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Genetic Evidence that Genes *fdhD* and *fdhE* Do Not Control Synthesis of Formate Dehydrogenase-N in *Escherichia coli* K-12

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Enterobacteria synthesize two formate dehydrogenases, formate dehydrogenase-N (encoded by *fdnGHI*) and formate dehydrogenase H (encoded by *fdhF*). Previous work has identified two *rha*-linked *Salmonella typhimurium* genes, *fdnB* and *fdnC*, which are required primarily for formate dehydrogenase-N activity. Analogous mutants, termed *fdhD* and *fdhE*, have been isolated in *Escherichia coli*. We used gene fusions between *fdnG*, the structural gene for the large subunit of formate dehydrogenase-N, and *lacZ*, the structural gene for β-galactosidase, to examine *E. coli fdnGHI* operon expression in *fdhD* and *fdhE* insertion mutants. Expression of the Φ(*fdnG-lacZ*) gene fusions was little affected by these insertions, suggesting that *fdhD* and *fdhE* do not control transcription or UGA decoding of the formate dehydrogenase-N structural genes. Our complementation tests, with cloned *E. coli fdhD* and *fdhE* genes, indicate that the *S. typhimurium fdnC* and *fdnB* genes are functionally homologous to the *E. coli fdhD* and *fdhE* genes, respectively.

Formate (HCO_2^-) is a product of anaerobic pyruvate cleavage. Enterobacteria have two pathways for anaerobic metabolism of formate (Fig. 1). A respiratory pathway, formate-nitrate oxidoreductase, allows for energy conservation via oxidative phosphorylation during anaerobic growth. This pathway involves two membrane-bound, multisubunit enzymes, formate dehydrogenase-N (FDH-N) and respiratory nitrate reductase. Synthesis of this enzyme complex is induced by nitrate during anaerobic growth. FDH-N and respiratory nitrate reductase are associated with specific cytochromes, cyt b_{556}^{Fdn} and cyt b_{556}^{Nar}, respectively. The strongly electronegative redox potential of the CO_2-HCO_2^- couple ($E_0' = -432$ mV) makes formate an efficient electron donor for respiratory nitrate reduction. The second pathway, formate-hydrogen lyase, operates anaerobically in the absence of nitrate. This pathway involves two enzymes, formate dehydrogenase-H (FDH-H) and hydrogenase 3. Synthesis of this enzyme complex is induced by anaerobiosis only in the absence of nitrate. The exact physiological role of formate-hydrogen lyase is not established. The H_2 and CO_2 produced by formate-hydrogen lyase accumulates in Durham tubes, so gas production provides a qualitative estimate of this activity. Formate metabolism in enterobacteria has been recently reviewed (33).

The three subunits of FDH-N are encoded by the *fdnGHI* operon at 32 min on the *Escherichia coli* genetic map (5). The FDH-H polypeptide is encoded by the *fdhF* gene at 92 min on the *E. coli* map (27). The FdnG and FdhF polypeptides each contain selenium in the form of selenocysteine, and each associates with molybdenum cofactor. Thus, mutants defective in selenocysteine (*sel*) or molybdenum cofactor (*chl*) synthesis or incorporation are devoid of formate dehydrogenase activity (for reviews, see references 7 and 33).

Analysis of *E. coli* and *Salmonella typhimurium* has identified four *sel* genes (originally termed *fdh*), lesions in which abolish the activities of FDH-N and FDH-H (2, 20, 24). The *selA* (80 min) and *selD* (38 min) gene products are involved in synthesis of selenocysteine (19), *selB* (80 min) encodes a specific elongation factor (14), and *selC* (82 min) encodes $tRNA_{UCA}^{Sec}$ (21), which inserts selenocysteine into FDH-H (40) and FDH-N (6) in response to unique UGA codons in the respective mRNAs (for a review, see reference 7).

Two additional genes affecting FDH-N activity have been identified in *E. coli* (*fdhD* and *fdhE*) and *S. typhimurium* (*fdnB* and *fdnC*) (2, 3, 23, 26, 28). In both organisms, these genes map at 88 min, between *rha* and *glnA* (23, 26). Strains with *fdhE* and *fdnB* lesions lack FDH-N activity but retain essentially wild-type levels of FDH-H activity and gas production. Thus, $fdhE^+$ ($fdnB^+$) may be specifically involved in the synthesis or activity of FDH-N. Strains with *fdhD* and *fdnC* lesions are also devoid of FDH-N activity, but they also exhibit defects in FDH-H activity and gas production, which are more or less severe depending on growth and assay conditions (23, 26). Bulk cytochrome *b* is poorly reduced by formate in both types of mutants, suggesting that they may have defects in electron transfer to or synthesis of cyt b_{556}^{Fdn} (3, 23). It was also suggested that $fdhD^+$ and/or $fdhE^+$ may be involved in regulation of FDH-N synthesis (28).

