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Escherichia coli Mutant with Altered Respiratory Control of the *frd* Operon

S. IUCHI, D. R. KURITZKES, AND E. C. C. LIN*

Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

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In wild-type *Escherichia coli*, fumarate reductase encoded by the *frd* operon is inducible by its substrate in the absence of molecular oxygen and nitrate. Synthesis of this enzyme under permissive conditions requires the *fnr*$^+$ gene product, which is believed to be a pleiotropic regulatory protein that activates transcription. A spontaneous mutant was isolated in which the expression of the *frd* operon no longer depended on the presence of fumarate or the *fnr*$^+$ gene product. Aerobic repression of the operon was abolished, but nitrate repression remained intact. Transductional analysis showed that the mutation was closely linked to the *frd* locus. The mutant phenotype strongly suggests that repression by molecular oxygen and nitrate is mediated by different mechanisms.

Fumarate reductase of *Escherichia coli* K-12 is an inducible membrane-bound flavoprotein complex that constitutes the terminal portion of one of the anaerobic respiratory chains. Reduction of fumarate energizes the cell membrane, providing the proton-motive force necessary for sugar and amino acid transport (16), ATP synthesis (24), and flagellar motility (2). The fumarate reductase complex comprises four subunits, all of which are encoded by the *frd* operon at min 94 of the chromosome (8, 18, 19). Aerobic growth or anaerobic growth in the presence of nitrate prevents induction of the enzyme complex by fumarate (12, 30). Thus, it seems that the complex is inducible by its substrate only in the absence of electron acceptors more redox positive than fumarate. The mechanisms of respiratory control of gene expression remain poorly understood, although it has been discovered that a positive regulatory protein Fnr (product of the *fnr*$^+$ gene) is required for effective transcription of a number of operons that encode anaerobic respiratory enzymes, including the *frd* operon (6, 7, 18, 26).

Fumarate reductase is necessary for anaerobic growth on glycerol and *sn*-glycerol 3-phosphate (G3P) with fumarate as the sole electron acceptor. Under such a growth condition, the *glpAB* operon, encoding the membrane-associated anaerobic G3P dehydrogenase, also becomes highly induced (20). A previous report described the use of *lac* fusions for studying the respiratory regulation of *glpAB* expression. Like *frd*, the *glpAB* operon is inducible by the substrate, is subject to respiratory repression by oxygen and nitrate, and requires the *fnr*$^+$ gene product for full expression (17). During the course of isolation and characterization of mutants with altered respiratory control of the *glpAB-lac* operon (unpublished data), a strain with alterated regulation of the *frd* operon was also discovered. The nature of the mutation affecting the behavior of the *frd* operon is the subject of this report.

MATERIALS AND METHODS

Materials. Phenazine methosulfate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, flavin adenine dinucleotide, flavin mononucleotide (grade I), benzyl viologen, *o*-nitrophenyl-β-D-galactopyranoside, and DL-dithiothreitol were obtained from Sigma Chemical Co., St. Louis, Mo.; 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside was from Bachem, Inc., Torrance, Calif. MacConkey medium, tryptone, and yeast extract were obtained from Difco Laboratories, Detroit, Mich. Vitamin-free casein acid hydrolysate was obtained from ICN Nutritional Biochemicals, Cleveland, Ohio. All other reagents used were commercial products of the highest grade available.

Bacterial and phage strains. The genotypes of the *E. coli* K-12 strains and bacteriophages used are given in Table 1.

Growth conditions. Bacteria were cultivated and phages were propagated as previously described (17). Growth was carried out at 30°C in experiments with cells bearing phage Mu dl; otherwise, incubations were carried out at 37°C. LB medium (1.0% tryptone, 0.5% yeast extract, 0.5% NaCl) was used for routine liquid cultures. Glucose was added to a concentration of 10 mM for growth of *glpD* strains (9). For enzyme assays, cells were grown in a standard minimal medium (SM) buffered at pH 7.0 by 0.1 M phosphate (33). Where indicated, glycerol was added to 20 mM, xylose was added to 10 mM, and lactose was added to 5 mM as carbon and energy sources. As hydrogen acceptors, fumarate was added to 20 mM, and potassium nitrate was added to 10 mM. Casein hydrolysate was added to 0.03% in liquid SM or minimal agar medium to stimulate anaerobic growth. Media were supplemented with vitamins and amino acids when needed.

