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The *frdR* Gene of *Escherichia coli* Globally Regulates Several Operons Involved in Anaerobic Growth in Response to Nitrate

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Fumarate reductase catalyzes the terminal step of anaerobic electron transport with fumarate as a terminal electron acceptor. Transcription of the fumarate reductase (*frdABCD*) operon in *Escherichia coli* is repressed in the presence of the preferred terminal electron acceptors, oxygen and nitrate. To identify *trans*-acting genes involved in regulation by nitrate, a number of *E. coli* mutants were generated in which expression of a *frdA'-'lacZ* protein fusion was no longer fully repressed by nitrate. One of these mutants, strain LK23R35, exhibited 17-fold higher β-galactosidase activity than the wild-type strain when grown anaerobically in the presence of nitrate. When grown aerobically in the presence of nitrate, it contained three- to fourfold more β-galactosidase activity than the wild-type strain did. Oxygen regulation of *frd* expression, however, was unaffected by the mutation, since the level of β-galactosidase activity in both strains was nearly identical when they were grown in the absence of nitrate either aerobically or anaerobically. To confirm that the mutation acts in *trans* to *frdABCD*, we measured fumarate reductase levels and found them to parallel FrdA'–β-galactosidase activity under all growth conditions tested. The effect of the mutation is pleiotropic, since the levels of nitrate reductase in LK23R35 were not induced by the addition of nitrate. The *frdR* mutant was also derepressed for nitrate control of the trimethylamine-*N*-oxide reductase and alcohol dehydrogenase enzymes. The mutation maps in a region between *trp* and *hemA* at 27 min on the *E. coli* chromosome. This gene, which we call *frdR*, is involved in both positive and negative regulation of electron transport and fermentation associated genes. A cloned 4.9-kilobase fragment of chromosomal DNA was found to complement the *frdR* mutation; both repression of fumarate reductase gene expression and activation of nitrate reductase gene expression were restored.

Escherichia coli can obtain energy via oxidative phosphorylation reactions by reducing a variety of terminal electron acceptors including, in order of decreasing potential energy yield, oxygen, nitrate, trimethylamine-*N*-oxide (TMAO), and fumarate (14). The enzyme fumarate reductase catalyzes the reduction of fumarate to succinate, the terminal step of electron transfer during anaerobic respiration of fumarate. The enzyme is composed of four polypeptides. FrdA, a 67-kilodalton flavoprotein, and FrdB, a 27-kilodalton iron-sulfur protein, together make up the catalytic domain (9, 10, 23, 33). The hydrophobic FrdC and FrdD subunits of 15 and 13 kilodaltons, respectively, anchor the FrdA and FrdB subunits to the inner surface of the cytoplasmic membrane (5, 7, 13, 19) and mediate electron transfer from menaquinone (5, 6). Levels of the enzyme are elevated in cells grown anaerobically in the absence of nitrate, but are low in cells grown in the presence of the preferred electron acceptors oxygen and nitrate (15, 17). Thus, the level of fumarate reductase in the cell appears to be regulated such that optimal energy is generated during anaerobic growth.

The genes that encode these four polypeptides, *frdABCD*, are located at 94.5 min on the *E. coli* chromosome (2). They have been cloned (6, 11, 20), and their DNA sequence has been determined (9, 10, 13). The genes make up an operon transcribed from a single strong promoter (17). Transcription of the fumarate reductase genes is regulated by the presence of the alternative terminal electron acceptors. By using *frdA'-'lacZ* gene fusions, it was shown that *frd* operon expression occurs at a low noninduced basal level under aerobic growth conditions (18). Expression increases 10-fold during anaerobiosis, and a further 1.5-fold when fumarate, the substrate, is added to the cell culture medium (18). However, the addition of nitrate results in repression of *frdA'-'lacZ* gene expression during either anaerobic (23-fold) or aerobic (3-fold) cell growth. Thus, nitrate control of *frd* gene expression appears to function independently of oxygen. We have also shown that strains defective in *fnr*, a gene necessary for normal anaerobic induction of *frd* gene expression, are unaffected for nitrate regulation (18). Additionally, Iuchi et al. report an oxygen regulatory mutation which maps to *frd* and is relieved of control by oxygen but not by nitrate (15, 16). These findings indicate that oxygen control and nitrate control of *frdABCD* gene expression occur by different mechanisms and that there must be different regulatory gene products involved in nitrate repression of *frd* gene transcription.

To study the mechanism for nitrate repression, strains containing *trans*-acting mutations defective in nitrate control of *frdABCD* operon expression were isolated and characterized. The results of these studies suggest that nitrate repression of *frd* operon expression occurs via a gene designated *frdR* that may encode a nitrate-sensing DNA-binding protein and that this regulatory gene product also globally affects the production of other enzymes involved in anaerobic respiration and fermentation.

