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Mutations in *trans* That Affect Formate Dehydrogenase (*fdhF*) Gene Expression in *Salmonella typhimurium*

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Expression of the *fdhF* locus of *Salmonella typhimurium* is shown to be dependent upon *ntrA* and *oxrB*. However, the *oxrB8* mutation is pleiotropic and also affects the expression of *hyd*, *pepT*, and *chlC*, whereas a mutation in *ntrA* does not. Insertional inactivation with Tn*10* and localized mutagenesis permitted the definition and partial characterization of two new genes, *fdhS* and *fdhR*, which appear to be involved in the positive regulation of *fdhF* expression. Both genes were mapped to the 71- to 72-min region of the *S. typhimurium* chromosome with the gene order *fdhS-crp-fdhR-rpsL*. Mutations in *fdhS* specifically affect *fdhF* expression without affecting the expression of the other anaerobically induced genes or enzymes that were tested, including *hyd*, *pepT*, *chlC*, nitrite reductase, sulfite reductase, and trimethylamine-*N*-oxide reductase. Both *fdhR* and *fdhS* may be involved in *fdhF* regulation vis-à-vis oxygen, since localized mutagenesis produced alleles of both genes that permitted the aerobic expression of *fdhF*. However, *fdhR* may more directly interact with *fdhF*, since insertional inactivation of *fdhS* does not abolish aerobic expression of *fdhF* in *fdhR* mutant strains. Taken together, these results suggest that *fdhS* and *fdhR* act in concert under anaerobic conditions to activate *fdhF* transcription.

Much attention has been focused in recent years on detailing the molecular mechanisms involved in the genetic regulation of anaerobic metabolism in members of the family *Enterobacteriaceae* (33). In particular the mechanisms that enable *Enterobacteriaceae* to switch from aerobic respiration to anaerobic metabolism (fermentation or anaerobic respiration) have attracted much interest. Under anaerobic growth conditions, these facultative bacteria are able to ferment glucose to produce lactate, acetate, ethanol, succinate, and formate (10). The formate that is produced can be converted into carbon dioxide and hydrogen by the formate-hydrogen lyase complex. This complex consists of a formate dehydrogenase (coded for by *fdhF*), redox carriers, and hydrogenase isoenzyme 3 (6). *Salmonella typhimurium* and *Escherichia coli* contain at least three hydrogenase isoenzymes (16, 17).

The formate dehydrogenase gene (*fdhF*) is located at min 93 in *S. typhimurium* (27), and several operons containing genes coding for hydrogenase synthesis and activity are probably located in the 58- to 59-min region, according to recent studies performed with *E. coli* (6, 28–30). Induction of *fdhF* synthesis requires the presence of formate and molybdate and the absence of electron acceptors like oxygen and nitrate (5, 9, 31, 36, 37). There is also a need for an acidic pH in the extracellular environment (23). At the transcriptional level, anaerobic expression of *fdhF* has been shown (4) to require an alternate enterobacterial sigma factor (NtrA) (14) and a *cis*-acting upstream activating sequence (UAS) (2) located at about 100 bp 5′ to the transcription start site *fdhF*. Deletion or mutagenesis of this region shows that it is absolutely required for formate induction of the *fdhF* gene (2). Indeed, it has not yet been possible to find physically separable sequences in or near the promoter region of *fdhF* that respond to the three distinct stimuli (oxygen, nitrate, and formate) known to regulate *fdhF* expression. In addition, the transcription of several genes involved in the synthesis of active hydrogenase has been shown to require NtrA (4, 6, 18). Unlike the case with other enterobacterial sigma factors, activation of transcription by NtrA has been shown to require accessory, DNA-binding, *trans*-acting factors that bind to specific UASs that have many of the properties of eucaryotic enhancers (7, 8, 26). The activity of these *trans*-acting factors is usually regulated by phosphorylation and dephosphorylation. Since the *fdhF* UAS mediates repression by nitrate and oxygen and induction by formate and molybdate, it would appear to be the site at which a putative transcriptional activator, homologous to *ntrC* or *nifA*, would interact. Additionally, it has been shown, by using a chimeric *fdhF* gene in which the *fdhF* UAS was exchanged for a *nif* UAS, that the UAS is solely responsible for the transcriptional regulation of *fdhF* (3), again strongly implying the binding of a *trans*-activating factor at this side.

What are the genes whose products activate transcription of *fdhF*? Do they act directly by binding at the *fdhF* UAS or indirectly as part of a regulatory cascade? Recent studies with *E. coli* (20, 32, 34) have demonstrated three factors that could potentially play a role, either direct or indirect, in the regulation of *fdhF*. However, their exact role and at what step they act in the regulation of *fdhF* are not clear. One of these factors, HydG, shows a high degree of homology with the transcriptional activator NtrC from *Klebsiella pneumoniae* (34). It is apparently required for the activity of the labile hydrogenase isoenzyme 3 (34). However, it has yet to be determined what factors serve to activate HydG or whether HydG is involved in the regulation of *fdhF*.

Another factor, *fhlA*, is located in the 59-min region of the *E. coli* chromosome, codes for a protein of 78 kDa, FhlA (29), and is capable, when cloned into a multicopy plasmid, of complementing a mutant strain that lacks formate-dependent expression of *fdhF* and *hyd-17*, a gene that is necessary for the activity of hydrogenase isoenzyme 3.

