Structural Genes for Nitrate-Inducible Formate Dehydrogenase in *Escherichia coli* K-12

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ABSTRACT

Formate oxidation coupled to nitrate reduction constitutes a major anaerobic respiratory pathway in *Escherichia coli*. This respiratory chain consists of formate dehydrogenase-N, quinone, and nitrate reductase. We have isolated a recombinant DNA clone that likely contains the structural genes, *fdnGHI*, for the three subunits of formate dehydrogenase-N. The *fdnGHI* clone produced proteins of 110, 32 and 20 kDa which correspond to the subunit sizes of purified formate dehydrogenase-N. Our analysis indicates that *fdnGHI* is organized as an operon. We mapped the *fdn* operon to 32 min on the *E. coli* genetic map, close to the genes for cryptic nitrate reductase (encoded by the *narZ* operon). Expression of Φ(*fdnG-lacZ*) operon fusions was induced by anaerobiosis and nitrate. This induction required *fnr*$^+$ and *narL*$^+$, two regulatory genes whose products are also required for the anaerobic, nitrate-inducible activation of the nitrate reductase structural gene operon, *narGHJI*. We conclude that regulation of *fdnGHI* and *narGHJI* expression is mediated through common pathways.

THE facultative aerobe *Escherichia coli* synthesizes a number of anaerobic respiratory chains. Formate, produced from pyruvate during anaerobiosis, serves as an efficient electron donor for nitrate respiration. The oxidation of formate during nitrate respiration is catalyzed by formate dehydrogenase-N. A major anaerobic respiratory chain consists of formate dehydrogenase-N, cytochrome b_{556}^{FDN}, quinone, cytochrome b_{556}^{NAR}, and nitrate reductase (ENOCH and LESTER 1974; RUIZ-HERRERA and DEMOSS 1969; reviewed by STEWART 1988).

Purified formate dehydrogenase-N consists of three subunits (α, β and γ) of 110, 32 and 20 kDa, respectively (ENOCH and LESTER 1975). The α subunit contains selenocysteine and molybdenum cofactor, and is likely to form the active site. The function of the β subunit is unknown, and the γ subunit is probably cytochrome b_{556}^{FDN}. Nitrate reductase also consists of three subunits and contains molybdenum cofactor and cytochrome b_{556}^{NAR}. Formate dehydrogenase-N and nitrate reductase are both cytoplasmic membrane-bound enzyme complexes (ENOCH and LESTER 1974; reviewed by STEWART 1988). The structural genes for nitrate reductase are encoded by the *narGHJI* operon at 27 min on the *E. coli* genetic map (reviewed by STEWART 1988). In contrast, the structural genes for formate dehydrogenase-N have not been characterized.

Synthesis of formate dehydrogenase-N and nitrate reductase is induced by nitrate during anaerobic growth. Figure 1 illustrates our current model for regulation of *narGHJI* transcription by anaerobiosis and nitrate. Anaerobic induction is mediated by FNR, an activator of anaerobic respiratory genes (LAMBDEN and GUEST 1976; NEWMAN and COLE 1978; reviewed by STEWART 1988). Induction by nitrate requires NarL, the product of the regulatory gene *narL* (STEWART 1982; STEWART and PARALES 1988). Transposon insertions in *narX* have only subtle effects on the induction of *narGHJI* by nitrate (STEWART and PARALES 1988). Mutations in *narL* and *narX* also affect nitrate repression of other anaerobic enzymes, including fumarate reductase, dimethylsulfoxide reductase, and pyruvate-formate lyase (COTTER and GUNSALUS 1989; IUCHI and LIN 1987; KALMAN and GUNSALUS 1989; SAWERS and BÖCK 1988; STEWART and BERG 1988). Comparisons of predicted amino acid seqences show that NarX (NOHNO *et al.* 1989; STEWART, PARALES and MERKEL 1989) and NarL (GUNSALUS, KALMAN and STEWART 1989; NOHNO *et al.* 1989; STEWART, PARALES and MERKEL 1989) are similar to other prokarotic regulatory proteins known as "two component regulatory systems" (STOCK, NINFA and STOCK 1989).

A second formate dehydrogenase, formate dehydrogenase-H, is a component of the formate-hydrogen lyase complex (PECK and GEST 1957; reviewed by STEWART 1988). Formate dehydrogenase-H is also a selenoenzyme, and is synthesized anaerobically only in the absence of nitrate. The structural gene for formate dehydrogenase-H, *fdhF*, encodes a UGA codon which directs selenocysteine incorporation by selenocysteyl-tRNA$_{UCA}^{Ser}$ (LEINFELDER, STADTMAN and BÖCK 1989; ZINONI *et al.* 1986, 1987).

E. coli expresses only two selenoproteins, formate dehdrogenase-N and formate dehydrogenase-H, as

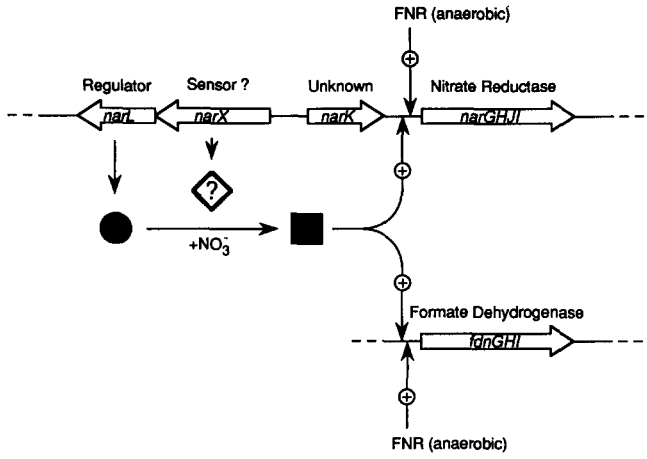

FIGURE 1.—Model for regulation of *fdnGHI* and *narGHJI* transcription. The *fdn* and *nar* regions are diagramed schematically (not to scale). Open arrows indicate protein coding regions; arrowheads indicate the direction of transcription. The *narL* gene product is hypothesized to be a nitrate-responsive DNA-binding protein which activates transcription of *fdnGHI* and *narGHJI*. The *fnr* gene product is required for the anaerobic induction of *fdnGHI* and *narGHJI* (reviewed by STEWART 1988). The precise functions of the *narX* and *narK* gene products are unknown.

detected by *in vivo* labeling with ^{75}Se (COX, EDWARDS and DEMOSS 1981). These proteins can be distinguished by their differences in electrophoretic mobility on SDS-polyacrylamide gels. Formate dehydrogenase-N is synthesized only in the presence of nitrate, while formate dehydrogenase-H is synthesized only in its absence (COX, EDWARDS and DEMOSS 1981). At least four genes (*selA–D*) are required for synthesis of both formate dehydrogenase-N and formate dehydrogenase-H (LEINFELDER *et al.* 1988a). The *selA* and *selD* gene products are required for formation of selenocysteine (LEINFELDER, STADTMAN and BÖCK 1989), *selC* encodes $tRNA^{Ser}_{UCA}$ (LEINFELDER *et al.* 1988b), and *selB* encodes a translation factor specific for selenocysteyl-$tRNA^{Ser}_{UCA}$ (FORCHHAMMER, LEINFELDER and BÖCK 1989).