In *E. coli*, *fdhE* mutants do not accumulate the 110- and 32-kDa subunits of FDH-N (as assayed by immunoprecipitation) while *fdhD* mutants accumulate essentially normal levels of these polypeptides (23). This contrasts with the reported situation in *S. typhimurium*, where *fdnB* and *fdnC* mutants were both found to accumulate these polypeptides (as assayed by gel staining; 3). This difference, along with differences in the details of the cytochrome *b* spectra, led to the conclusion that *fdhD* and *fdhE* lesions in *E. coli* and *fdnB* and *fdnC* lesions in *S. typhimurium* do not identify functionally homologous genes (23) despite their similar phenotypic properties and map positions.

Recently, Mandrand and co-workers reported their molecular genetic characterization of *fdhD* and *fdhE* in *E. coli* (28). They demonstrated that $fdhD^+$ and $fdhE^+$ encode proteins of 30.5 and 32 kDa, respectively. They also showed that the two genes are transcribed divergently and are separated by approximately 4 kb of DNA.

We are studying the regulation of the FDH-N structural

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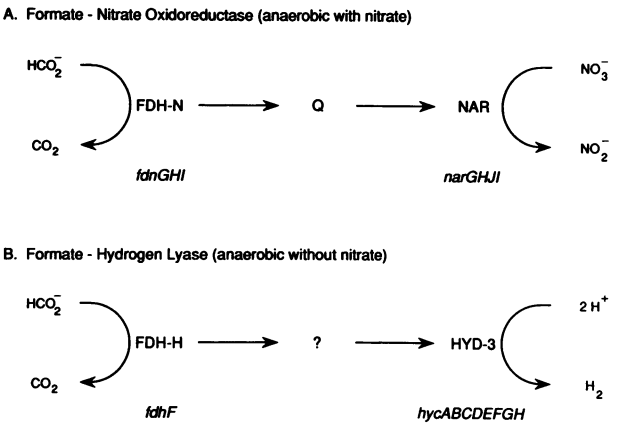

FIG. 1. Pathways for formate metabolism in enterobacteria. (A) Formate-nitrate oxidoreductase. FDH-N (encoded by *fdnGHI*) oxidizes formate and transfers electrons to a quinone pool (Q), which then transfers electrons to nitrate reductase (NAR; encoded by *narGHJI*) (for a review, see reference 33). (B) Formate-hydrogen lyase. FDH-H (encoded by *fdhF*) (27) oxidizes formate and transfers electrons to an unknown component(s), which then transfers electrons to hydrogenase-3 (HYD-3; encoded by *hycABCDEFGH*) (8).

gene operon *fdnGHI* (5). Thus, we wished to examine the effects of *fdhD* and *fdhE* lesions on *fdnGHI* expression. We report here our use of Φ(*fdnG-lacZ*) gene fusions to analyze *fdnGHI* operon expression in *fdhD* and *fdhE* insertion mutants. The *fdhD* and *fdhE* lesions had little effect on *fdnGHI* expression, suggesting that these gene products act posttranslationally to control formate dehydrogenase assembly or activity. We also found that $fdhD^+$ clones complemented *S. typhimurium fdnC* mutants and that $fdhE^+$ clones complemented *fdnB* mutants. This indicates that *fdhD-fdnC* and *fdhE-fdnB* are functionally homologous genes in *E. coli* and *S. typhimurium*.

MATERIALS AND METHODS

Strains. Strains are listed in Table 1. Strains were constructed by generalized transduction by using bacteriophage P1 *kc* (25) for *E. coli* and bacteriophage P22 HT105/1 *int-201* (22) for *S. typhimurium*.

Gene fusions between *fdnG* and *lacZ* were constructed on plasmid pRS414 and crossed to λRS45 as described by Simons et al. (29). The Φ(*fdnG-lacZ*)*121*(Hyb) [$\Phi(fdnG\text{-}lacZ)_{UGA}$] gene fusion consists of the *fdnGHI* promoter regulatory region and the first 793 codons of *fdnG* fused to codon 9 of *lacZ*. Thus, expression of hybrid β-galactosidase requires translation of the *fdnG* sequence. The Φ(*fdnG-lacZ*)*122*(Hyb) [$\Phi(fdnG\text{-}lacZ)_{UCA}$] gene fusion is identical except that the selenocysteine codon (TGA) at position 196 has been converted to a serine codon (TCA) by site-specific mutagenesis. These fusions will be described in greater detail elsewhere (4).

The *fdhE*::Tn*10* and *rha*::Tn*10* alleles were isolated by mutagenizing *E. coli* with Tn*10* as described by Way et al. (37) and screening Tc^r colonies for Fdn^- and Rha^- phenotypes on MacConkey-nitrate and MacConkey-rhamnose media, respectively. The *fdhE* mutant was differentiated from *sel* mutants by testing for gas production. The *rha*::MudJ allele was isolated by mutagenizing *S. typhimurium* with MudJ as described by Hughes and Roth (16) and screening Km^r colonies for the Rha^- phenotype on rhamnose-tetrazolium agar (25).

