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 produces 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 fdnGHI and narGHJI 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.

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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<sub>NAR</sub> 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<sub>NAR</sub>. Nitrate reductase also consists of three subunits and contains molybdenum cofactor and cytochrome b<sub>NAR</sub>. 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 sequences show that NarX (Nohno et al. 1989; Parales and Merkel 1989) and NarL (Gunsalus, Kalman and Stewart 1989; Nohno et al. 1989; Stewart, Parales and Merkel 1989) are similar to other prokaryotic 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 Guest 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 selenocystein-tRNA<sub>Ec</sub> (Leinfeld, Stadtman and Böck 1989; Zinoni et al. 1986, 1987).

E. coli expresses only two selenoproteins, formate dehydrogenase-N and formate dehydrogenase-H, as
Our data suggest that the fdnCHI operon
product of a truncated 110-kDa selenopeptide. At
both formate dehydrogenase-N and formate dehydro-
ene is required for the anaerobic induction of
specificity on SDS-polyacrylamide gels. Formate dehydro-
reactions. The donor strain for cloning was VJS773.
Purification from a pEG5005-derived clone into pHG329, a me-
mainly through common pathways (Figure 1).

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

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 dlp', 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-Bertelot 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 dlp' lac). This clone contained three genes, which we designate fdnGHI. Our data suggest that the fdnGHI operon encodes all three subunits of formate dehydrogenase-
E. coli strains

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<td>Leinfelder et al. (1988a)</td>
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Plasmids

- pACYC184: Cm' Te'
- pCHM1: Ap', narG501 in SacI-EcoRI sites of pBR322
- pEG5005: Ap' Km'
- pGEM3: Ap', T7 φl promoter
- pGP1-2: Km', T7 gene 1 (RNA polymerase)
- pHP5: Ap' Sp' (Ω)
- pT7-3: Ap', T7 φl promoter
- pHG165: Ap'
- pHC329: Ap'
- pVJS102: Cm', fadGHI in PstI site of pVJS104
- pVJS103: T7 φl promoter in BamHI-PstI sites of pBR322
- pVJS104: Cm', T7 φl promoter in EcoRI-HindIII sites of pACYC184

**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; Na₂MoO₄, 1 μM; and Na₂SeO₃, 1 μM. TYGN was TYG with 40 mM NaNO₃. Defined media contained Na₂MoO₄ (1 μM), Na₂SeO₃ (1 μM), and L-tryptophan (0.2 mM). NaNO₃ (40 mM) and NaHCO₃ (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 MacGregor 1982).
GREGOR 1982). Agar and dehydrated media were from Difco Laboratories (Detroit, Michigan). Other components were from Sigma (St. Louis, Missouri).

**Culture conditions:** Cultures for $\beta$-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 NaPO₄ buffer. Cell pellets were stored overnight at -20°C.

**Enzyme assays:** $\beta$-Galactosidase assays were done at room temperature, approximately 21°C. Cell pellets were suspended in 4 ml of Z buffer (MILLER 1972) and stored on ice. Activity was measured in CHCl₃-SDS-permeabilized cells by monitoring the hydrolysis of o-nitrophenyl-$\beta$-D-galactopyranoside. Activities are expressed in Miller units (MILLER 1972).

Formate dehydrogenase-N activity was assayed at 30°C by measuring phenazine methosulfate (PMS)-mediated reduction of dichlorophenol indophenol (DCPIP) essentially as described by LESTER and DEMOS (1971). All insertions were prepared with a French pressure cell. The cuvettes described by LESTER and DEMOS (1971). Cell-free extracts were grown in TYG or TYGN in 250-ml flasks fitted with rubber stoppers. Cultures 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 medium prior to recombination in a recBC shbGC 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 $\Omega$) resistance. We mapped insertions to the fdnGHZ region by demonstrating linkage to zdc-2092::Tn10. This Tn10 insertion, which was 95% linked to fdh-24::Mu dl(Ap'), was isolated from a pool of random Tn10 insertions by transducing WL24 [fdh-24::Mu dl(Ap', 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::Tn10. The nar insertions were similarly mapped, using zeg-622::Tn10 (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 $\phi$10 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 $\lbrack^{35}S\rbrack$-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 fdnGHZ plasmid pVJS105 grew slowly, and we had difficulty isolating Tn5 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 fdnGHZ 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 Tn5 insertions. pVJS103, which carries T7 gene 1, is compatible with pVJS102. Cultures were grown anaerobically in TYG medium prior to forming 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 $\lbrack^{35}S\rbrack$-methionine resulted in selective labeling of plasmid-encoded gene products.