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TABLE 1. *E. coli* strains, phages, and plasmids

Strain, phage, or plasmid	Parent	Genotype or phenotype	Source or reference[a]
Bacteria			
MC4100		F⁻ Δ(*argF-lac*)*U169 araD139 rpsL150 deoC1 relA1 flbB5301 rbsR ptsF25*	25
LK2001	MC4100(λJ100)	*frdR1*	This work
LK2002	MC4100(λJ100)	*frdR2*	This work
LK2003	MC4100(λJ100)	*frdR3*	This work
LK2004	MC4100(λJ100)	*frdR4*	This work
LK2006	MC4100(λJ100)	*frdR6*	This work
LK2007	MC4100(λJ100)	*frdR7*	This work
LK23R35	MC4100(λJ100)	*frdR2* Tn*10*Δ16Δ17::*kan*	R. Humbert
RH67		F⁻ *thi asnA31 asnB32 rpsL trpC*::Tn*10*	
BW5660		Hfr Δ(*gpt-lac*)*5 supE44* λ⁻ *srlC300*::Tn*10 relA1*? *spoT1*? *thi-1* (Hfr origin P066 of Hfr PK19)	31
BW6161		Hfr *lac42* λ⁻ *zed*::Tn*10 supD relA1 spoT1 thi-1* (Hfr origin P042 of Hfr KL99)	31
SASX41B		Hfr *hemA41 metB1 relA1*	R. Simons
LK3001	LK23R35	*frdR2* Tn*10*Δ16Δ17::*kan trpC*::Tn*10*	This work
P90C		F⁻ Δ(*lac pro*) *ara thi*	12
LK3005	P90C(λLK1)	*frdR2* Tn*10*Δ16Δ17::*kan*	This work
Phages			
P1*cam*		P1::Tn*9clr-100*	25
λJ100		Φ(*frdA'- 'lacZ*)(Hyb) *lacY*⁺ *bla*⁺	18
λ1105		Tn*10*Δ16Δ17::*kan*	32
λLK1		Φ(*frdA'-'lacZ*)(Hyb) *lacY*⁺ *lacA*⁺	This work
Plasmids			
pLK2	pBR322	*frdR*⁺	This work
pLK9	pBR322	*frdR*⁺	This work

[a] All Hfr Tn*10* strains were kindly provided by B. Bachmann.

Kalman and R. P. Gunsalus, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, H165, p. 167].)

MATERIALS AND METHODS

Bacterial strains, plasmids, and phage. The *E. coli* K-12 strains, phage, and plasmids used in this study are listed in Table 1. Phage λLK1 was constructed from λRS45 (26) and pJ100 (18) by previously described methods (26). To construct strain LK3005, strain P90C was lysogenized with λLK1 and single lysogens were isolated. The *frdR2* allele and a linked Tn*10* (mini-*kan*) element from strain LK23R35 were then cotransduced into strain P90C(λLK1), and a red, kanamycin-resistant colony was isolated on MacConkey-lactose-fumarate-nitrate-kanamycin indicator plates. A plasmid-containing a DNA fragment which complements the *frdR* gene was identified from a genomic *Hin*dIII pBR322 library by screening LK3005 transformants on MacConkey-lactose-fumarate-nitrate-ampicillin indicator plates. Complementation of the mutant FrdR phenotype in strain LK3005 was indicated by the presence of white colonies in a background of red noncomplementing transformants. Plasmids containing the 4.9-kilobase insert in opposite orientations were designated pLK2 and pLK9 (see Fig. 2).

Media. For liquid cell culture, basal minimal medium (27) was supplemented with thiamine, Casamino Acids (Difco Laboratories, Detroit, Mich.), and glucose at final concentrations of 0.1 mg/ml, 0.5 mg/ml, and 40 mM, respectively. Sodium fumarate (40 mM [pH 7]), sodium nitrate (40 mM), or both were added when indicated. MacConkey agar (Difco)–1% lactose plates containing either fumarate (40 mM) or nitrate (40 mM), or both, were used for mutant isolation and strain construction. Vogel-Bonner (VB) medium was as previously described (30). Kanamycin, ampicillin, tetracycline, and streptomycin were used at final concentrations of 50, 40, 20, and 150 μg/ml, respectively.

Chemicals. *O*-nitrophenyl-β-D-galactopyranoside (ONPG), tetracycline, ampicillin, streptomycin, kanamycin, Δ-aminolevulinic acid, and nitrosoguanidine were purchased from Sigma Chemical Co., St. Louis, Mo. All other reagents used were commercial products of the highest grade available.

Nitrosoguanidine mutagenesis. Mutants altered for nitrate control of *frdA'-'lacZ* gene expression were generated by treatment of MC4100(λJ100) cells with nitrosoguanidine for 35 min (22). To prevent the formation of siblings, mutagenized cells were plated without growth on MacConkey-lactose-fumarate-nitrate indicator plates and incubated overnight at 37°C.

Bacteriophage and genetic methods. All P1 transductions, lysate preparations, and lysogenies were performed as previously described (25, 26). Hfr conjugation experiments were performed basically as previously described (22, 34). The exconjugates were plated on LB-streptomycin (to select against the donor)-tetracycline (to select for transfer of the Tn*10* which indicates mating has occurred) plates and incubated overnight. Colonies were then transferred to LB-streptomycin-tetracycline, LB-streptomycin-tetracycline-kanamycin, and LB plates. Proximity of the Tn*10* to *frdR* was determined by the frequency that the closely linked Tn*10*Δ16Δ17::*kan* (mini-*kan*) element was replaced by the Tn*10* transferred from the Hfr strain.