Plasmids. Plasmids are listed in Table 1, and plasmid inserts are diagrammed in Fig. 2. The in vivo cloning vector pEG5005 of Groisman and Casadaban (15) was used to isolate clones that complemented the *fdhE*::Tn*10* mutant as judged by phenotype on MacConkey-nitrate medium. pEG5005 is an in vivo cloning vector based on bacteriophage Mu; clones generated by transposition are packaged into Mu particles and can be screened by transduction (15). Fragments from one such clone (pVJS115) were subcloned into the general-purpose vectors pHG165 (31) and pACYC184 (12) to yield the clones described in Table 1 and Fig. 2. Standard methods were used for restriction endonuclease digestion, ligation, and transformation (13). Restriction enzymes and T4 DNA ligase were from New England Biolabs, Inc. (Beverly, Mass.)

The *fdhD*::Km allele was constructed by inserting a *Pst*I-digested Km^r cassette (1) into *Nsi*I-digested pVJS608 to form pVJS610 (Fig. 2). This allele was transferred to the chromosome of *E. coli* in a *recBC sbcBC* strain as described previously (38) and then crossed to other strains by transduction.

Culture media. Cultures for β-galactosidase assays of Φ(*fdnG-lacZ*) strains were grown in 3-[*N*-morpholino]propanesulfonic acid (MOPS)-buffered minimal medium with glucose as the sole carbon source (36). The initial pH of this medium was set at 7.8. L-Tryptophan (0.2 mM) was added to all defined media, and $NaNO_3$ (40 mM) was added as indicated.

Media for tests of gas production in Durham tubes were tryptone-yeast extract-glucose (TYG; 5) and FHL broth, which consisted of MES (morpholineethanesulfonic acid) culture medium (34) supplemented with 1 μM Na_2MoO_4, 1 μM Na_2SeO_3, 1 μM $NiCl_2$, 0.5% proteose peptone, 0.1% acid-hydrolyzed casein, and 80 mM glucose (39). Gas accumulation was estimated after 48 h of incubation. The benzyl viologen overlay test for FDH-H activity was done as described previously (24).

Defined complex and indicator media for routine genetic manipulations were used as described previously (13, 25). Ampicillin, chloramphenicol, kanamycin, spectinomycin, and tetracycline were used at 200, 25, 75, 15, and 20 μg/ml, respectively. The MacConkey-nitrate medium of Barrett and Riggs (2) was slightly modified (36). Agar and dehydrated media were from Difco Laboratories (Detroit, Mich.). Other components were from Sigma Chemical Co. (St. Louis, Mo.).

Culture conditions. Culture densities were monitored with a Klett-Summerson photoelectric colorimeter (Klett Mfg. Co., New York) equipped with a no. 66 (red) filter. Cultures were grown at 37°C. Anaerobic cultures for β-galactosidase assays were grown in screw-cap tubes as described previously (36). Plates were incubated anaerobically in Brewer jars (9).

β-Galactosidase assay. Assays were carried out at room temperature, approximately 21°C. Cell pellets were resuspended in 4 ml of Z buffer (25) 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 (25). Each culture was assayed in duplicate. Reported values are averaged from two independent experiments.

Plasmid complementation. Transformants were selected directly on anaerobically incubated MacConkey-nitrate

