Mutants of *Escherichia coli* Specifically Deficient in Respiratory Formate Dehydrogenase Activity

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Escherichia coli K12 mutants lacking phenazine-methosulphate-linked formate dehydrogenase (FDH-PMS) activity, but still capable of producing normal levels of benzyl-viologen-linked formate dehydrogenase (FDH-BV) and nitrate reductase activities, have been isolated following P1 localized mutagenesis. The relevant mutations mapped with the same cotransduction frequency close to the *rhaD* gene, at 88 min on the *E. coli* chromosome. They were further subdivided into two classes. Class I consisted of six *fdhD* mutants which synthesized an inactive FDH-PMS protein with the same subunit composition as the wild-type enzyme. In contrast, class II contained four *fdhE* mutants totally devoid of this antigen. Construction of merodiploid strains harbouring various combinations of the mutated alleles, *fdhE* on the episome and *fdhD* on the chromosome, led to the restoration of FDH-PMS activity by complementation of the products encoded by the respective wild-type alleles. Difference spectroscopy suggested that both *fdhD* and *fdhE* mutants contained normal amounts of the cytochrome *b*₅₅₉ associated with FDH-PMS although the cytochrome had lost its capacity for formate-dependent reduction.

INTRODUCTION

Growth of *Escherichia coli* anaerobically in the presence of nitrate leads to the formation of a membrane-bound multifunctional respiratory complex that catalyses the energy-conserving reduction of nitrate to nitrite by formate. This electron-transport pathway consists of formate dehydrogenase (EC 1.2.2.1) and nitrate reductase (EC 1.7.99.4) (both molybdenum-containing iron-sulphur proteins), *b*-type cytochromes and quinone (Haddock & Jones, 1977; Ruiz-Herrera & DeMoss, 1969). Formate dehydrogenase has been purified to homogeneity and shown to contain three polypeptides, α (*M*_r 110 000), β (*M*_r 32 000) and γ (*M*_r 20 000). Selenium is incorporated into the 110 000 *M*_r peptide and the haem is thought to be associated with the γ subunit (Enoch & Lester, 1975). In the absence of an exogenous electron acceptor, *E. coli* can synthesize a different formate dehydrogenase activity involved in hydrogen evolution as a component of the formate hydrogenlyase system (Peck & Gest, 1957). Nitrate-reductase-linked formate dehydrogenase (FDH-PMS) preferentially reduces phenazine methosulphate but not benzyl viologen, while hydrogenase-linked formate dehydrogenase (FDH-BV) has the opposite specificity (Lester & DeMoss, 1971). The distinction and cellular location of the two activities have been clearly resolved on the basis of the complete separation of two different selenium-containing peptides (Cox *et al.*, 1981) with no immunological relationship (Giordano *et al.*, 1983).

Abbreviations: FDH-PMS, phenazine-methosulphate-linked formate dehydrogenase; FDH-BV, benzyl-viologen-linked formate dehydrogenase.

A large variety of mutants have been isolated in *E. coli* with defects in their ability to reduce nitrate. Most of them have been selected for by their resistance to chlorate under anaerobiosis: they are pleiotropic mutants, since they have lost the activities of the three molybdoenzymes FDH-PMS, FDH-BV and nitrate reductase (Glaser & DeMoss, 1972; Stewart & MacGregor, 1982). Mutants specifically deficient in the two formate dehydrogenase activities have also been described, both in *E. coli* (Graham *et al.*, 1980; Haddock & Mandrand-Berthelot, 1982) and *Salmonella typhimurium* (Chippaux *et al.*, 1977; Barrett *et al.*, 1979). More recently, Barrett and others (Barrett *et al.*, 1979; Barrett & Riggs, 1982) have reported the characterization of new classes of *fdn* mutants in *S. typhimurium* specifically defective in FDH-PMS activity.

We have identified two distinct phenotypic classes of mutants in *E. coli* which lack only FDH-PMS activity and have lesions in a region equivalent to that of *S. typhimurium*. However, their biochemical, immunological and genetic properties suggest that they do not correspond to the mutant classes previously described (Barrett & Riggs, 1982).

METHODS

Bacterial strains. The *E. coli* K12 strains used in this study are listed in Table 1. Merodiploid strains carrying a derivative of episome F14 were constructed as follows. F14 was transferred into strains FD171 (*fdhD1*), FD191 (*fdhD3*), FD161 (*fdhE1*) and FD221 (*fdhE4*), selecting for the presence of the *ilv*⁺ and *arg*⁺ markers. Recombination between chromosome and episome, occurring at a frequency of 0.1% (Miller, 1972), resulted in the integration of the *fdhD* or *fdhE* alleles on the episome. This event was scored by examination of the colonies on MacConkey-nitrate plates under anaerobiosis (Barrett *et al.*, 1979): very rare red colonies of small size exhibiting an FDH-PMS⁻ phenotype (homogenotes *fdhD/fdhD* or *fdhE/fdhE*) were clearly distinguished from the bulk of large pale pink colonies of the wild-type (heterogenotes *fdhD*⁺/*fdhD* or *fdhE*⁺/*fdhE*). The former were purified and checked. The mutated episome was then transferred to *recA* Tet^R derivatives of strains FD171, FD191, FD161 and FD221 selecting for *ilv*⁺ *arg*⁺ Tet^R.

