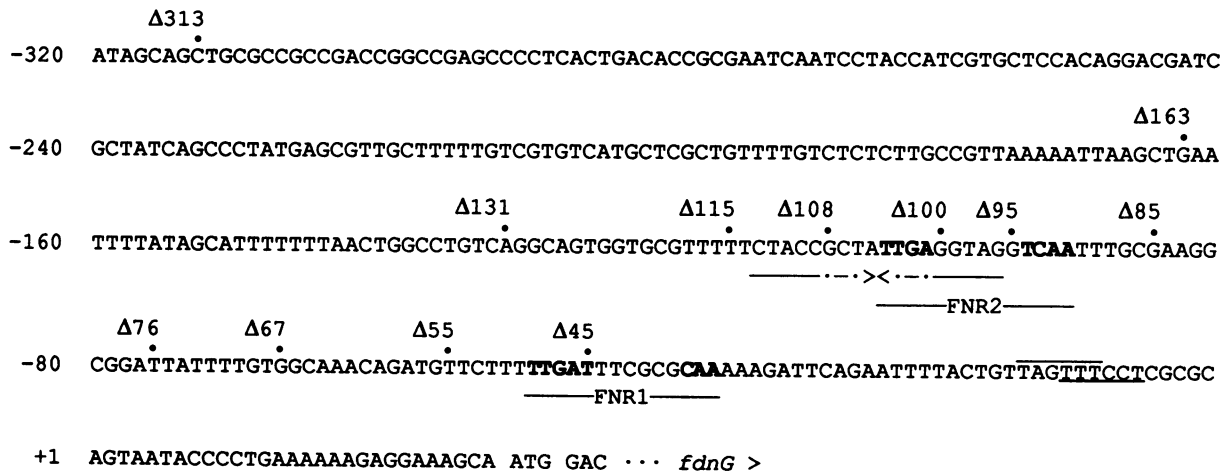

FIG. 3. Nucleotide sequence of the *fdn* operon control region. Deletion endpoints are indicated by numbered dots. Nucleotides in the FNR1 and FNR2 sites that share similarity with the FNR protein binding site core consensus are shown in boldface. The imperfect dyad symmetry involved in nitrate induction is indicated by the converging arrows. Potential −10 sites are indicated by overlining and underlining.

The results revealed no differences in the regulation of these fusions (data not shown). This shows that the *cis*-acting sites for *fdn* operon regulation are located within 163 bp upstream of the transcription initiation site.

Deletion analysis of the *fdn* operon control region. In order to determine the nucleotide sequences required for anaerobic induction and nitrate regulation, the control region of the *fdn* operon was subjected to 5′ deletion analysis. A series of control region deletions was fused to *lacZ* in order to construct gene fusions. Each of these fusions was carried on λ bacteriophage and was integrated into *E. coli* chromosome in single copy at the λ attachment site, and β-galactosidase activity was measured after growth under different conditions. Results are shown in Table 2.

The deletion constructs Δ131 and Δ115 expressed β-galactosidase in patterns similar to those of the Δ313 and Δ163 constructs. In all four cases, there was no detectable aerobic expression, low anaerobic expression, and full expression in the presence of nitrate. In the constructs Δ108, Δ100, Δ95, Δ85, and Δ76, β-galactosidase synthesis was induced normally by anaerobiosis but only weakly by nitrate. Thus, deletion to position −108 eliminated at least part of the DNA sequence required for nitrate induction.

TABLE 2. Expression of Φ(*fdnG-lacZ*) gene fusion constructs bearing control region deletions

Fusion construct[a]	β-Galactosidase sp act[b]			
	−O_2		+O_2	
	−NO_3^-	+NO_3^-	−NO_3^-	+NO_3^-
Δ313	8	560	UD	UD
Δ163	4	530	UD	UD
Δ131	3	420	UD	UD
Δ115	3	370	UD	UD
Δ108	6	11	UD	—
Δ100	18	79	UD	UD
Δ95	21	39	UD	UD
Δ85	24	30	UD	—
Δ76	17	24	—	—
Δ67	3	6	—	—
Δ55	7	9	UD	UD
Δ45	9	6	3	2

[a] See Fig. 3 for positions of deletions.
[b] Determined as described in Materials and Methods and expressed in arbitrary (Miller) units. Cultures were grown with glucose anaerobically or aerobically without or with nitrate as indicated. UD, undetectable activity (<0.05 Miller units); —, not determined.