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The FhlA protein has been proposed to be a transcriptional activator for the production of the formate-hydrogen lyase complex, since it shows a high degree of homology with other transcriptional activators that function in concert with NtrA, such as NtrC, HydG, and *Klebsiella pneumoniae* NifA (20, 32). The *hyp* operon, which contains *fhlA* and five other genes that are necessary for the activity of all three hydrogenases, is expressed both aerobically and anaerobically (19). What is the function of FhlA, and what factor in turn regulates its activity? FhlA has recently been implicated in the regulation of the expression of *fhlB* (21), which is located in the 95-min region of the *E. coli* chromosome. *fhlB* has been suggested as a third potential regulator of *fdhF*, since it is required for the formate dehydrogenase activity associated with formate hydrogenlyase and since mutations in *fhlB* pleiotropically affect all three hydrogenase isoenzymes (21). FhlB is maximally expressed under anaerobic conditions in the presence of formate, and it has been proposed that formate interacts with the FhlA protein to initiate transcription of *fhlB* (21). Thus, the picture of how *fdhF* is regulated in *E. coli* is far from clear.

As part of an ongoing interest in the regulation of anaerobic metabolism in *S. typhimurium*, we are attempting to define the genetic factors required for the regulation of *fdhF* expression in this organism. In *S. typhimurium*, very little is presently known about potential *fdhF* regulatory factors. In this report, we present evidence for a set of *fdhF* regulatory loci, which differ from *fhlA* and *fhlB* of *E. coli* and for which we propose the names *fdhR* and *fdhS*. *fdhR* and *fdhS* have been mapped in the 71- to 72-min region of the *S. typhimurium* genome. Our results suggest that at least one of these genes (*fdhR*) may be specifically and directly involved in the regulation of *fdhF* expression vis-à-vis oxygen, since mutations in this gene cause constitutive expression of *fdhF* under aerobic conditions.

MATERIALS AND METHODS

Strains, media, and growth conditions. The bacterial strains used in this study are listed in Table 1. For the determination of the correction of auxotrophies, a minimal medium (M9) containing lactose (0.2%) or glucose (0.2%) was prepared as previously described (12). Amino acids and vitamins, when needed, were used at the following concentrations: arginine (0.6 mM), proline (2 mM), cysteine (0.3 mM), tryptophan (0.1 mM), phenylalanine (0.5 mM), *para*-aminobenzoate (0.1 mM), and tyrosine (0.1 mM). MacConkey agar medium contained lactose (1%) or mannitol (1%). Tetrazolium agar was formulated as NB agar with the addition of 50 μg of 2,3,5-triphenyl-2-tetrazolium chloride per ml and lactose (final concentration, 1%) (22). Antibiotics were added at the following final concentrations: ampicillin (50 μg/ml), kanamycin (25 μg/ml), tetracycline (20 μg/ml), and streptomycin (500 μg/ml). Minimal media were formulated with one-half of these concentrations. When needed, MacConkey agar and tetrazolium agar contained 8 μg of tetracycline per ml. Strains were tested for TMAO (trimethylamine-*N*-oxide) reductase by streaking on MacConkey agar base that contained glucose (0.15%) and TMAO (0.1%) and incubating anaerobically. Strains were tested for H_2S production from sulfite by using nutrient agar that contained $FeCl_2$ (1 mM), glucose (10 mM), and Na_2SO_3 (12 mM) (11). Strains were tested for nitrite reductase activity by scoring for anaerobic growth on Gutnick medium (13) containing 0.2% galactose and 10 mM $NaNO_2$. When necessary, plates were incubated anaerobically at 37°C in Gas-Pak jars.

TABLE 1. List of strains

Strain	Genotype or phenotype	Source or reference[a]
EB137[b]	*fdhF101*::Mu d1 *lac* (Ap^r)	1
EB138[c]	*hyd-101*::Mu d1 *lac* (Ap^r)	E. L. Barrett, H. S. Kwan
EB222	*phs-101*::Mu d1 *lac* (Ap^r)	10
EB424	*chlC101*::Mu d1 *lac* (Ap^r)	E. L. Barrett
LT2(Z)	Wild type	B. N. Ames
PH229	*ntrA209*::Tn*10 fdhF101*::Mu d1	P22 (SK284) × EB137
PH244	*oxrB8 zhb-895*::Tn*5 phs-101*::Mu d1	P22 (TN2064) × EB222
PH245	*oxrB8 zhb-895*::Tn*5 fdhF-101*::Mu d1	P22 (TN2064) × EB137
PH246	*oxrB8 zhb-895*::Tn*5 hyd-101*::Mu d1	P22 (TN2064) × EB138
PH254	*fdhF101*::Mu d1 *zhb-895*::Tn*5*	P22 (TN2064) × EB137
PH264	*fdhS101*::Tn*10 fdhF101*::Mu d1	P22 (RnTn10) × EB137
PH271	*pepT7*::Mu d1	P22 (TN2064) × LT2
PH301	*oxrB8 zhb-895*::Tn*5 chlC101*::Mu d1	P22 (TN2064) × EB424
PH306	*fdhS101*::Tn*10 pepT7*::Mu d1	P22 (PH264) × PH271
PH315	*fdhS101*::Tn*10 chlC101*::Mu d1	P22 (PH264) × EB424
PH316	*fdhS101*::Tn*10 hyd-101*::Mu d1	P22 (PH264) × EB138
PH317	*fdhS101*::Tn*10 phs-101*::Mu d1	P22 (PH264) × EB222
PH359	*fdhF101*::Mu d1 *zhb-895*::Tn*5 fdhR101*	P22* (PH254) × EB137
PH360	*fdhF101*::Mu d1 *zhb-895*::Tn*5 fdhS103*	P22* (PH254) × EB137
PH361	*fdhF101*::Mu d1 *zhb-895*::Tn*5 fdhR102*	P22* (PH254) × EB137
PH437	*fdhF101*::Mu d1 *zhb-895*::Tn*5 fdhS104*	P22* (PH254) × EB137
PH456	Pro$^-$, *zhb-6755*::Tn*10*	P22 (RnTn10) × SL3715
PH457	*fdhF101*::Mu d1 *fdhR105*	P22* (PH456) × EB137
PH458	*fdhF101*::Mu d1 *fdhR106*	P22* (PH456) × EB137
PH459	*fdhF101*::Mu d1 *fdhR107*	P22* (PH456) × EB137
PH460	*fdhF101*::Mu d1 *fdhR108*	P22* (PH456) × EB137
PH502	*fdhF101*::Mu d1 *fdhR101 fdhS101*::Tn*10*	P22 (PH264) × PH359
PH503	*fdhF101*::Mu d1 *fdhR102 fdhS101*::Tn*10*	P22 (PH264) × PH361
SK284	*ntrA209*::Tn*10 hisF645*	SGSC
SK398	*ntrB137*::Tn*10 hisF645*	SGSC
SK835	*ntrC352*::Tn*10 hisF645*	SGSC
TN2064[d]	*pepT7*::Mu d1 LeuBCD485 *oxrB8 zhb-895*::Tn*5*	48
TT2398	DP118[*lys*(*serA cysG1542*::Tn*5*) (*serA cysG*$^+$)*ilv*]	J. R. Roth
SL3715	*argD pro*	
STM540	*aroE*	
PP1037	*crp-773*::Tn*10 trpB223*	SGSC