LB agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (40 μg/liter) and the appropriate inducer were used to screen for β-galactosidase activity in isolated colonies. When used, ampicillin was added to 50 μg/ml, and tetracycline was added to 20 μg/ml. Anaerobic incubation was carried out in sealed jars made anaerobic by an H_2-CO_2 generator (GasPak Anaerobic System; BBL Microbiology Systems, Cockeysville, Md.). GasPak anaerobic indicators (BBL) were used to document anaerobiosis.

For assays of anaerobic G3P dehydrogenase and fumarate reductase activities, cells were grown aerobically in 40-ml cultures that were vigorously agitated in 300-ml flasks and harvested in mid-exponential phase of growth (approximately 100 Klett units, no. 42 filter) or were grown anaerobically in 150-ml screw-capped flasks filled to the top and left undisturbed for 16 h. For β-galactosidase assay, cells grown aerobically with vigorous agitation in 5-ml cultures in 50-ml tubes were harvested at mid-exponential phase, and cells

* Corresponding author.

TABLE 1. *E. coli* and bacteriophage strains

Strain	Derived from strain	Genotype or phenotype	Source or reference
Bacteria			
MC4100		F⁻ *thi araD139 ΔlacU169 rpsL relA fldB ptsF25*	4
ECL126		F⁻ *frd thr-1 leu-6 thi-1 lacY1 malA1 xyl-7 ara-13 mtl-2 str-135 azi-8 tonA2* λ⁻ *supE44*	23
ECL322	MC4100	F⁻ *thi araD139 ΔlacU169 zjd*::Tn*10 rpsL relA fldB ptsF25*	This work
ECL323	MC4100	F⁻ *thi araD139 ΔlacU169 fnr zci*::Tn*10 rpsL relA fldB ptsF25*	17
ECL324	MC4100	F⁻ *srl*::Tn*10 recA*	C. Kumamoto
ECL371		F⁻ *thi sdh-9 araD139 ΔglpD102 ΔlacU169 rpsL relA fldB ptsF25*	17
ECL380	ECL371	F⁻ *frd-101*::Mu d1	This work
ECL381	ECL371	F⁻ *glpA101*::Mu d1	17
ECL389	ECL392	F⁻ *glpA101*::λp1(209) ΔMu *srl*::Tn*10 recA*	This work
ECL392	ECL381	F⁻ *glpA101*::λp1(209) ΔMu	This work
ECL503	ECL389	F⁻ *glpA101*::λp1(209) *frd*^{c} aerobic inducible Lac⁺	This work
ECL508	ECL380	F⁻ Δ*frd-101* ΔMu	This work
ECL509	ECL380	F⁻ Δ*frd-102* ΔMu	This work
ECL510	ECL508	F⁻Δ*frd-101 zjd*::Tn*10*	This work
ECL511	ECL509	F⁻ *frd*^{c}	This work
ECL512	ECL371	F⁻ *sdh-9 ΔglpD102 fnr zci*::Tn*10*	This work
ECL513	ECL511	F⁻ *frd*^{c} *fnr zci*::Tn*10*	This work
Phages			
P1 *vir*			
Mu d1		*cts trp'CBA' lac'OZYA* 'Tn*3* Ap^r	5
λNK55		*cI857 b221 oam cIII*::Tn*10*	14
λp1(209)		*attPP' int xis lac'AYZO'-ΔW209-trp'AB'*::(+Mu')	4

grown anaerobically were harvested after 16 h of incubation as described above. As inducers, fumarate was added to 20 mM, but glycerol was added only to 2 mM to avoid aerobic growth inhibition of *glpD* strains (9).

Preparation of cell extracts. Cells harvested for anaerobic G3P dehydrogenase and fumarate reductase assays were washed in 10 mM potassium phosphate buffer (pH 7.0) plus 1 mM DL-dithiothreitol and suspended in 4 volumes of the same solution. The suspended cells were disrupted with cooling in a 60-W sonic disintegrator (Measuring & Scientific Equipment, Ltd., London, England), allowing 1 min of treatment per ml of suspension. Cellular debris was removed by centrifugation at 10,000 × *g* for 30 min.

Enzyme assays. Anaerobic G3P dehydrogenase activity was assayed at 30°C by the phenazine methosulfate-mediated reduction of 3-(4,5-dimethylthiazol-2-ly)-2,5-diphenyl tetrazolium bromide monitored at 570 nm in the presence of added flavin adenine dinucleotide and flavin mononucleotide (13). Fumarate reductase activity was assayed at 30°C in an anaerobic cuvette by monitoring the reoxidation of reduced benzyl viologen at 500 nm (17). Protein concentrations in cell extracts were estimated with bovine serum albumin as the standard (21). Specific activities of anaerobic G3P dehydrogenase and fumarate reductase were expressed in nanomoles per minute per milligram of protein.