The deletion constructs from Δ108 to Δ76 exhibited weak (2-fold or less) induction by nitrate except for construct Δ100, which exhibited 5- to 10-fold induction by nitrate in different experiments (Table 2 and data not shown). To determine the basis for this residual induction, we introduced a *narL*::Tn*10* insertion into strains carrying the Δ313, Δ108, Δ100, Δ95, Δ85, Δ76, and Δ67 fusion constructs and measured β-galactosidase expression after growth in the absence and presence of nitrate. The *narL* insertion reduced nitrate induction in the Δ313 strain from greater than 100-fold to about 4-fold (data not shown). This residual NARL protein-independent nitrate induction of *fdn* operon expression has been noted previously, and its basis is not understood (4). The *narL* insertion had no effect on the slight nitrate induction in all other deletion constructs tested except for Δ100, where the 5- to 10-fold induction was reduced to about 2-fold by *narL*::Tn*10* (data not shown). We do not know why the Δ100 construct retained this NARL protein-dependent nitrate induction. The DNA sequence upstream of the Δ100 deletion endpoint is identical to that upstream of most other deletions, including Δ108, so differences in upstream regions cannot account for this difference.

The deletions Δ100, Δ95, and Δ85, which progressively removed the FNR2 site centered at position −97.5, caused only a subtle effect; anaerobic expression was slightly elevated. This suggests that the FNR2 site does not control anaerobic induction and that it might play a negative role in *fdn* expression.

β-Galactosidase synthesis was still induced by anaerobiosis in the deletion constructs Δ67 and Δ55. However, the deletion Δ45, which removed part of the FNR1 site centered at position −42.5, resulted in constitutive β-galactosidase synthesis under both anaerobic and aerobic conditions. A similar phenomenon was noted in a deletion analysis of the *nar* operon control region (16). This suggests that expression of the Δ45 construct was no longer dependent on the FNR

protein. Thus, the FNR1 site at position −42.5 is required for anaerobic regulation of the *fdn* operon.

Mutational analysis of the FNR1 site. To further study the function of the putative FNR protein binding site centered at position −42.5, we constructed a series of site-specific mutations in this site. Previous work with synthetic binding sites has suggested that CRP and FNR protein binding sites can be interconverted by simple substitutions (1, 33). The FNR1 site at position −42.5 is not identical to the core consensus sequence for FNR protein binding. Thus, we introduced a series of changes at the FNR1 site that converted it into the core consensus FNR protein binding site (FF construct) or into the core consensus CRP protein binding site (CC construct [Fig. 4A]). Intermediates in the mutagenesis contained FNR protein binding sequences in one dyad and CRP protein binding sequences in the other (CF and FC constructs [Fig. 4A]).

We examined expression of these Φ(*fdnG-lacZ*) fusion constructs by measuring β-galactosidase activity. The results are shown in Table 3. The FF fusion construct had increased expression under both anaerobic and aerobic conditions. This suggests that the wild-type sequence at position −42.5 is optimum for controlling anaerobic induction of the *fdn* operon. The CF and FC fusion constructs were only expressed under anaerobic conditions, albeit at significantly reduced levels. Thus, changing half of the FNR protein binding site into half of the CRP protein binding site lowered but did not abolish anaerobic induction of the *fdn* operon. These changes (CF and FC) did not cause aerobic *fdn* expression. Finally, the CC fusion construct had severely decreased anaerobic expression but weak aerobic expression in the presence of nitrate (Table 3).

We further examined expression of these Φ(*fdnG-lacZ*) fusion constructs by measuring β-galactosidase activity in *fnr*::Tn*10* derivatives. Loss of FNR protein function abolished expression from the wild-type, FF, CF, and FC fusion constructs (data not shown). Thus, expression from these fusion constructs was wholly dependent upon the FNR protein. The *fnr*::Tn*10* insertion significantly decreased anaerobic expression of the CC fusion construct but had no influence on its aerobic expression (Table 4).

We hypothesized that the residual FNR protein-independent expression of the CC fusion construct was due to activation by the CRP protein. Growth with glucose causes catabolite repression, one manifestation of which is lowered CRP protein activity (due to lowered cyclic AMP [for a review, see reference 21]). We used two approaches to further test this possibility. First, we substituted sorbitol for glucose as the sole carbon source. Sorbitol is less effective than glucose in eliciting catabolite repression. Results are shown in Table 4. This carbon source substitution had little effect on expression from the wild-type *fdn* control region, but it increased expression from the CC *fdn* control region. This was most clear in the *fnr*::Tn*10* strain, in which sorbitol caused at least fivefold greater expression from the CC *fdn* control region.