Media, growth conditions and genetic techniques. The strains were grown in L-broth medium supplemented with glucose (0.2%, w/v), sodium selenite (2 µM) and ammonium molybdate (2 µM) (Wu & Mandrand-Berthelot, 1986). When indicated, potassium nitrate (1%, w/v) was added. For enzyme assays, anaerobic growth was accomplished at 37 °C in non-agitated closed vessels filled almost to the top. For ³⁵S-labelling experiments, Casamino acids were omitted from the basal medium used and the ammonium sulphate was replaced with NH₄Cl (Giordano *et al.*, 1983). Minimal medium was M63 (Miller, 1972) supplemented with thiamin (0.5 µg ml⁻¹). When required, L-amino acids (100 µg ml⁻¹) and tetracycline hydrochloride (10 µg ml⁻¹) were added.

Interrupted mating experiments, transductions with phage P1kc and episome transfers were performed according to Miller (1972).

Isolation and identification of the FDH-PMS⁻ mutants. A P1kc lysate was prepared on strain HfrH and concentrated to 5 × 10¹¹ phage ml⁻¹ by centrifugation. It was then mutagenized with 0.4 M-hydroxylamine hydrochloride for 7 h at 37 °C, according to the procedure described by Murgola & Yanofsky (1974). Once sedimented by centrifugation, the surviving phage (2% of the original stock) were resuspended in L-broth medium supplemented with 5 mM-CaCl₂. The mutated lysate was used to transduce strain D54 (*rhaD metB*), selecting for *rhaD*⁺ colonies which had coinherited the FDH-PMS⁻ phenotype, identified by means of the soft agar dye overlay methods described previously (Wu & Mandrand-Berthelot, 1986).

Preparation of extracts and enzyme assays. Cultures were harvested at the mid- to late-exponential phase of growth and suspended, after being washed twice, in a buffer containing 25 mM-KH₂PO₄/25 mM-Na₂HPO₄ (pH 6.8) and 1 mM-benzamidinium hydrochloride, to a final concentration close to 10 mg protein ml⁻¹. Bacteria were ruptured in a French pressure cell at 10000 p.s.i. (69 MPa). Cellular debris was removed by centrifugation at 12000 g for 15 min and the supernatant containing the crude extract was used directly for enzyme assays. FDH-PMS, FDH-BV, nitrate reductase and hydrogenase activities were measured spectrophotometrically at 600 nm and at 30 °C as described previously (Wu & Mandrand-Berthelot, 1986). Measurements on whole cells were performed in the same way.

Gas production was estimated in Durham tubes filled with rich medium supplemented with glucose (0.8%, w/v), sodium selenite (2 µM) and ammonium molybdate (2 µM). Formate hydrogenlyase activity was followed manometrically by measuring formate-dependent evolution of hydrogen according to the procedure of Azoulay & Marty (1970). Protein was measured by the Lowry method, using bovine serum albumin (fraction V, NBC) as standard.

Immunological analysis. Rocket immunoelectrophoresis was performed on crude extracts solubilized with (1%, v/v) Triton X-100 as described by Graham *et al.* (1980), using an antiserum specific for the FDH-PMS enzyme purified essentially following the method of Enoch & Lester (1975).