β-Galactosidase activity was assayed in whole cells (rendered permeable by the addition of 1 drop of 0.1% sodium dodecyl sulfate and 2 drops of chloroform to the suspension) at 30°C by monitoring the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside at 420 nm, and the specific activity was expressed in units by the method of Miller (25).

P1 transduction. P1 *vir* was used for transductions (3). Lysates were prepared in liquid medium, except for those from *recA* cells which were grown in soft agar. All transductants were purified on the same selective medium and replica plated to screen for unselected markers. Antibiotic-resistant transductants were selected on LB agar with the appropriate drug. To screen for *recA* transductants, cells streaked on LB agar were exposed to UV light (General Electric model #G1BTB, 15 W) for 10, 30, or 50 s and then incubated in the dark at 37°C. Transductants sensitive to greater than 10 s of UV light exposure were classified as RecA⁻.

Construction of *glpA*-λ *lac* derivatives of *glpA*-Mu d1 fusions. The high frequency of transposition of bacteriophage Mu makes Mu d1 (*lac*) fusions unsuitable for the selection of regulatory mutants with altered expression of β-galactosidase. Therefore, the method of Komeda and Iino (15) was used to convert the *glpA*-Mu *lac* fusion to *glpA*-λ *lac* fusions. To avoid duplications or deletions in *glpA*-λ *lac* fusions (10), a *recA* mutant allele was introduced into the strain by P1 transduction. Strain ECL324, a *recA* strain which carries Tn*10* inserted into the closely linked *srl* gene, was used as the donor; strain ECL392 was used as the recipient. Strain ECL389, a tetracycline-resistant transductant which inherited UV sensitivity, was used for the isolation of mutants with altered respiratory control.

Construction of *frd* strains. First, strain ECL380 with an *frd*::Mu d1 fusion was constructed from strain ECL371 by the method of Casadaban and Cohen (5) with the procedures described for the isolation of *glpA-lac* fusions (17). Strain ECL380 failed to grow anaerobically on glycerol when fumarate was provided as the terminal hydrogen acceptor; anaerobic growth on glycerol and nitrate was unimpaired, as was anaerobic growth on glucose alone. Analysis of cell extracts showed an absence of fumarate reductase activity but presence of anaerobic G3P dehydrogenase activity. The position of the Mu d1 insertion in strain ECL380 was confirmed by P1 transduction.

Mutants of strain ECL380 deleted in *frd* were isolated after growth in liquid SM at 42°C to induce the Mu prophage and subsequent screening for white colonies after growth on SM–xylose–5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside agar supplemented with 0.2 mM glycerol and 1 mM fumarate. Presumptive Lac⁻ clones were purified on the same medium and tested for ampicillin sensitivity and anaerobic growth on glycerol-fumarate medium. Two sponta-

TABLE 2. β-Galactosidase and fumarate reductase activities in mutant and wild-type strains[a]

Strain	β-Galactosidase activity (U) in conditions:				Fumarate reductase activity (nmol/min per mg of protein) in conditions:			
	Aerobic		Anaerobic		Aerobic		Anaerobic	
	−GF	+GF	−GF	+GF	−GF	+GF	−GF	+GF
ECL 389	3	47	19	420	0	14	320	1,100
ECL 503	35	490	120	2,500	650	670	1,100	1,300

[a] Cells were grown aerobically on SM-xylose medium or anaerobically on SM-xylose-casein hydrolysate. +GF, Glycerol and fumarate added; −GF, glycerol and fumarate omitted.

neous Ap^s Frd$^-$ derivatives of strain ECL380, strains ECL508 and ECL509, were shown to have a deletion in *frd* by their failure to give rise to Frd$^+$ revertants.

