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Three Classes of *Escherichia coli* Mutants Selected for Aerobic Expression of Fumarate Reductase

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Fumarate reductase (encoded by *frd*) and succinate dehydrogense (encoded by *sdh*) of *Escherichia coli* are both known to catalyze the interconversion of fumarate and succinate. Fumarate reductase, however, is not inducible aerobically and therefore cannot participate in the dehydrogenation of succinate. Three classes of suppressor mutants, classified as *frd* oxygen-resistant [*frd*(Oxr)], constitutive [*frd*(Con)], and gene amplification [*frd*(Amp)] mutants, were selected from an *sdh* strain as pseudorevertants that regained the partial ability to grow aerobically on succinate. All contained increased aerobic levels of fumarate reductase activity. In *frd*(Oxr) mutants expression of the operon showed increased resistance to aerobic repression. Under anaerobic conditions expression of the operon became less dependent on the *fnr*$^+$ gene product, a pleiotropic activator protein for genes encoding anaerobic respiratory enzymes. Exogenous fumarate, however, was still required for full induction, and repression by nitrate was undiminished. Thus, aerobic repression and anaerobic nitrate repression appear to involve separate mechanisms. In *frd*(Con) mutants expression of the operon became highly resistant to aerobic repression. Under anaerobic conditions expression of the operon no longer required the *fnr*$^+$ gene product or exogenous fumarate and became immune to nitrate repression. In partial diploids bearing an *frd*(Oxr) or an *frd*(Con) allele and Φ(*frd*$^+$-*lac*) there was no mutual regulatory influence between the two genetic loci. Thus, the *frd* mutations act in *cis* and hence are probably in the promoter region. In *frd*(Amp) mutants the *frd* locus was amplified without significant alteration in the pattern of regulation.

Two flavoprotein complexes catalyze the interconversion of succinate and fumarate in *Escherichia coli* K-12: fumarate reductase and succinate dehydrogenase (9, 32). Fumarate reductase has a higher apparent affinity for fumarate than for succinate, whereas the reverse is true for succinate dehydrogenase (9). The genes encoding these enzymes probably share a common line of descent (36). Even in contemporary organisms the physiological specialization of these enzymes still appears to be critically dependent on the regulation of gene expression, as illustrated by the partial remedy of the phenotypic defect resulting from the loss of succinate dehydrogenase gene function by amplification of the genes for fumarate reductase (8). Fumarate reductase, encoded by the *frdABCD* operon, is highly inducible anaerobically, provided that nitrate, a more effective electron acceptor, is absent (9, 32, 35). The normal function of this enzyme is to catalyze the terminal electron transfer from nonfermentable compounds like *sn*-glycerol 3-phosphate (G3P) or D-lactate to fumarate as the acceptor. The coupling of the anaerobic oxidation of G3P (15–17) to the reduction of fumarate energizes the cell membrane for sugar and amino acid transport and ATP synthesis (10, 18, 24; M. Miki and E. C. C. Lin, Abstr. Annu. Meet. Fed. Am. Soc. Exp. Biol., p. 632, 1973). Succinate dehydrogenase, encoded by the *sdhCDAB* operon (35), is highly inducible only aerobically (29, 32). This enzyme is not only a member of the tricarboxylic acid cycle but is also responsible for aerobic growth on succinate as the sole source of carbon and energy (9).

As in the case of fumarate reductase, the induced synthesis of anaerobic G3P dehydrogenase is curtailed by molecular oxygen or nitrate (7, 16). Even under permissive conditions full induction of these enzymes requires a positive regulatory protein encoded by *fnr* (11, 19, 20). This protein functions as a pleiotropic activator of genes specifying anaerobic respiratory pathways (3, 4, 26, 30). Codon analysis of the *fnr*$^+$ gene revealed homology between the Fnr protein and the cyclic AMP receptor protein involved in the regulation of catabolic pathways (31).

Recently, it was discovered that in an *sdh* strain a mutation rendering the *frd* operon resistant to aerobic repression (11) permitted growth on succinate. Advantage then was taken of this property to select for more mutants affected in the *frd* operon. In addition to the example reported above, two additional classes of mutants with elevated aerobic levels of fumarate reductase activity were isolated. The genetic nature of the three classes of mutations are reported here.

MATERIALS AND METHODS

Materials. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was obtained from Bachem, Inc. (Torrance, Calif.). Vitamin-free casein acid hydrolysate was obtained from ICN Nutritional Biochemicals (Cleveland, Ohio). All other reagents used in this study were commercial products of the highest grade available.