Table 1. *Strains of E. coli K12*

Strain	Genotype	Source or reference
HfrH	Hfr	Low (1972)
D54	F ⁺ <i>metB1 rhaD54</i>	Power (1967)
EMG29	F ⁻ <i>metB his trpB lac rpsL</i>	NCIB*
mal38	F ⁻ <i>proB his trpB lac rpsL</i>	T. Silhavy
CGSC4443†	F ⁻ <i>thi thr leu argH his pro purE chlB lacY mtl xyl malA ara gal rpsL tonA</i>	F. Casse
GY05	F ⁻ as EMG29 also <i>fdhD6</i>	Mandrand-Berthelot <i>et al.</i> (1978)
FD17	F ⁺ <i>fdhD1 metB1</i>	This work
FD18	F ⁺ <i>fdhD2 metB1</i>	This work
FD19	F ⁺ <i>fdhD3 metB1</i>	This work
FD24	F ⁺ <i>fdhD4 metB1</i>	This work
FD25	F ⁺ <i>fdhD5</i>	This work
FD16	F ⁺ <i>fdhE1</i>	This work
FD20	F ⁺ <i>fdhE2 metB1</i>	This work
FD21	F ⁺ <i>fdhE3</i>	This work
FD22	F ⁺ <i>fdhE4 metB1</i>	This work
161R	F ⁻ <i>hisG1 ilvD16 glpK1 argH1 thi-1 lacY1</i> or <i>Z4</i> or <i>20 gal-6 malA1 mtl-2 rpsL8</i> , <i>9</i> or <i>17 tonA2</i> or <i>22 tsx-7 λ^R λ⁻ supE44 rhaD54</i>	<i>rhaD metB⁺</i> derivative of LIN161, by transduction with P1
F14	F' F14 <i>ilvD⁺ argEH⁺/ilv-8 pro-2 his-4 lacY1 galK rpsL</i>	F. Jacob (Pasteur Institute)
FD171	F ⁻ as 161R, but <i>rhaD⁺ metB1 fdhD1</i>	P1(FD17) × 161R <i>rha⁺</i> selection
FD191	F ⁻ as 161R, but <i>rhaD⁺ fdhD3</i>	P1(FD19) × 161R <i>rha⁺</i> selection
FD161	as 161R, but <i>rhaD⁺ fdhE1</i>	P1(FD16) × 161R <i>rha⁺</i> selection
FD221	F' s 161R, but <i>rhaD⁺ fdhE4</i>	P1(FD22) × 161R <i>rha⁺</i> selection
F14-1/FD171rec	F' F14 <i>fdhD1</i> /FD171 <i>src300::Tn10 recA56</i>	This work
F14-2/FD191rec	F' F14 <i>fdhD3</i> /FD191 <i>src300::Tn10 recA56</i>	This work
F14-3/FD161rec	F' F14 <i>fdhE1</i> /FD161 <i>src300::Tn10 recA56</i>	This work
F14-4/FD221rec	F' F14 <i>fdhE4</i> /FD221 <i>src300::Tn10 recA56</i>	This work

*NCIB, National Collection of Industrial Bacteria, Aberdeen, UK.

†CGSC, Coli Genetic Stock Center, Yale University, via B. Bachmann.

For the analysis of the subunit composition of FDH-PMS, crude extracts of cells labelled with K₂³⁵SO₄ were centrifuged at 170000 *g* for 90 min to separate them into a soluble and a membrane fraction. The membrane fraction containing the FDH-PMS activity was immunoprecipitated by the addition of 30 µl antiserum per mg membrane protein solubilized with Triton X-100. Antigenic material was analysed by determining the distribution of radioactivity in polyacrylamide gels (10%, w/v) in the presence of SDS as described previously (Giordano *et al.*, 1978).

Cytochrome scans. Absorption spectra of the frozen-thawed cell preparations were recorded at room temperature in a double-beam spectrophotometer whose specifications have been reported by Denis & Richaud (1982). Samples were introduced into one cuvette and they were first oxidized by potassium ferricyanide, then sequentially reduced by addition of ascorbate, formate and dithionite.

RESULTS

Characterization of a mutant devoid of FDH-PMS activity

Mutant GY05 had been previously isolated following *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment of strain EMG29 and detected by its absence of colour using the formate-benzyl viologen dye overlay procedure (Mandrand-Berthelot *et al.*, 1978). It lacked FDH-PMS activity but retained the nitrate reductase molybdoenzyme. Surprisingly, it exhibited normal levels of FDH-BV activity after cell breakage (Table 2). These biochemical properties led us to

Table 2. *Enzyme activities in FDH-PMS⁻ mutants grown under anaerobiosis in the presence or absence of nitrate*

Bacteria were grown anaerobically in L-broth medium as described in Methods. The activities of FDH-PMS and FDH-BV are expressed as $\mu\text{mol formate min}^{-1} (\text{mg protein})^{-1}$; the results are mean values from two or three independent experiments. '0' indicates values smaller than 0.001.

Strain	<i>fdh</i> genotype	FDH-PMS		FDH-BV	
		-NO ₃ ⁻	+ NO ₃ ⁻	-NO ₃ ⁻	+ NO ₃ ⁻
D54	Wild-type	0.04	0.89	0.24	0
FD17	<i>fdhD</i>	0	0.005	0.13	0
FD18	<i>fdhD</i>	0	0.004	0.05	0
FD19	<i>fdhD</i>	0	0	0.09	0
FD24	<i>fdhD</i>	0	0	0.2	0
FD25	<i>fdhD</i>	0	0.02	0.21	0
FD16	<i>fdhE</i>	0	0	0.16	0
FD20	<i>fdhE</i>	0	0.002	0.17	0
FD21	<i>fdhE</i>	0	0	0.24	0
FD22	<i>fdhE</i>	0	0	0.13	0
EMG29	Wild-type	0.018	0.45	0.30	0.001
GY05	<i>fdhD</i>	0	0.001	0.42	0

Table 3. *P1-mediated transduction mapping of *fdhD* and *chlB* mutations*

The *fdhD* allele was scored using the dye overlay procedures for FDH-BV and FDH-PMS activities (Mandrand-Berthelot *et al.*, 1978; Wu & Mandrand-Berthelot, 1986). The *chlB* allele was scored using the dye overlay methods for FDH-BV and nitrate reductase activities (Wu & Mandrand-Berthelot, 1986).