Construction of a Δ*frd zjd*::Tn*10* strain. To obtain a cell line with Tn*10* inserted close to *frd*, strain ECL126 (*frd*) was transduced with a P1 lysate of a population of strain MC4100 harboring about 15,000 random Tn*10* insertions (resulting from infection with λNK55). Transductants were selected anaerobically on glycerol-fumarate agar supplemented with tetracycline. The linked *frd*$^+$ and Tn*10* in one clone were transduced to a fresh genetic background (strain MC4100) to eliminate possible extraneous copies of the transposon. The resulting transductant ECL322 donated Tn*10* with about 80% linkage to *frd*$^+$. (Cotransduction of Tn*10* and *frd*$^+$ in crosses with strain ECL126 established for the first time the locus of this original fumarate reductase mutation [12, 23].) Strain ECL322 was used as a Tn*10* donor to strain ECL508, and a Δ*frd zjd*::Tn*10* transductant, strain ECL510, was identified by its inability to grow anaerobically on glycerol-fumarate medium and its resistance to tetracycline.

Construction of *fnr* strains. Strain ECL323 (*fnr zci*::Tn*10*) was used as the standard transduction donor of *fnr* in strain constructions by selecting for Tc^r and screening for the loss of ability to grow anaerobically on glycerol-fumarate and glycerol-nitrate agar. In this manner, strains ECL512 (*frd*$^+$ *fnr*) and ECL513 (*frd*^{c} *fnr*) were isolated.

Selection of *glpAB* regulatory mutants. Spontaneous mutants in which the *glpAB-lac* fusion operon was well expressed aerobically were isolated from strain ECL389. About 10^9 cells from a clone of this strain were plated on SM–lactose–5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside agar containing 0.2 mM glycerol and 1 mM fumarate as inducers. After 2 to 4 days of aerobic incubation at 37°C, blue colonies were picked as Lac$^+$ mutants (spontaneous mutation frequency was approximately 5×10^{-6}). (There was a background of tiny pale blue or colorless colonies which were too numerous to count.) Mutant colonies were purified twice on the same selective medium and assayed for β-galactosidase activity under aerobic and anaerobic conditions.

RESULTS

Mutant strain with altered control of *glpAB* and *frd*. Strain ECL503 was selected from strain ECL389 as a mutant with increased aerobic expression of *glpAB-lac*. Analysis of cells grown under different conditions showed that β-galactosidase activity in this mutant was inducible by glycerol to levels higher than those of the parent strain both aerobically and anaerobically (Table 2). Unexpectedly, high levels of fumarate reductase activity were also synthesized under aerobic conditions.

Genetic characterization of the mutation affecting *frd* expression. The altered regulatory patterns of *glpAB-lac* and *frd* in strain ECL503 were revealed by transductional analysis to be the result of two separate mutations. When a P1 lysate of this strain was used to infect strain ECL509, which has a deletion in the *frd* operon, all 11 transductants selected for anaerobic growth on glycerol-fumarate medium were found to possess high levels of fumarate reductase when grown aerobically. Thus, the mutation that altered the expression of the *frd* operon, hereafter referred to as *frd*^{c}, cotransduced with the structural genes. One of these transductants, strain ECL511, was used for further study. The *frd* genes in strain ECL511 were shown to be in the expected location by another P1 transduction experiment in which strain ECL510, which harbors a Tn*10* insertion in the neighborhood of an *frd* deletion (Δ*frd zjd*::Tn*10*), was used as donor. Transductants selected for Tc^r were scored for anaerobic growth on glycerol-fumarate agar. *Frd*$^-$ was found to be 74% linked with Tc^r (Table 3). A control experiment showed a similar linkage between the Tc^r marker and the *frd*$^+$ locus when the wild-type strain ECL371 served as recipient. Thus, the *frd*^{c} mutation was either within or close to the operon.

Constitutivity of fumarate reductase in strain ECL511 and escape from oxygen effect. The regulation of fumarate reductase and anaerobic G3P dehydrogenase levels in strain ECL511 was compared with that in strain ECL371, which bears wild-type *frd* and *glpAB* operons (Table 4). As ex-

TABLE 3. Mapping of *frd*^{c} in strain ECL511 with strain ECL510 (Δ*frd-101 zjd*::Tn*10*) as the donor[a]

Recipient strain	No. of Tc^r transductants	No. of unselected markers (Frd$^-$)	% Cotransduction of Frd$^-$ with Tc^r
ECL371 (*frd*$^+$)	48	34	71
ECL511 (*frd*^{c})	50	37	74

[a] Transductants were selected for Tc^r and scored for Frd$^-$ as described in the text.