Second, we also examined expression of the wild-type and CC fusion constructs in an *fnr*::Tn*10* Δ*crp* strain background. The results presented in Table 4 show that the residual FNR protein-independent expression of the CC fusion construct was eliminated by the Δ*crp* mutation, irrespective of growth conditions. Taken together, these results suggest that the CRP-cyclic AMP complex activated gene expression from the CC control region.

Mutational analysis of the FNR2 site. To further examine the function of the FNR2 site at position −97.5, we disrupted

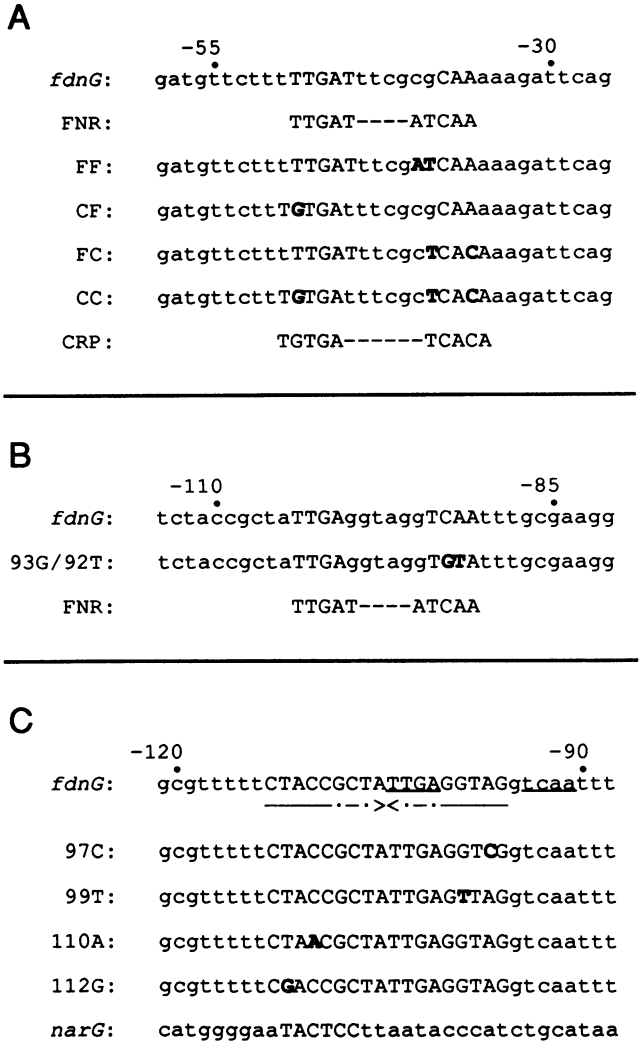

FIG. 4. Site-specific mutational alterations in the *fdn* operon control region. (A) Changes in the FNR1 site. The core consensus sequences for FNR and CRP protein binding are shown. Uppercase letters indicate matches to the core consensus sequences. Boldface letters represent mutational changes. (B) Changes at the FNR2 site. The core consensus for FNR protein binding is shown. Uppercase letters indicate matches to the consensus sequence for the FNR2 site. Boldface letters represent mutational changes. (C) Changes at the NARL protein binding site. The imperfect dyad symmetry (uppercase letters) is indicated by converging arrows. Boldface letters represent mutational changes. The NARL protein binding site in the *nar* operon control region is shown for comparison; uppercase letters represent the 6-bp critical region (7).

the right half-site by site-specific mutagenesis. The FNR2 site alterations 93G/92T (Fig. 4B) did not affect anaerobic induction of *fdn* operon expression (Table 5). However, the basal level expression during anaerobic growth was elevated. These results, which are consistent with the deletion analysis (Table 2), suggest that the FNR2 site is not involved in anaerobic induction of the *fdn* operon but that it has a role in lowering the basal level of expression during anaerobic growth in the absence of nitrate.

Mutational analysis of the NARL protein binding site. Our deletion analysis suggested that the region around position −110 is essential for nitrate induction of the *fdn* operon

TABLE 3. Expression of Φ(*fdnG-lacZ*) gene fusion constructs bearing FNR1 site point mutations

Fusion construct[a]	β-Galactosidase sp act[b]			
	$-O_2$		$+O_2$	
	$-NO_3^-$	$+NO_3^-$	$-NO_3^-$	$+NO_3^-$
FF	14	670	UD	45
WT	5	580	UD	UD
CF	0.1	150	UD	UD
FC	0.3	320	UD	UD
CC	UD	36	UD	2

[a] See Fig. 4A for positions of point mutations. All constructs are based on the Δ313 deletion (Fig. 3).