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

Growth conditions. Bacteria were cultivated and phages were propagated as described previously (19). Cells for enzyme assays were grown at 37°C in a standard minimal medium (SM) buffered at pH 7.0 with 0.1 M phosphate (34). To test anaerobic repression by nitrate, SM buffered at pH 7.6 with 0.2 M phosphate (2PSM) was used as the growth medium. As carbon and energy sources, glycerol, succinate, or DL-lactate was added to 20 mM; D-xylose or glucose was added to 10 mM; and lactose was added to 5 mM. As electron acceptors fumarate was added to 20 mM and

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TABLE 1. *E. coli* strains used in this study

Strain	Derived from:	Genotype or phenotype	Source or reference
RK4918		F⁻ *thi araD139 ΔlacU169 zbh-620*::Tn*10 rpsL gyrA non*	33
RK4921		F⁻ *thi araD139 ΔlacU169 zbh-623*::Tn*10 rpsL gyrA non*	33
ECL322		F⁻ *thi araD139 ΔlacU169 zjd*::Tn*10 rpsL relA fldB ptsF25*	11
ECL323		F⁻ *thi araD139 ΔlacU169 fnr-1 zci*::Tn*10 rpsL relA fldB ptsF25*	19
ECL371		F⁻ *thi sdh-9 araD139 ΔglpD102 ΔlacU169 rpsL relA fldB ptsF25* Gal⁻	19
ECL380	ECL371	F⁻ *frd-101*::Mu dl	11
ECL381	ECL371	F⁻ *frd*⁺ *glpA101*::Mu dl	19
ECL388	ECL380	F⁻ *frd-101*::λ pl(209) ΔMu	This study
ECL509		F⁻ Δ*frd-102* ΔMu	11
ECL510		F⁻ Δ*frd-101 zjd*::Tn*10*	11
ECL511		F⁻ *frd*(Con)	11
ECL514	ECL381	F⁻ *frd*⁺ Φ(*glpA101-lacZ*⁺ *Y*) ΔMu	This study
ECL515	ECL514	F⁻ *frd-4*(Oxr)	This study
ECL516	ECL514	F⁻ *frd-5*(Oxr)	This study
ECL517	ECL514	F⁻ *frd-2*(Con)	This study
ECL518	ECL514	F⁻ *frd-3*(Con)	This study
ECL520	ECL514	F⁻ *frd*⁺ *fnr-1 zci*::Tn*10*	This study
ECL521	ECL515	F⁻ *frd-4*(Oxr) *fnr-1 zci*::Tn*10*	This study
ECL522	ECL516	F⁻ *frd-5*(Oxr) *fnr-1 zci*::Tn*10*	This study
ECL523	ECL517	F⁻ *frd-2*(Con) *fnr-1 zci*::Tn*10*	This study
ECL524	ECL518	F⁻ *frd-3*(Con) *fnr-1 zci*::Tn*10*	This study
ECL528	ECL514	F⁻ *frd-6*(Amp)	This study
ECL529	ECL514	F⁻ *frd-7*(Amp)	This study
ECL530	ECL514	F⁻ *frd-8*(Amp)	This study
ECL531	ECL528	F⁻ *frd-6*(Amp) *fnr-1 zci*::Tn*10*	This study
ECL532	ECL529	F⁻ *frd-7*(Amp) *fnr-1 zci*::Tn*10*	This study
ECL533	ECL530	F⁻ *frd-8*(Amp) *fnr-1 zci*::Tn*10*	This study
ECL567	ECL515	F⁻ *frd-4*(Oxr) *zjd*::Tn*10*	This study
ECL568	ECL516	F⁻ *frd-5*(Oxr) *zjd*::Tn*10*	This study
ECL569	ECL511	F⁻ *frd-1*(Con) *zjd*::Tn*10*	This study
ECL570	ECL517	F⁻ *frd-2*(Con) *zjd*::Tn*10*	This study
ECL571	ECL518	F⁻ *frd-3*(Con) *zjd*::Tn*10*	This study
ECL572	ECL528	F⁻ *frd-6*(Amp) *zjd*::Tn*10*	This study
ECL573	ECL529	F⁻ *frd-7*(Amp) *zjd*::Tn*10*	This study
ECL574	ECL530	F⁻ *frd-8*(Amp) *zjd*::Tn*10*	This study
ECL575	ECL371	F⁻ Gal⁺	This study
ECL576	ECL575	F⁻ λ⁺	This study
ECL577	ECL576	F⁻ *frd*⁺ *frd-101*::λ pl(209)	This study
ECL578	ECL577	F⁻ *frd-4*(Oxr) *frd-101*::λ pl(209)	This study
ECL579	ECL577	F⁻ *frd-1*(Con) *frd-101*::λ pl(209)	This study

