JOURNAL OF BACTERIOLOGY, July 1987, p. 3340–3349
0021-9193/87/073329-10$02.00/0

Vol. 169, No. 7

Regulation of *Escherichia coli* Fumarate Reductase (*frdABCD*) Operon Expression by Respiratory Electron Acceptors and the *fnr* Gene Product

HELEN M. JONES[1]† AND ROBERT P. GUNSALUS[1,2]*

Department of Microbiology[1] and the Molecular Biology Institute,[2] University of California at Los Angeles, Los Angeles, California 90024

Received 16 June 1986/Accepted 6 April 1987

The fumarate reductase enzyme complex, encoded by the *frdABCD* operon, allows *Escherichia coli* to utilize fumarate as a terminal electron acceptor for anaerobic oxidative phosphorylation. To analyze the expression of fumarate reductase, protein and operon fusions were constructed between the *frdA* and the *lacZ* genes and introduced onto the *E. coli* chromosome at the lambda attachment site. Expression of β-galactosidase from either fusion was increased 10-fold during anaerobic versus aerobic cell growth, increased an additional 1.5-fold by the presence of fumarate, the substrate, and decreased 23-fold by nitrate, a preferred electron acceptor. The addition of trimethylamine-*N*-oxide as an electron acceptor did not significantly alter *frdA'-'lacZ* expression. Control of *frd* operon expression is therefore exerted at the transcriptional level in response to the availability of the electron acceptors oxygen, fumarate, and nitrate. Anaerobic induction of *frdA'-'lacZ* expression was impaired in an *fnr* mutant and was restored when the *fnr*$^+$ gene was provided in *trans*, thus establishing that the *fnr* gene product, Fnr, is responsible for the anaerobic activation of *frd* operon expression. Nitrate repression of *frdA'-'lacZ* expression was observed under either aerobic or anaerobic cell growth conditions in both wild-type and *fnr* mutant strains, demonstrating that the mechanism for nitrate repression is independent of nitrate respiration and oxygen control imparted by Fnr. Studies performed with a *fnr'-'lacZ* protein fusion confirmed that the *fnr* gene is expressed both aerobically and anaerobically. A model is proposed for the regulation of *frdABCD* operon expression in response to the availability of the alternate terminal electron acceptors oxygen, nitrate, and fumarate.

Escherichia coli can utilize a number of terminal electron acceptors for electron transport-coupled oxidative phosphorylation (24). These terminal oxidants, in order of decreasing theoretical energy yield, include oxygen, nitrate, trimethylamine-*N*-oxide, and fumarate (4, 24). Fumarate reductase catalyzes the reduction of fumarate to succinate in the terminal step of anaerobic electron transfer to fumarate. This enzyme is a membrane-bound complex composed of four nonidentical polypeptides designated FrdA, FrdB, FrdC, and FrdD. The FrdA subunit is a 69-kilodalton (kDa) protein which contains a covalently bound flavin adenine dinucleotide (12, 51). The 27-kDa FrdB protein appears to contain the iron-sulfur centers of the enzyme (6, 11, 35). The FrdC and FrdD polypeptides of 15 and 13 kDa, respectively, are integral membrane proteins which bind the catalytic FrdAB domain to the inner side of the cytoplasmic membrane (5, 6, 20, 31).

The genes encoding the four individual fumarate reductase polypeptides are located at 94 min on the *E. coli* genetic map (30). The *frd* locus has been cloned (6, 13, 17, 21, 32), and the DNA sequence of the genes has been determined (11, 12, 20). We have previously reported the location of a single promoter for the expression of the *frdABCD* genes (27). The *frdABCD* mRNA was found to initiate with an adenine residue 93 base pairs before the first codon of *frdA* and terminate in a uridine-rich region following a proposed stem-and-loop structure located 3′ of *frdD* (27). By using a *frd* promoter-*galK* operon fusion plasmid, it was demonstrated that oxygen, nitrate, and fumarate alter fumarate reductase levels by regulating transcription of the *frd* operon (27). However, the degree of regulation imparted by each of the alternate electron acceptors on *frd* operon expression could not be quantitatively evaluated at either the transcriptional or translational level because multicopy plasmids were used in these experiments.