[b] Determined as described in Materials and Methods and expressed in arbitrary (Miller) units. Cultures were grown with glucose anaerobically or aerobically without or with nitrate as indicated. UD, undetectable activity (<0.05 Miller units).

(Table 2). Mutational analysis of the *narGHJI* operon control region has shown that the sequence TACTTC, located at about 200 bp upstream of the transcription initiation site, is critical for nitrate induction (7, 16). Comparison of the *fdn* −110 and *nar* −200 regions reveals a partial similarity (Fig. 4C). This sequence in the *fdn* control region is part of an imperfect dyad symmetry (Fig. 3 and 4C). To test the dependence of the DNA sequence for nitrate regulation in *fdnGHI* operon, we introduced several changes by site-specific mutagenesis and constructed Φ(*fdnG-lacZ*) fusions containing these changes. We examined expression of these fusion constructs in the absence and presence of nitrate under anaerobic conditions (Table 5). The 112G and 97C changes dramatically decreased expression in the presence of nitrate. These data establish that the 112T and 97A positions are critical for nitrate induction of the *fdn* operon. By contrast, the 110A and 99T changes had little effect on nitrate induction. Thus, further mutagenesis will be necessary to precisely delineate the positions that are essential for NARL protein action.

The 97C change caused elevated *fdn* operon expression in the absence of nitrate (Table 5). This effect was similar to that of the 93G/92T alterations, which were designed to disrupt the FNR2 site. Apparently the 97C change similarly disrupts this site in addition to its effect on the NARL protein binding site.

TABLE 4. Effects of carbon source, *fnr*, and *crp* on expression of Φ(*fdnG-lacZ*) gene fusion constructs bearing FNR1 site point mutations

Fusion construct and allele[a]	β-Galactosidase sp act[b]			
	$-O_2$, $+NO_3^-$		$+O_2$, $+NO_3^-$	
	Glc	Srl	Glc	Srl
WT, *fnr*$^+$ *crp*$^+$	580	700	UD	UD
WT, *fnr*::Tn*10* *crp*$^+$	UD	UD	UD	UD
WT, *fnr*::Tn*10* Δ*crp*	UD	UD	UD	UD
CC, *fnr*$^+$ *crp*$^+$	36	50	1	6
CC, *fnr*::Tn*10* *crp*$^+$	2	15	1	5
CC, *fnr*::Tn*10* Δ*crp*	UD	UD	UD	UD

[a] Carries WT or CC sequence at the FNR1 site (Fig. 4A). All constructs are based on the Δ313 deletion (Fig. 3). Strains carry the indicated *fnr* and *crp* alleles.

[b] Determined as described in Materials and Methods and expressed in arbitrary (Miller) units. Cultures were grown anaerobically or aerobically in the presence of nitrate as indicated. UD, undetectable activity (<0.05 Miller units).

TABLE 5. Expression of Φ(*fdnG-lacZ*) gene fusion constructs bearing FNR2 or NARL protein binding site point mutations

Fusion construct[a]	β-Galactosidase sp act[b]			
	$-O_2$		$+O_2$	
	$-NO_3^-$	$+NO_3^-$	$-NO_3^-$	$+NO_3^-$
WT	3	400	UD	UD
93G/92T	16	340	UD	UD
97C	15	55	—	—
99T	6	390	—	—
110A	7	390	—	—
112G	5	13	—	—

[a] Carries the indicated change at the FNR2 site or the NARL protein binding site (Figs. 4B and C). All constructs are based on the Δ313 deletion (Fig. 3).

[b] Determined as described in Materials and Methods and expressed in arbitrary (Miller) units. Cultures were grown with glucose anaerobically or aerobically without or with nitrate as indicated. UD, undetectable activity (<0.05 Miller units); —, not determined.

DISCUSSION

The synthesis of formate dehydrogenase-N and nitrate reductase is coordinately regulated by anaerobiosis and nitrate. The FNR protein is required for *fdnGHI* and *narGHJI* operon expression during anaerobic growth, and the NARL protein is required for induction by nitrate (4; for a review, see reference 25). The organization of the *nar* operon control region has been determined by mutational analysis and was found to contain three *cis*-acting elements (Fig. 1): an FNR protein binding site centered at position −41.5 (30), an integration host factor binding site centered at approximately −125 (19), and a presumed NARL protein binding site located at approximately −198 to −193 (7). We report here our mutational analysis of the *fdn* operon control region, which has a very different architecture despite the coordinate regulation of the *fdn* and *nar* operons (Fig. 1).