[a] When strains are transductants, this is indicated by P22 (*Y*) × *Z*, where *Y* is the strain used to prepare the phage lysate and *Z* is the recipient strain. RnTn10 indicates a random pool of Tn*10* insertions. * indicates that the phage were subjected to chemical mutagenesis as described in Materials and Methods. SGSC, Salmonella Genetic Stock Center.

[b] The mutation in EB137, formerly *fhl-101*::Mu d1 *lac* (Ap^r), has been redesignated *fdhF101*::Mu d1 *lac* (Ap^r) (37).

[c] The mutation in EB138 [*hyd-101*::Mu d1 *lac* (Ap^r)] pleiotropically affects all three hydrogenases (17).

[d] The Tn*5* insertion in TN2064 has been redesignated *zhb-895*::Tn*5* (25).

Genetic techniques. Phage P22 *HT105/int4* lysates were prepared and transductions were performed as previously described (12). Tn*10* insertion mutants that decreased expression of *fdhF101*::Mu d1 (Ap^r) were isolated from a random pool of Tn*10* insertions kindly provided by John Roth. Specifically, phage P22 lysates of these pools were used to transduce strain EB137, and transductants were

screened on MacConkey-tetracycline plates. When grown anaerobically on MacConkey medium, strain EB137 produces bright red colonies. Therefore, transductants with an apparently decreased color were kept and further characterized. In some cases, the identical procedure was followed with tetrazolium agar, except that the expected color change was reversed. Localized mutagenesis (15) was used to isolate mutants altered in the regulation of *fdhF* as follows: P22 grown on PH254 (selection for aerobic expression) or PH456 (selection for absence of nitrate repression) was mutagenized and used to infect EB137 on, respectively, aerobically incubated minimal lactose medium containing kanamycin or anaerobically incubated minimal lactose medium containing nitrate (10 mM) and tetracycline. Since *S. typhimurium* is naturally Lac$^-$ and since *fdhF101*::Mu d1 is solely expressed under anaerobic conditions in the absence of nitrate (1), only transductants possessing putative mutations allowing the altered expression of *fdhF* should grow under the selection conditions used. Deletion mutants were generated by using the previously described medium for isolation of tetracycline-sensitive mutants (20).

Assays of β-galactosidase activity. Anaerobic culture densities were monitored with a Spectronic 20 spectrophotometer (Milton Roy Co.). Aerobic culture densities were monitored with a Hewlett-Packard Diode Array Spectrophotometer 8452A. Inocula for liquid cultures were aerated overnight at 275 rpm in 2.5 ml of LB medium (23) that was buffered (pH 6.5) with 80 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) and contained 0.2% glucose. Anaerobic growth was initiated by inoculation (84 μl) of screw-cap tubes (13 by 100 mm) filled to the top (8.4 ml) with LB-MES-KOH (pH 6.5) and glucose (80 mM). Cultures were placed in an incubator at 37°C. When needed, formate (final concentration, 0.2%) or nitrate (final concentration, 10 mM) was added to the medium. Aerobic growth was initiated by inoculating 50 μl of an overnight culture into loosely capped tubes (14 by 120 mm) that contained 5 ml of LB-MES-KOH (pH 6.5) and glucose (80 mM). Cultures were incubated at 37°C in a shaker (275 rpm). Tests indicated that under these conditions the cultures were not O_2 limited (data not shown). β-Galactosidase activities of aerobic and anaerobic cultures were determined with mid-exponential-phase cultures. Cells were lysed as previously described (24), and β-galactosidase activities were measured as described by Miller (22). All assays were conducted in duplicate. The reported values are averaged from at least three independent experiments. Activities are expressed in terms of nanomoles of *o*-nitrophenol per minute per unit of optical density at 600 nm.