TABLE 1. Strains and plasmids

Strain or plasmid	Genotype	Reference or source
E. coli K-12		
MC4100	*araD139* Δ(*argF-lac*)*U169 deoC1 flhD5301 ptsF25 relA1 rpsL150*	11
RK4353	As MC4100 but *gyrA219 non-9*	35
VJS691	Δ(*argF-lac*)*U169* Δ(*trpEA*)*2*	36
VJS1938	As VJS691 but λΦ(*fdnG-lacZ*)$_{UGA}$	4
VJS1945	As VJS691 but λΦ(*fdnG-lacZ*)$_{UCA}$	4
VJS2216	As RK4353 but *fdhD201*::Km	This study
VJS2217	As RK4353 but *fdhE202*::Tn*10*	This study
VJS2218	As VJS691 but λΦ(*fdnG-lacZ*)$_{UGA}$ *fdhE202*::Tn*10*	This study
VJS2219	As VJS691 but λΦ(*fdnG-lacZ*)$_{UCA}$ *fdhE202*::Tn*10*	This study
VJS2220	As VJS691 but λΦ(*fdnG-lacZ*)$_{UGA}$ *fdhD201*::Km	This study
VJS2221	As VJS691 but λΦ(*fdnG-lacZ*)$_{UCA}$ *fdhD201*::Km	This study
VJS2222	As VJS691 but λΦ(*fdnG-lacZ*)$_{UGA}$ *fdnG119*::Ω	This study
VJS2223	As VJS691 but λΦ(*fdnG-lacZ*)$_{UCA}$ *fdnG119*::Ω	This study
VJS2224	As VJS691 but λΦ(*fdnG-lacZ*)$_{UGA}$ *fdhD201*::Km *fdhE202*::Tn*10*	This study
VJS2226	As VJS691 but λΦ(*fdnG-lacZ*)$_{UGA}$ *fdhD201*::Km *fdnG119*::Ω	This study
VJS2227	As VJS691 but λΦ(*fdnG-lacZ*)$_{UCA}$ *fdhD201*::Km *fdnG119*::Ω	This study
VJS2228	As VJS691 but λΦ(*fdnG-lacZ*)$_{UGA}$ *fdhE202*::Tn*10 fdnG119*::Ω	This study
VJS2229	As VJS691 but λΦ(*fdnG-lacZ*)$_{UCA}$ *fdhE202*::Tn*10 fdnG119*::Ω	This study
VJS2234	As VJS691 but λΦ(*fdnG-lacZ*)$_{UCA}$ *fdhD201*::Km *fdhE202*::Tn*10*	This study
W18	As MC4100 but *fdh-8*::Mu d1(Ap^r *lac*)	20
S. typhimurium LT2		
EB26	*fdnB5*	2
EB264	*fdnB14*(Am)	26
EB276	*fdnC26*(Am)	26
LB5010	*galE856 hsdL6 hsdSA29 hsdSB ilv-452 leu metA22 metE551 rpsL120 trpC2 xyl-404*	10[a]
TB78	Prototroph	R. Bauerle
TC183	*fdnC6*	2
VJSS054	As TB78 but *rha*::MudJ	This study
VJSS055	As VJSS054 but *rha*$^+$ *fdnC6*	This study
VJSS056	As VJSS054 but *rha*$^+$ *fdnB5*	This study
VJSS057	As VJSS054 but *rha*$^+$ *fdnB14*(Am)	This study
VJSS058	As VJSS054 but *rha*$^+$ *fdnC26*(Am)	This study
Plasmids		
pACYC184	Cm^r Tc^r	12
pEG5005	Km^r	15
pHG165	Ap^r; *lacZ* α polylinker	31
pVJS115	*E. coli fdhD*$^+$ *fdhE*$^+$; ~20-kb insert in pEG5005	This study
pVJS601	*E. coli fdhD*$^+$ *fdhE*$^+$; ~20-kb *Pst*I insert in pHG165	This study
pVJS604	*E. coli fdhE*$^+$; ~1.5-kb *Eco*RI-*Hin*dIII insert in pHG165	This study
pVJS605	*E. coli fdhD*$^+$; ~6-kb *Pst*I-*Sal*I insert in pHG165	This study
pVJS608	*E. coli fdhD*$^+$; ~4-kb *Bam*HI-*Cla*I insert in pACYC184	This study
pVJS610	As pVJS608 but *fdhD201*::Km	This study

[a] Courtesy of K. E. Sanderson (University of Calgary, Alberta, Canada).

plates supplemented with ampicillin or chloramphenicol. Complemented strains formed large, pale-colored colonies, while noncomplemented strains formed smaller, dark-red colonies. Plasmids were introduced into *S. typhimurium* by first transforming them into LB5010, an r$^-$ m$^+$ strain (10). Plasmids were reisolated from LB5010 for subsequent transformation into *fdn* mutants.

RESULTS

Isolation of an *fdhE*::Tn*10* mutant. Barrett and co-workers (2) described a differential medium, MacConkey-nitrate medium, which contains glycerol, formate, nitrate, and pH indicators. Wild-type colonies respire nitrate, with formate as electron donor, and form large, light-colored (relatively alkaline) colonies. Mutants unable to metabolize formate form smaller, dark-red (relatively acidic) colonies. This medium has been extensively used to isolate and characterize *sel*, *fdh*, and *fdn* mutants (2, 3, 20, 26). We used MacConkey-nitrate medium to screen Tn*10*-mutagenized colonies of *E. coli*. One mutant retained the ability to produce gas from glucose, and the Tn*10* insertion was 100% linked to the Fdh$^-$ phenotype in backcrosses. This lesion was approximately 50% linked to *rha* in cotransduction experiments, indicating that it was probably an allele of *fdhD* or *fdhE*. Subsequent work, described below, shows that this lesion is in *fdhE*, and we refer to this allele as *fdhE202*::Tn*10*.

Molecular cloning of *fdhD*$^+$ and *fdhE*$^+$. We used the in vivo method of Groisman and Casadaban (15) to isolate clones that complemented the *fdhE202*::Tn*10* lesion. A lysate containing pEG5005-generated clones of *E. coli* chromosomal DNA, packaged into bacteriophage Mu particles, was used to infect a strain carrying *fdhE202*::Tn*10*. Colonies were selected on MacConkey-nitrate-kanamycin plates incubated anaerobically. Several Fdh$^+$ (large and pale in color) colonies were identified, and plasmid DNA was prepared from each and used to retransform the *fdhE202*::Tn*10* strain. One