The FNR1 site. The 5′ end of *fdn* operon mRNA, as determined by primer extension analysis (Fig. 2), is located downstream of a site (FNR1) that shares similarity with the core consensus sequence for FNR protein binding (Fig. 3). Most FNR protein-dependent promoters examined to date have an FNR protein binding site centered around 40 to 45 nucleotides upstream of the transcription initiation site (23); the FNR1 site in the *fdn* control region is centered at position −42.5. Additionally, the promoter −10 site, but not the −35 site, is essential for FNR protein-dependent *nar* operon expression (31). The *fdn* control region contains two potential −10 sites, but the −35 region shares little similarity with the consensus −35 site (Fig. 3) (12).

The FNR1 site matches the core consensus for FNR protein binding at 8 of 10 positions. We used site-specific mutagenesis to convert the FNR1 site into a perfect match with the core consensus (Fig. 4A). The mutant site (FF) caused mildly elevated *fdn* operon expression during anaerobic growth in the absence or presence of nitrate (Table 3). Surprisingly, the FF site also led to significant *fdn* operon expression during aerobic growth, at least in the presence of nitrate (Table 3). This suggests that the FNR protein can bind to DNA and activate transcription during aerobic growth if the *cis*-acting elements (FNR site and promoter −10 site) have appropriate sequences. An analogous obser-

vation was reported for the *frdABCD* (fumarate reductase operon) control region, in which a mutation causing aerobic *frd* expression changed the FNR protein binding site into a closer match to the core consensus (10).

The FNR and CRP proteins share sequence similarity and probably activate transcription by analogous mechanisms (for a review, see reference 23). Furthermore, the core consensus sequences for the FNR and CRP protein binding sites are quite similar. Studies with synthetic FNR and CRP protein binding sites in the normally CRP-dependent *mel* and *lac* promoters have suggested that a single nucleotide substitution in each symmetric half-site is sufficient to interconvert FNR and CRP protein binding sites (1, 33). We attempted to convert the FNR1 site into a CRP protein binding site as a way to demonstrate the importance of the FNR1 site for anaerobic *fdn* expression. Changing the FNR1 site into the CRP protein binding site core consensus drastically lowered anaerobic expression and did confer weak CRP protein-dependent expression (Tables 3 and 4). However, even during growth with sorbitol, expression from the CC fusion construct was much lower than from the wild-type, FF, CF, and FC constructs, each of which was fully dependent on the FNR protein. Surprisingly, the majority of CC fusion construct expression was dependent on the FNR protein (Table 4). Thus, in the context of the *fdn* operon control region, the conversion of an FNR protein binding site into a CRP protein binding site was only partially successful and did not match the simple predictions drawn from studies of synthetic sites (1, 33).

The NARL protein binding site. Our deletion analysis identified the region in the vicinity of position −110 as essential for NARL protein-mediated nitrate induction of *fdn* operon expression (Table 2). Recently, Dong, Li, and DeMoss (7) isolated point mutations in the −200 region of the *nar* operon control region. Their analysis identified a sequence, TACTCC, that is essential for NARL protein-dependent nitrate induction of *nar* operon expression.

The *fdn* operon control region contains a similar sequence, TACCGC, in the vicinity of position −110. This sequence is part of an imperfect dyad symmetry (Fig. 3). To assess the role of this dyad symmetry in *fdn* operon regulation, we made several site-specific changes (Fig. 4C). The changes 112G and 97C virtually eliminated nitrate induction (Table 5), suggesting that the wild-type dyad serves as a NARL protein binding site for the *fdn* control region. Interestingly, the changes 110A and 99T had little influence on nitrate control (Table 5). Further mutational analysis of the *fdn* operon control region will more precisely define the sequence elements necessary for NARL protein action.

The NARL protein binding sites are located at very different positions in the *nar* and *fdn* operon control regions (Fig. 1). The *nar* operon control region requires the DNA-bending protein integration host factor for NARL protein-mediated nitrate induction, while the *fdn* operon control region is indifferent to the presence of integration host factor (19). Thus, the different locations of these NARL protein binding sites reflect the very different overall architectures of the two control regions.

The FNR2 site. A second site with substantial similarity to the FNR protein binding site core consensus is centered at position −97.5 (Fig. 3). Our deletion and site-specific mutational analyses of this site suggest that it is not a major element in regulating expression from the *fdn* operon control region (Tables 2 and 5). However, deletion or alteration of the FNR2 site resulted in slightly elevated anaerobic *fdn* operon expression, at least in the absence of nitrate. This site may play a role in suppressing basal-level *fdn* operon expression during anaerobic growth. Whether the FNR protein acts at this site remains to be determined.

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