RESULTS

Mutations that negatively affect *fdhF* expression. Previously, it was reported that in *E. coli* the expression of *fdhF* (4) and various hydrogenase genes (6, 18, 30) required a functional *ntrA*. We have confirmed that *fdhF* expression in *S. typhimurium* also requires *ntrA* by using transposon insertional inactivation, i.e., introduction of *ntrA209*::Tn*10*. As is the case for *E. coli*, *ntrA* may be specific for fermentative pathways, since insertional inactivation of *ntrA* did not affect the expression of *phs* (necessary for the production of hydrogen sulfide), *chlC* (the nitrate reductase operon), and *pepT* (an anaerobically induced peptidase). To search for potential regulators that could act in concert with NtrA to activate *fdhF* expression, we used transposon mutagenesis. This approach has at least two advantages: the creation of a null phenotype and the introduction of a marker that facilitates further genetic analysis. Therefore, pools of random Tn*10* insertions were screened for a decrease in *fdhF* expression by transducing strain EB137 (*fdhF101*::Mu d1) with phage lysates of these pools and selecting for tetracycline resistance on MacConkey-tetracycline plates. Colonies showing the desired phenotype, i.e., a paler color than that of the parent strain EB137, were isolated, and the phenotype (decreased *fdhF101*::Mu d1 expression) was verified by β-galactosidase assays. One insertion that showed an apparent decrease in *fdhF* expression was retained for further characterization. As shown below, this insertion is in a new locus for which we propose the name *fdhS*. Thus, this insertion will be referred to as *fdhS101*::Tn*10*. The effects of this mutation on the expression of *fdhF* and other anaerobically induced genes were determined (Table 2). The insertional inactivation of *fdhS* caused a strong reduction of *fdhF* expression (~10-fold) and a smaller decrease in *phs* expression (~2-fold). However, assays of β-galactosidase activities of strains carrying *fdhS101*::Tn*10* showed that the Tn*10* insertion had no effect on the expression of *chlC101*, *pepT7*, and *hyd-101*. (The *hyd-101* operon fusion does not appear to be in a hydrogenase structural gene, because it pleiotropically affects all three hydrogenases [16, 17].) All strains carrying *fdhS101*::Tn*10* were incapable of gas production. Thus insertional inactivation of *fdhS* has the same effects as does insertional inactivation of *ntrA* on the fusions tested.

TABLE 2. Effect of *oxrB8* and *fdhS*::Tn*10* on the expression of some anaerobically inducible genes

Mu d1 fusion	Growth conditions[a]	β-Galactosidase activity (nmol min^{-1} OD_{600}^{-1})[b]		
		No mutation	Background	
			oxrB8	*fdhS101*::Tn*10*
fdhF101	0.2% formate, 80 mM glucose	360	31	32
hyd-101	0.2% formate, 80 mM glucose	55	22	46
phs-101	80 mM glucose	40	36	20
pepT7	80 mM glucose	80	28	84
chlC101	10 mM nitrate, 80 mM glucose	416	56	388

[a] All strains were grown under anaerobic growth conditions in LB-MES-KOH (pH 6.5) with the indicated additions.
[b] OD_{600}, optical density at 600 nm.

Previously, it was reported that *oxrB8*, for which no counterpart in *E. coli* has yet been described, decreased the anaerobic expression of the peptidase *pepT* as well as several unidentified operon fusions (35). Since preliminary mapping data had placed *fdhS* in the same chromosomal region as *oxrB*, it was of interest to determine whether *oxrB8* would have the same effect as a mutation in *fdhS*. We found that the expression of *fdhF* was reduced about 10-fold in a strain carrying *oxrB8* (Table 2) but that, unlike *fdhS101*::Tn*10*, it does not seem to specifically affect *fdhF* expression because strains carrying *oxrB8* also showed decreased expression of *hyd101*, *pepT7*, and *chlC101*. Thus, the spectrum of effects shown by *oxrB8* is different than that shown by *fdhS101*::Tn*10*: *oxrB8* is pleiotropic, whereas *fdhS* appears to be specific for *fdhF*. Furthermore, *ntrA* is required for the expression of *fdhF* but not for the other genes studied, strongly suggesting that *ntrA* and *fdhS* are working in concert to activate *fdhF* expression.