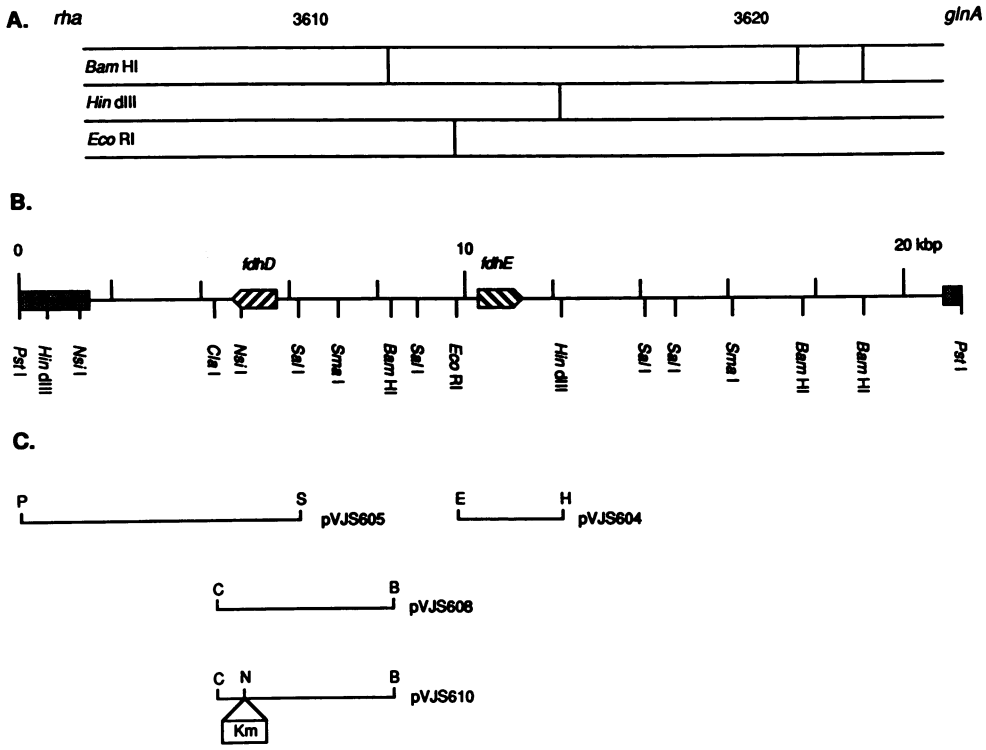

FIG. 2. Physical map of the *fdnDE* region and subclones used in this study. (A) Portion of the Kohara restriction map (18) between *rha* and *glnA*. Only sites for *Hin*dIII, *Bam*HI, and *Hin*dIII are depicted here. (B) Restriction map of the pVJS601 *fdhD*$^+$*E*$^+$ insert. Sequences derived from pEG5005 are shown as gray boxes; a polylinker region from pEG5005 is shown as a black box. The location and orientation of the *fdhD* and *fdhE* genes are shown as described by Schlindwein et al. (28). (C) Inserts of subclones used for complementation analysis.

clone, pVJS115, which contained approximately 20 kb of insert DNA, was chosen for further analysis.

Several subclones were generated from pVJS115 and tested for complementation of the *fdhE202*::Tn*10* lesion on MacConkey-nitrate medium. A 1.5-kb *Eco*RI-*Hin*dIII fragment (pVJS604; Fig. 2) fully complemented *fdhE202*::Tn*10* and also complemented two *fdnB* mutants of *S. typhimurium*. Two other subclones, containing a 6-kb *Pst*I-*Sal*I fragment (pVJS605) or a 4-kb *Cla*I-*Bam*HI fragment (pVJS608), complemented two *fdnC* mutants of *S. typhimurium*. This latter result located the *E. coli* homolog of *fdnC* (presumably *fdhD*) to the 2-kb region between *Cla*I and *Sal*I (Fig. 2). Complementation results are summarized in Table 2.

Construction of *fdhD*::Km. We wished to construct an *fdhD* allele for further genetic and phenotypic analysis. Restriction mapping had located an *Nsi*I site roughly in the middle of the 2-kb *Cla*I-*Sal*I region that presumably carried *fdhD*$^+$ (Fig. 2). Insertion of a kanamycin cassette into the *Nsi*I site of pVJS608 abolished its ability to complement the *fdnC* mutants of *S. typhimurium*, so this cassette presumably interrupted the *fdhD* coding sequence. We used an allele

TABLE 2. Phenotypes and complementation of *fdhD*, *fdhE*, *fdnB*, and *fdnC* strains

Strain	Allele	Gas production in:[a]		BV test[b]	Complementation by:[c]		
		TYG	FHL broth		pVJS604	pVJS605	pVJS608
E. coli							
RK4353	*fdh*$^+$	++++	++++	+	ND[d]	ND	ND
VJS2216	*fdhD201*::Km	+	−	−	−	+	+
VJS2217	*fdhE202*::Tn*10*	++++	++++	+	+	−	−
WL8	*fdh-8*::Mu d1	++++	++++	+	ND	ND	−
S. typhimurium							
TB78	*fdn*$^+$	++++	++++	+	ND	ND	ND
VJSS055	*fdnC6*	++++	++++	−	−	+	+
VJSS056	*fdnB5*	++++	++++	+	+	−	−
VJSS057	*fdnB14*(Am)	++++	++++	+	+	−	−
VJSS058	*fdnC26*(Am)	++++	++++	−	−	+	+

[a] Qualitative estimate of total gas accumulation in Durham tubes after 48 h of incubation in the indicated medium.
[b] Ability to mediate formate-dependent benzyl viologen reduction as determined in plate overlays.
[c] Complementation of Fdh$^-$ (Fdn$^-$) phenotype by the indicated plasmid as tested on MacConkey-nitrate medium.
[d] ND, not determined.

replacement method (38) to place the kanamycin insertion into the *E. coli* chromosome. Phenotypic analysis (see below) indicated that the resulting strains were indeed *fdhD* mutants. Genetic mapping, using *rha*::Tn*10*, established that the Km^r and Fdh$^-$ phenotypes were 100% linked to each other and approximately 50% linked to *rha*.