TABLE 4. Fumarate reductase and anaerobic G3P dehydrogenase activities in *frd*^{c} and wild-type strains[a]

Strain	Fumarate reductase activity (nmol/min per mg of protein) in conditions:				Anaerobic G3P dehydrogenase activity (nmol/min per mg of protein) in conditions:			
	Aerobic		Anaerobic		Aerobic		Anaerobic	
	−GF	+GF	−GF	+GF	−GF[b]	+GF	−GF	+GF
ECL371	0	17	460	1,100	—[b]	1	1	20
ECL571	550	460	1,000	1,000	—	1	0	20

[a] Growth conditions and abbreviations are as given in Table 2.
[b] —, Not done.

TABLE 5. Effect of nitrate on fumarate reductase levels in aerobically and anaerobically grown strains[a]

Strain	Fumarate reductase activity (nmol/min per mg of protein) in conditions:			
	Aerobic		Anaerobic	
	$-NO_3$	$+NO_3$	$-NO_3$	$+NO_3$
ECL371	20	20	1,500	40
ECL511	630	610	1,300	27

[a] Cells were grown with fumarate plus glycerol as described in Table 2. $+NO_3$, Nitrate added; $-NO_3$, nitrate omitted.

pected, fumarate reductase activity was negligible in strain ECL371 grown aerobically in the presence or absence of glycerol and fumarate, but a high level of the activity was found when the strain was grown anaerobically on xylose alone. The addition of glycerol plus fumarate to the growth medium increased the anaerobic activity another twofold. In contrast, the level of fumarate reductase activity in strain ECL511 grown aerobically in the presence of glycerol and fumarate was 50% of that of anaerobically induced cells. Moreover, no inducer effect was observed either aerobically or anaerobically. Thus, *frd* expression in strain ECL511 was constitutive with respect to substrate control, as was the case for strain ECL503 (Table 2). No difference was observed in the pattern of anaerobic G3P dehydrogenase activity of strain ECL511 when compared with strain ECL371. Together these results suggest that the alteration of the expression of the *glpAB-lac* operon in strain ECL503 was caused by a separate mutation.

Retention of nitrate repressibility of fumarate reductase in the *frd*^{c} mutant. Since expression of *frd*$^+$ can also be anaerobically repressed by nitrate, the question arose whether the *frd*^{c} mutation likewise lifted this control. Nitrate retained its ability to prevent anaerobic induction of fumarate reductase in strain ECL511 (Table 5). However, nitrate was ineffective in preventing the aerobic expression of fumarate reductase in this mutant. Apparently, nitrate repression occurred only under conditions permitting induction of the nitrate reductase system, i.e., anaerobically in the presence of nitrate.

Effect of the *fnr* allele on fumarate reductase synthesis in the *frd*^{c} mutant. The product of the *fnr*$^+$ gene is required for effective anaerobic induction of fumarate reductase (18) and anaerobic G3P dehydrogenase (17). The introduction of a defective *fnr* allele into strain ECL511 (to give strain ECL513) had no effect on anaerobic constitutive expression of its *frd* operon (Table 6). That the *fnr* allele was indeed introduced was shown by the lowered induced level of anaerobic G3P dehydrogenase. The introduction of the same *fnr* allele into a strain bearing the wild-type *frd*$^+$ allele (to give strain ECL512) reduced the anaerobically induced level of fumarate reductase by about fourfold.

DISCUSSION

A hierarchy of respiratory control is established in some facultative anaerobes whereby terminal oxidants of more positive redox potential (hence, more favorable energetically) repress hydrogen transfer systems of more negative potential. For example, ethanol dehydrogenase is repressed by fumarate, fumarate reductase is repressed by nitrate, and nitrate reductase is repressed by oxygen (22, 34). Respiratory control is therefore analogous to catabolite repression and inducer exclusion, which establish a hierarchy for carbohydrate utilization.

Two models of hierarchal control of respiratory and fermentation enzymes have been envisaged. The first invokes intracellular redox potential, E_h, as the key signal (for examples, see references 1 and 34). According to this model, terminal hydrogen acceptors, having relatively positive standard redox potentials, prevent the synthesis of proteins that function with hydrogen acceptors of more negative standard redox potentials. This occurs by the influence of terminal hydrogen reactions on the relative concentrations of a common effector which can exist in an oxidized or reduced state, such as a coenzyme.