Insertional inactivation of *fdhS* or *ntrA* does not directly

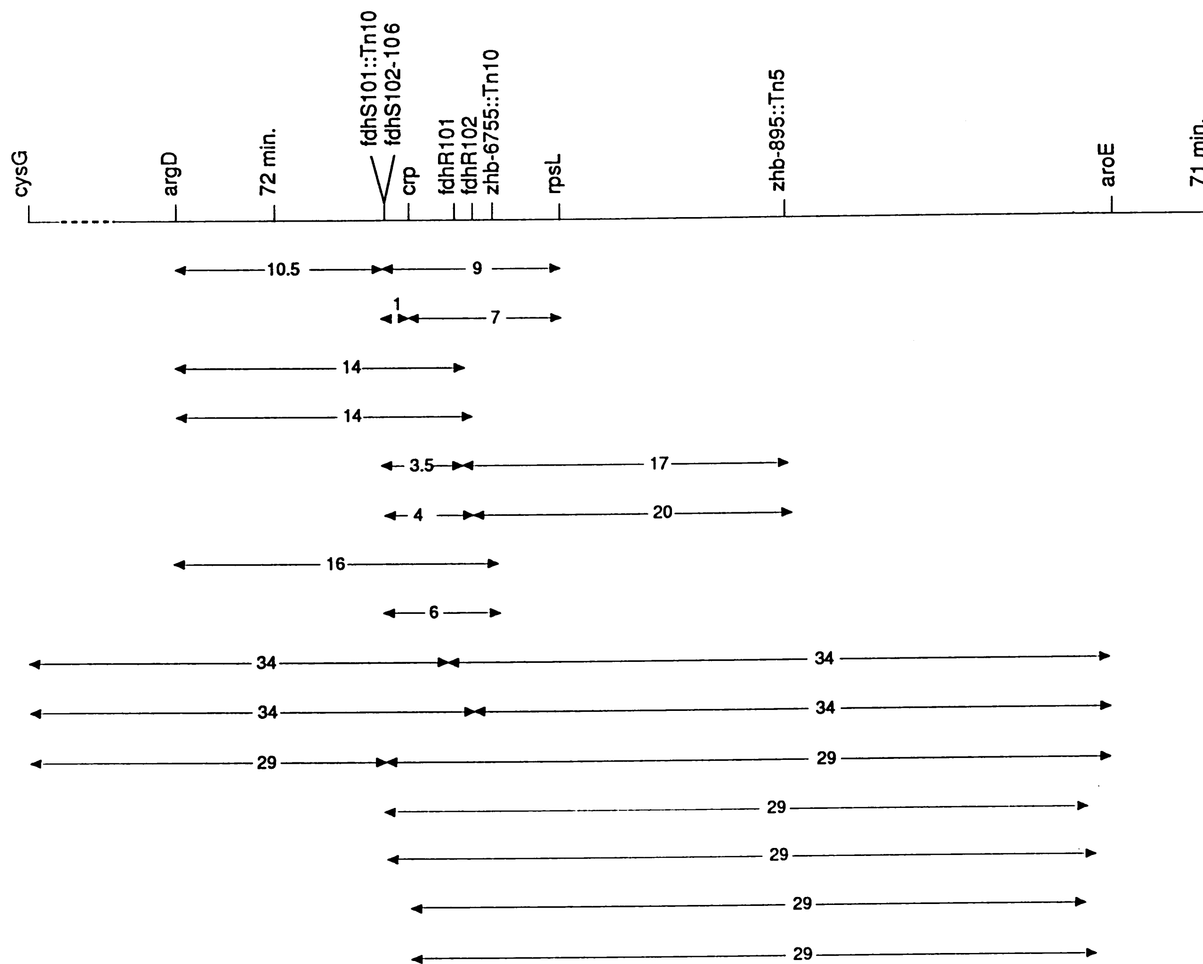

FIG. 1. Genetic map of the 71- to 72-min region of the *Salmonella* chromosome. The numbers below the line are kilobase pairs calculated from P22 cotransduction frequencies according to the formula of Sanderson and Roth (27). The positions of *cysG*, *argD*, *rpsL*, and *aroE* are those of Sanderson and Roth (27). *oxrB8* maps between *zhb-895*::Tn*5* and *aroE* (unpublished data).

affect TMAO, nitrite, fumarate, or sulfite reductase. The question arose as to whether *fdhS* and/or *ntrA* was involved in the regulation of other anaerobic genes. There are a variety of anaerobic metabolic pathways whose regulation in *S. typhimurium* is presently not understood. For example, unlike *fnr* mutants of *E. coli*, *oxrA* mutants of *S. typhimurium* have been reported to be capable of anaerobic growth with fumarate as the electron acceptor or nitrite as the nitrogen source (35). Likewise, the genes whose products regulate anaerobic sulfite reduction have yet to be identified. Therefore a variety of tests were carried out comparing the phenotypes of strains carrying either *fdhS101*::Tn*10* or *ntrA209*::Tn*10* with the wild-type strain, *S. typhimurium* LT2(Z). By using the appropriate indicator media (see Materials and Methods), it was determined that insertional inactivation of *fdhS* had no effect on either TMAO or sulfite reduction. Likewise, a strain carrying *fdhS101*::Tn*10* was still capable of anaerobic growth with nitrite as the sole nitrogen source. On the other hand, growth of strains carrying mutations in either *fdhS* or *ntrA* was greatly decreased over that of the wild type on glycerol-fumarate medium. However, these effects are probably secondary, since essentially the same result (poor growth) was obtained with strain EB137 (*fdhF101*::Mu d1). Thus, *fdhS101*::Tn*10* appears to affect *fdhF* expression specifically with essentially no direct effect on TMAO reductase, nitrite reductase, fumarate reductase, or sulfite reductase (data not shown).

***fdhS* is located near min 72 of the *Salmonella* map.** The *fdhS* gene was mapped in the 71- to 73-min region of the *S. typhimurium* genome by P22 transduction analysis with the following markers: *argD*, *cysG*, *rpsL*, *crp*, *aroE*, and *zhb-895*::Tn*5*. The results, summarized in Fig. 1, are presented in kilobase pairs; we used the formula given by Sanderson and Roth (27) to take into account the influence of the sizes of the Tn*10* and Tn*5* elements on apparent transduction frequencies. These results show that *fdhS* is highly linked (98.4%) to *crp*. It was also found to be linked (40%) to a streptomycin resistance marker (*rpsL*) near min 71.5 and linked (41%) to *argD* near min 72. Three-factor crosses (data not shown) established the gene order *fdhS-crp-rpsL*. *fdhS* was unlinked to either *cysG* (min 73) or *aroE* (min 71). Since *crp* and *fdhS* were found to be highly linked, they were tested for possible identity by two different methods. First, a *crp* mutant strain was tested for its ability to produce gas under anaerobic conditions with nutrient agar stabs containing glucose (final concentration, 0.5%); production of gas was normal. Likewise, *fdhS* mutant strains were tested for their *crp* character by examining their ability to ferment