Construction of an MC4100 pool with random Tn*10* insertions. An MC4100 pool with random Tn*10*Δ16Δ17::*kan* in-

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sertions was constructed by infection of MC4100 with λ1105 which carries the Tn*10*Δ16Δ17::*kan* element (mini-*kan* [32]), and Km^r colonies were selected as previously described (32). Approximately 40,000 such colonies were pooled and used for growth of P1*cam*.

Cell growth and enzyme assay. Cells were grown and cell extracts were prepared as previously described (17, 18). Fumarate reductase, alcohol dehydrogenase, and nitrate reductase assays were performed as previously described (3, 5, 21). TMAO reductase activity was assayed spectrophotometrically with reduced benzyl viologen as the electron donor in a manner similar to that used for the fumarate reductase assay. Inside an anaerobic chamber, 0.94 ml of 50 mM Tris hydrochloride buffer (pH 7), 10 μl of benzyl viologen (10 mg/ml), and 2.5 μl of $Na_2S_2O_4$ (10 mg/ml) were added to each cuvette to give an initial optical density of reduced benzyl viologen of 2.0 at 550 nm. After the solution was mixed, the cuvette was sealed with a serum stopper and subsequently removed from the anaerobic chamber. Cell extract was injected into the cuvette, and the reaction was initiated by the addition of 10 μl of 1 M TMAO. Oxidation of benzyl viologen was monitored at 550 nm. The molar extinction coefficient used for reduced benzyl viologen was 7.8×10^3/cm (5). The protein concentration was determined by the method of Bradford (4).

β-Galactosidase was assayed as previously described (12) with the following modifications. Cells were harvested at an optical density of 0.4 at 600 nm (Uvikon 210 Spectrophotometer; Kontron AG, Zurich, Switzerland) and permeabilized by the addition of sodium dodecyl sulfate and chloroform as previously indicated (22). Protein was estimated by assuming that the cell density at an optical density of 1.4 at 600 nm is equal to 150 μg of protein per ml (22). Units of β-galactosidase are expressed as nanomoles of ONPG hydrolyzed per minute per milligram of protein. An extinction coefficient for ONPG of 0.0045 was used (22).

RESULTS

Isolation of mutants. Previous studies have shown that nitrate repression of *frd* operon expression is independent of oxygen regulation and *fnr* (15, 16, 18). To study the mechanism for nitrate control, we used nitrosoguanidine mutagenesis of *E. coli* MC4100(λJ100) to generate mutants defective in nitrate repression. This strain is lysogenized with a λ phage containing a *frdA'-'lacZ* protein fusion constructed in vitro (18). It exhibits a white colony phenotype when plated aerobically on MacConkey-lactose-fumarate-nitrate indicator plates. However, if nitrate is omitted from the medium, red colonies result. Thus, mutants defective in normal nitrate control of *frdA'-'lacZ* gene expression may be identified by the presence of red or pink colonies on the nitrate-containing indicator medium. Of approximately 3,500 colonies examined, 10 were red. These mutants were purified and designated strains LK2001 to LK2010; which the properties of six of these strains are reported here.

TABLE 2. Levels of β-galactosidase in strains altered for nitrate control of *frdA'-'lacZ* gene expression

Strain	β-Galactosidase activity[a]	
	$+NO_3^-$	$-NO_3^-$
MC4100(λJ100)	123	2,333
LK2001	2,315	2,738
LK2002	2,209	2,552
LK2003	1,484	1,813
LK2004	622	1,505
LK2006	952	1,230
LK2007	2,224	3,027

[a] Activity is expressed in nanomoles of ONPG cleaved per minute per milligram of protein. Cells were grown anaerobically in minimal glucose medium supplemented with yeast extract (0.5%), tryptone (1%), and fumarate (40 mM). Nitrate (40 mM) was added where indicated.

The extent to which nitrate is able to repress *frdA'-'lacZ* gene expression in six of the independently isolated mutants was examined by measuring β-galactosidase levels in cells grown anaerobically in either the presence or absence of this respiratory substrate (Table 2). Although nitrate exerted approximately 20-fold repression of *frdA'-'lacZ* expression in the parental strain MC4100(λJ100), only a 1.1- to 2.4-fold effect was seen in the mutants.

One mutant, LK2002, was chosen at random for detailed genetic and phenotypic analysis. To determine whether the mutation responsible for the derepressed nitrate control phenotype in this mutant strain was located in the *frdA'-'lacZ* region contained on λJ100, or elsewhere on the chromosome, λJ100 was isolated from LK2002 and lysogenized into the parental strain MC4100. The phage was found to be wild type. Thus, the mutation that confers the nitrate-derepressed phenotype in strain LK2002 is not contained on the λJ100 phage and must therefore act in *trans*.