potassium nitrate was added to 10 mM. As an inducer of the Φ(*glpA*⁺-*lac*) hybrid operon, glycerol was added at a low concentration (0.2 mM) to avoid toxic accumulation of G3P (21). Casein acid hydrolysate was added to 0.03% in SM or 2PSM liquid medium, as well as in agar medium, to stimulate anaerobic growth. When used, ampicillin was added to 50 μg/ml, tetracycline to 20 μg/ml, and chloramphenicol to 40 μg/ml. Anaerobic incubation of agar plates 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 assay of fumarate reductase activity, cells were grown aerobically in 40-ml cultures vigorously agitated in 300-ml flasks and harvested in mid-exponential phase (approximately 100 Klett units, no. 42 filter) or anaerobically in 50-ml screw-cap test tubes filled to the top and left undisturbed for 16 h. For the β-galactosidase assay, cells were grown aerobically in 5-ml cultures vigorously agitated in 50-ml tubes and harvested in mid-exponential phase or anaerobically in filled tubes as described above.

Determination of ampicillin resistance. Cultures growing exponentially on LB medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) were diluted with 0.85% NaCl and spread (about 300 cells) on LB agar containing different concentrations of ampicillin (2).

Enzyme assays. Cell extracts, prepared as described previously (11), were assayed for fumarate reductase activity at 30°C in an anaerobic cuvette by monitoring the reoxidation of reduced benzyl viologen at 500 nm (19). Protein concentrations were estimated with bovine serum albumin as the standard (22). Specific activity of fumarate reductase was 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 cell 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).

Isolation of stable derivative of Mu dl(Ap^r *lac*) fusion to *glpA*. To isolate a stable Φ(*glpA*⁺-*lac*) from the original fusion in strain ECL381 the thermal inducible Mu prophage was deleted by heating the culture to 42°C for 7 h (23). The survivors were plated on a medium containing xylose, glycerol, fumarate, and X-Gal. Blue colonies were purified on the same medium and screened for the inability to grow anaerobically on glycerol-fumarate and glycerol-nitrate agar and for sensitivity to ampicillin. Strain ECL514 obtained by

TABLE 2. Expressions of *frd* and Φ(*glpA*$^+$-*lacZ*) in parent and mutant strains

Strain (genotype)	Fumarate reductase activity (nmol/min per mg of protein) under the following growth conditions[a]:				β-Galactosidase activity (U) under the following growth conditions[a]:	
	Aerobic		Anaerobic		Aerobic +GF	Anaeobic +GF
	−GF	+GF	−GF	+GF		
ECL514 (*frd*$^+$)	14 (0.01)	13 (0.01)	270 (0.25)	1,100 (1.0)	38 (0.10)	390 (1.0)
ECL515 [*frd-4*(Oxr)]	260 (0.08)	300 (0.09)	880 (0.27)	3,300 (1.0)	38 (0.11)	360 (1.0)
ECL516 [*frd-5*(Oxr)]	130 (0.06)	220 (0.10)	730 (0.32)	2,300 (1.0)	37 (0.09)	430 (1.0)
ECL511 [*frd-1*(Con)]	550 (0.55)	460 (0.46)	1,000 (1.0)	1,000 (1.0)		
ECL517 [*frd-2*(Con)]	250 (0.28)	290 (0.32)	840 (0.93)	900 (1.0)	37 (0.08)	440 (1.0)
ECL518 [*frd-3*(Con)]	400 (0.58)	340 (0.49)	870 (1.26)	690 (1.0)	37 (0.09)	430 (1.0)
ECL528 [*frd-6*(Amp)]	200 (0.03)	310 (0.06)	1,600 (0.20)	8,000 (1.0)	42 (0.12)	360 (1.0)
ECL529 [*frd-7*(Amp)]	140 (0.02)	240 (0.04)	1,300 (0.21)	6,300 (1.0)	45 (0.12)	390 (1.0)
ECL530 [*frd-8*(Amp)]	45 (0.01)	73 (0.02)	910 (0.25)	3,600 (1.0)	28 (0.07)	380 (1.0)

[a] Cells were grown aerobically or anaerobically on SM-xylose-casein hydrolysate medium. −GF, glycerol and fumarate omitted; +GF, glycerol and fumarate added. For each strain data in parentheses give its relative enzyme activities normalized with respect to those found in cells grown anaerobically with GF. Data on strain ECL511 are cited from a previous study (11).

this procedure sustained a deletion in *lacY*, as indicated by its failure to accumulate methyl-β-D-thiogalactopyranoside. The inducibility of the β-galactosidase specified by the stabilized Φ(*glpA*$^+$-*lac*) and aerobic repression was not changed.