TABLE 3. Mutations in *fdhR* allow aerobic expression of *fdhF101*::Mu d1 or relieve anaerobic nitrate repression

Mutant selection conditions	Strain	Relevant genotype	β-Galactosidase activity (nmol min^{-1} OD_{600}^{-1})[a]		
			$+O_2$	$-O_2$	$-O_2$ + nitrate
	EB137	*fdhF101*::Mu d1	12	326	28
Aerobic	PH360	*fdhF101*::Mu d1 *fdhS103*	57	54	58
	PH437	*fdhF101*::Mu d1 *fdhS104*	52	45	45
	PH359	*fdhF101*::Mu d1 *fdhR101*	130	170	172
	PH361	*fdhF101*::Mu d1 *fdhR102*	96	195	134
	PH502	*fdhF101*::Mu d1 *fdhR101 fdhS101*::Tn*10*	100	ND[b]	ND
	PH503	*fdhF101*::Mu d1 *fdhR102 fdhS101*::Tn*10*	95	ND	ND
Anaerobic, with NO_3^-	PH457	*fdhF101*::Mu d1 *fdhR105*	76	1,016	149
	PH458	*fdhF101*::Mu d1 *fdhR106*	41	727	152
	PH459	*fdhF101*::Mu d1 *fdhR107*	76	1,600	216
	PH460	*fdhF101*::Mu d1 *fdhR108*	79	818	155

[a] OD_{600}, optical density at 600 nm.
[b] ND, not determined.

mannitol on MacConkey-mannitol plates; they were capable of fermenting mannitol. These results strongly suggest that *fdhS* and *crp* are not the same gene. However, the possibility that *fdhS* is a highly unusual allele of *crp* has not yet been rigorously excluded. No analogous mutations have yet been mapped to this position in *E. coli*.

Mutations that allow aerobic expression of *fdhF*. As detailed above, a null mutation in *fdhS* caused a drastic reduction in anaerobic *fdhF* expression. Thus *fdhS* appeared to be necessary for the positive control of *fdhF* expression. We reasoned that if this were indeed the case and if *fdhS* were involved in the response to the lack of oxygen, then it might be possible to find mutations in *fdhS* that allow the aerobic expression of *fdhF*. To search for such mutations, localized mutagenesis (12, 15) was carried out in this region with a P22 phage lysate of strain PH254 (*zhb-895*::Tn*5* *fdhF101*::Mu d1). The mutagenized phage lysate was used to transduce strain EB137 (*fdhF101*::Mu d1), and transductants capable of expressing *fdhF101*::Mu d1 aerobically were selected on minimal lactose-kanamycin medium (*S. typhimurium* is naturally Lac$^-$). The mutants obtained could be classified into three groups according to the linkage of the mutation allowing aerobic *fdhF* expression to the *fdhS*::Tn*10* insertion. Group I contains five strains that have a mutation highly (97 to 100%) linked to *fdhS101*::Tn*10*, and presumably were in the *fdhS* gene. Group II contains four strains that have a mutation that is 55 to 85% linked to *fdhS101*::Tn*10*. The results of P22 transductional linkage analysis together with the results of β-galactosidase activity measurements (see below) suggest that these group II mutations lie in a second gene regulating *fdhF*. We propose the name *fdhR* for this locus. Mapping of these mutations with P22 established the gene order *fdhS-crp-fdhR-rpsL* (Fig. 1). This gene order was confirmed by three-factor crosses (data not shown). Finally, group III contains two strains with mutations that are 15 to 20% linked to *fdhS101*::Tn*10*. These mutations have not been further characterized.

To quantitate the effect of *fdhS* (group I) and *fdhR* (group II) mutations on *fdhF* expression, β-galactosidase assays of mid-exponential-phase cultures of two isolates of each locus (*fdhS*, PH360 and PH437; *fdhR*, PH359 and PH361) were carried out (Table 3). As expected from the selection procedure used, the aerobic β-galactosidase activities of the mutant strains examined were considerably higher than that of the parent fusion (EB137). Interestingly, these mutations also diminished the anaerobic induction of *fdhF*. Even more striking was the marked alleviation of nitrate repression of *fdhF* expression in these mutants. Nitrate caused a 12-fold decrease in *fdhF* expression in the parent strain EB137, whereas there was no decrease found with nitrate with strains *PH360*, *PH437*, and *PH359* and *fdhF* expression in strain PH361 was only decreased 1.4-fold. Thus, the effect of the mutations in these strains was to cause, under the conditions used here, an apparent constitutive expression of *fdhF*. The major difference observed between the mutations in *fdhS* (strains PH360 and PH437) and the mutations in *fdhR* (strains PH359 and PH361) that were examined was the higher level of *fdhF* expression in strains with *fdhR* mutations. Thus, under aerobic conditions, *fdhF* expression by strains PH361 and PH359 was 8- to 11-fold greater than that of strain EB137, whereas *fdhF* expression by strains PH360 and PH437 was only 4- to 5-fold greater. (These differences are statistically significant.) These results suggest that both *fdhR* and *fdhS* may be involved, either directly or indirectly, in the regulation of *fdhF* expression vis-à-vis oxygen. The introduction of *fdhS101*::Tn*10* into group II strains PH359 and PH361 had no effect on aerobic *fdhF* expression as ascertained with β-galactosidase assays (compare the results for strains PH359 and PH502 with those for strains PH361 and PH503 in Table 3). This suggests that the *fdhR* locus plays a more direct role than does the *fdhS* locus in the regulation of *fdhF*; i.e., *fdhR* is epistatic to *fdhS*.