To facilitate genetic mapping, a chromosomally located Km^r marker from a random Tn*10*Δ16Δ17::*kan E. coli* MC4100 pool was placed in close proximity to the *trans*-acting mutation that confers the red colony phenotype in LK2002. A P1 lysate was prepared from an MC4100 pool of random Tn*10*Δ16Δ17::*kan* (mini-*kan* [32]) insertions (see Materials and Methods). Kanamycin-resistant P1 transductants of LK2002 were selected directly on MacConkey-lactose-fumarate-nitrate-kanamycin plates, and white Km^r colonies were isolated. A P1 lysate was made from this strain and used to transduce strain LK2002 to a red Km^r phenotype. The mini-*kan* element cotransduced with *frdR* at a frequency of approximately 55%. Finally, to move the mutated gene and the mini-*kan* element to an unmutated parental background, we prepared a P1 lysate from a red Km^r colony and used it to transduce MC4100(λJ100) to the red Km^r phenotype. This strain was designated LK23R35. The mutation responsible for the nitrate-derepressed *frdA'-'lacZ* phenotype was named *frdR*.

To determine whether the other mutants that exhibit the FrdR phenotype (Table 2) contain defects that are linked to *frdR*, we performed P1 transduction experiments. The mutations in all strains tested were found to cotransduce with the mini-*kan* element at a frequency of 53%.

Mapping of the *frdR* locus. Genetic mapping of *frdR* was done by Hfr mapping procedures as previously described (22, 34). The results of matings between LK23R35 and strain BW6161 or BW5660 indicate that *frdR* lies in a 20-min region between the origin of transfer in the two Hfr strains. P1 transduction experiments were then performed to locate the mutation to within 1 or 2 min on the genetic map. A P1 lysate was made of strain RH67 which has a Tn*10* element inserted in *trpC* at 27 min and was used to transduce strain LK23R35 to Tc^r. The transductants were plated on MacConkey-lactose-fumarate-nitrate-tetracycline plates, and Tc^r transductants were selected and subsequently screened for the FrdR$^+$, FrdR, Km^r, or Km^s phenotype. Both *frdR*$^+$ and mini-*kan* cotransduced with *trpC*::Tn*10*, and the frequency indicates that both markers are within about 0.8 min of the *trp* operon at 27 min (Table 3) (35). The frequency of a Tc^r *frdR*$^+$ Km^r class of transductants indicates that the gene

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TABLE 3. Three-factor cross analysis for *hemA*, *frdR*, mini-*kan*, and *trp*

Cross	Donor / Recipient	Selected marker (no. scored)	Resulting characteristics				Relative frequency (% of total)	Min. no. of crossovers for orders[a]		
			hemA	*frdR*	mini-*kan*	*trp*		I	II	III
1	RH67 + + *trpC*::Tn*10* / LK23R35 *frdR* Km^r +	*trpC*::Tn*10* (320)		−	−	−	14.4	2	4	2
				+	−	−	20.3	2	2	2
				−	+	−	65.0	2	2	2
				+	+	−	0.31	4	2	2
2	SASX41B *hemA* + + + / LK3001 + *frdR* mini-*kan* *trpC*::Tn*10*	*trp*$^+$ (152)	+	−		+	75.7	2	2	
			−	−		+	2.0	4	2	
			+	+		+	11.8	2	2	
			−	+		+	10.5	2	2	
3	SASX41B *hemA* + + + / LK3001 + *frdR* mini-*kan* *trpC*::Tn*10*	*trp*$^+$ (140)		−	+	+	47.9	2	2	
				−	−	+	26.4	2	2	
				+	+	+	4.3	4	4	
				+	−	+	21.4	2	2	
4	SASX41B *hemA* + + + / LK3001 + *frdR* mini-*kan* *trpC*::Tn*10*	*trp*$^+$ (133)	+		+	+	43.6	2	2	
			−		+	+	0.75	4	2	
			+		−	+	40.6	2	2	
			−		−	+	15.0	2	2	

[a] This column represents the number of crossover events required to produce the characteristics indicated from a given order. Order for cross 1: I = *frdR*–mini-*kan*–*trp*; II = mini-*kan*–*frdR*–*trp*; III = mini-*kan*–*trp*–*frdR*. Order for crosses 2, 3, and 4: I = *hemA*–*frdR*–mini-*kan*–*trp*; II = *hemA*–*trp*–mini-*kan*–*frdR*.

order is *frdR-kan-trpEDCBA* (Table 3), although the orientation with respect to *trp* could not be determined from this experiment. To determine the side of *trp* on which *frdR* and Tn*10*Δ16Δ17::*kan* lie, three-factor crosses were performed. A P1 lysate was prepared from SASX41B which bears a *hemA* mutation at about 26 min and used to transduce LK3001 to Trp$^+$. The phenotypes of the colonies were then scored on MacConkey–lactose–fumarate–nitrate–Δ-aminolevulinic acid (40 μg/ml) indicator plates (FrdR$^+$ or FrdR), VB–glycerol (40 mM) plates (HemA$^+$ or HemA), or LB–glucose (40 mM)–Δ-aminolevulinic acid–kanamycin (mini-*kan*). The frequency of classes of transductants which require quadruple crossover events for a particular gene order indicate the gene order to be *hemA-frdR-kan-trpEDCBA* (Fig. 1; Table 3).