Phenotypic characterization of mutants. Work by Mandrand-Berthelot and co-workers has shown that *fdhD* and *fdhE* mutants have distinct phenotypes with respect to gas production and ability to catalyze the formate-dependent reduction of benzyl viologen in plate overlays (23). We used these tests to characterize our *fdhD201*::Km and *fdhE202*::Tn*10* mutants, as well as *S. typhimurium fdn* mutants isolated by Barrett and co-workers (26), and a *rha*-linked *fdh*::Mu dl(Ap^r *lac*) mutant isolated by Leinfelder et al. (5, 20). Results are shown in Table 2.

We used two different media to estimate gas production by Fdh$^+$ and mutant strains. TYG broth is a rich medium supplemented with glucose and is unbuffered. FHL broth is a semidefined medium supplemented with glucose and is strongly buffered at pH 6.5. Fdh$^+$ and *fdhE* (*fdnB*) mutants produced equivalent amounts of gas in both media (Table 2). The response of *fdhD* (*fdnC*) mutants was more complicated. The *E. coli fdhD201*::Km mutant accumulated approximately 25% as much gas as the Fdh$^+$ parent in TYG broth, in agreement with previous results (23). However, the *E. coli fdhD201*::Km mutant produced no detectable gas in FHL broth. In contrast, the *S. typhimurium fdnC* mutants produced essentially wild-type levels of gas in both media.

We also used an in situ assay for FDH-H activity, as described by Mandrand-Berthelot et al. (24). For this assay, colonies of the strains to be tested were cultured anaerobically overnight on nutrient agar. The plates were then overlaid with a mixture of benzyl viologen and formate. FDH-H will catalyze the formate-dependent reduction of benzyl viologen; reduced benzyl viologen is purple, while oxidized benzyl viologen is colorless. Thus, Fdh$^+$ and *fdhE* colonies are purple, and *fdhD* colonies are white. Results are shown in Table 2. The Fdh$^+$ and *fdhE* (*fdnB*) strains were purple, while the *fdhD* (*fdnC*) strains were white.

The physiological characterization of our *fdhD201*::Km and *fdhE202*::Tn*10* mutants is fully consistent with these gene assignments on the basis of the phenotypes of the *fdhD* and *fdhE* mutants studied by Mandrand-Berthelot and coworkers (23). Analysis of the *fdh-8*::Mu dl(Ap^r *lac*) mutant of Leinfelder et al. (20) suggests that it carries an *fdhE* allele (Table 2).

Physical map location of *fdhD* and *fdhE*. After the subcloning and physiological experiments described above were completed, Schlindwein et al. published their molecular analysis of the *fdhDE* region (28). Our restriction mapping and subcloning results are in perfect agreement with those of Schlindwein et al. By the definitions of Mandrand and co-workers (23, 28), the *fdh* gene common to both pVJS605 and pVJS608 is *fdhD*, and the *fdh* gene located on pVJS604 is *fdhE*. Thus, these comparisons further confirm the genetic assignments of our mutants as *fdhD201*::Km and *fdhE202*::Tn*10*.

We compared our restriction map with the region between *rha* and *glnA* on the *E. coli* physical map of Kohara et al. (18). The alignment is unambiguous; sites for *Bam*HI, *Hin*dIII, and *Eco*RI on the Kohara map are shown in Fig. 2. The *fdhD* gene is located at approximately 3,608 kb, and the *fdhE* gene is located at approximately 3,614 kb. Thus, *fdhD* is closer to *rha*, in agreement with the cotransduction data of Paveglio et al. (26), who found that *fdnC* (*fdhD*) is more tightly linked to *rha* than is *fdnB* (*fdhE*).

TABLE 3. Expression of Φ(*fdnG-lacZ*) gene fusions in *fdhD*::Km, *fdhE*::Tn*10*, and *fdnG*::Ω mutants

Strain[a]	β-Galactosidase sp act[b]			
	Φ(*fdnG-lacZ*)$_{UGA}$		Φ(*fdnG-lacZ*)$_{UCA}$	
	$-NO_3^-$	$+NO_3^-$	$-NO_3^-$	$+NO_3^-$
Wild type	5	770	7	1,440
fdhD201::Km	5	920	6	1,330
fdhE202::Tn*10*	2	870	1	1,330
fdhD201::Km *fdhE202*::Tn*10*	2	850	2	1,390
fdnG119::Ω	8	1,150	14	1,740
fdhD201::Km *fdnG119*::Ω	11	1,140	14	1,630
fdhE202::Tn*10* *fdnG119*::Ω	6	1,080	5	1,420

[a] See Table 1 for full strain designations.
[b] Determined as described in Materials and Methods and expressed in arbitrary (Miller) units. Cultures were grown anaerobically with or without nitrate as indicated.