Mutations that relieve anaerobic nitrate repression of *fdhF* expression. As mentioned above, mutants selected for aerobic *fdhF* expression were also found to be affected in anaerobic nitrate repression. One possible explanation for this effect is that these mutations are such that the gene product of *fdhR* or *fdhS* is locked into an active configuration and no longer responds to its normal effectors. Thus it was of interest to directly select for mutants that had lost anaerobic nitrate repression of *fdhF* expression. To do this, localized mutagenesis of the *fdhR* (*fdhS*) region was carried out with a P22 phage lysate of PH456 (*zhb-6755*::Tn*10*). The mutagenized phage lysate was used to transduce strain EB137 (*fdhF101*::Mu d1), and transductants were selected on an anaerobically incubated lactose minimal medium containing nitrate (10 mM) and tetracycline.

Four putative mutants (strains PH457 through PH460)

TABLE 4. β-Galactosidase activities of merodiploid strains[a]

Strain	Relevant genotype	β-Galactosidase activity (nmol min^{-1} OD_{600}^{-1})
EB137	*fdhF101*::Mu d1	10
PH359	*fdhF101*::Mu d1 *fdhR101*	270
PH361	*fdhF101*::Mu d1 *fdhR102*	228
PH422	*fdhF101*::Mu d1 Dp118 [*lys*(*serA cysG1542*::Tn*5*) (*serA cysG*$^+$)*ilv*]	4
PH463	*fdhF101*::Mu d1 *fdhR101* DP118 [*lys* (*serA cysG1542*::Tn*5*) (*serA cysG*$^+$)*ilv*]	165
PH464	*fdhF101*::Mu d1 *fdhR102* DP118 [*lys* (*serA cysG1542*::Tn*5*) (*serA cysG*$^+$)*ilv*]	148

[a] Cultures were grown aerobically in Gutnick medium (12) containing 0.4% glucose, 20 mM NH_4Cl, and 12.5 μg of kanamycin per ml to the mid-exponential phase, and β-galactosidase activity was determined as described under Materials and Methods. Strains PH422, PH463, and PH464 were derived from duplication strain TT2398 (obtained from J. R. Roth) by transduction. OD_{600}, optical density at 600 nm.

were isolated and further characterized. In all cases linkage (42 to 58%) of the mutations in these strains to *zhb-6755*::Tn*10* was confirmed. The results of β-galactosidase assays conducted on cultures grown aerobically, anaerobically, and anaerobically with nitrate are shown in Table 3. As might be expected from the selection procedure used, anaerobic expression of *fdhF* in the presence of nitrate was increased five- to eightfold over that found with the parent strain (EB137). Additionally, in all cases the aerobic expression of *fdhF* was also 3.5- to 6.5-fold greater than that of the parent strain. These increases might be at least partly explained by a general overall increase in the expression of *fdhF*. For example, even when these mutant strains (PH457 through PH460) were incubated anaerobically in the absence of nitrate, their β-galactosidase activities increased two- to fivefold. The linkage to *zhb-6755*::Tn*10* found for the mutations in these strains was essentially the same as that in the mutations in strains PH359 and PH361 described above. This indicated that the mutations in strains PH457 through PH460 are in *fdhR*. This was confirmed by fine structure mapping with P22 (Fig. 1).

Aerobic regulation in diploid strains. Since *fdhR* interacts more directly than does *fdhS* with *fdhF*, we were interested in studying the effects of the presence of the wild-type *fdhR* allele (*fdhR*$^+$) on *fdhF* expression when mutant alleles of *fdhR* were also present. To do this, strains that were diploid for the *fdhR* locus, i.e., *fdhR101*/*fdhR*$^+$ *fdhR102*/*fdhR*$^+$, were constructed by transduction of the required genes into strain TT2398 (obtained from J. R. Roth), which carries a duplication of the chromosomal region between 62 and 73 min. Diploid strains were maintained and cultured for β-galactosidase assays by simultaneously selecting for kanamycin resistance and Cys$^+$. (Diploidy was verified by segregation of the appropriate markers [data not shown].) As might be expected, the aerobic expression of *fdhF*::Mu d1 was unchanged in the *fdhR*$^+$/*fdhR*$^+$ diploid strain PH422 (Table 4). For unknown reasons, the β-galactosidase activities of strains PH359 and PH361 were significantly higher in minimal medium than in buffered LB. The β-galactosidase activities of aerobically grown strains diploid for mutant and wild-type alleles of *fdhR* (PH463, PH464) were 60% of those of haploid strains (PH359, PH361). These results demonstrate that *fdhR101* and *fdhR102* are partially dominant over *fdhR*$^+$.

DISCUSSION

The current knowledge of the physiology of *fdhF* regulation and the molecular architecture of the region upstream of the FdhF coding region presents two salient features: (i) NtrA is required for transcription initiation at the *fdhF* promoter, and (ii) another factor that presumably binds at the UAS is also required. In addition, all of the regulatory signals (absence of oxygen, nitrate concentration, and formate induction) are integrated at the level of the UAS, the additional factor, or both. The present study has attempted to further elaborate upon these points.