In their screen for *sel* mutants, LEINFELDER *et al.* (1988a) recovered a mutant with a lesion in the gene encoding the α subunit of formate dehydrogenase-N. The mutation, *fdh-24*::Mu dl(Ap^r *lac*), results in the production of a truncated 110-kDa selenopeptide. At least two *rha*-linked genes, termed *fdhD* and *fdhE*, specifically affect formate dehydrogenase-N activity (BARRETT and RIGGS 1982; MANDRAND-BERTHELOT *et al.* 1988; PAVEGLIO *et al.* 1988; B. L. BERG, J. T. LIN and V. STEWART, unpublished observations). The functions of *fdhD* and *fdhE* are not understood, but it is unlikely that they encode any of the three formate dehydrogenase-N subunits.

We report here our isolation of a recombinant DNA clone that complemented *fdh-24*::Mu dl(Ap^r *lac*). This clone contained three genes, which we designate *fdnGHI*. Our data suggest that the *fdnGHI* operon encodes all three subunits of formate dehydrogenase-N. We mapped *fdnGHI* to approximately 32 min on the *E. coli* genetic map (1565 kb on the physical map of KOHARA, AKIYAMA and ISONO 1987). Curiously, *fdnGHI* is closely linked to the *narZ* operon, encoding cryptic nitrate reductase. We found that expression of the *fdnGHI* operon was induced anaerobically by nitrate, and required *fnr*$^+$ and *narL*$^+$. We conclude that regulation of *fdnGHI* and of *narGHJI* transcription is mediated through common pathways (Figure 1).

MATERIALS AND METHODS

Nomenclature: Previously identified genes required for formate dehydrogenase activity have been designated "*fdh*." Many *fdh* mutations (*fdhA–C*) are pleiotropic, affecting both formate dehydrogenase-N and formate dehydrogenase-H. Recently, these genes have been renamed "*sel*" (*selA–D*) to reflect their role in selenium metabolism (LEINFELDER *et al.* 1988a). Mutations in *fdhD* and *fdhE* specifically affect formate dehydrogenase-N (MANDRAND-BERTHELOT *et al.* 1988); analogous mutations in *Salmonella typhimurium* have been designated "*fdn*" (*fdnB* and *fdnC*) to reflect this phenotype (PAVEGLIO *et al.* 1988). Finally, *fdhF* designates the structural gene for formate dehydrogenase-H (PECHER, ZINONI and BÖCK 1985). We have named the structural genes for formate dehydrogenase-N *fdnG*, *H* and *I*, to avoid confusion with the previously used *fdhA–F* and *fdnB-C* designations.

BARRETT and co-workers (1979) devised a differential medium, MacConkey nitrate agar, to identify formate dehydrogenase mutants. Wild-type strains form large, salmon-colored colonies on MacConkey nitrate agar, while strains with defects in formate metabolism form medium-sized, dark red colonies. Strains carrying *selA–D*, *fdhDE*, *fdnGHI* or *narL* mutations exhibit essentially identical phenotypes on this medium, and cannot be differentiated on this basis alone. Thus, we use "Fdh$^+$" to designate the wild-type phenotype on MacConkey nitrate agar, and "Fdh$^-$" to designate the mutant phenotype, irrespective of the specific genetic lesion.

Strains, plasmids and genetic methods: *E. coli* K-12 strains and plasmids used in this study are listed in Table 1. Genetic crosses were performed using bacteriophage P1 *kc*-mediated transduction (MILLER 1972).

The mini-Mu cloning vector pEG5005 (GROISMAN and CASADABAN 1986) was used to isolate clones which complemented *fdh-24*::Mu dl(Ap^r *lac*) as judged on MacConkey nitrate agar. The donor strain for cloning was VJS773. pVJS101 was constructed by subcloning an 8-kb *Pst*I fragment from a pEG5005-derived clone into pHG329, a medium copy-number cloning vector (STEWART *et al.* 1986). pVJS104 was constructed by cloning the *Pvu*II-*Hin*dIII fragment (containing the ϕ10 promoter) from pT7-3 into *Eco*RV- and *Hin*dIII-digested pACYC184. pVJS103 was constructed by cloning the *Bam*HI-*Pst*I fragment (containing T7 gene *1*) from pGP1-2 into pHG165. pGEM3 was from Promega Corp. (Madison, Wisconsin). Standard methods were used for restriction endonuclease digestion, ligation, and transformation of DNA (DAVIS, BOTSTEIN and ROTH 1980). Restriction enzymes and T4 DNA ligase were from New England Biolabs, Inc. (Beverly, Massachusetts).