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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°C.

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' 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 PsiI 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' 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' 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' 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 zdc-235::Tn9 (approximately 24% linkage) and zdc-235::Tn10 (approximately 50% linkage) in P1 transduction crosses. zdc-235::Tn10 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::Tn10 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' lac) and fdh-25::Mu dl(Ap' 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' 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; PAVEGLO et al. 1988). fdh-25::Mu dl(Ap' 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, 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 Tn5 insertions in pVJS102, and examined these insertion plasmids for their ability to complement fdnG108::Mu dl1734. pVJS102 contains the 8-kb PsiI fragment encoding fdnGHI cloned in a low copy-number T7 expression vector. The locations of the Tn5 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 Tn5 insertions.

Figure 4 shows the proteins produced from the Tn5 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::Tn5, 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 Tn5 inserts in-
dicated in Figure 2 were analyzed for their protein products; representative examples are shown in Figure 4. Insertion fdnG109::Tn5 (lane 2) abolished production of the 110-kDa polypeptide, while insertions fdnG112::Tn5 (lane 3), fdnG110::Tn5, and fdnG111::Tn5 (data not shown) produced truncated 110-kDa polypeptides. Insertions fdnH114::Tn5 and fdnH115::Tn5 (lanes 4 and 5) abolished production of the 32-kDa polypeptide. Insertions fdnI117::Tn5 (lane 6) and fdnI116::Tn5 (data not shown) abolished production of the 20-kDa polypeptide, while insertion fdnI118::Tn5 produced a truncated 20-kDa polypeptide (lane 7). Based on the map positions of the Tn5 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 Tn5 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 Gumpert 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 O interposon (Prentki and Krisch 1984) into the BglII site of pVJS101 and crossed this insertion back to the E. coli chromosome as described 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, pVSJ105 (fdnGHI); 3, pGEM5. “G,” “H” and “I” indicate the presumed α, β and γ subunits of formate dehydrogenase-N; “bla” indicates β-lactamase. Molecular mass markers were carboxic 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 Tn5-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. Tn5 insertions were in pVJS102, which contained the 8-kb Pst fdnGHI fragment cloned into pVSJ104 (Figure 2). Lanes: 1, zdc-2091::Tn5; 2, fdnG109::Tn5; 3, fdnG112::Tn5; 4, fdnH114::Tn5; 5, fdnH115::Tn5; 6, fdnI117::Tn5; 7, fdnI118::Tn5; 8, pVSJ104 (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 (described in the text). “IS50” indicates the IS50 gene products (Rothstein et al. 1980); “Cam” indicates chloramphenicol acetyltransferase (Shaw et al. 1979); “Kan” indicates kanamycin phosphotransferase (Beck et al. 1982). Expression of kanamycin phosphotransferase and various IS50 proteins was dependent on the orientation of the Tn5 with respect to the φ10 promoter. Lanes 2, 3, 4 and 7 represent Tn5 insertions oriented with respect to the φ10 promoter such that the kan gene product and the truncated IS50L gene products were synthesized. Lanes 5 and 6 represent Tn5 insertions in the opposite orientation, such that the full-sized products of IS50R were synthesized (see Rothstein et al. 1980). Molecular mass markers (not shown) were the same as those indicated in Figure 3.
E. coli fdnGHI Operon

### MATERIALS AND METHODS

Ω, which confers resistance to spectinomycin, contains strong transcription terminators at each of its ends. We transduced Fdn-G119::Ω into three strains which contained Φ(fdn-g-lacZ) 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 Fdn-G108::Mu d1734. By contrast, FdnG119::Ω abolished expression from the downstream operon fusions formed by FdnI103::Mu d1734 and FdnI102::Mu d1734.

### Regulation of Φ(fdn-g-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(fdn-g-lacZ) expression. Maximum induction of β-galactosidase required anaerobiosis plus nitrate. Expression of Φ(fdn-g-lacZ) was unaffected by addition of formate or by growth at low pH (Table 2).