Fnr has been proposed to be responsible for anaerobic induction of *frd* gene expression, since *fnr* mutants have reduced levels of fumarate reductase activity (9, 30, 36). Strains defective in *fnr* are also deficient in the anaerobic respiratory enzymes nitrate reductase, *sn*-glycerol-3-phosphate dehydrogenase, and formate dehydrogenase, as well as the fermentation enzymes formate hydrogen lyase and hydrogenase (26a, 28). Gene expression studies with *lac* operon fusions to the nitrate reductase genes (8, 18, 48) and to an *sn*-glycerol-3-phosphate gene (28) in *fnr*$^+$ and *fnr* strains showed that the Fnr protein positively activates anaerobic gene transcription at these loci. The *fnr* gene, which is located at 29 min on the *E. coli* genetic map, has been cloned, and its DNA sequence has been determined (40–42). It is likely, although untested, that Fnr acts in a similar fashion to positively activate *frd* transcription in response to anaerobiosis. Whereas nitrate inhibition of fumarate reductase expression has been hypothesized to be mediated by a locus designated *narK* (49), the effect of this mutation on either fumarate reductase enzyme activity or *frd* operon expression has not been determined.

We wish to understand the molecular mechanisms which allow *E. coli* to preferentially utilize one respiratory pathway over another and to use respiratory pathways before fermentation pathways. We examined the expression of the anaer-

* Corresponding author.
† Present address: Department of Botany, University of California at Berkeley, Berkeley, CA 94720.

TABLE 1. *E. coli* K-12 strains, bacteriophages, and plasmids

Strain, phage, or plasmid	Origin	Genotype and phenotype	Source or reference
Strains			
MC4100		F⁻ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	44
RK4353	MC4100	MC4100 *gyrA non*	49
RK5288	RK4353	λ p1(209) Δ(Mu) Φ(*nar-lac*)*218 fnr-250 zcj-637*::Tn*10* (Fnr⁻)	48
RG100	MC4100	MC4100 *fnr-250 zcj-637*::Tn*10* (Fnr⁻)	This work
RK5266	RK4353	RK4353 *nar-203*::Tn*10* (NarK⁻)	49
Phages			
λRZ5		λ *'bla 'lacZ lacY*⁺	R. Zagursky, unpublished
λJ100	λRZ5	λ Φ(*frdA'-'lacZ*)(Hyb) *lacY*⁺ *bla*⁺	This work
λJ110	λRZ5	λ Φ(*frdA'-lacZ*) *lacY*⁺ *bla*⁺	This work
λJ415	λRZ5	λ *lacZ*⁺ *lacY*⁺ *bla*⁺	This work
λJ115	λRZ5	λ Φ(*fnr'-lacZ*) *lacY*⁺ *bla*⁺	This work
W30 (λ *cI*)		λ *b2 cI*	44
W14 (λ *vir*)		λ *v2 v1 v3*	44
P1 *cam*		P1::Tn*9 clr-100*	44
Plasmids			
pMC1396		*'lacZ lacY*⁺ *bla*⁺	3
pJ100	pMC1396	Φ(*frdA'-'lacZ*)(Hyb) *lacY*⁺ *bla*⁺	This work
pRS415		*lacZ*⁺ *lacY*⁺ *bla*⁺	45
pJ110	pRS415	Φ(*frdA'-lacZ*⁺) *lacY*⁺ *bla*⁺	This work
pfnr2	pBR322	*fnr*⁺	This work
pfnr3	pACYC184	*fnr*⁺	This work
pRS414		*'lacZ lacY*⁺ *bla*⁺	45
pJ113	pRS114	*'lacZ lacY*⁺ *bla*⁺	This work
pJ115	pJ113	Φ(*fnr'-'lacZ*)(Hyb) *lacY*⁺ *bla*⁺	This work

obic respiratory enzyme fumarate reductase as a model system. To rigorously examine *frd* operon expression in response to the alternative electron acceptors, we constructed *frdA'-'lacZ* protein and *frdA'-lacZ*⁺ operon fusions and introduced them in single copy at the lambda attachment site on the *E. coli* chromosome. We assayed the *frd*-directed expression of β-galactosidase in the presence or absence of the alternate terminal electron acceptors oxygen, nitrate, fumarate, and trimethylamine-*N*-oxide. Additionally, we assayed the effect of the *fnr* and *narK* mutations on *frdA'-'lacZ* expression. Finally, we constructed and assayed the expression of a chromosomal *fnr'-'lacZ* fusion and confirmed that the *fnr* gene is expressed under both aerobic and anaerobic growth conditions (38). On the basis of the results of these studies, we propose a model by which *frd* gene expression is controlled in response to the preferred electron acceptors oxygen and nitrate.