β-Galactosidase activity. To determine the degree to which nitrate represses *frdA'-'lacZ* gene expression in *frdR*$^+$ and *frdR* strains, levels of β-galactosidase activities were measured in MC4100(λJ100) and LK23R35, respectively. The wild-type and mutant strains were grown under four different conditions: anaerobically in the presence and absence of nitrate and aerobically in the presence and absence of nitrate (Table 4). In the absence of nitrate, under either aerobic or anaerobic conditions, MC4100(λJ100) and LK23R35 contained nearly identical levels of β-galactosidase activity. Thus, the degree of anaerobic *frdA'-'lacZ* expression in both strains was the same in the absence of nitrate. Anaerobically the level of *frdA'-'lacZ* expression in strain MC4100(λJ100) in the presence of nitrate was 27-fold less than that in cells grown without nitrate. However, the *frdR* mutant had 17-fold higher levels of activity than the *frdR*$^+$ strain when grown in the presence of nitrate under anaerobic conditions. When grown aerobically, the parent strain [MC4100(λJ100)] exhibited 3.8-fold-higher levels of β-galactosidase activity in the absence than in the presence of nitrate (Table 4). Repression by nitrate was not seen in the *frdR* strain aerobically, although the same low basal level of β-galactosidase was seen in both strains when nitrate was absent from the growth medium. These results further demonstrate that oxygen regulation of *frdA'-'lacZ* expression is independent of nitrate control.

Fumarate reductase activity. To show that the *frdR* mutation functions in *trans* to the chromosomal *frdABCD* locus, fumarate reductase enzyme assays were performed on strains MC4100(λJ100) and LK23R35 grown under the same conditions as those used for the β-galactosidase assay (Table 4). An identical pattern of expression was seen for fumarate reductase levels as for *frdA'-'lacZ* expression under all cell culture conditions tested. Anaerobically the level of expression in MC4100(λJ100) in the presence of nitrate was 23-fold less than that in cells grown without nitrate. Anaerobically, in the presence of nitrate, fumarate reductase levels in strain LK23R35 were 14-fold higher than those seen in MC4100 (λJ100) grown in the presence of nitrate. Together, the results of both assays indicate that the *frdR* mutation in strain LK23R35 acts in *trans* to regulate *frdABCD* expression.

***frdR* affects the production of other respiratory and fermentation enzymes.** Nitrate reductase and TMAO reductase assays were performed on anaerobically grown MC4100 (λJ100) and LK23R35 to determine the effect of *frdR* on the production of these respiratory enzymes (Table 5). When

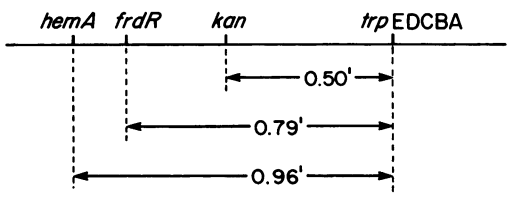

FIG. 1. Map of region around 27 min. Approximate cotransduction frequencies are indicated in map distances (35).

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TABLE 4. Levels of β-galactosidase and fumarate reductase in mutant and wild-type strains

Strain	β-Galactosidase activity[a]				Fumarate reductase activity[b]			
	Aerobic		Anaerobic		Aerobic		Anaerobic	
	$+NO_3^-$	$-NO_3^-$	$+NO_3^-$	$-NO_3^-$	$+NO_3^-$	$-NO_3^-$	$+NO_3^-$	$-NO_3^-$
MC4100(λJ100)	50	190	109	2,989	0.01	0.01	0.03	0.70
LK23R35	186	183	1,868	2,998	0.03	0.03	0.41	0.60

[a] Units are expressed in nanomoles of ONPG hydrolyzed per minute per milligram of protein. Cells were grown in minimal-glucose-fumarate medium in the presence or absence of oxygen as described in Materials and Methods. Where indicated, nitrate was present in the medium at an initial concentration of 40 mM.
[b] Units are expressed in micromoles of benzyl viologen oxidized per minute per milligram of protein. Cells were grown as described above.

grown anaerobically without nitrate, LK23R35 and MC4100(λJ100) contained similar low levels of nitrate reductase. Strain MC4100(λJ100) grown in the presence of nitrate contained the expected 25-fold increase in nitrate reductase typical of nitrate induction (24). In contrast, nitrate reductase levels in the *frdR* mutant (LK23R35) grown in the presence of nitrate were similar to the low, noninduced levels of activity typical of either *frdR* or *frdR*$^+$ cells grown in the absence of nitrate. Thus, the *frdR* strain (LK23R35) is not able to respond to the addition of nitrate to the medium and can neither repress production of fumarate reductase nor induce levels of nitrate reductase within the cell.

The production of TMAO reductase in *frdR*$^+$ and *frdR* strains grown either in the presence or in the absence of nitrate was also measured. Although the wild-type strain [MC4100(λJ100)] contained roughly 1.5-fold-lower levels of this enzyme when grown in the presence of TMAO and nitrate than when grown without nitrate, the *frdR* strain was derepressed for nitrate regulation of TMAO reductase expression (Table 5). It is interesting that in the presence of nitrate, the amount of activity in the mutant was elevated above that seen in cells grown without nitrate, although we do not know the significance of this observation.