The second model invokes a series of regulatory proteins that assure utilization of the preferred hydrogen acceptor (27, 31). For instance, in the presence of molecular oxygen, the most preferred electron acceptor, an aerobic regulator protein (e.g., a heme protein) allows transcription of the genes encoding not only enzymes of the aerobic respiratory chain but also a pleiotropic repressor of genes encoding all anaerobic respiratory proteins. In the absence of molecular oxygen, nitrate, the most preferred electron acceptor, is able to induce its reductase system. When this system is induced, another pleiotropic repressor with fewer target operons than the aerobic repressor is synthesized. This repressor prevents the induction of other anaerobic respiratory proteins, such as the fumarate reductase complex. Similarly, when the fumarate reductase system is expressed, the genes for formate hydrogenlyase and ethanol dehydrogenase are not inducible. Such regulatory proteins with ranking order dispense with a control mechanism that has to sense the intracellular redox potential. Although such a model is unattractive in that it requires many regulatory sites for operons at the bottom of the hierarchy, support for it is provided by the observation that mutations in *narK* prevent nitrate repression of tertiary amine *N*-oxide reductase expression. It thus appears that the *narK*$^+$ gene, which is induced together with the *narGHI* genes and whose product has no role in the nitrate reductase complex, is the pleiotropic repressor associated with the nitrate system (31, 32).

To characterize the respiratory control of the genes encoding the fumarate reductase complex, we constructed a *lac* fusion (strain ECL353) in which β-galactosidase activity was induced anaerobically by fumarate. From this mutant, several derivatives with altered properties of regulation were isolated (28). When a mapping experiment was carried out with the parental strain, the *lac* genes were found, surprisingly, to be located in the *trp* operon (unpublished data). Evidently, homology between the *trp* base sequences carried on λp1(209) and the chromosomal *trp* genes provided a site for recombination (4). Therefore, no further study of these *lac* fusion strains was carried out. In their stead, a new

TABLE 6. Fumarate reductase and anaerobic G3P dehydrogenase activities in *fnr* derivatives of *frd*^{c} and wild-type strains[a]

Strain	Fumarate reductase activity (nmol/min per mg of protein)	Anaerobic G3P dehydrogenase activity (nmol/min per mg of protein)
ECL371	1,200	26
ECL512 (*fnr*)	340	7
ECL511 (*frd*^{c})	1,100	29
ECL513 (*frd*^{c} *fnr*)	1,100	8

[a] Cells were grown anaerobically on SM–xylose–casein hydrolysate medium supplemented with glycerol plus fumarate.

frd-Mu *lac* mutant (strain ECL380 described above) was isolated. After verifying the position, the fusion was converted to a *frd*-λ *lac* fusion. To stabilize the hybrid operon further, a *recA* allele was then introduced to the strain. From this strain, mutants capable of synthesizing high levels of β-galactosidase under aerobic conditions were isolated. However, the instability of these mutations during transduction has impeded genetic analysis (work in progress). Unexpectedly, a parallel study of a *glpAB-lac* strain afforded a view of how respiratory repression of the *frd* operon is exerted by molecular oxygen and nitrate.

Transduction of the regulatory characteristics of the frd^c allele from strain ECL503 into a host strain deleted in *frd* without altering the control pattern of the *glpAB* of the recipient showed that the aerobic inducibility of Φ(*glpAB-lac*) and the aerobic constitutivity of *frd* in strain ECL503 were consequences of two separate mutations. Since the experimental protocol was designed for the aerobic inducibility of the *glpAB* hybrid operon, it is likely that an frd^c cell arose from the primary mutant clone that became aerobically inducible in Φ(*glpAB-lac*). Moreover, it is likely that the secondary frd^c mutation conferred an additional growth advantage on lactose so that the double mutant constituted a significant fraction of the population by the time the colony was picked for single-cell isolation. This postulated growth advantage of frd^c over frd^+ was confirmed by the following test. Strain ECL503 (Φ[*glpAB-lac*] frd^c) was plated on lactose agar side by side with an frd^+ isogenic strain constructed by transduction. After incubation for 36 h at 37°C, frd^c colonies were 1.2 ± 0.04 (SE) mm and frd^+ colonies were 0.71 ± 0.02 (SE) mm in diameter. Apparently in the absence of succinic dehydrogenase (for our studies, an *sdh* mutation was introduced to the parental strain because the enzyme can contribute to fumarate reductase activity when assayed in vitro [12]), fumarate reductase can act as a substitute during growth. A similar suppressor effect was reported in a study of *frd* gene amplification (11).