TABLE 1

Strains and plasmids

Strain	Genotype	Reference or source
E. coli strains		
MC4100	*araD139* Δ(*argF-lac*)*U169 deoC1 flhD5301 ptsF25 relA1 rpsL150*	CASADABAN (1976)
M9	As MC4100 but *fdhF9*::Mu dl(Ap^r *lac*)	PECHER, ZINONI and BÖCK (1985)
JCB315	*fnr-21*::Tn*10 ana-1 fhuA22 lacY1 leu-6 nirD82 nirH rpsL thi-1 thr-1*	MACDONALD, POPE and COLE (1985)
PK1241	*zdd-230*::Tn*9 gal-25 nirR1 pyrF287 rac rpsL195 trpA9761* Δ*trpE*	BITNER and KUEMPEL (1981)
PK1273	*zdc-235*::Tn*10 gal-25 nirR1 pyrF287 rac rpsL195 trpA9761* Δ*trpE*	BITNER and KUEMPEL (1981)
RK4353	As MC4100 but *gyrA219 non-9*	STEWART and MACGREGOR (1982)
RK4920	As RK4353 but *zcg-622*::Tn*10* (50% linked to *nar*)	STEWART and MACGREGOR (1982)
RK5266	As RK4353 but *narK203*::Tn*10*	STEWART and MACGREGOR (1982)
RK5268	As RK4353 but *narG205*::Tn*10*	STEWART and MACGREGOR (1982)
RK5278	As RK4353 but *narL215*::Tn*10*	STEWART and MACGREGOR (1982)
VJS482	Δ(*argF-lac*)*U169 gal hsdR metB1 recA56 supE44 supF58 trpR*	STEWART and PARALES (1988)
VJS632	Prototroph	STEWART and PARALES (1988)
VJS691	As VJS632 but Δ(argF-*lac*)*U169* Δ(*trpEA*)*2*	STEWART and PARALES (1988)
VJS773	As VJS632 but *mal*::Mu *cts recA938*::*cat*/pEG5005	Laboratory collection
VJS882	As VJS691 but *narG234*::Mu dl1734	This work
VJS1032	As M9 but Δ(*trpEA2*) Ap^r Mu^s (thermostable)	STEWART and BERG (1988)
VJS1048	As MC4100 but *zdc-2092*::Tn*10* (95% linked to *fdnGHI*)	This work
VJS1224	As VJS691 but *fdnG104*::Mu dl1734	This work
VJS1250	As VJS691 but *fdnG108*::Mu dl1734	This work
VJS1260	As VJS691 but *fdnG108*::Mu dl1734 *narL215*::Tn*10*	This work
VJS1262	As VJS691 but *fdnG108*::Mu dl1734 *narG205*::Tn*10*	This work
VJS1264	As VJS691 but *fdnG108*::Mu dl1734 *narX236*::Ω	This work
VJS1263	As VJS691 but *fdnG108*::Mu dl1734 *narK203*::Tn*10*	This work
VJS1266	As VJS691 but *fdnG108*::Mu dl1734 *fnr-21*::Tn*10*	This work
VJS1311	As VJS691 but *narX236*::Ω	This work
VJS1312	As VJS691 but *narG234*::Mu dl1734 *narX236*::Ω	This work
VJS1338	As VJS691 but *narG234*::Mu dl1734 *narL215*::Tn*10*	This work
VJS1339	As VJS691 but *narG234*::Mu dl1734 *fnr-21*::Tn*10*	This work
VJS1611	As MC4100 but *fdnG108*::Mu dl1734	This work
VJS1612	As VJS1032 but *fdnG108*::Mu dl1734	This work
VJS1625	As VJS691 but *fdnI102*::Mu dl1734 *fdnG119*::Ω	This work
VJS1626	As VJS691 but *fdnI103*::Mu dl1734 *fdnG119*::Ω	This work
VJS1627	As VJS691 but *fdnG108*::Mu dl1734 *fdnG119*::Ω	This work
WL8	As MC4100 but *fdh-8*::Mu dl(Ap^r *lac*)	LEINFELDER *et al.* (1988a)
WL24	As MC4100 but *fdh-24*::Mu dl(Ap^r *lac*)	LEINFELDER *et al.* (1988a)
WL25	As MC4100 but *fdh-25*::Mu dl(Ap^r *lac*)	LEINFELDER *et al.* (1988a)
Plasmids		
pACYC184	Cm^r Tc^r	CHANG and COHEN (1978)
pCHM1	Ap^r, *narKGHJI* in *Sal*I-*Eco*RI sites of pBR322	CAROLYN MACGREGOR
pEG5005	Ap^r Km^r	GROISMAN and CASADABAN (1986)
pGEM3	Ap^r, T7 φ10 promoter	CARL SCHNAITMAN
pGP1-2	Km^r, T7 gene *1* (RNA polymerase)	TABOR and RICHARDSON (1985)
pHP45	Ap^r Sp^r (Ω)	PRENTKI and KRISCH (1984)
pT7-3	Ap^r, T7 φ10 promoter	TABOR and RICHARDSON (1985)
pHG165	Ap^r	STEWART *et al.* (1986)
pHG329	Ap^r	STEWART *et al.* (1986)
pVJS101	Ap^r, *fdnGHI* in *Pst*I site of pHG329	This work
pVJS102	Cm^r, *fdnGHI* in *Pst*I site of pVJS104	This work
pVJS103	Ap^r, T7 gene *1* in *Bam*HI-*Pst*I sites of pHG165	This work
pVJS104	Cm^r, T7 φ10 promoter in *Eco*RV-*Hin*dIII sites of pACYC184	This work
pVJS105	Ap^r, *fdnGHI* in *Pst*I site of pGEM3	This work

Culture media: Cultures for β-galactosidase assays were grown in 3-[*N*- morpholino]propanesulfonic acid (MOPS)-buffered medium with glucose as the sole carbon source (STEWART and PARALES 1988). The initial pH of this medium was 7.8. For medium with a lower pH, we used the 2-[*N*-morpholino]ethanesulfonic acid (MES)-buffered medium (pH 6.5) described previously (STEWART and BERG 1988). TYG broth contained (per liter) Bacto-tryptone, 8 g; Bacto-yeast extract, 5 g; NaCl, 5 g; glucose, 20 mM; Na_2MoO_4, 1 μM; and Na_2SeO_3, 1 μM. TYGN was TYG with 40 mM $NaNO_3$. Defined media contained Na_2MoO_4 (1 μM), Na_2SeO_3 (1 μM), and L-tryptophan (0.2 mM). $NaNO_3$ (40 mM) and $NaHCO_2$ (20 mM) were added as indicated.

Defined, complex, and indicator media for routine genetic manipulations were used as described previously (DAVIS, BOTSTEIN and ROTH 1980; MILLER 1972). MacConkey nitrate agar (BARRETT *et al.* 1979) was used to identify Fdh and Nar phenotypes (STEWART and MAC-

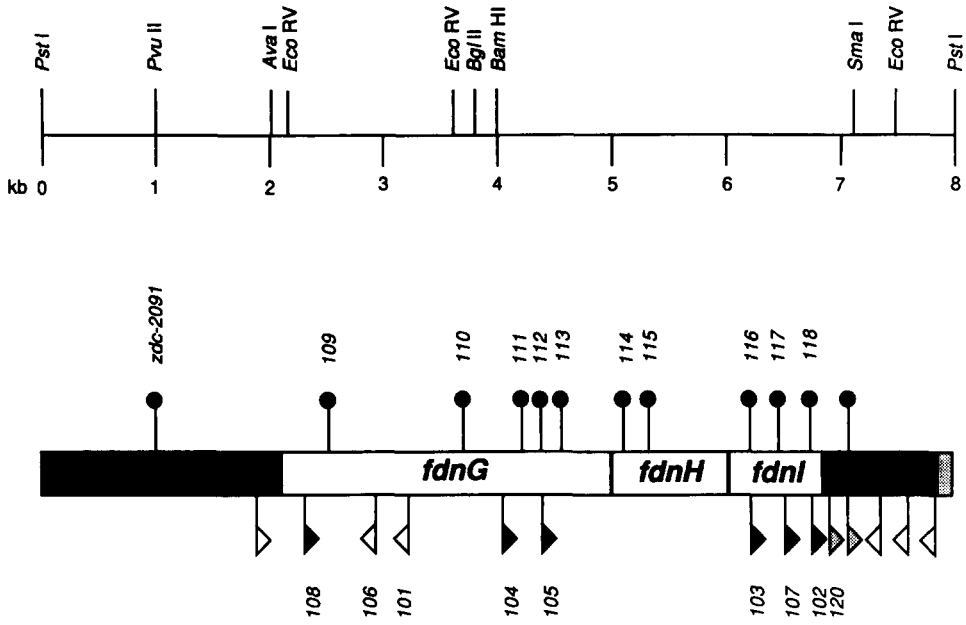

FIGURE 2.—Physical map of the *fdn* region. The restriction map of the pVJS101 insert is shown at the top portion of the figure. No cleavage was observed with *Eco*RI, *Hin*dIII, *Kpn*I, *Nco*I, *Nsi*I or *Xho*I. The bottom portion of the figure shows insertions and location of the genes. Mu dl1734 insertions are represented by triangles. Lac$^+$ insertions are black and define the direction of transcription of *fdnGHI* (rightward). Lac$^-$ insertions are white, and weakly Lac$^+$ insertions are gray (described in the text). Tn*5* insertions are represented by filled circles. The gray region on the right end defines DNA from Mu sequences derived from the cloning vector pEG5005 (see text).