As detailed in the introduction, recent studies with *E. coli* have implicated three potential regulatory genes, *hydG* (34), *fhlA* (31), and *fhlB* (20), in the regulation of *fdhF* expression. What is their mode of action, and with what metabolic signals might they interact? *hydG*, when cloned into a multicopy plasmid, causes the expression of hydrogenase 3 in a pleiotropic hydrogenase mutant (34). Whether it controls hydrogenase 3 expression in wild-type cells or has any effect on *fdhF* expression is presently unknown. Likewise, the specific role of a second potential regulator, *fhlA*, in the control of *fdhF* expression is presently unclear. Although *fhlA* has been described as part of the *hyp* operon that is expressed both aerobically and anaerobically (19), studies with *fhlA*::*cat* fusions have suggested that it is only expressed anaerobically (29). Thus, whether one of the signals for *fdhF* expression is required for *fhlA* expression in the absence of oxygen or whether oxygen interacts with FhlA has yet to be determined. A potential role for FhlA in sensing formate has been proposed, since it appears to be necessary for the anaerobic, formate-mediated induction of *fhlB* (21). Mutational analysis indicates that an intact *fhlB* is absolutely necessary for *fdhF* expression. Thus, any model for *fdhF* regulation must include *fhlA* and *fhlB*. Obviously one potential role is that of a formate sensor. However, all the signals known to regulate *fdhF* may not interact with *fhlA* and *fhlB*. Since *fhlB* expression is not repressed by nitrate, nitrate repression of *fdhF* expression must occur either by interaction with FhlB or via another set of regulatory factors. Further work will be required to obtain a clear picture of *fdhF* regulation in *E. coli*.

Much less is known about *fdhF* regulation in *S. typhimurium*. Using transposon and localized mutagenesis, we have in the present study identified two potential factors, *fdhS* and *fdhR*, that are implicated in the regulation of *fdhF* expression in *S. typhimurium* and that differ from the previously described *E. coli* genes. These genes were mapped to the 71- to 72-min region of the *Salmonella* chromosome with the gene order *fdhS-crp-fdhR-rpsL*. No analogous mutations have yet been mapped to this region of the *E. coli* chromosome. Two lines of evidence implicate *fdhS* and *fdhR* in the regulation of *S. typhimurium fdhF* expression. (i) A Tn*10* insertion in *fdhS* specifically diminishes *fdhF* expression without affecting the expression of *chlC*, *pepT*, or *hyd*. Also, *fdhS* mutant strains are unaffected in TMAO, nitrite, or sulfite reduction. In addition, the introduction of a Tn*10* insertion in *ntrA* has the same effect. (ii) Localized mutagenesis of this region produces mutations (mapped to *fdhR* and *fdhS*) that permit, in mutant strains, the aerobic expression of *fdhF* or its anaerobic expression in the presence of nitrate. Both types of selection procedure used, the presence of oxygen and the presence of nitrate under anaerobic conditions, produced alleles of *fdhR* with increased aerobic expression and decreased anaerobic repression by nitrate of *fdhF* expression. However, the nitrate selection procedure yielded mutants

that still showed anaerobic induction of *fdhF*, whereas the oxygen selection procedure did not. These differences merit further study. One possible explanation is that the increased anaerobic induction of *fdhF* in these strains is due to the increased expression of *fdhR*.

Merodiploid analysis showed that mutant alleles of *fdhR* that show increased expression of *fdhF* aerobically were partially dominant to *fdhR*$^+$. Among several possible explanations for the lack of full dominance are (i) mutationally altered FdhR and FdhR$^+$ competitively binding to a site involved in *fdhF* regulation and (ii) autoregulation of the *fdhR* locus with FdhR$^+$ decreasing aerobic expression of *fdhR101* and *fdhR102*. Further experiments will be necessary to differentiate between these and other possibilities. Nevertheless, these results provide further evidence for the participation of *fdhR* in the positive control of *fdhF* expression.

The roles of *fdhS*, *fdhR*, *fhlA*, and *fhlB* in the regulation of *fdhF* expression are presently unclear. *fhlA* has been shown to possess an *ntrA*-dependent promoter (31) and may be expressed only in the absence of oxygen (29). Thus, it is possible that *fdhR* and *fdhS* act in concert to regulate *fhlA* expression. Another possibility that cannot be ruled out at present is that *fdhR* interacts directly with the *fdhF* UAS to activate transcription. According to this model, *fhlB* (or *fhlA*) could communicate the formate status of the cell to *fdhR* and *fdhS* could communicate to *fdhR* the oxygen status (and possibly the nitrate status) of the cell. Such a model is consistent with a number of observations. Both *fdhR* and *fdhS* appear to be involved in the anaerobic expression of *fdhF*, since mutant alleles of both genes can be obtained that permit the aerobic expression of *fdhF*. *fdhR* may interact with *fdhF* more directly than does *fdhS*, since insertional inactivation of *fdhS* does not abolish the aerobic expression of *fdhF* in *fdhR* strains. Single-point mutations in *fdhR* can cause apparent constitutive expression of *fdhF*, suggesting that three of the signals known to affect *fdhF* expression (the absence of oxygen or nitrate and the presence of formate) interact at or before *fdhR*. Although this model can explain the effects of all presently known regulatory factors, obviously at present other models, or the interaction with other factors, such as *hydG* (34), cannot be excluded. Further studies are in progress to characterize *fdhS* and *fdhR* on a molecular level.

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ADDENDUM IN PROOF

oxrB8 has been shown to be a mutant allele of *rpoA* (M. J. Lombardo, D. Bagga, and C. G. Miller, Abstr. 91st Gen. Meet. Am. Soc. Microbiol., H-148, p. 179, 1991).

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