Strain constructions. Strain ECL323 (*fnr zci*::Tn*10*) was used as the standard donor in P1 *vir* transductions (1) of the *fnr* allele (10% cotransduction with Tn*10*). Transductants were selected for tetracycline resistance (Tc^r) and screened for loss of the ability to grow anaerobically on SM agar containing glycerol-fumarate, glycerol-nitrate, or DL-lactate–nitrate. Strains ECL520, ECL521, ECL522, ECL523, ECL524, ECL531, ECL532, and ECL533 were thus isolated.

Strain ECL510 (Δ*frd zjd*::Tn*10*) was used as the standard donor to place Tn*10* near a mutant *frd* allele (80% cotransduction) in recipients also harboring the *sdh* mutation. Transductants were selected for Tc^r and screened for aerobic growth on succinate (Suc$^+$). Strains ECL567, ECL568, ECL569, ECL570, ECL571, ECL572, ECL573, and ECL574 were thus isolated.

Isolation of regulatory mutants. Mutants with altered regulation of the *frd* operon were selected from ethyl methanesulfonate-mutagenized and nonmutagenized clones of strain ECL514. Approximately 10^9 cells were plated on SM-succinate agar (supplemented with 1 mM fumarate in case induction priming was required) and incubated aerobically. Colonies appearing after 1 week were purified on the same selective medium. To test whether derepression of *frd* was accompanied by increased expression of Φ(*glpA*$^+$-*lac*), the purified colonies were then streaked on agar containing X-Gal, glycerol (as inducer), and xylose. None of the clones showed a darker blue color than the parental control ECL514.

Construction of *frd* Φ(*frd*$^+$-*lac*) merodiploid strains. The parental strain ECL371 was discovered to be Gal$^-$. Because with this defect the induction of Φ(*frd*$^+$-*lac*) would not allow growth on lactose, a Gal$^+$ revertant, strain ECL575, was isolated to construct the merodiploid strains. From this revertant a lambda lysogen, strain ECL576, was obtained. Strain ECL388, bearing *frd-101*:: λ pl(209), was induced by UV irradiation, and the lambda lysate was used to transfer the Φ(*frd*$^+$-*lac*) fusion to the lysogenized strain ECL576. Infected cells were placed on SM-lactose agar medium supplemented with fumarate as the inducer and incubated anaerobically. Lac$^+$ colonies were purified on the same agar. The anaerobic induction of β-galactosidase by fumarate confirmed the presence of Φ(*frd*$^+$-*lac*). A double lysogen thus isolated, strain ECL577, had the fusion at the *att* site for lambda. The insertion of Φ(*frd*$^+$-*lac*) at this site was confirmed by transduction mapping. The Tn*10* transposon near *chlA* in strain RK4918 and Tn*10* near *chlD* in strain RK4921 genes are both closely linked to *att*. P1 grown on these strains was used to infect strain ECL577. Among purified Tc^r transductants, 60% were Lac$^-$ when strain RK4918 was the donor and 80% were Lac$^-$ when strain RK4921 was the donor.

P1 lysates of strains ECL567 [*frd-101*(Oxr) *zjd*::Tn*10*] and ECL569 [*frd-101*(Con) *zjd*::Tn*10*] were used to transfer the mutant *frd* alleles into strain ECL577 by cotransduction with *zjd*::Tn*10*. Transductants were selected for Tc^r and screened for Suc$^+$. Strains ECL578 [*frd-101*(Oxr) Φ(*frd*$^+$-lac)] and ECL579 [*frd-101*(Con) Φ(*frd*$^+$-*lac*)] were thus isolated.