The close linkage of the regulatory mutation to the *frd* locus suggests a base sequence change in the operator region of the *frd* operon. The observation that the frd^c mutation could free the expression of the *frd* operon from repression by molecular oxygen but not nitrate, which has a redox potential less positive than that of oxygen, favors the multitiered model for respiratory control. Moreover, it should be noted that the effect of nitrate in the frd^c mutant was observed only when the nitrate reductase system could be induced, that is, anaerobically but not aerobically. In addition, in an frd^c *fnr* double mutant (strain ECL513), nitrate had no effect on the expression of frd^c even anaerobically (data not shown).

Codon analysis of the cloned fnr^+ gene predicted homology between the Fnr protein and other regulatory proteins such as the catabolite activator protein. This homology has led to the suggestion that Fnr functions as a gene activator by interacting with an unidentified effector molecule in a manner analogous to that involving catabolite activator protein and cAMP (29). However, exposure to O_2 during growth could simply convert the Fnr product into a protein that fails to bind to target operators or into a repressor.

The simultaneous dispensability of both the specific inducer and the Fnr protein by a single mutation in frd^c would suggest that the site for the specific regulatory protein overlaps or is adjacent to that for the general regulatory protein. An independent site is likely to exist for nitrate repression. More mutant phenotypes need to be discovered before this crude model can be refined.

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LITERATURE CITED

1. **Ashcroft, J. R., and B. A. Haddock.** 1975. Synthesis of alternative membrane-bound redox carriers during aerobic growth of *Escherichia coli* in the presence of potassium cyanide. Biochem. J. **148**:349–352.
2. **Bar Tana, J., B. J. Howlett, and D. E. Koshland, Jr.** 1977. Flagellar formation in *Escherichia coli* electron transport mutants. J. Bacteriol. **130**:787–792.
3. **Berman, M. L., L. W. Enquist, and T. H. Silhavy.** 1981. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
4. **Casadaban, M. J.** 1976. Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophages lambda and Mu. J. Mol. Biol. **104**:541–555.
5. **Casadaban, M. J., and S. N. Cohen.** 1979. Lactose genes fused to exogenous promoters in one step using a Mu-*lac* bacteriophage: *in vivo* probe for transcriptional control sequences. Proc. Natl. Acad. Sci. U.S.A. **76**:4530–4533.
6. **Chippaux, M., D. Giudici, A. Abou-Jaudé, F. Casse, and M.-C. Pascal.** 1978. A mutation leading to the lack of nitrite reductase activity in *Escherichia coli* K12. Mol. Gen. Genet. **160**:225–229.
7. **Cole, J. A., and F. B. Ward.** 1973. Nitrite reductase-deficient mutants of *Escherichia coli* K-12. J. Gen. Microbiol. **76**:21–29.
8. **Cole, S. T., T. Grundstrom, B. Jaurin, J. J. Robinson, and J. H. Weiner.** 1982. Location and nucleotide sequence of *frdB*, the gene coding for the iron-sulphur protein of the fumarate reductase of *Escherichia coli*. Eur. J. Biochem. **126**:211–216.
9. **Cozzarelli, N. R., J. P. Koch, S. Hayashi, and E. C. C. Lin.** 1965. Growth stasis by accumulated L-α-glycerophosphate in *Escherichia coli*. J. Bacteriol. **90**:1325–1329.
10. **Gowrishankar, J., and J. Pittard.** 1982. Construction from Mu d1(*lac* Ap^r) lysogens of lambda bacteriophage bearing promoter-*lac* fusions: isolation of λ p*pheA-lac*. J. Bacteriol. **150**: 1122–1129.
11. **Guest, J. R.** 1981. Partial replacement of succinate dehydrogenase function by phage and plasmid-specified fumarate reductase in *Escherichia coli*. J. Gen. Microbiol. **122**:171–179.
12. **Hirsch, C. A., M. Rasminsky, B. D. Davis, and E. C. C. Lin.** 1963. A fumarate reductase in *Escherichia coli* distinct from succinate dehydrogenase. J. Biol. Chem. **238**:3770–3774.
13. **Kistler, W. S., and E. C. C. Lin.** 1971. Anaerobic L-α-glycerophosphate dehydrogenase of *Escherichia coli*: its genetic locus and its physiological role. J. Bacteriol. **108**:1224–1234.
14. **Kleckner, N., D. Baker, D. G. Ross, D. Botstein, J. A. Swan, and M. Zaben.** 1978. Properties of the tetracycline-resistant element Tn*10* in *Escherichia coli* and bacteriophage lambda. Genetics **90**:427–461.
15. **Komeda, Y., and T. Iino.** 1979. Regulation of expression of the flagellin gene (*hag*) in *Escherichia coli* K-12: analysis of *hag-lac* gene fusions. J. Bacteriol. **139**:721–729.
16. **Konings, W. N., and H. R. Kaback.** 1973. Anaerobic transport in *Escherichia coli* membrane vesicles. Proc. Natl. Acad. Sci. U.S.A. **79**:3376–3381.
17. **Kuritzkes, D. R., X.-Y. Zhang, and E. C. C. Lin.** 1984. Use of Φ(*glp-lac*) in studies of respiratory regulation of the *Escherichia coli* anaerobic *sn*-glycerol-3-phosphate dehydrogenase genes (*glpAB*). J. Bacteriol. **157**:591–598.
18. **Lambden, P. R., and J. R. Guest.** 1976. Mutants of *Escherichia coli* unable to use fumarate as an anaerobic electron acceptor. J. Gen. Microbiol. **97**:145–160.
19. **Lemire, B. D., J. J. Robinson, and J. H. Weiner.** 1982. Identification of membrane anchor polypeptides of *Escherichia coli* fumarate reductase. J. Bacteriol. **152**:1126–1131.
20. **Lin, E. C. C.** 1976. Glycerol dissimilation and its regulation in bacteria. Annu. Rev. Microbiol. **30**:535–578.
21. **Lowry, O. H., N. J. Rosebough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J.