GREGOR 1982). Agar and dehydrated media were from Difco Laboratories (Detroit, Michigan). Other components were from Sigma (St. Louis, Missouri).

Culture conditions: Cultures for β-galactosidase assays were grown exactly as described by STEWART and PARALES (1988), care being taken to maintain cultures in balanced growth. Anaerobic cultures for formate dehydrogenase-N assays were grown in TYG or TYGN in 250-ml flasks fitted with rubber stoppers. Cultures were grown with gentle agitation to late-exponential phase (approximately 80 Klett units), chilled on ice, and washed with 50 mM $NaPO_4$ buffer. Cell pellets were stored overnight at −20°.

Enzyme assays: β-Galactosidase assays were done at room temperature, approximately 21°. Cell pellets were suspended in 4 ml of Z buffer (MILLER 1972) and stored on ice. Activity was measured in $CHCl_3$-SDS-permeabilized cells by monitering the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside. Activities are expressed in Miller units (MILLER 1972).

Formate dehydrogenase-N activity was assayed at 30° by measuring phenazine methosulfate (PMS)-mediated reduction of dichlorophenol indophenol (DCPIP) essentially as described by LESTER and DEMOSS (1971). Cell-free extracts were prepared with a French pressure cell. The cuvettes were sealed with rubber stoppers and the headspace was flushed with 95%N_2-5%CO_2 for 1 min before the reaction was initiated by the addition of formate. Activities are expressed as μmol DCPIP reduced min^{-1} mg $protein^{-1}$. Protein concentrations were determined by the method of BRADFORD (1976).

Reported values for enyme assays are averaged from at least two independent experiments.

Insertions in the *fdnGHI* and *nar* regions: Mu dl1734 insertions were isolated in pVJS101 (*fdnGHI*$^+$) and pCHM1 (*narKGHJI*$^+$) as described by CASTILHO, OLFSON and CASADABAN (1984) and STEWART and PARALES (1988). Tn*5* insertions were isolated in pVJS102 (*fdnGHI*$^+$) as described by BERG, SCHMANDT and LOWE (1983) and STEWART and PARALES (1988). *fdnG119*::Ω was constructed by cloning *Bam*HI-excised Ω into *Bcl*I-digested pVJS101 (see Figure 2). *narX236*::Ω was constructed by replacing the large central *Bcl*I fragment in *narX* (STEWART, PARALES and MERKEL 1989) with *Bam*HI-excised Ω.

All insertions were crossed back to the chromosome by recombination in a *recBC sbcBC* strain as described by WINANS *et al.* (1985) and STEWART and PARALES (1988). The insertions were then moved into strain VJS691 via P1 transduction by selecting for kanamycin (or spectinomycin, for Ω) resistance. We mapped insertions to the *fdnGHI* region by demonstrating linkage to *zdc-2092*::Tn*10*. This Tn*10* insertion, which was 95% linked to *fdh-24*::Mu dl(Ap^r *lac*), was isolated from a pool of random Tn*10* insertions by transducing WL24 [*fdh-24*::Mu dl(Ap^r *lac*)] to tetracycline resistance and screening for Fdh$^+$ colonies (KLECKNER, ROTH and BOTSTEIN 1977). All of the backcrossed *fdn*-region insertions were linked to *zdc-2092*::Tn*10*. The *nar* insertions were similarly mapped, using *zcg-622*::Tn*10* (STEWART and MACGREGOR 1982).

Detection of plasmid-encoded proteins: Plasmid-encoded proteins were detected in strain VJS482 using an *in vivo* T7 expression system (TABOR and RICHARDSON 1985). This method involved cloning genes of interest downstream from a phage T7 promoter (the φ10 promoter) and transforming this plasmid into a strain which carried the gene for T7 RNA polymerase (gene *1*) under the control of a heat-inducible promoter. After heat induction of T7 RNA polymerase, rifampicin was added to inhibit host RNA polymerase. Subsequent addition of [^{35}S]methionine resulted in selective labeling of plasmid-encoded gene products.

We initially used pGEM3 as the expression vector in this system. However, we found that strains containing the *fdnGHI*$^+$ plasmid pVJS105 grew slowly, and we had difficulty isolating Tn*5* insertions in this plasmid. We presume that this was due to the high copy-number of pGEM3 (approximately 200 per cell). Therefore, we constructed the *fdnGHI*$^+$ plasmid pVJS102, which has a copy-number of less than 20 per cell. Cells containing pVJS102 grew well, and we used this plasmid to isolate Tn*5* insertions. pVJS103, which carries T7 gene *1*, is compatible with pVJS102. Cultures were grown anaerobically in TYG medium prior to

induction and labeling. Samples were electrophoresed on Laemmli gels (HAMES and RICKWOOD 1981), fixed, stained, treated with sodium salicylate (CHAMBERLIN 1979), dried and exposed to X-ray film at −70°.

RESULTS

Molecular cloning of *fdnGHI*: LEINFELDER *et al.* (1988a) isolated a mutant, WL24, which contains a transposon insertion in the structural gene for the α subunit of formate dehydrogenase-N. The strain carrying this mutation, *fdh-24*::Mu dl(Ap^r *lac*), produces a truncated 110-kDa selenopeptide and exhibits the characteristic Fdh$^-$ phenotype on MacConkey nitrate agar. We used the *in vivo* cloning vector pEG5005 (GROISMAN and CASADABAN 1986) to isolate six clones which complemented the Fdh$^-$ phenotype of WL24. DNA fragments were subcloned into pHG329. One resulting plasmid, pVJS101, contained an 8-kb *Pst*I fragment and was used for all subsequent experiments and constructions. The restriction map of the pVJS101 insert is shown in Figure 2.

Insertion mutagenesis of *fdnGHI*: Fourteen Mu dl1734 insertions (CASTILHO, OLFSON and CASADABAN 1984) were isolated in pVJS101. Mu dl1734 is a bacteriophage transposon which confers kanamycin resistance and contains a promoterless *lac* operon which can be used to form operon fusions. Figure 2 shows the location and orientation of these insertions within the pVJS101 insert. The resulting plasmids were examined for their ability to complement *fdh-24*::Mu dl(Ap^r *lac*) on MacConkey nitrate agar. The region of DNA necessary for complementation was approximately 4.5 kb (Figure 2).

We backcrossed the Mu dl1734 insertions to the *E. coli* chromosome and mapped them to the vicinity of the *fdh-24*::Mu dl(Ap^r *lac*) insertion as described in MATERIALS AND METHODS. These insertions were examined for their Lac phenotype on MacConkey lactose medium plus nitrate. Within the 4.5-kb region essential for complementation of *fdh-24*::Mu dl(Ap^r *lac*), all Lac$^+$ insertions were in one orientation, while all Lac$^-$ insertions were in the opposite orientation (Figure 2). An exceptional insertion, *fdn-120*::Mu dl1734, conferred a weak Lac$^+$ phenotype but did not affect complementation. We conclude that this insertion was located just downstream of the *fdnGHI* operon.