RESULTS

Mutants with altered control of *frd*. When cells of strain ECL514 (*sdh frd*$^+$) were plated on succinate agar, Suc$^+$ pseudorevertants appeared with a spontaneous frequency of about 10^{-8}. (Spontaneous reversions in *sdh* occurred at a frequency of less than 10^{-9}.) Mutagenized cultures gave pseudorevertants at a frequency of about 4×10^{-6}. Three independent spontaneous mutants (ECL515, ECL528, ECL529) and four independent induced mutants (ECL516, ECL517, ECL518, ECL530) were examined for their profile of enzyme activities. All produced increased aerobic levels of fumarate reductase (Table 2). Fumarate reductase of strains ECL515 and ECL516, classified as *frd* oxygen-resistant [*frd*(Oxr)] mutants, remained inducible by fumarate anaerobically. In contrast, fumarate reductase of strains ECL517 and ECL518 was expressed constitutively. This phenotype is similar to that observed in strain ECL511 described above, which harbors the *frd*-constitutive [*frd*(Con)] allele (11). In both *frd*(Oxr) and *frd*(Con) mutants anaerobic growth still gave higher fumarate reductase activities. In *frd*(Oxr) mutants the anaerobically induced levels of the enzyme exceeded that of the wild-type strains more than twofold.

Strains ECL528 and ECL529 showed greatly elevated anaerobic levels of fumarate reductase activity, especially when induced by the substrate. These features are reminiscent of the properties of ampicillin-resistant mutants discov-

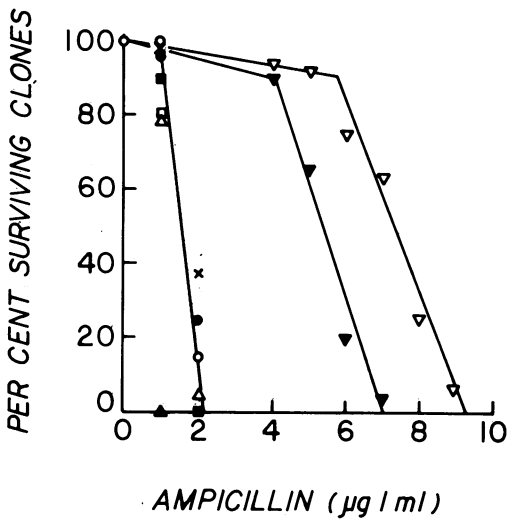

FIG. 1. Approximately 300 viable cells of each strain were spread on LB agar plates supplemented with different concentrations of ampicillin. The number of colonies was counted after overnight incubation at 37°C. Symbols: ○, ECL514; △, ECL515; □, ECL516; ●, ECL511; ▲, ECL517; ■, ECL518; ▽, ECL528; ▼, ECL529; and ×, ECL530.

ered to have multiplications of the overlapping *ampC-frd* genes (6). Therefore, the two strains were tested for resistance to the drug. Whereas 2 μg of ampicillin per ml significantly inhibited the growth of the parent strain ECL514 and the *frd*(Oxr) and *frd*(Con) mutants, 6 to 8 μg of ampicillin per ml was necessary for a similar effect on strains ECL528 and ECL529 (Fig. 1). The results indicate that strains ECL528 and ECL529 have multiple *ampC-frd* genes, and therefore constitute a third genotype, *frd* gene amplification [*frd*(Amp)] mutants.

Strain ECL530, in which the enzyme level was elevated more than threefold without a striking change in the regulatory pattern (see also other phenotypic characterizations presented below), remained normally sensitive to ampicillin. These features suggest that an amplification of the *frd* locus not encompassing *ampC* took place.

Assays of the β-galactosidase encoded by the indicator operon Φ(*glpA*$^+$-*lac*) did not reveal any pleiotropic effect in the mutants studied (Table 2).

Mapping of mutant *frd* alleles. Mutant *frd* alleles were first mapped by P1 transduction with strain ECL322 (*frd*$^+$*zjd*::Tn*10*) as donor. Transductants were selected for tetracycline resistance and scored for growth on succinate. The expected linkage between Tc^r and Suc$^-$ (67 to 85%) was found in all cases. A control with a strain doubly defective in *frd* and *sdh* as the recipient gave no Suc$^+$ clones among 100 Tc^r transductants. Thus, the *frd*(Oxr), *frd*(Con), and *frd*(Amp) mutations are either within or close to the *frd* operon.

In a second transduction experiment, the *frd* mutants bearing *zjd*::Tn*10* were used as donors, and strain ECL509 Δ*frd* was used as the recipient. Transductants were selected for tetracycline resistance and scored for aerobic growth on succinate and anaerobic growth on glycerol and fumarate (Table 3). The *frd*(Oxr) and *frd*(Con) alleles were again closely linked to *zjd*::Tn*10*. Moreover, there was nearly complete concordance between the two growth characteristics. (The single GF$^+$ [growth on glycerol-fumarate] and Suc$^-$ clones in three transductional crosses indicate that the deletion in the *frd* recipient did not extend into the promoter and that a recombination event occurred between the donor and recipient *frd* promoters.) By contrast, in the cases of strains ECL528, ECL529, and ECL530, the linkage between the *frd*(Amp) allele and *zjd*::Tn*10* showed progressive diminution, correlating with increasing gene dosage. Furthermore, aerobic growth ability on succinate (requiring inheritance of multicopies of *frd*$^+$) and anaerobic growth ability on glycerol-fumarate (with inheritance of a single copy of *frd*$^+$ being sufficient) could be dissociated by transduction.