Donor strain	Recipient strain	Selected marker	Colonies scored	Percentage cotransduction	
GY05 (<i>fdhD</i>)	mal38 (<i>metE ilv</i>)	<i>ilv</i> ⁺	143	0 <i>fdhD</i>	8 <i>metE</i> ⁺
		<i>metE</i> ⁺	149	1 <i>fdhD</i>	44 <i>ilv</i> ⁺
GY05	D54 (<i>rhaD metB</i>)	<i>rhaD</i> ⁺	241	68 <i>fdhD</i>	22 <i>metB</i> ⁺
		<i>metB</i> ⁺	296	8 <i>fdhD</i>	10 <i>rhaD</i> ⁺
CGSC4443 (<i>chlB</i>)	mal38	<i>ilv</i> ⁺	145	0 <i>chlB</i>	3 <i>metE</i> ⁺
		<i>metE</i> ⁺	149	14 <i>chlB</i>	40 <i>ilv</i> ⁺
CGSC4443	D54	<i>rhaD</i> ⁺	132	6 <i>chlB</i>	12 <i>metB</i> ⁺
		<i>metB</i> ⁺	148	1 <i>chlB</i>	10 <i>rhaD</i> ⁺

distinguish it from other mutants of *E. coli* that we isolated previously and which had lost both formate dehydrogenase activities simultaneously (*fdhA*, *B* and *C*: Mandrand-Berthelot *et al.*, 1978; Haddock & Mandrand-Berthelot, 1982). We thus decided to determine the map location of the relevant locus, designated *fdhD*. Mutant GY05 was used as a recipient strain in uninterrupted mating experiments with HfrH: among the *pro*⁺, *lac*⁺, *trp*⁺ or *his*⁺ selected recombinants, none had inherited the *fdhD* allele. Mutant GY05 was then tested for P1-mediated cotransduction of the *fdhD* mutation with known genetic markers located in the opposite position on the circular map. No cotransduction was found with *xyl*, *mtl*, *pyrE* or *ilv*, but a low linkage was observed with *metE* (Table 3). Coinheritance of adjacent markers *rhaD* and *metB* was subsequently tested to locate *fdhD* more accurately. In this area of the bacterial chromosome lies the *chlB* gene, whose mutations lead to impairment in the synthesis of both formate dehydrogenases and nitrate reductase (Haddock & Jones, 1977). To rule out the possibility that the *fdhD* mutation was a particular allelic form of the *chlB* gene, we compared the cotransduction frequency of the *chlB* mutation carried by strain CGSC4443 with the same selected markers (Table 3). Our data clearly show that *fdhD* and *chlB* lesions belong to two separate loci which are distant from each other. Additional evidence was obtained by the direct

Table 4. P1-mediated transduction mapping of *fdhD* and *fdhE* mutations

Strain 161R (*rhaD54 glpK argH*) was used as recipient for transductions, except with P1(FD21), where strain D54 (*rhaD54 metB*) was used.

Selected marker ...	<i>rhaD</i> ⁺					<i>glpK</i> ⁺				
	Percentage cotransduction of unselected markers					Percentage cotransduction of unselected markers				
	Colonies scored	<i>fdhD,E</i> ⁺	<i>glpK</i> ⁺	<i>metB</i>	<i>argH</i> ⁺	Colonies scored	<i>fdhD,E</i> ⁺	<i>rhaD</i> ⁺	<i>metB</i>	<i>argH</i> ⁺
FD17 (<i>fdhD1 metB1</i>)	150	69	27	21	5	224	8	11	53	21
FD18 (<i>fdhD2 metB1</i>)	150	65	31	18	6	150	10	11	53	20
FD19 (<i>fdhD3 metB1</i>)	150	57	39	23	6	151	9	11	60	25
FD24 (<i>fdhD4 metB1</i>)	76	55	29	21	5	150	13	19	64	20
FD25 (<i>fdhD5</i>)	86	62	36	—	8	150	17	20	—	21
FD16 (<i>fdhE1</i>)	150	57	27	—	6	150	7	12	—	19
FD20 (<i>fdhE2 metB1</i>)	75	64	35	13	—	150	9	11	49	11
FD21 (<i>fdhE3</i>)	152	46	—	21	—	—	—	—	—	—
FD22 (<i>fdhE4 metB1</i>)	50	64	46	28	8	148	8	9	57	24

* *fdhD* and *fdhE* markers were scored using the dye overlay procedures for FDH-BV and FDH-PMS activities (Wu & Mandrand-Berthelot, 1986).

selection of *chlB*⁺ transductants in a cross between strain GY05 (*fdhD*) as donor and strain CGSC4443 (*chlB*) as recipient (data not shown): only 8% of the *ChlB*⁺ clones had inherited the donor FDH-PMS⁻, FDH-BV⁻ phenotype, suggesting that the *chlB* and *fdhD* alleles are at least one minute apart on the *E. coli* genome.