Alcohol dehydrogenase is also repressed by nitrate in anaerobically grown *E. coli* cells (8). We therefore wished to determine whether cells defective for *frdR* are also defective for nitrate control of this enzyme. Although alcohol dehydrogenase levels were regulated approximately 34-fold by nitrate in the anaerobically grown wild-type strain, MC4100(λJ100) (0.8 U with and 27.0 U without nitrate, where 1 U is defined as 1 nmol/min per mg), this effect was not seen in the *frdR* strain, LK23R35 (21.9 U with and 22.4 U without nitrate). Thus, nitrate control of at least one gene (*adhE*) involved in fermentative growth is also negatively regulated by *frdR*.

Complementation of the *frdR* mutation. To confirm that the *frdR* gene product acts in *trans* to *frdABCD* and to determine the dominance of the *frdR* mutation, we transformed strain LK3005 with a plasmid library of *E. coli* genomic DNA and screened for colonies that exhibit wild-type nitrate repression of *frdA'-'lacZ* expression (i.e., white colonies on MacConkey-lactose-fumarate-nitrate indicator plates). We identified 32 such colonies from a total of approximately 9,000 transformants. Plasmids from all colonies contained an identical 4.9-kb *Hin*dIII insert. Both orientations were identified by restriction analysis and designated pLK2 and pLK9 (Fig. 2). Strain LK3005, containing either pLK2 or pLK9, exhibited repressed levels of FrdA'-β-galactosidase in the presence of nitrate in the cell growth medium (Table 6). In contrast, strain LK3005 alone or with the plasmid vector pBR322 showed little nitrate repression of *frdA'-'lacZ* expression. Induction of nitrate reductase expression in LK3005 containing pLK2 was also restored (data not shown). These findings establish that the *frdR* gene product acts as both a negative and positive activator of gene expression in response to nitrate and that the *frdR* allele is recessive to the wild-type gene.

TABLE 5. Nitrate reductase and TMAO reductase levels in mutant and wild-type strains

Strain	Nitrate reductase activity[a]		TMAO reductase activity[b]	
	$+NO_3^-$	$-NO_3^-$	$+NO_3^-$	$-NO_3^-$
MC4100(λJ100)	572	23	4,405	5,798
LK23R35	13	14	11,251	6,290

[a] Units are expressed in nanomoles of nitrite produced per minute per milligram of protein. Cells were grown anaerobically in minimal-glucose-fumarate medium with the addition of nitrate (40 mM) where indicated.
[b] Units are expressed in nanomoles of benzyl viologen oxidized per minute per milligram of protein. Cells were grown in the minimal glucose medium described above, except that TMAO was added at a final concentration of 40 mM. Potassium nitrate (40 mM) was added where indicated.

DISCUSSION

To elucidate the mechanism for nitrate repression of *frdABCD* operon expression and thus the ability of cells to respire anaerobically by using the appropriate electron acceptor, we isolated *E. coli* mutants that exhibit nitrate-independent expression of a *frdA'-'lacZ* gene fusion on MacConkey-lactose-fumarate-nitrate indicator plates. These mutants are derepressed for fumarate reductase gene expression. *frdA'-'lacZ* gene expression in mutant LK23R35 was

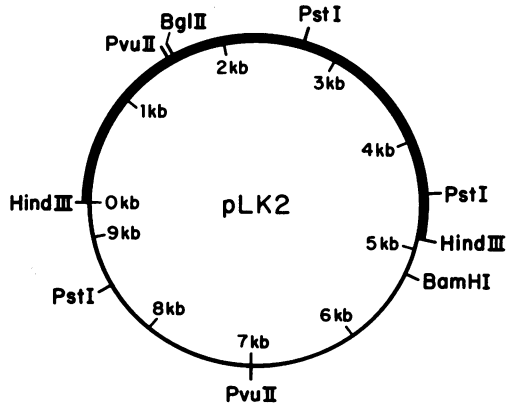

FIG. 2. Map of plasmid pLK2 that complements the *frdR* mutation in *E. coli* LK3005. Symbols: —, 4.9-kilobase chromosomal DNA insert; ——, vector portion. The approximate locations of several restriction sites within the 9.3-kilobase plasmid are indicated. Plasmid pLK9 is identical to pLK2 but contains the *Hin*dIII insert in the opposite orientation.

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TABLE 6. Plasmid complementation of the *frdR* mutation

Strain	β-Galactosidase activity[a]	
	$+NO_3^-$	$-NO_3^-$
P90C(λLK1)	162	4,459
LK3005	3,943	4,658
LK3005(pLK2)	770	4,227
LK3005(pLK9)	3,007	4,232
LK3005(pBR322)	4,408	4,774

[a] Units are expressed in nanomoles of ONPG hydrolyzed per minute per milligram of protein. Cells were grown in minimal-glucose-fumarate medium in the absence of oxygen as described in Materials and Methods. Where indicated, nitrate was present in the medium at an initial concentration of 40 mM.

increased 17-fold relative to that in the wild-type strain when each was grown anaerobically and in the presence of nitrate. When grown aerobically in the presence of nitrate, expression of the *frdA'-'lacZ* fusion in mutant LK23R35 was increased 3.7-fold over that in the wild-type strain. However, the mutant and wild-type strains had the same levels of *frdA'-'lacZ* expression when grown in the absence of nitrate either aerobically or anerobically. A similar pattern was observed for the levels of fumarate reductase activity in the mutant versus the wild-type strain. These results confirm that oxygen control is independent of nitrate control as previously suggested (15, 16, 18; H. M. Jones, manuscript in preparation). These findings are consistent with a model in which *frdABCD* operon expression is negatively controlled by a *trans*-acting gene that presumably encodes a nitrate-sensing regulatory protein that acts independently of oxygen.