Effect of *fnr* on expression of *frd* alleles. Because the Fnr protein is required for full expression of *frd*$^+$ and *glpA*$^+$, the effect of transducing an *fnr* mutant allele into the *frd*(Oxr) and *frd*(Con) strains was tested. Table 4 shows that the *fnr* mutation only slightly lowered the anaerobic fumarate reductase level specified by *frd-4*(Oxr) but reduced by one-third the enzyme level specified by *frd-5*(Oxr). As in the case of *frd-1*(Con) (11), the *fnr* mutation exerted no appreciable effect on the fumarate reductase levels specified by the *frd-2*(Con) and *frd-3*(Con) alleles. By contrast, the *fnr* mutation reduced by three- to fourfold the fumarate reductase level specified by the *frd*(Amp) alleles. In all strains the the *fnr* mutation reduced anaerobic expression of the Φ(*glpA*$^+$-*lac*) threefold, as observed in a previous study (19).

Effect of nitrate on anaerobic expression of *frd* alleles. Fumarate reductase activity in an *frd*(Con) mutant strain ECL511 was previously reported to be repressed 50-fold anaerobically by 10 mM nitrate (11). It was subsequently realized that the pH of the culture dropped to 6 when the cells were harvested. When nitrate, chloramphenicol, and xylose were introduced anaerobically into a fully grown culture on glycerol-fumarate and incubated for 10 h, a similar decline in pH occurred. The specific activity of fumarate reductase decreased 20-fold during this period. A control in which nitrate was omitted showed that no inactivation occurred, despite the drop in pH. When the test was carried out with the concentration of the phosphate buffer in the medium increased from 0.1 to 0.2 M and the initial pH adjusted to 7.6 instead of 7.0 (2PSM), the pH dropped to 7.0 at the end of a similar incubation period. Under such a condition there was no inactivation of fumarate reductase even in the presence of nitrate. Perhaps the presence of nitrate in a low-pH medium resulted in the oxidative inactivation of fumarate reductase. Therefore, all subsequent experiments on nitrate repression were carried out with the

TABLE 3. Transduction of *frd* mutations into strain ECL509 (Δ*frd*)

Donor (genotype)	No. of transductants with unselected markers when grown on[a]:	
	GF$^+$	Suc$^+$
ECL567 [*frd-4*(Oxr) *zjd*::Tn*10*]	83	82
ECL568 [*frd-5*(Oxr) *zjd*::Tn*10*]	81	81
ECL569 [*frd-1*(Con) *zjd*::Tn*10*]	93	92
ECL570 [*frd-2*(Con) *zjd*::Tn*10*]	90	89
ECL571 [*frd-3*(Con) *zjd*::Tn*10*]	88	88
ECL572 [*frd-6*(Amp) *zjd*::Tn*10*]	22	8
ECL573 [*frd-7*(Amp) *zjd*::Tn*10*]	38	10
ECL574 [*frd-8*(Amp) *zjd*::Tn*10*]	56	38

[a] In each experiment 100 Tc^r transductants were scored for anaerobic growth on glycerol-fumarate agar and for aerobic growth on succinate agar.

2PSM medium. Under this protocol fumarate reductase in the *frd*$^+$ strain ECL514 was repressed fivefold by nitrate, whereas the enzyme in the *frd*(Con) strain ECL511 was repressed less than twofold (Table 5). Fumarate reductase in the other *frd*(Con) mutant strains ECL517 and ECL518 was also repressed less than twofold. In contrast, the enzyme in *frd*(Oxr) mutant strains ECL515 and ECL516 was repressed at least fourfold. The enzyme in the three *frd*(Amp) mutant strains ECL528, ECL529, and ECL530 were also strongly repressed.