Schlindwein et al. used *lacZ* operon fusions and protein expression experiments to determine the direction of *fdhD* and *fdhE* transcription (28); these directions are indicated in Fig. 2. Thus, *fdhD* is transcribed toward *rha* (clockwise on the standard *E. coli* map), and *fdhE* is transcribed toward *glnA* (counterclockwise on the standard *E. coli* map).

Regulation of Φ(*fdnG-lacZ*) expression in *fdhD*::Km and *fdhE*::Tn*10* strains. Our primary interest in studying *fdhD* and *fdhE* mutants was to determine whether or not these genes play a role in FDH-N (*fdnGHI*) gene expression. We have recently shown that *fdnGHI* encodes the three subunits of FDH-N (5), and we have determined the nucleotide sequence of *fdnGHI* (4). We have constructed gene fusions, carried on bacteriophage λ prophages, in which most of the *fdnG* coding region is fused in-frame to *lacZ*. Thus, β-galactosidase synthesis in strains carrying these fusions requires transcription and translation of most of *fdnG*. One of the fusions, termed Φ(*fdnG-lacZ*)$_{UGA}$, carries the wild-type *fdnG* sequence at codon 196, which is a TGA codon encoding selenocysteine (see reference 40). In this construct, β-galactosidase synthesis requires both selenium and *sel*$^+$ genes (4, 6). The other fusion, termed Φ(*fdnG-lacZ*)$_{UCA}$, has a serine codon at position 196. In this latter construct, β-galactosidase synthesis is independent of selenium and *sel*$^+$ genes (4, 6).

We transduced *fdhD201*::Km and *fdhE202*::Tn*10* into strains carrying the gene fusions, cultured the strains anaerobically in the absence and presence of nitrate, and determined β-galactosidase–specific activities (Table 3). Overall expression of the Φ(*fdnG-lacZ*)$_{UGA}$ fusion was consistently lower than that of the Φ(*fdnG-lacZ*)$_{UCA}$ fusion, reflecting the inherent inefficiency of UGA decoding as selenocysteine (4).

Neither *fdhD201*::Km nor *fdhE202*::Tn*10* had a large effect on Φ(*fdnG-lacZ*) expression. We suspected that any difference seen might represent an indirect effect caused by the absence of FDH-N enzyme activity rather than a specific regulatory effect of the *fdhD201*::Km or *fdhE202*::Tn*10* lesions. To test this idea, we also examined the effect of *fdnG119*::Ω (5), which is an insertion in the structural gene for the selenopolypeptide of FDH-N. Indeed, β-galactosidase–specific activities were slightly but consistently higher in the *fdnG*::Ω strains than those in the *fdhD* and *fdhE* strains (Table 3). Finally, we also constructed double mutants in all possible combinations of *fdhD*, *fdhE*, and *fdnG*.

None of the double mutants had phenotypes strikingly different from those of the single mutants (Table 3).

DISCUSSION

FDH-N synthesis is induced anaerobically in the presence of nitrate. We recently identified the structural genes for this enzyme complex, *fdnGHI* (5), and studied their regulation. Anaerobic induction requires the *fnr* gene product, a transcriptional regulator of anaerobic respiratory enzyme synthesis (for a review, see reference 30). Nitrate induction requires the *narL* gene product, a transcriptional regulator that mediates nitrate induction and repression of anaerobic respiratory enzyme synthesis (17, 32, 34, 36). Transcription of the *fdnGHI* operon is not controlled by formate or by catabolite repression (5). Thus, all known regulation of *fdnGHI* expression is mediated by fnr^+ and $narL^+$.

The roles of the *fdhD* and *fdhE* genes in the formation of FDH-N activity are unknown. We imagined that these genes could possibly be involved in any of the following three steps in the process of expressing FDH-N: (i) transcriptional regulation, (ii) decoding the *fdnG* UGA codon as a selenocysteine sense codon, or (iii) posttranslational events (such as enzyme or cofactor processing or assembly). Our analysis of $\Phi(fdnG\text{-}lacZ)_{UGA}$ and $\Phi(fdnG\text{-}lacZ)_{UCA}$ gene fusion expression in the *fdhD201*::Km and *fdhE202*::Tn*10* strains indicates that these genes are not involved in possibilities i or ii, i.e., transcription initiation or UGA decoding (Table 3).

Functional homology of *fdhD-fdnC* and *fdhE-fdnB*. The similar map positions and phenotypes of *fdhDE* in *E. coli* and *fdnBC* in *S. typhimurium* suggested that these genes are homologous in the two species. However, differences in reported phenotypes, notably accumulation of FDH-N polypeptides and spectral analyses of cytochrome content, led to the suggestion that the genes were nonhomologous and played different roles in the two species (23).