Biol. Chem. **193**:265–275.
22. **McPhedran, P., B. Sommer, and E. C. C. Lin.** 1961. Control of ethanol dehydrogenase levels in *Aerobacter aerogenes*. J. Bacteriol. **81**:852–857.
23. **Miki, K., and E. C. C. Lin.** 1973. Enzyme complex which couples glycerol-3-phosphate dehydrogenation to fumarate reduction in *Escherichia coli*. J. Bacteriol. **114**:767–771.
24. **Miki, K., and E. C. C. Lin.** 1975. Anaerobic energy-yielding reaction associated with transhydrogenation from glycerol 3-phosphate to fumarate by an *Escherichia coli* system. J. Bacteriol. **124**:1282–1287.
25. **Miller, J. H. (ed.).** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Newman, B. M., and J. A. Cole.** 1978. The chromosomal location and pleiotropic effects of mutations of the *nirA*$^+$ gene of *Escherichia coli* K12: the essential role of *nirA*$^+$ in nitrite reduction and in other anaerobic redox reactions. J. Gen. Microbiol. **106**:1–12.
27. **Pascal, M.-C., J.-F. Burini, J. Ratouchniak, and M. Chippaux.** 1982. Regulation of the nitrate reductase operon: effect of mutations in *chlA*, *B*, *D*, and *E* genes. Mol. Gen. Genet. **188**:103–106.
28. **Ruch, F. E., D. R. Kuritzkes, and E. C. C. Lin.** 1979. Use of *lac* operon fusions to isolate *Escherichia coli* mutants with altered expression of the fumarate reductase system in response to substrate and respiratory controls. Biochem. Biophys. Res. Commun. **91**:1365–1370.
29. **Shaw, D. J., D. W. Rice, and J. R. Guest.** 1983. Homology between CAP and Fnr, a regulator of anaerobic respiration in *Escherichia coli*. J. Mol. Biol. **166**:241–247.
30. **Spencer, M. E., and J. R. Guest.** 1973. Isolation and properties of fumarate reductase mutants of *Escherichia coli*. J. Bacteriol. **114**:563–570.
31. **Stewart, V.** 1982. Requirement of Fnr and NarL functions for nitrate reductase expression in *Escherichia coli* K-12. J. Bacteriol. **151**:1320–1325.
32. **Stewart, V., and C. H. MacGregor.** 1982. Nitrate reductase in *Escherichia coli* K-12: involvement of *chlC*, *chlE*, and *chlG* loci. J. Bacteriol. **151**:788–799.
33. **Tanaka, S., S. A. Lerner, and E. C. C. Lin.** 1967. Replacement of a phosphoenolpyruvate-dependent phosphotransferase by a nicotinamide adenine dinucleotide-linked dehydrogenase for the utilization of mannitol. J. Bacteriol. **93**:642–648.
34. **Wimpenny, J. W. T.** 1969. The effect of E_h on regulatory processes in facultative anaerobes. Biotechnol. Bioeng. **11**: 623–629.