Gene expression in merodiploid *frd* and Φ(*frd*$^+$-*lac*) strains. The levels of fumarate reductase and β-galactosidase were tested in strains bearing an *frd*$^+$, *frd*(Oxr), or *frd*(Con) allele at its proper locus and Φ(*frd*$^+$-*lac*) integrated with the prophage at the *att* locus. The aerobic and anaerobic levels of β-galactosidase in cells grown in the presence or absence of glycerol and fumarate were not significantly affected by the introduction of *frd*(Oxr) or *frd*(Con) alleles. On the other hand, the elevated levels of fumarate reductase in *frd*(Oxr) and *frd*(Con) cells were not reduced to the wild-type level by the presence of the hybrid *frd*$^+$-*lac* operon (data not shown). The *cis* dominance of the *frd*(Oxr) and *frd*(Con) mutations indicates that they occurred in the promoter-operator region.

Attempts to isolate *trans*-regulatory mutants. One objective of this study was to use the *sdh* and Φ(*glpA-lac*) strain ECL514 to isolate pleiotropic regulatory mutations, e.g., those in the *fnr* gene that permit aerobic expression of its target operons. However, among 300 Suc$^+$ pseudorevertants scored on xylose agar supplemented with glycerol (to induce the β-galactosidase encoded by the fusion) and X-Gal (a chromogenic substrate), no pleiotropic mutant was detected.

Attempts to exploit the Φ(*frd*$^+$-*lac*) strain for the isolation of regulatory mutants resistant to aerobic repression by selecting for growth on lactose in the presence of fumarate and molecular oxygen were also unsuccessful. When analyzed genetically, several mutants with the desired phenotype were found to have the fusion translocated. It is likely that the constitutive expression of the *lac* genes resulted from fusion to some unknown constitutive promoters.

TABLE 4. Anaerobic expression of *frd* alleles and Φ(*glpA*$^+$-*lacZ*) in *fnr*$^+$ and *fnr* backgrounds[a]

Strain (genotype)	Fumarate reductase activity		β-Galactosidase activity	
	nmol/min per mg of protein	*fnr*/*fnr*$^+$	U	*fnr*/*fnr*$^+$
ECL514 (*frd*$^+$ *fnr*$^+$)	980		360	
ECL520 (*frd*$^+$ *fnr*)	280	0.29	140	0.39
ECL515 [*frd-4*(Oxr) *fnr*$^+$]	3,300		320	
ECL521 [*frd-4*(Oxr) *fnr*]	3,000	0.91	100	0.31
ECL516 [*frd-5*(Oxr) *fnr*$^+$]	2,400		370	
ECL522 [*frd-5*(Oxr) *fnr*]	1,500	0.63	150	0.41
ECL517 [*frd-2*(Con) *fnr*$^+$]	710		370	
ECL523 [*frd-2*(Con) *fnr*]	780	1.1	150	0.41
ECL518 [*frd-3*(Con) *fnr*$^+$]	830		380	
ECL524 [*frd-3*(Con) *fnr*]	940	1.1	140	0.37
ECL528 [*frd-6*(Amp) *fnr*$^+$]	7,000		290	
ECL531 [*frd-6*(Amp) *fnr*]	2,600	0.37	110	0.38
ECL529 [*frd-7*(Amp) *fnr*$^+$]	6,000		300	
ECL532 [*frd-7*(Amp) *fnr*]	1,500	0.25	120	0.40
ECL530 [*frd-8*(Amp) *fnr*$^+$]	3,700		300	
ECL533 [*frd-8*(Amp) *fnr*]	900	0.24	120	0.40

[a] The cells were grown anaerobically in the presence of glycerol and fumarate as described in footnote *a* of Table 2.

TABLE 5. Anaerobic effect of nitrate on fumarate reductase levels in *frd*$^+$, *frdO*, *frdC*, and *frdM* strains

Strain (genotype)	Fumarate reductase activity (nmol/min per mg of protein) when grown on[a]:	
	+GF	+GFN
ECL514 (*frd*$^+$)	1,000 (1.0)	210 (0.21)
ECL515 [*frd-4*(Oxr)]	3,500 (1.0)	810 (0.23)
ECL516 [*frd-5*(Oxr)]	2,300 (1.0)	580 (0.25)
ECL511 [*frd-1*(Con)]	1,100 (1.0)	820 (0.75)
ECL517 [*frd-2*(Con)]	890 (1.0)	470 (0.53)
ECL518 [*frd-3*(Con)]	870 (1.0)	730 (0.84)
ECL528 [*frd-6*(Amp)]	10,000 (1.0)	590 (0.06)
ECL529 [*frd-7*(Amp)]	6,700 (1.0)	1,100 (0.16)
ECL530 [*frd-8*(Amp)]	3,300 (1.0)	1,200 (0.36)

[a] Cells were grown anaerobically on 2PSM-xylose-casein hydrolysate medium in the presence of glycerol-fumarate (+GF) or glycerol-fumarate-nitrate (+GFN). For each strain data in parentheses are its relative enzyme activities normalized with respect to the activity found in cells grown anaerobically in the presence of glycerol-fumarate.