The mutation in strain LK23R35 has been localized to 27 min on the *E. coli* chromosome by cotransduction of *frdR* with the Tn*10*::*trpC* insertion in strain RH67 and by three-factor cross analysis. The data indicate that the gene order is *hemA-frdR-kan-trpEDCBA*. This mutation maps near the structural genes of nitrate reductase (*narGHI*), *narL*, and *adhE* (2, 8, 29).

The mutation in strain LK23R35 is not restricted to the regulation of fumarate reductase (*frdABCD*) operon expression by nitrate, since this mutant is also defective for nitrate regulation of other genes necessary for anaerobic respiration. Nitrate reductase levels were low in *frdR* cells grown anaerobically in the presence of nitrate, in contrast to the 44-fold-higher levels in the parental strain, MC4100(λJ100) (Table 5). The mutant is apparently unable to induce expression of the nitrate reductase structural genes anaerobically upon the addition of nitrate. Previous studies have shown that the *narL* gene functions to positively regulate nitrate reductase (*narGHI*) expression (28, 29). It is therefore not clear whether the *frdR* lesions described here affect the *narL* gene or some other, closely linked, regulatory locus necessary for induction of nitrate reductase by nitrate. The *frdR* mutation characterized here also has pleiotropic effects on other nitrate-controlled genes involved in anaerobic respiration and fermentation (Table 5). Levels of TMAO reductase were elevated twofold in the *frdR* strain grown in the presence of nitrate. Alcohol dehydrogenase levels were no longer repressed 34-fold by the addition of nitrate to *frdR* cell cultures compared with *frdR*$^+$ cells. These results suggest that the genes which encode these enzymes are also negatively regulated through *frdR*. Another mutation affecting the production of alcohol dehydrogenase and nitrate reductase that maps near *narGHI* has been identified in *E. coli* (8). The *frdR* strain exhibits a similar phenotype.

A regulatory scheme has been proposed for *frdABCD* operon regulation in which a pleiotropic repressor exerts transcriptional control on genes required for the use of less-preferred electron acceptors (e.g., operons for fumarate reductase and TMAO reductase enzymes) when cells are grown in the presence of the preferred electron acceptor, nitrate (15, 18). This *frd* repressor may function in a manner analogous to the Trp repressor-corepressor (36) by binding either nitrate or another signal molecule as a ligand. This activated regulatory complex would then bind to sites in the promoter region of the nitrate-regulated operons and thereby block transcription (for fumarate reductase, alcohol dehydrogenase, and TMAO reductase) or stimulate transcription (for nitrate reductase). From these studies it is not clear whether the putative FrdR regulatory protein senses nitrate by directly binding nitrate or whether a signal molecule that is analogous to cyclic AMP-cyclic AMP receptor protein regulation of sugar operons is required (1). *frdR* may, alternatively, be involved in the production of a signal molecule that reports nitrate levels within the cell (e.g., *cya*). All models for nitrate control of respiratory gene expression must account for the >20-fold positive and negative regulation of the respiratory and fermentative enzymes in response to nitrate (Tables 4 and 5).

As shown by these studies, *frdR* is involved in the global regulation of at least three sets of genes that encode anaerobic respiratory enzymes (fumarate reductase, nitrate reductase, and TMAO reductase) and one gene related to fermentation functions (alcohol dehydrogenase). Whether additional genes under nitrate control are regulated by *frdR* is unknown but is currently under study.

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LITERATURE CITED

1. **Adhya, S., and S. Garges.** 1982. How cyclic AMP and its receptor protein act in *Escherichia coli*. Cell **29**:287–298.
2. **Bachmann, B. J.** 1983. Linkage map of *Escherichia coli* K-12, edition 7. Microbiol. Rev. **47**:180–230.
3. **Bergmeyer, H. U. (ed.)** 1974. Methods of enzymatic analysis, vol. 1, p. 428–429. Verlag chemie, Weinheim, Federal Republic of Germany.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
5. **Cecchini, G., B. A. C. Ackrell, J. O. Deshler, and R. P. Gunsalus.** 1986. Reconstitution of quinone reduction and characterization of *Escherichia coli* fumarate reductase activity. J. Biol. Chem. **261**:1808–1814.
6. **Cecchini, G., B. A. C. Ackrell, E. B. Kearney, and R. P. Gunsalus.** 1984. Fumarate reductase from *Escherichia coli* requires the *frdC* and *frdD* gene products for quinone reductase activity, p. 555–558. *In* R. C. Bray, P. C. Engel, and S. G. Mayhew (ed.), Flavins and flavoproteins. Water de Gruyter, New York.
7. **Cecchini, G., C. Thompson, B. A. C. Ackrell, D. J. Westenberg, N. Dean, and R. P. Gunsalus.** 1986. Oxidation of reduced menaquinone by fumarate reductase complex in *Escherichia*