Genetic map location of *fdnGHI*: We used Hfr time of entry experiments to determine that *fdnG108*::Mu dl1734 was located between *his* and *trp* on the *E. coli* genetic map. We further localized *fdnG108*::Mu dl1734 by demonstrating linkage to *zdd-230*::Tn*9* (approximately 24% linkage) and *zdc-235*::Tn*10* (approximately 50% linkage) in P1 transduction crosses. *zdc-235*::Tn*10* is located at 32 min on the *E. coli* genetic map (HENSON and KUEMPEL 1983), and comparing the restriction maps of the *narZ* region (BONNEFOY *et al.* 1987) and the terminus region (HENSON and KUEMPEL 1983) reveals that *zdc-235*::Tn*10* is located within the *narZ* operon. *fdnGHI* was located at approximately 1565 kb on the physical map of KOHARA, AKIYAMA and ISONO (1987), within 10 kb of the *narZ* operon.

Genetic map location of other *fdh* mutations: We also mapped the positions of *fdh-8*::Mu dl(Ap^r *lac*) and *fdh-25*::Mu dl(Ap^r *lac*), two other formate dehydrogenase-N-specific mutations recovered in the screen for strains defective in selenium metabolism (LEINFELDER *et al.* 1988a). *fdh-8*::Mu dl(Ap^r *lac*) was linked to *rha* and therefore resides in a cluster of genes known to affect formate dehydrogenase-N activity (BARRETT and RIGGS 1982; MANDRAND-BERTHELOT *et al.* 1988; PAVEGLIO *et al.* 1988). *fdh-25*::Mu dl(Ap^r *lac*) was an allele of *narL*. This mutation conferred the characteristic NarL$^-$ phenotype, mapped to the *nar* region, and was complemented in *trans* by *narL*$^+$ (data not shown).

Organization of the *fdnGHI* operon: We used an *in vivo* T7 expression system (TABOR and RICHARDSON 1985) to detect the proteins encoded by *fdnGHI*. Figure 3 shows the proteins encoded by pVJS105, which contains the 8-kb DNA fragment from pVJS101 subcloned into the T7 expression vector pGEM3. This plasmid produced proteins of approximate M_r 110,000, 32,000 and 20,000. These sizes are consistent with the known molecular masses of the three subunits (α, β and γ) of purified formate dehydrogenase-N (ENOCH and LESTER 1975). The FdnH (β) polypeptide in Figure 3 is difficult to see because it migrated very close to β-lactamase, but it was visible on all gels we have run.

To determine the order of the three genes in the *fdn* region, we isolated and mapped 12 Tn*5* insertions in pVJS102, and examined these insertion plasmids for their ability to complement *fdnG108*::Mu dl1734. pVJS102 contains the 8-kb *Pst*I fragment encoding *fdnGHI* cloned in a low copy-number T7 expression vector. The locations of the Tn*5* insertions are shown in Figure 2. We then examined the proteins produced by these insertion plasmids, and we correlated the missing polypeptides with the positions of the Tn*5* insertions.

Figure 4 shows the proteins produced from the Tn*5* insertion plasmids. In this figure, the FdnI (γ) band is more difficult to see because it migrated very close to chloramphenicol acetyltransferase. The clone containing insertion *zdc-2091*::Tn*5*, which mapped outside of the presumed *fdnGHI* coding region, produced all three *fdn*-specific polypeptides (Figure 4, lane 1).

All of the plasmids containing the Tn*5* inserts in-

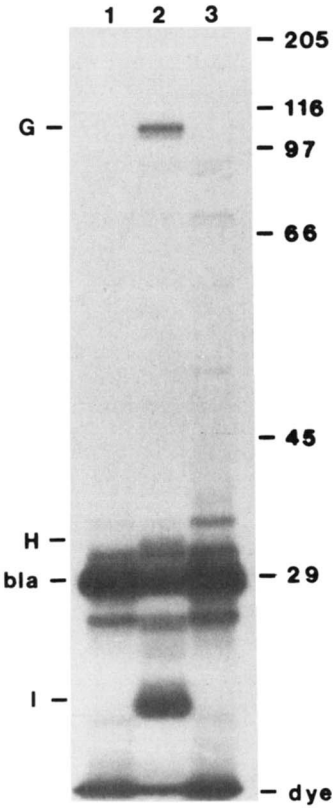

FIGURE 3.—*fdnGHI*-encoded polypeptides. Polypeptides encoded by an *fdnGHI*-containing plasmid were detected with a T7 expression system (described in the text). Samples were electrophoresed on a 10% polyacrylamide Laemmli gel. Lanes: 1, pGEM3 (vector); 2, pVJS105 (*fdnGHI*$^+$); 3, pGEM3. "G," "H" and "I" indicate the presumed α, β and γ subunits of formate dehydrogenase-N; "bla" indicates β-lactamase. Molecular mass markers were carbonic anhydrase (29 kD), egg albumin (45 kD), bovine albumin (66 kD), phosphorylase *b* (97 kD), β-galactosidase (116 kD) and myosin (205 kD).