Isolation and preliminary study of additional mutants lacking FDH-PMS activity

In the light of the close proximity of the *fdhD* and *rhaD* loci (see Table 3), we sought other similar mutants, using a P1 localized mutagenesis procedure to transduce strain D54 (*rhaD*) and select *Rha*⁺ recombinants (see Methods). A total of 2300 transductants were screened for their ability to reduce PMS with formate by the dye overlay test. FDH-PMS⁻ potential candidates were purified and scored for the presence of both formate dehydrogenases and nitrate reductase using dye indicator media (Wu & Mandrand-Berthelot, 1986). Nine independent FDH-PMS⁻ mutants were thus identified. They could be further separated into two classes. Like mutant GY05, the five mutants of class I, FD17, FD18, FD19, FD24 and FD25, additionally lacked the ability to reduce BV with formate (as detected on Petri dishes), while the four other mutants, FD16, FD20, FD21 and FD22, belonging to class II, remained unaffected. In addition, formate hydrogenlyase activity (consisting of FDH-BV and hydrogenase activities) was estimated in Durham tubes by gas production from glucose in rich medium. Our results indicate that it was considerably diminished (to about 25% of the level found in the wild-type strain) only in the *fdhD* mutants of class I. The mutants of class II showed the same activity as the wild-type. This latter class was further designated *fdhE*.

To confirm the map location of the mutants, each *fdhD* or *fdhE* mutant was used as donor in P1-mediated transduction with strain 161R (*rhaD glpK argH*), except FD21 which was crossed with strain D54 (*rhaD metB*). Our data showed an average linkage of 60% with *rhaD*, in agreement with that previously reported for GY05 (Table 3), and 10% with *glpK* (Table 4). Three-factor cross analysis of the *glpK*⁺ transductants gave the following order: *fdh(DE)-rhaD-glplK* at 88 min on the *E. coli* chromosome. A 16% cotransduction frequency was also observed with the *glnA* marker at 87 min (data not shown). Thus *fdhD* and *fdhE* behave as closely-linked genes lying midway between *glnA* and *glpK*.

Biochemical characteristics of the mutants

The levels of the two formate dehydrogenase activities were determined in crude extracts of each FDH-PMS⁻ mutant, after anaerobic growth on rich medium in the presence or absence of

Table 5. *Formate dehydrogenase activities of the merodiploid fdhE/fdhD strains*

Bacteria were grown anaerobically in L-broth medium as described in Methods. The activities of FDH-PMS and FDH-BV are expressed as $\mu\text{mol formate min}^{-1} (\text{mg protein})^{-1}$; the results are mean values from triplicate experiments.

Strain	Merodiploid episome/chromosome	FDH-PMS		FDH-BV	
		-NO ₃ ⁻	+ NO ₃ ⁻	-NO ₃ ⁻	+ NO ₃ ⁻
F14-1/FD171rec	<i>fdhD1/fdhD1</i>	0.001	0.001	0.02	0
F14-2/FD191rec	<i>fdhD3/fdhD3</i>	0.002	0.003	0.014	0
F14-3/FD161rec	<i>fdhE1/fdhE1</i>	0	0.001	0.046	0
F14-4/FD221rec	<i>fdhE4/fdhE4</i>	0	0	0.05	0
F14-3/FD171rec	<i>fdhE1/fdhD1</i>	0.037	0.37	0.028	0
F14-3/FD191rec	<i>fdhE1/fdhD3</i>	0.075	0.38	0.029	0
F14-3/FD221rec	<i>fdhE1/fdhE4</i>	0	0.001	0.04	0
F14-4/FD171rec	<i>fdhE4/fdhD1</i>	0.038	0.28	0.01	0
F14-4/FD191rec	<i>fdhE4/fdhD3</i>	0.102	0.40	0.023	0
F14-4/FD161rec	<i>fdhE4/fdhE1</i>	0	0	0.035	0
161R	Wild-type	0.068	0.50	0.066	0

nitrate. Activities of nitrate reductase (a component of the formate nitrate reductase complex) and of hydrogenase (a component of the formate hydrogenlyase system) were simultaneously assayed. As previously outlined for mutant GY05, all the *fdhD* and *fdhE* mutants were totally devoid of FDH-PMS activity but they displayed nearly wild-type specific activities of FDH-BV, except FD18 and FD19, which showed a three- to fivefold reduction in the level of this enzyme. On the other hand, nitrate reductase and hydrogenase activities were comparable to those found in the wild-type strain (data not shown). Growth of cells under anaerobic conditions with or without nitrate allowed us to check that formation of enzymes in the mutants followed a pattern identical to that known for the wild type: FDH-BV and hydrogenase were synthesized in the absence of nitrate and completely repressed in its presence (Table 2 and data not shown); nitrate reductase was induced about tenfold by nitrate (not shown).