DISCUSSION

Three kinds of suppressor mutants able to grow aerobically on succinate were isolated from in an *sdh* (Suc$^-$) strain. All the mutants contained increased aerobic levels of fumarate reductase activity. Two classes of mutations, *frd*(Oxr) and *frd*(Con), changed the regulatory pattern of the operon. In *frd*(Oxr) mutants expression of the operon showed increased resistance to aerobic repression. Anaerobically, dependence on the *fnr*$^+$ gene product was reduced, exogenous induction by fumarate still occurred, and nitrate repression remained intact. In *frd*(Con) mutants expression of the operon was more strongly resistant to aerobic repression than in *frd*(Oxr) mutants. Anaerobically, the *fnr*$^+$ gene product became completely dispensable, exogenous fumarate became superfluous for induction, and repression by nitrate was moderated.

The close linkage of the *frd*(Oxr) and *frd*(Con) traits to *frdABCD* and the *cis* dominance of the regulatory mutations indicate that they occur in the promoter region. The mutations that relieve the dependence on the *fnr* gene product might be the counterparts of the class III promoter mutations of the *lac* operon that relieves its dependence on the cyclic AMP receptor protein for expression (28). Correlation of the relief from aerobic repression with reduced anaerobic dependence on the Fnr protein in *frd*(Oxr) and *frd*(Con) mutants suggests that aerobic repression is effected by lowering the concentration of functional Fnr protein, either through conversion of the protein into its inactive form or through limitation of its synthesis. It should be noted, however, that even in *frd*(Con) mutants the fumarate reductase level was still lower aerobically than anaerobically by a factor of 2 to 3. It is possible that the enzyme itself is unstable during aerobic metabolism. Resolution of this problem might have to await the construction of hybrid operons with the promoter region of the *frd*(Con) gene fused to an extrinsic structural gene.

Nitrate has been shown to block induction of galactose kinase in Φ(*frd*$^+$-*galK*)-bearing strains (12). A similar effect of nitrate was observed in Φ(*frd*$^+$-*lacZ*) strains (unpublished data). In theory the nitrate may act indirectly by excluding the inducer fumarate or more directly at the level of transcription. Inducer exclusion is a well-documented regulatory mechanism for selective utilization of carbohydrates (5, 27), but such a mechanism does not seem to be involved in the

regulation of *frd* expression by nitrate. Fumarate is known to be a substrate and inducer for the C_4-dicarboxylic acid permease (13, 14). When cells induced in this permease were assayed for the rate of transport of labeled 0.1 mM fumarate, less than twofold inhibition was observed with 10 mM nitrate (unpublished data). Results of a recent study revealed that nitrate in combination with the *narL* product blocks *frd* expression (S. Iuchi, and E. C. C. Lin, Abstr. Annu. Meet. Soc. Microbiol. 1986, H80, p. 140).

Substrate inducibility of fumarate reductase implies the existence of a specific regulator protein. (The high anaerobic basal level of the enzyme is probably attributable to elevated endogenous induction.) However, extragenic mutations causing specific constitutivity or noninducibility of frd^+ have not yet been demonstrated. An *frdR* gene encoding a specific repressor for the operon has been postulated on the basis of a disproportionate increase in aerobic levels of fumarate reductase activity relative to β-lactamase activity in cells amplified in *ampC-frd*. It was argued that the increased copies of the *frd* operator resulted in repressor titration (6, 8). Other explanations, however, are possible. For instance, there may be an *frdR* gene encoding the specific activator protein which is closely linked to *frdABCD*. Amplification of the region would increase the cellular concentration of the positive regulatory protein.

Tandem duplications of the overlapping *ampC-frd* genes have been isolated by selecting for high-level ampicillin resistance. Increased levels of resistance to ampicillin paralleled increased levels of fumarate reductase activity (6). In this study similar mutants were isolated by selection for growth on succinate. The reduced linkage of the *frd*(Amp) alleles to *zjd*::Tn*10* from 80% when the mutants served as recipients to 8 to 38% when the mutants served as donors is consistent with the elongation of the *frd* region for the following reasons. An amplified chromosomal locus of a mutant recipient should retain its expected transduction linkage to a nearby marker because the extra genetic material may "loop out" during homologous recombination. On the other hand, when such a mutant serves as donor packaging constraints of P1 would more frequently exclude the marker outside the amplified region, resulting in lower linkage.

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