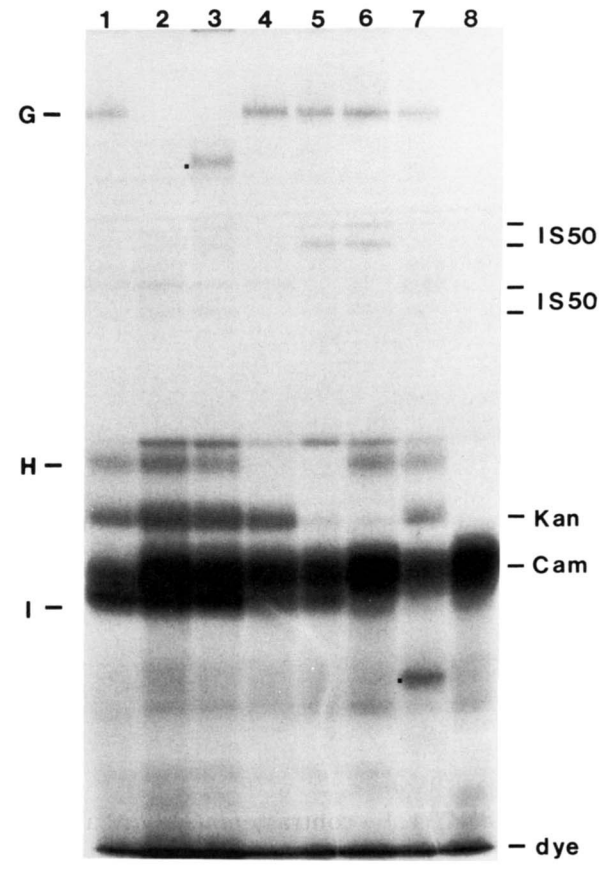

FIGURE 4.—*fdnGHI* gene-product relationships. Polypeptides encoded by Tn*5*-mutagenized *fdnGHI*-containing plasmids were detected with a T7 expression system (described in the text). Samples were electrophoresed on a 12.5% polyacrylamide Laemmli gel. Tn*5* insertions were in pVJS102, which contained the 8-kb *Pst*I *fdnGHI*$^+$ fragment cloned into pVSJ104 (Figure 2). Lanes: 1, *zdc-2091*::Tn*5*; 2, *fdnG109*::Tn*5*; 3, *fdnG112*::Tn*5*; 4, *fdnH114*::Tn*5*; 5, *fdnH115*::Tn*5*; 6, *fdnI117*::Tn*5*; 7, *fdnI118*::Tn*5*; 8, pVJS104 (vector). "G," "H" and "I" indicate the presumed α, β and γ subunits of formate dehydrogenase-N. The dots in lanes 3 and 7 indicate truncated FDN proteins (dexcribed in the text). "IS*50*" indicates the IS*50* gene products (ROTHSTEIN *et al.* 1980); "Cam" indicates chloramphenicol acetylytransferase (SHAW *et al.* 1979); "Kan" indicates kanamycin phosphotransferase (BECK *et al.* 1982). Expression of kanamycin phosphotransferase and various IS*50* proteins was dependent on the orientation of the Tn*5* with respect to the ϕ10 promoter. Lanes 2, 3, 4 and 7 represent Tn*5* insertions oriented with respect to the ϕ10 promoter such that the *kan* gene product and the truncated IS*50*L gene products were synthesized. Lanes 5 and 6 represent Tn*5* insertions in the opposite orientation, such the full-sized products of IS*50*R were synthesized (see ROTHSTEIN *et al.* 1980). Molecular mass markers (not shown) were the same as those indicated in Figure 3.

dicated in Figure 2 were analyzed for their protein products; representative examples are shown in Figure 4. Insertion *fdnG109*::Tn*5* (lane 2) abolished production of the 110-kDa polypeptide, while insertions *fdnG112*::Tn*5* (lane 3), *fdnG110*::Tn*5*, and *fdnG111*::Tn*5* (data not shown) produced truncated 110-kDa polypeptides. Insertions *fdnH114*::Tn*5* and *fdnH115*::Tn*5* (lanes 4 and 5) abolished production of the 32-kDa polypeptide. Insertions *fdnI117*::Tn*5* (lane 6) and *fdnI116*::Tn*5* (data not shown) abolished production of the 20-kDa polypeptide, while insertion *fdnI118*::Tn*5* produced a truncated 20-kDa polypeptide (lane 7). Based on the map positions of the Tn*5* insertions and the direction of transcription determined from the Mu dl1734 operon fusions, we assign the gene order *fdnGHI* (Figure 2).

We did not observe any polar effects of the Tn*5* insertions in *fdnGHI*. Indeed, previous work indicates that T7 RNA polymerase does not recognize many *E. coli* transcription terminators (DUNN and STUDIER 1983; JENG, GARDNER and GUMPORT 1990; STUDIER and MOFFATT 1986; TABOR and RICHARDSON 1985). Therefore, we wished to determine whether *fdnGHI* is organized in an operon with no internal promoters, or whether the three genes are transcribed from independent promoters. To do this, we cloned the highly polar Ω interposon (PRENTKI and KRISCH 1984) into the *Bgl*II site of pVJS101 and crossed this insertion back to the *E. coli* chromosome as described in

TABLE 2

Effects of pH, formate and anaerobiosis on Φ(*fdnG-lacZ*) and Φ(*narG-lacZ*) expression

	β-Galactosidase specific activity					
	Φ(*fdnG-lacZ*)			Φ(*narG-lacZ*)		
Culture medium	$+O_2$	$-O_2$	$+NO_3^- - O_2$	$+O_2$	$-O_2$	$+NO_3^- - O_2$
MOPS (pH 7.8)	<1	14	1280	<1	7	825
MOPS + formate	<1	12	1380	<1	16	860
MES (pH 6.5)	<1	10	1010	<1	26	670

Strains VJS1224 [Φ(*fdnG-lacZ*)*104*] and VJS882 [Φ(*narG-lacZ*)*234*] were cultured aerobically or anaerobically in the indicated media as described in the text. Nitrate was added as indicated. β-Galactosidase specific activities were determined in permeabilized cells; units are arbitrary (Miller units).

TABLE 3

Effects of *fnr*, *narL* and *narX* insertions on Φ(*fdnG-lacZ*) and φ(*narG-lacZ*) expression

	β-Galactosidase specific activity			
	Φ(*fdnG-lacZ*)		Φ(*narG-lacZ*)	
Genotype	$-O_2$	$+NO_3^- - O_2$	$-O_2$	$+NO_3^- - O_2$
Wild-type	6	880	11	805
fnr-21::Tn*10*	<1	<1	<1	<1
narL215::Tn*10*	7	28	7	10
narX236::Ω	5	310	7	505

Strains VJS1250 [Φ(*fdnG-lacZ*)*108*], VJS882 [Φ(*narG-lacZ*)*234*] and their derivatives (see Table 1 for descriptions) were cultured anaerobically in MOPS medium as described in the text. Nitrate was added as indicated. β-Galactosidase specific activities were determined in permeabilized cells; units are arbitrary (Miller units).

MATERIALS AND METHODS. Ω, which confers resistance to spectinomycin, contains strong transcription terminators at each of its ends. We transduced *fdnG119*::Ω into three strains which contained Φ(*fdnlacZ*) operon fusions. If *fdnH* and *fdnI* expression is due to transcription that initiates upstream of *fdnG*, we would expect operon fusions located downstream of the Ω element to be silent due to polarity (CIAMPI and ROTH 1988). *fdnG119*::Ω had no effect on expression of the upstream operon fusion formed by *fdnG108*::Mu dl1734. By contrast, *fdnG119*::Ω abolished expression from the downstream operon fusions formed by *fdnI103*::Mu dl1734 and *fdnI102*::Mu dl1734.

Regulation of Φ(*fdnG-lacZ*) expression: We examined the regulation of *fdnGHI* expression under various growth conditions. Table 2 shows the effects of pH, formate, aeration and nitrate on Φ(*fdnG-lacZ*) expression. Maximum induction of β-galactosidase required anaerobiosis plus nitrate. Expression of Φ(*fdnG-lacZ*) was unaffected by addition of formate or by growth at low pH (Table 2).

Transcription activation of the nitrate reductase structural gene operon (*narGHJI*) requires the FNR protein for induction by anaerobiosis and the NarL protein for induction by nitrate (STEWART 1982). Since Φ(*fdnG-lacZ*) was also induced anaerobically by nitrate (Table 2), we reasoned that induction of *fdnGHI* might also be mediated by FNR and NarL. To test this, we examined expression of Φ(*fdnG-lacZ*) in strains which contained transposon insertions in *fnr* and *narL*. For comparison, we also measured induction of Φ(*narG-lacZ*).

fnr-21::Tn*10* abolished anaerobic induction of both Φ(*fdnG-lacZ*) and Φ(*narG-lacZ*) and *narL215*::Tn*10* prevented induction by nitrate (Table 3). The *narX236*::Ω allele caused a subtle decrease of Φ(*fdnG-lacZ*) and Φ(*narG-lacZ*) induction. These results are fully consistent with previous observations (STEWART and PARALES 1988; STEWART and BERG 1988).