We used complementation analysis to directly address this issue. A plasmid containing the $fdhE^+$ gene of *E. coli* fully complemented two *fdnB* mutants of *S. typhimurium*, while plasmids containing the $fdhD^+$ gene of *E. coli* fully complemented two *fdnC* mutants of *S. typhimurium* (Table 2). Indeed, we originally identified our $fdhD^+$ plasmids on the basis of their complementation patterns in the *S. typhimurium* mutants; subsequent comparison of our plasmids with those of Schlindwein et al. (28) showed that this strategy correctly identified the *E. coli* $fdhD^+$ *gene*.

Further evidence for this homology comes from genetic map position. Paveglio et al. (26) found that *fdnC* lesions are more tightly linked to *rha* in generalized transduction crosses, suggesting a gene order of *rha–fdnC–fdnB–glnA*. Our comparison of the physical map of the *fdhDE* region of *E. coli* with the restriction map of Kohara et al. (18; Fig. 2) reveals a genetic order of *rha–fdhD–fdhE–glnA*. Thus, the functionally homologous genes in the two organisms also have homologous map positions.

It seems most likely that *fdhD-fdnC* and *fdhE-fdnB* are structurally as well as functionally homologous. We suggest that the phenotypic differences observed between the two sets of mutants reflect species differences in addition to differences in experimental design and analysis.

Role of the *fdhE* (*fdnB*) wild-type gene. Mutants lacking *fdhE* (*fdnB*) have relatively clear-cut phenotypes; they are devoid of FDH-N enzyme activity but retain essentially wild-type levels of FDH-H enzyme activity and gas production (23, 26). What is not clear, however, is whether these mutants accumulate FDH-N polypeptides. Barrett and Riggs (3), studying two *fdnB* mutants, observed for each a band that comigrated with FDH-N in nondenaturing polyacrylamide gels. By contrast, Mandrand-Berthelot et al. (23), by using rocket immunoelectrophoresis, failed to detect FDH-N polypeptides in each of three tested *fdhE* mutants. Leinfelder et al. (20) observed reduced but detectable levels of the FDH-N selenopolypeptide in their *fdh-8*::Mu d1 mutant, which may carry and *fdhE* allele (Table 2). Finally, as assayed by $\Phi(fdnG\text{-}lacZ)$ gene fusions (Table 3), *fdhE* apparently does not play a role in FDH-N synthesis. Taken together, these results suggest that FDH-N polypeptides are synthesized in *fdhE* (*fdnB*) mutants in near-normal amounts, but that they may be degraded relatively rapidly.

Schlindwein et al. (28) found that β-galactosidase synthesis in a $\Phi(fdhE\text{-}lacZ)$ gene fusion was greatly reduced in a *selA* mutant. The *selA* gene product is involved in synthesis of selenocysteine, so this observation suggested that the *fdhE* (*fdnB*) gene product might be involved in decoding UGA as a selenocysteine codon in the *fdnG* mRNA. However, our comparison of β-galactosidase synthesis in $\Phi(fdnG\text{-}lacZ)_{UGA}$ and $\Phi(fdnG\text{-}lacZ)_{UCA}$ gene fusions failed to reveal a role for $fdhE^+$ in UGA decoding (Table 3).

Finally, it has been suggested that *fdnB* encoded a structural component of cyt b_{556}^{Fdn} (26). However, our analysis of the *fdnGHI* operon of *E. coli* has shown that the structural gene for this cytochrome is encoded by *fdnI* (4, 5). Thus, the posttranslational role of the *fdhE* (*fdnB*) gene product in formation of FDH-N activity remains to be established.

Role of the *fdhD* (*fdnC*) wild-type gene. The phenotypes of *fdhD* (*fdnC*) mutants are even more complicated. Again, these mutants are virtually devoid of FDH-N activity, but they also have various defects in FDH-H activity. These mutants are unable to catalyze the formate-dependent reduction of benzyl viologen in plate overlays (23; Table 2), an activity that is generally thought to be associated with FDH-H. Furthermore, *E. coli fdhD* mutants generally have reduced FDH-H activity as assayed in extracts and produce only about 25% of the wild-type level of gas (23; Table 2). Thus, these mutants have defects in both formate dehydrogenases, although the defects are more severe for FDH-N than for FDH-H.

Further analysis of *S. typhimurium fdnC* mutants has led to a more complicated view (26). First, these mutants produce gas in media that contain relatively low amounts of glucose (0.1% versus 0.5%) or in media supplemented with thiosulfate (26). In contrast, we observed essentially wild-type gas production by *fdnC* mutants in media containing relatively high levels of glucose (Table 2). In any case, it is clear that the gas phenotypes of *S. typhimurium fdnC* mutants are quite different from those of *E. coli fdhD* mutants (26; Table 2). The basis for this difference is unknown.

Second, extracts of *S. typhimurium fdnC* mutants regain FDH-N activity after incubation (26). This suggests that the in vivo role of the *fdhD* (*fdnC*) gene product is in enzyme assembly or maturation. Much more work is necessary to determine the roles of *fdhD* (*fdnC*) and *fdhE* (*fdnB*) in the establishment of formate dehydrogenase activity in enterobacteria.

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