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coli requires the hydrophobic FrdD peptide. Proc. Natl. Acad. Sci. USA **83**:8898–8902.
8. **Clark, D., and J. Cronan.** 1980. *Escherichia coli* mutants with altered control of alcohol dehydrogenase and nitrate reductase. J. Bacteriol. **141**:177–183.
9. **Cole, S.** 1982. Nucleotide sequence coding for the flavoprotein subunit of the fumarate reductase of *Escherichia coli*. Eur. J. Biochem. **122**:479–484.
10. **Cole, S., T. Grundstrom, B. Jaurin, J. J. Robinson, and J. H. Weiner.** 1982. Location and nucleotide sequence of *frdB*, the gene coding for the iron-sulfur protein subunit of the fumarate reductase of *Escherichia coli*. Eur. J. Biochem. **126**:211–216.
11. **Cole, S. T., and J. R. Guest.** 1980. Genetic and physical characterization of lambda transducing phages (λ*frdA*) containing the fumarate reductase gene of *Escherichia coli* K-12. Mol. Gen. Genet. **178**:409–418.
12. **Grove, C. L., and R. P. Gunsalus.** 1987. Regulation of the *aroH* operon of *Escherichia coli* by the Trp repressor. J. Bacteriol. **169**:2158–2164.
13. **Grundstrom, T., and B. Jaurin.** 1982. Overlap between *ampC* and *frd* operons on the *Escherichia coli* chromosome. Proc. Natl. Acad. Sci. USA **79**:1111–1115.
14. **Ingledew, W. J., and R. K. Poole.** 1984. The respiratory chains of *Escherichia coli*. Microbiol. Rev. **48**:222–271.
15. **Iuchi, S., D. R. Kuritzkes, and E. C. C. Lin.** 1985. *Escherichia coli* mutant with altered respiratory control of the *frd* operon. J. Bacteriol. **161**:1023–1028.
16. **Iuchi, S., D. R. Kuritzkes, and E. C. C. Lin.** 1986. Three classes of *Escherichia coli* mutants selected for aerobic expression of fumarate reductase. J. Bacteriol. **168**:1415–1421.
17. **Jones, H. M., and R. P. Gunsalus.** 1985. Transcription of the *Escherichia coli* fumarate reductase genes (*frdABCD*) and their coordinate regulation by oxygen, nitrate, and fumarate. J. Bacteriol. **164**:1100–1109.
18. **Jones, H. M., and R. P. Gunsalus.** 1987. Regulation of *Escherichia coli* fumarate reductase (*frdABCD*) operon expression by respiratory electron acceptors and the *fnr* gene product. J. Bacteriol. **169**:3340–3349.
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. **Lohmeier, E., D. S. Hagen, P. Dickie, and J. H. Weiner.** 1981. Cloning and expression of the fumarate reductase genes of *Escherichia coli*. Can. J. Biochem. **59**:158–164.
21. **MacGregor, C. H., C. A. Schnaitman, D. E. Normansell, and M. G. Hodgins.** 1974. Purification and properties of nitrate reductase from *Escherichia coli* K12. J. Biol. Chem. **249**:5321–5327.
22. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Morningstar, J. E., M. K. Johnson, G. Cecchini, and B. A. C. Ackrell.** 1985. The high potential iron-sulphur center in *Escherichia coli* fumarate reductase is a three iron cluster. J. Biol. Chem. **261**:1808–1814.
24. **Showe, M. K., and J. A. DeMoss.** 1968. Location and regulation of synthesis of nitrate reductase in *Escherichia coli*. J. Bacteriol. **95**:1305–1313.
25. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Simons, R. W., F. Houman, and N. Kleckner.** 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene **53**:85–96.
27. **Spencer, M. E., and J. R. Guest.** 1973. Isolation and properties of fumarate reductase mutants of *Escherichia coli*. J. Bacteriol. **114**:563–570.
28. **Stewart, V.** 1982. Requirement of Fnr and NarL functions for nitrate reductase expression in *Escherichia coli* K-12. J. Bacteriol. **151**:1320–1325.
29. **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.
30. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithinease of *E. coli*; partial purification and some properties. J. Biol. Chem. **218**:97–106.
31. **Wanner, B.** 1986. Novel regulatory mutants of the phosphate regulon in *Escherichia coli* K-12. J. Mol. Biol. **191**:39–58.
32. **Way, J. C., M. A. Davis, D. Morisato, D. E. Roberts, and N. Kleckner.** 1984. New Tn*10* derivatives for transposon mutagenesis and for construction of *lacZ* operon fusions by transposition. Gene **32**:369–379.
33. **Weiner, J. H., and P. Dickie.** 1979. Fumarate reductase of *Escherichia coli*: elucidation of the covalent-flavin component. J. Biol. Chem. **254**:8590–8593.
34. **Winkelman, J. W., and D. P. Clark.** 1986. Anaerobically induced genes in *Escherichia coli*. J. Bacteriol. **167**:362–367.
35. **Wu, T. T.** 1966. A model for three-point analysis of random general transduction. Genetics **54**:405–410.
36. **Yanofsky, C.** 1971. Tryptophan biosynthesis in *Escherichia coli*. J. Am. Med. Assoc. **218**:1026–1035.

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