In order to find the reason for the difference in the phenotypic behaviour of *fdhD* and *fdhE* classes relative to the *in situ* BV dye overlay test on Petri dishes, we compared FDH-BV, nitrate reductase and hydrogenase activities in intact whole cells and in crude extracts after cell breakage by a French press. We were surprised to detect no differences in any of the mutants tested. In particular, FDH-BV activity of *fdhD* mutants was the same in whole cells and crude extracts and it was comparable to that measured in the wild-type and *fdhE* mutants (data not shown). In addition, formate hydrogenlyase activity assayed manometrically by the Warburg procedure gave identical results with whole cells of mutants FD17 (*fdhD*) and FD16 (*fdhE*) (data not shown), although, as reported above, gas production in Durham tubes was strongly decreased in all *fdhD* mutants. The reason for this discrepancy is unknown, but it was of considerable help to discriminate between the two groups of mutants.

Analysis of fdhD/fdhE merodiploid strains

In order to assess further the existence of two distinct *fdhD* and *fdhE* genes, we constructed a series of merodiploid strains harbouring various combinations of the *fdhD1*, *fdhD3*, *fdhE1* and *fdhE4* alleles originating from strains FD17, FD19, FD16 and FD22 respectively (see Methods and Table 1). Both formate dehydrogenase activities were then assayed in every merodiploid strain after anaerobic growth on rich medium in the presence or absence of nitrate (Table 5). As expected, crude extracts of reference merodiploids bearing the same type of mutation on the episome as well as on the chromosome (i.e. *fdhDx/fdhDy* or *fdhEx/fdhEy*) failed to produce FDH-PMS activity, like the original haploid mutants. In contrast, in merodiploids carrying any combination between an *fdhE* allele on the episome and an *fdhD* allele on the chromosome (i.e. *fdhEx/fdhDy*), FDH-PMS activity was restored to a level similar to that found in the parental

Fig. 1. Analysis by rocket immunoelectrophoresis of FDH-PMS protein in *fdhD* and *fdhE* mutants. Crude extracts (8 mg protein ml⁻¹) were obtained from cells grown anaerobically without nitrate (a) or with nitrate (b). Each well received 7.5 µl crude extract solubilized with Triton X-100. Plates were poured with 3.3 ml 1% agarose containing (a) 15 µl or (b) 30 µl anti-FDH-PMS serum. 1, Wild-type strain EMG29; 2, *fdhD* mutant GY05; 3, wild-type strain D54; 4, *fdhD* mutant FD17; 5, *fdhD* mutant FD19; 6, *fdhD* mutant FD24; 7, *fdhE* mutant FD16; 8, *fdhE* mutant FD21; 9, *fdhE* mutant FD22.

strain 161R. The reason for the decrease in FDH-PMS activity is likely to be that loss of the F' factor can reach 50% in some cultures. In all cases, the FDH-BV activity in the merodiploid strains varied from 10 to 25% of the values obtained with the primary mutants (compare Table 5 with Table 2). This could result from the transfer of the various *fdhD* and *fdhE* alleles in the multiply-marked strain 161R, which served as recipient for the construction of all merodiploids and also shows decreased FDH-BV activity (Table 5).

These results demonstrate that pairs of *fdhD* and *fdhE* mutations are able to complement with each other to restore a wild-type phenotype of FDH-PMS activity. Thus they belong to two different closely-linked genes.

FDH-PMS protein synthesis in FDH-PMS mutants

To determine whether the *fdhD* and *fdhE* mutants produce an FDH-PMS protein identical to that of the wild-type, we analysed them immunochemically, employing antiserum which had been raised to the purified FDH-PMS enzyme.

Crude extracts of mutant and parental strains, solubilized with Triton X-100, were submitted to 'rocket' immunoelectrophoretic analysis (Giordano *et al.*, 1983) (Fig. 1). Although they have lost FDH-PMS activity, *fdhD* mutants (GY05, FD17, FD19 and FD24) still synthesize amounts of FDH-PMS protein comparable to either of the wild-type proteins in strains EMG29 and D54. In contrast, *fdhE* mutants (FD16, FD21 and FD22) did not make any FDH-PMS antigen, whatever the growth conditions. This additional biochemical difference gives further support to the distinction of two groups in the FDH-PMS⁻ mutants.

The distribution of immunoprecipitable FDH-PMS protein after cell fractionation was similar for the *fdhD* mutants and the wild-type strain (75% in the membrane fraction and 25% in the soluble fraction). This ruled out the possibility that the mutation affecting *fdhD* resulted in the accumulation of a soluble (inactive) form of FDH-PMS enzyme.

To characterize the structure of the FDH-PMS protein produced in the *fdhD* mutants, we compared its subunit composition with that of the protein from the parental strain. Membrane fractions labelled after growth of cells in the presence of K₂³⁵SO₄ were prepared from the representative mutant FD17 and the wild-type strain D54. Immunoprecipitated material obtained following incubation with antiserum raised to purified FDH-PMS enzyme was analysed by SDS-PAGE (Fig. 2). In both cases, the antigen recovered had a polypeptide content characteristic of the FDH-PMS enzyme, with equimolar amounts of α subunit (M_r 110 000) and β subunit (M_r 32 000) (1 α :1 β for D54; 0.95 α :1 β for FD17). Except for the γ subunit, which we were not able to identify in this experiment as previously described (Giordano *et al.*, 1980), we can conclude that the molecular composition of FDH-PMS protein remains unchanged in the *fdhD* mutants.