Transcription activation of the nitrate reductase structural gene operon (narGHJIH) requires the FNR protein for induction by anaerobiosis and the NarL protein for induction by nitrate (Stewart 1982). Since Φ(fdn-g-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(fdn-g-lacZ) in strains which contained transposon insertions in fnr and narL. For comparison, we also measured induction of Φ(nar-g-lacZ).

fnr-21::Tn10 abolished anaerobic induction of both Φ(fdn-g-lacZ) and Φ(nar-g-lacZ) and narL215::Tn10 prevented induction by nitrate (Table 3). The narX236::Ω allele caused a subtle decrease of Φ(fdn-g-lacZ) and Φ(nar-g-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(fdn-g-lacZ) expression was only weakly induced by nitrite, and essentially not induced by trimethylamine N-oxide or fumarate (Table 4).

Confoundingly, Φ(nar-g-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(fdn-g-lacZ) expression in a narG::TnlO strain was also efficiently induced by nitrite (Table 4).

### fnGHI* 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 fnrG 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-

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### Table 2

<table>
<thead>
<tr>
<th>Culture medium</th>
<th>Φ(fdn-g-lacZ)</th>
<th>Φ(nar-g-lacZ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOPS (pH 7.8)</td>
<td>&lt;1 14 1280</td>
<td>&lt;1 7 825</td>
</tr>
<tr>
<td>MOPS + formate</td>
<td>11 12 1380</td>
<td>11 16 860</td>
</tr>
<tr>
<td>MES (pH 6.5)</td>
<td>&lt;1 10 1010</td>
<td>&lt;1 26 670</td>
</tr>
</tbody>
</table>

Strains VJS1224 [Φ(fdn-g-lacZ)104] and VJS882 [Φ(nar-g-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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Φ(fdn-g-lacZ)</th>
<th>Φ(nar-g-lacZ)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-O2 +NO3 -O2</td>
<td>-O2 +NO3 -O2</td>
</tr>
<tr>
<td>Wild-type</td>
<td>6 880</td>
<td>11 805</td>
</tr>
<tr>
<td>fnr-21::Tn10</td>
<td>&lt;1 &lt;1</td>
<td>&lt;1 &lt;1</td>
</tr>
<tr>
<td>narL215::Tn10</td>
<td>7 28</td>
<td>7 10</td>
</tr>
<tr>
<td>narX236::Ω</td>
<td>5 310</td>
<td>7 505</td>
</tr>
</tbody>
</table>

Strains VJS1250 [Φ(fdn-g-lacZ)108], VJS882 [Φ(nar-g-lacZ)234] and their derivatives 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).
organization of the fdnGHZ operon: We identified a 4.5-kb region of DNA that was essential for complementing the fdh-24::Mu dl(Ap 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 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::Tn10, 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 nitrate, and it has been

**TABLE 4**

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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>−O₂</th>
<th>+NO₂ − O₂</th>
<th>+TMAO⁺ − O₂</th>
<th>Fumarate − O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Φ(fdnG-lacZ)108</td>
<td>5</td>
<td>1040</td>
<td>42</td>
<td>17</td>
</tr>
<tr>
<td>Φ(narG-lacZ)234</td>
<td>7</td>
<td>940</td>
<td>440</td>
<td>7</td>
</tr>
<tr>
<td>Φ(fdnG-lacZ)108 narG205::Tn10</td>
<td>2</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Strains VJS1250 [Φ(fdnG-lacZ)108], VJS882 [Φ(narG-lacZ)234] and VJS1262 [Φ(fdnG-lacZ)108 narG205::Tn10] 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).

**TABLE 5**

Formate dehydrogenase-N activities in fdnG and fdhF mutant strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>DCPNP/PMS-formate dehydrogenase specific activity</th>
<th>−O₂</th>
<th>+NO₂ − O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC4100</td>
<td>fdhF⁺ fdnG⁺</td>
<td>0.09</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>VJS1052</td>
<td>fdhF⁺::Mu fdnG⁺</td>
<td>0.09</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>VJS1611</td>
<td>fdhF⁺::fdnG108::Mu &lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>VJS1612</td>
<td>fdhF⁺::Mu fdnG108::Mu &lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td></td>
</tr>
</tbody>
</table>