Formate oxidation can be coupled to the reduction of other anaerobic electron acceptors in addition to nitrate (ABOU-JAOUDÉ, CHIPPAUX and PASCAL 1979; POPE and COLE 1982; YAMAMOTO and ISHIMOTO 1977). It had seemed likely that formate dehydrogenase-N mediates electron flow from formate to the electron acceptors nitrite, trimethylamine *N*-oxide and fumarate. However, Φ(*fdnG-lacZ*) expression was only weakly induced by nitrite, and essentially not induced by trimethylamine *N*-oxide or fumarate (Table 4).

Confoundingly, Φ(*narG-lacZ*) expression was significantly induced by nitrite (Table 4). However, this appeared to be an indirect consequence of the fact that these strains are *narG*; Φ(*fdnG-lacZ*) expression in a *narG*::Tn*10* strain was also efficiently induced by nitrite (Table 4).

***fdnGHI*$^+$ encodes formate dehydrogenase-N:** Formate dehydrogenase-N activity can be assayed by measuring the PMS-mediated reduction of DCPIP (RUIZ-HERRERA and DEMOSS 1969). Strains with mutations in *fdnGHI* are expected to be defective in DCPIP/PMS-linked activity, while strains with mutations in *fdhF*, the structural gene for formate dehydrogenase-H, retain wild-type levels of DCPIP/PMS-linked activity.

Table 5 shows DCPIP/PMS-linked activity in extracts of such strains grown anaerobically in the presence and absence of nitrate. The wild-type strain expressed nitrate-inducible DCPIP/PMS-linked activity, while a strain which contained both *fdhF* and *fdnG* insertions expressed no activity with or without nitrate. An *fdnGHI*$^+$ strain with an *fdhF* insertion expressed activity comparable to that of the wild type. As expected, activity in extracts of an *fdhF*$^+$ strain carrying an *fdnG* insertion was undetectable, suggesting that all measurable DCPIP/PMS-linked formate dehydrogenase activity is contributed exclusively by formate dehydrogenase-N.

Formate dehydrogenase-H is a component of formate-hydrogen lyase. This enzyme complex is respon-

TABLE 4

Effects of electron acceptors on Φ(*fdnG-lacZ*) and Φ(*narG-lacZ*) expression

Genotype	β-Galactosidase specific activity				
	$-O_2$	$+NO_3^- - O_2$	$+NO_2^- - O_2$	+TMAO[a] $- O_2$	+Fumarate $- O_2$
Φ(*fdnG-lacZ*)*108*	5	1040	42	17	7
Φ(*narG-lacZ*)*234*	7	940	440	7	17
Φ(*fdnG-lacZ*)*108 narG205*::Tn*10*	2	—[b]	175	—	—

Strains VJS1250 [Φ(*fdnG-lacZ*)*108*], VJS882 [Φ(*narG-lacZ*)*234*] and VJS1262 [Φ(*fdnG-lacZ*)*108 narG205*::Tn*10*] were cultured anaerobically in MOPS medium as described in the text. Electron acceptors (40 mM) were added as indicated. β-Galactosidase specific activities were determined in permeabilized cells; units are arbitrary (Miller units).

[a] TMAO, trimethylamine *N*-oxide.

[b] —, not determined in this experiment. In other experiments, β-galactosidase synthesis was fully induced (approximately 1000 units) in this strain cultured in the presence of nitrate.

TABLE 5

Formate dehydrogenase-N activities in *fdnG* and *fdhF* mutant strains

Strain	Genotype	DCPIP/PMS-formate dehydrogenase specific activity	
		$-O_2$	$+NO_3^- - O_2$
MC4100	*fdhF*$^+$ *fdnG*$^+$	0.09	0.27
VJS1032	*fdhF9*::Mu *fdnG*$^+$	0.09	0.48
VJS1611	*fdhF*$^+$ *fdnG108*::Mu	<0.01	<0.01
VJS1612	*fdhF9*::Mu *fdnG108*::Mu	<0.01	<0.01

Strains were cultured anaerobically in TYG medium; nitrate was added as indicated. Formate dehydrogenase-N specific activities were determined in cell-free extracts as described in the text. Units are μmol DCPIP reduced min^{-1} mg $protein^{-1}$.

sible for anaerobic production of gas (hydrogen) in glucose-grown cultures. Formate-hydrogen lyase activity can be estimated by measuring gas accumulation with Durham tubes (GUEST 1969). As expected, cultures of the *fdhF* strains failed to accumulate gas, while cultures of the *fdnG* strains accumulated wild-type levels of gas. This further demonstrates that the products of the *fdnGHI* operon are exclusively involved in respiratory formate oxidation.

None of the three *fdnG*::Mu dl1734 strains we examined expressed detectable formate dehydrogenase-N activity (data not shown). Our standard prototrophic strain, VJS632, had activity comparable to that of MC4100. We also wished to demonstrate that a plasmid containing *fdnGHI*$^+$ could restore DCPIP/PMS-linked activity to one of the mutant strains. The *fdn* mutant strain VJS1250, when carrying pVJS102, produced about one-half of the wild-type activity, irrespective of added nitrate (data not shown).

DISCUSSION

Organization of the *fdnGHI* operon: We identified a 4.5-kb region of DNA that was essential for complementing the *fdh-24*::Mu dl(Ap^r *lac*) mutation (Figure 2). This region encoded the three formate dehydrogenase-N subunits of 110, 32 and 20 kDa (ENOCH and LESTER 1975; Figures 3 and 4). All of our insertions were genetically linked to the *fdh-24*::Mu dl(Ap^r *lac*) mutation, so we are confident that we have cloned the chromosomal region affected by this mutation.

The orientation of Lac$^+$ *fdn* insertions (Figure 2) indicates that all three genes are transcribed in the same direction, clockwise with respect to the *E. coli* genetic map. *fdnG119*::Ω had no effect on expression of the upstream Φ(*fdnG-lacZ*) operon fusion, but it abolished expression of the downstream Φ(*fdnI-lacZ*) operon fusions, indicating that *fdnGHI* is organized as an operon. Furthermore all Lac$^+$ insertions throughout *fdnGHI* were regulated virtually identically (data not shown).