Fig. 2. Subunit composition of membrane-bound FDH-PMS in wild-type strain D54 and in mutant strain FD17 (*fdhD*). Cells were grown anaerobically in the presence of nitrate. Triton X-100 solubilized membrane fractions, labelled with $K_2^{35}SO_4$, of (a) strain D54 [2×10^7 c.p.m. (mg protein) $^{-1}$] and (b) strain FD17 [0.8×10^{-6} c.p.m. (mg protein) $^{-1}$] were incubated with anti-FDH-PMS serum as described in Methods. The immunoprecipitates were subjected to electrophoresis in SDS/10% polyacrylamide gels. The distribution of radioactivity was determined by scintillation counting of 1 mm slices of gels.

Spectral analysis of *b*-type cytochromes

The components of the formate-nitrate reductase pathway, formate dehydrogenase and nitrate reductase, are each associated with an anaerobic *b*-type cytochrome (Enoch & Lester, 1974). Cytochrome *b* (FDH) has been suggested to be associated with the γ -subunit (M_r 20 000) of FDH-PMS by analogy with cytochrome *b* (NR), which constitutes the γ -subunit of nitrate reductase. These cytochromes show a single α -absorption peak at 559 nm at 25 °C. Moreover, cytochrome *b* (NR) has been resolved into two distinct components with midpoint oxidation-reduction potentials of +20 mV and +120 mV whereas cytochrome *b* (FDH) titrates with a redox potential of about -110 mV (Hackett & Bragg, 1983*a, b*). A clear distinction between the two specific cytochromes *b* can be achieved on the basis of their differential response to certain electron donors. Ascorbate reduces only cytochrome *b* (NR) whereas formate reduces both cytochrome *b* (FDH) and cytochrome *b* (NR) by the sequential transfer of electrons (Ruiz-Herrera & DeMoss, 1969). *fdn* mutants of *Salmonella typhimurium* specifically defective in the formation of FDH-PMS activity exhibited strong similarities with the *fdhD* mutants described here (Barrett & Riggs, 1982). Their map position has been assigned to an equivalent site on the *S. typhimurium* chromosome, close to the *rha* locus, and all *fdn* mutants produce an FDH-PMS protein comparable to that of the wild-type. However, spectral analysis revealed that they are defective in the anaerobic cytochrome b_{559} associated with FDH-PMS. In order to compare these mutants with our own mutants, we examined their cytochrome *b* composition by the sequential addition of ascorbate, formate and dithionite (the last causing the complete reduction of the cytochrome *b* present) to cell preparations from the wild-type D54, the *fdhD* mutant FD17 and the *fdhE* mutant FD16 grown anaerobically with nitrate (Fig. 3). In strain D54, ascorbate reduced about 47% of the total cytochrome *b* and the addition of formate resulted in a comparable increment of reduction. Dithionite led to a further 6% reduction, which could be attributable to the presence of cytochromes *b* characteristic of aerobic respiratory pathways

Fig. 3. Differential spectra of cytochrome *b* reduced by ascorbate (●-●-●-●), formate (---) and dithionite (—). Bacteria were grown anaerobically in L-broth medium containing 1% potassium nitrate, washed and suspended in 50 mM-Tris/HCl (pH 7.6) plus 1 mM-benzamidine, then frozen and thawed three times. Absorption spectra were recorded at room temperature with a double-beam spectrophotometer (Denis & Richaud, 1982). A 2.5 ml sample of each cell suspension (about 16 mg protein ml⁻¹) was introduced into one cuvette. The sample was first oxidized by addition of 10 mM-potassium ferricyanide, and sequentially reduced by addition of 20 mM-ascorbate, 20 mM-formate and a few grains of dithionite. The height of the α -peak of cytochrome *b* was measured above the base line at 580 nm: it allowed the determination of the percentage of reduced cytochrome *b*.

(Hackett & Bragg, 1983*a*). In mutant FD17 (*fdhD*), ascorbate also reduced about 46% of the cytochrome *b* and the addition of formate promoted a further reduction of only 10%. However, the total amount of cytochrome *b* reduced by dithionite was of the same order of magnitude as that of the wild-type. Mutant FD16 (*fdhE*) differed from both the wild-type and the *fdhD* mutant in that its cytochrome content showed no reduction by formate, although normal levels of reduction by ascorbate and dithionite were attained.

These results suggest that the cytochrome *b*₅₅₉ (FDH) is present in normal amounts in the *fdhD* and *fdhE* mutants of *E. coli*, but that it has lost in part (*fdhD*) or totally (*fdhE*) its ability to be reduced by formate. These observations are also consistent with the hypothesis that the mutants produce an altered FDH-PMS apoprotein which is impaired in the transfer of electrons to cytochrome *b* (FDH).