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 DCPNP reduced min⁻¹ mg protein⁻¹. Possible 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 fdnG 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 DCPNP/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**
hypothesized that formate dehydrogenase-N is a component of the nitrite respiratory chain (ABOU-JAOUDÉ, CHIPPAX AND PASCAL 1979; ABOU-JAOUDÉ, PASCAL AND CHIPPAUX 1979; see also POPE AND COLE 1982). We therefore examined whether $\text{fdn}GH$ expression was induced by alternate electron acceptors. In contrast to the approximately 200-fold induction by nitrate, $\Phi(\text{fdn}G$-$\text{lac}Z)$ 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 $\Phi(\text{fdn}G$-$\text{lac}Z)$ and $\Phi(\text{nar}G$-$\text{lac}Z)$ expression in $\text{nar}G$ strains is puzzling. It is possible that our nitrite is contaminated by small amounts of nitrate. In $\text{nar}^+$ strains, this residual nitrate could cause a low level induction of $\text{fdn}$ expression. In $\text{nar}G$ strains, this residual nitrate would be only slowly metabolized, and could act as a gratuitous inducer.

All of the $\text{fdn}G$ insertion mutants we examined were devoid of DCP/IP/PMS-linked formate dehydrogenase-N enzyme activity, while an $\text{fdh}F$ mutant retained wild-type levels of activity (Table 5). These observations suggest that DCP/IP/PMS-linked formate dehydrogenase activity is exclusively due to formate dehydrogenase-N.

**Common mechanisms for regulating $\text{fdn}GH$ and $\text{nar}GHJ$ expression:** Previous work has shown that $\text{nar}GHJ$ 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 $\Phi(\text{fdn}G$-$\text{lac}Z)$ required $\text{fnr}^+$ for anaerobic induction, and required $\text{nar}L^+$ for complete induction by nitrate (Table 3). The subtle decrease in nitrate-induced $\Phi(\text{fdn}G$-$\text{lac}Z)$ expression observed in the $\text{nar}X236::\Omega$ mutant paralleled the effect of $\text{nar}X$ insertion mutations on $\Phi(\text{nar}G$-$\text{lac}Z)$ 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 $\text{fdn}GH$ and $\text{nar}GHJ$ was mediated through common trans-acting regulatory elements.

We consistently observed a slight nitrate induction of $\Phi(\text{fdn}G$-$\text{lac}Z)$ expression in $\text{nar}L_+::\text{Tn}10$ strains (Table 3). This induction was not observed with the $\Phi(\text{nar}G$-$\text{lac}Z)$ operon fusion; we do not know what was responsible for this residual induction of $\Phi(\text{fdn}G$-$\text{lac}Z)$.

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 $\Phi(\text{fdn}G$-$\text{lac}Z)$ operon fusions contradict this conclusion (Tables 2 and 3). Efficient $\text{fdn}GH$ expression requires nitrate (Tables 2–5; reviewed by STEWART 1988), which was not added to the cultures examined by GUEST and co-workers. The $\text{fnr}^+$ requirement for formate dehydrogenase-N expression was previously noted by BIRKMAN, 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 $\Phi(\text{fdn}G$-$\text{lac}Z)$ 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 $\text{fdn}GH$ and $\text{nar}GHJ$ transcription. The FNR protein activates transcription in response to anaerobiosis. The NarL protein, in the presence of nitrate, activates transcription of $\text{fdn}GH$ and $\text{nar}GHJ$. The cis-acting sites required for anaerobic induction and nitrate induction have been identified upstream of the $\text{nar}GHJ$ promoter (LI AND DEMOSS 1988). We presume that the $\text{fdn}GH$ 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 $\text{fdh}F$; involved in formate-hydrogen lyase) and formate dehydrogenase-N (encoded by $\text{fdn}GH$; involved in formate-nitrate oxidoreductase), are physiologically and genetically distinct. Not surprisingly, regulation of their synthesis occurs through separate pathways. Expression of $\text{fdh}F$ is repressed by nitrate, and is stimulated by low pH and added formate (BIRKMAN ET AL. 1987; WU AND MANDRAND-BERTHELOT 1987). Transcription of $\text{fdh}F$ requires $\sigma^+$ (the $\text{ntr}A$ gene product; KUSTU ET AL. 1989), and is independent of $\text{fnr}^+$ (BIRKMAN, SAWERS AND BÖCK 1987). Nitrate repression of $\text{fdh}F$ is independent of $\text{nar}L^+$ (STEWART AND BERG 1988). By contrast, expression of $\text{fdn}GH$ was induced by nitrate, while low pH and added formate had no effect (Table
2). Synthesis of formate dehydrogenase-N is independent of σ^4 (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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