Location of *fdnGHI* on the *E. coli* genetic map: The genetic map position of *fdnGHI* (32 min) was distinct from that of *fdhF*, the stuctural gene for formate dehydrogenase-H (92 min; PECHER, ZINONI and BÖCK 1985). By inspection, we were able to locate *fdnGHI* at coordinate 1565 kb on the Kohara physical map of the *E. coli* chromosome (KOHARA, AKIYAMA and ISONO 1987). Surprisingly, *fdnGHI* mapped very close to the *narZ* operon, which encodes a cryptic nitrate reductase. The *narZ* operon has DNA sequence homology to *narGHJI*, and multicopy plasmids carrying *narZ*$^+$ complement *narG* mutations (BONNEFOY *et al.* 1987). The physiological function of the *narZ*-encoded nitrate reductase is unknown. We estimate that *fdnGHI* is approximately 10 kb from *zdc-235*::Tn*10*, which maps within the *narZ* operon. The proximity of *fdnGHI* and *narZ* is of unknown significance.

Regulation of *fdnGHI* expression: Previous studies have shown that formate dehydrogenase-N activity is highest in extracts of cells grown anaerobically in the presence of nitrate (reviewed by STEWART 1988). Indeed, significant Φ(*fdnG-lacZ*) expression occurred only when cultures were grown anaerobically with nitrate (Tables 2–4).

Many strains of *E. coli* K-12 also couple formate oxidation to the reduction of nitrite, and it has been

hypothesized that formate dehydrogenase-N is a component of the nitrite respiratory chain (ABOU-JAOUDÉ, CHIPPAUX and PASCAL 1979; ABOU-JAOUDÉ, PASCAL and CHIPPAUX 1979; see also POPE and COLE 1982). We therefore examined whether *fdnGHI* expression was induced by alternate electron acceptors. In contrast to the approximately 200-fold induction by nitrate, Φ(*fdnG-lacZ*) expression was induced only about eightfold by nitrite, at least in our standard laboratory strain (Table 4). We entertain two ideas that might explain this observation. First, it is possible that formate dehydrogenase-N is not involved in the formate-nitrite respiratory chain. Second, it is possible that the formate-nitrite respiratory chain is only weakly induced by nitrite. Further work is required to identify the formate dehydrogenase that is involved in nitrite respiration.

The observation that nitrite is a relatively strong inducer of Φ(*fdnG-lacZ*) and Φ(*narG-lacZ*) expression in *narG* strains is puzzling. It is possible that our nitrite is contaminated by small amounts of nitrate. In *nar*$^+$ strains, this residual nitrate could cause a low level induction of *fdn* expression. In *narG* strains, this residual nitrate would be only slowly metabolized, and could act as a gratuitous inducer.

All of the *fdnG* insertion mutants we examined were devoid of DCPIP/PMS-linked formate dehydrogenase-N enzyme activity, while an *fdhF* mutant retained wild-type levels of activity (Table 5). These observations suggest that DCPIP/PMS-linked formate dehydrogenase activity is exclusively due to formate dehydrogenase-N.

Common mechanisms for regulating *fdnGHI* and *narGHJI* expression: Previous work has shown that *narGHJI* operon expression requires FNR for induction by anaerobiosis, and NarL for induction by nitrate (STEWART 1982; LI and DEMOSS 1988; reviewed by STEWART 1988). FNR shows sequence and functional similarity to CRP, the cyclic AMP receptor protein (BELL *et al.* 1989; SPIRO and GUEST 1987), although the mechanism by which FNR senses anaerobiosis is unknown. NarX and NarL show sequence similarity to the sensor and regulator classes of two component regulatory proteins, respectively (see STOCK, NINFA and STOCK 1989), although the role of NarX is unclear (STEWART and PARALES 1988; S. M. EGAN and V. STEWART, manuscript in preparation).

Expression of Φ(*fdnG-lacZ*) required *fnr*$^+$ for anaerobic induction, and required *narL*$^+$ for complete induction by nitrate (Table 3). The subtle decrease in nitrate-induced Φ(*fdnG-lacZ*) expression observed in the *narX236*::Ω mutant paralleled the effect of *narX* insertion mutations on Φ(*narG-lacZ*) expression (Table 3; STEWART and BERG 1988; STEWART and PARALES 1988; S. M. EGAN and V. STEWART, manuscript in preparation). Thus, anaerobic and nitrate regulation of *fdnGHI* and *narGHJI* was mediated through common *trans*-acting regulatory elements.

We consistently observed a slight nitrate induction of Φ(*fdnG-lacZ*) expression in *narL*::Tn*10* strains (Table 3). This induction was not observed with the Φ(*narG-lacZ*) operon fusion; we do not know what was responsible for this residual induction of Φ(*fdnG-lacZ*).

Previous studies led to the conclusion that formate dehydrogenase-N synthesis is not activated by FNR (LAMBDEN and GUEST 1976; SHAW and GUEST 1983). Our results with Φ(*fdnG-lacZ*) operon fusions contradict this conclusion (Tables 2 and 3). Efficient *fdnGHI* expression requires nitrate (Tables 2–5; reviewed by STEWART 1988), which was not added to the cultures examined by GUEST and co-workers. The *fnr*$^+$ requirement for formate dehydrogenase-N expression was previously noted by BIRKMANN, SAWERS and BÖCK (1987).

IUCHI and LIN (1988) reported that formate dehydrogenase-N is synthesized in aerated cultures with xylose as sole carbon source. Again, our results are inconsistent with this conclusion. We found that Φ(*fdnG-lacZ*) was not expressed at detectable levels in aerated cultures with either glucose (Table 2) or xylose (data not shown) as the sole carbon source.

Figure 1 illustrates our model for regulation of *fdnGHI* and *narGHJI* transcription. The FNR protein activates transcription in response to anaerobiosis. The NarL protein, in the presence of nitrate, activates transcription of *fdnGHI* and *narGHJI*. The *cis*-acting sites required for anaerobic induction and nitrate induction have been identified upstream of the *narGHJI* promoter (LI and DEMOSS 1988). We presume that the *fdnGHI* promoter region contains similar regulatory sequences.

Differential regulation of formate dehydrogenase-N and formate dehydrogenase-H synthesis: The two formate dehydrogenases of *E. coli*, formate dehydrogenase-H (encoded by *fdhF*; involved in formate-hydrogen lyase) and formate dehydrogenase-N (encoded by *fdnGHI*; involved in formate-nitrate oxidoreductase), are physiologically and genetically distinct. Not surprisingly, regulation of their synthesis occurs through separate pathways. Expression of *fdhF* is repressed by nitrate, and is stimulated by low pH and added formate (BIRKMANN *et al.* 1987; WU and MANDRAND-BERTHELOT 1987). Transcription of *fdhF* requires σ^{54} (the *ntrA* gene product; KUSTU *et al.* 1989), and is independent of *fnr*$^+$ (BIRKMANN, SAWERS and BÖCK 1987). Nitrate repression of *fdhF* is independent of *narL*$^+$ (STEWART and BERG 1988). By contrast, expression of *fdnGHI* was induced by nitrate, while low pH and added formate had no effect (Table

2). Synthesis of formate dehydrogenase-N is independent of σ^{54} (BIRKMANN, SAWERS and BÖCK 1987), and it requires *fnr*$^+$ (BIRKMANN, SAWERS and BÖCK 1987; Table 3). Finally, nitrate induction required *narL*$^+$ (Table 3).

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