DISCUSSION

We have described ten mutants of *E. coli* which specifically lack FDH-PMS activity, but which still produce functional nitrate reductase and formate hydrogenlyase activities. Although genetic analysis suggested that all the relevant mutations reside at the same site near the *rhaD* gene, these mutants have been clearly divided into two classes, *fdhD* and *fdhE*, on the basis of their ability to reduce benzyl viologen with formate using an *in situ* dye overlay test on Petri dishes. *fdhD* mutants appeared phenotypically FDH-BV⁻ while *fdhE* mutants were FDH-BV⁺, but all of them possessed a normal level of FDH-BV activity. It seems unlikely that this discrepancy could be attributed to difficulty in the access of benzyl viologen to its reaction site *in vivo* with FDH-BV, since (i) the non-permeant oxidized species of benzyl viologen is reduced by FDH-BV on the periplasmic side of the cytoplasmic membrane (Jones & Garland, 1977), and (ii) when FDH-BV activity is assayed directly in intact *fdhD* or *fdhE* cells, this difference is no longer detectable. As the same phenomenon is also seen with formate hydrogenlyase activity, depending upon its investigation by gas production in Durham tubes or its measurement in whole cells by the Warburg method, we speculate that aggregation of colonies on agar or sedimentation at the bottom of Durham tubes could lead to the secondary loss of FDH-BV activity in the *fdhD* mutants. In both cases, ageing of cells would result in this effect.

Analysis of merodiploid *fdhD*/*fdhE* strains, in which FDH-PMS activity is similar to the wild-type level, clearly supports the conclusion that both types of mutations are complementary to one another. In addition, immunological characterization with our antiserum raised against purified FDH-PMS indicated that, unlike the *fdhE* mutants, the *fdhD* mutants produced normal amounts of inactive membrane-bound FDH-PMS enzyme.

Several properties suggest that our mutants are different from the six *fdn* mutants described in the equivalent region of the *Salmonella typhimurium* map (Barrett & Riggs, 1982). Although they express a similar FDH-PMS⁻ phenotype, the *fdnA*, *B*, *C* mutants are distributed into at least three unlinked genetic sites between markers *glnA* and *glpK*. Moreover, all *fdn* mutants synthesize an FDH-PMS protein similar to the wild-type protein. In this respect, only our *fdhD* mutants would be likely to resemble *S. typhimurium* *fdn* mutants. *fdhE* mutants, which totally fail to make an FDH-PMS polypeptide, comprise a new class of mutants. Additional striking evidence allows us to separate the mutants isolated in *E. coli* and *S. typhimurium*. Spectral analysis of the cytochrome content of *fdhD* and *fdhE* mutants reveals the existence of both cytochromes *b*₅₅₉ (NR) and *b*₅₅₉ (FDH) in equal amounts to those found in the wild-type. In contrast, Barrett & Riggs (1982) reported that all *fdn* mutants of *S. typhimurium* had lost significant amounts or all cytochrome *b*₅₅₉ (FDH) and that they overproduced cytochrome *b*₅₅₉ (NR). They drew the conclusion that *fdn* genes are possibly involved in the synthesis of a functional cytochrome *b*₅₅₉ (FDH). Formate still reduces, although with a weaker effect, cytochrome *b*₅₅₉ (FDH) in *fdhD* mutants, but it has no effect in *fdhE* mutants, which are unable to produce an FDH-PMS protein. Thus, we tentatively propose the hypothesis that *fdhD* and *fdhE* genes might participate in the synthesis or the assembly of FDH-PMS apoprotein which consists of the three subunits α , β and γ . *fdhD* mutants would synthesize normal amounts of a modified FDH-PMS which fails to transfer electrons from formate to cytochrome *b*₅₅₉ (FDH), due to limited access or to difficulty in the attachment of cytochrome *b* to FDH-PMS. One could also imagine that the structure of FDH-PMS protein has been mutated in such a way that it has lost its affinity for formate and thus cannot liberate carbon dioxide and electrons. In fact, *fdhD* mutants might be defective in the structural gene of either the α or the β subunit, provided that the finding that cytochrome *b*₅₅₉ (FDH) is not essential for FDH-PMS activity assayed with PMS as electron acceptor still holds (Ruiz-Herrera & DeMoss, 1969). On the other hand, the behaviour of the *fdhE* mutants, totally devoid of FDH-PMS protein, is reminiscent of the loss of a positive regulator that would activate the anaerobic transcription of genes involved in the synthesis of FDH-PMS. Alternatively, the FdhE⁻ phenotype might result from the absence of a structural component required for the integrity of the enzyme, which would thus become degraded when it was lacking. By comparison, Barrett & Riggs (1982) did not detect any reduction of cytochrome *b*₅₅₉ (FDH) by formate in an *fdhA* mutant of *S. typhimurium* deficient in both FDH activities and in FDH-PMS protein: spectral analysis of the cytochrome content was identical to that of our *fdhE* mutant and its typical pattern was attributed to a faulty FDH-PMS enzyme itself.

Further genetic experiments, including molecular cloning and *in vivo* expression, are in progress to define the gene product-function relationship of *fdhD* and *fdhE*.

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