MATERIALS AND METHODS

Materials. Horseradish peroxidase-conjugated goat anti-rabbit antibody and the horseradish peroxidase color development reagent were obtained from Bio-Rad Laboratories, Richmond, Calif. Purified rabbit anti-β-galactosidase antibody was obtained from Cooper Biomedical, Inc., Malvern, Pa. Rabbit antiserum generated against the purified *E. coli* fumarate reductase complex (Frd) was a gift from Gary Cecchini. High-molecular-weight protein standard mixture (no. SDS-6H) and *o*-nitrophenyl-β-D-galactopyranoside were obtained from Sigma Chemical Co., St. Louis, Mo. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was obtained from International Biotechnologies, Inc., New Haven, Conn. All other reagents used were commercial products of the highest grade available.

Bacterial strains, bacteriophages, and plasmids. The genotypes of the *E. coli* K-12 strains, bacteriophages, and plasmids used are given in Table 1. Strain RG100 was constructed by moving the *fnr-250* mutation of RK5288 (48) into MC4100 via P1-mediated transduction (44). Transductants were identified by selecting for the transfer of tetracycline resistance ᵀ from the *fnr*-linked Tn*10* (*zcj-637*::Tn*10*) and by scoring for the *fnr-250* mutant phenotype (inability to grow anaerobically on glycerol minimal medium supplemented with either fumarate or nitrate). The *fnr* gene was cloned from a genomic *Hin*dIII library by selecting for complementation of the mutant Fnr⁻ phenotype in RK5288 (J. O. Deshler and R. P. Gunsalus, unpublished data). Restriction enzyme analysis confirmed that the isolated *fnr*⁺ DNA was identical to that previously described by Shaw and Guest (40, 42). A 1.6-kilobase *Hin*dIII-*Bam*HI DNA fragment from the 11.5-kilobase *Hin*dIII genomic clone was subcloned into the pACYC184 vector (7), creating pfnr3, or into pBR322 to generate pfnr2. The *lacZ* protein fusion plasmid pJ113 was constructed from pRS414 (45) by inserting a *Hin*dIII fragment containing oligonucleotide into the *Sma*I site positioned between the transcription terminators (see Fig. 4) and the *lacZ* gene.

Construction of *frdA'-lacZ* and *fnr'-'lacZ* fusion strains. Protein and operon fusions between the *frdA* gene and the *lacZ* gene were constructed on plasmids as described above and as shown in Fig. 1. (For construction of the protein fusion between the *fnr* gene and the *lacZ* gene, see Fig. 4.) Procedures for endonuclease digestion, phosphatase treatment, ligation, cell transformation, DNA preparation, and analysis of restriction fragments were performed as previously described (33). All fusions were subsequently transferred to the lambda transducing phage λRZ5 (gift of Robert Zagursky, unpublished data) by the following methods. MC4100 cells containing the *frdA'-'lacZ* protein fusion plasmid pJ100, the *frdA'-lacZ*⁺ operon fusion plasmid pJ110, or the *fnr'-'lacZ* protein fusion plasmid pJ115, were infected

with λRZ5, and the resulting phage lysates were used to transduce MC4100 cells to ampicillin resistance (Ap^r) (44). The Ap^r Lac^+ lysogens occurred at an approximate frequency of 0.001%. Cross-streaking against λ *cI* and λ *vir* confirmed that the Lac^+ Ap^r cells were lysogens. Phage was isolated from the Lac^+ Ap^r cells, and phage DNA was prepared and analyzed by restriction enzyme digestions to confirm that the respective *frdA'-lacZ* and *fnr'-'lacZ* fusions were transferred to λRZ5. All the Lac^+ Ap^r cells analyzed contained the intended phages λJ100 (see Fig. 3), λJ110, and λJ115 (not shown). The λJ100, λJ110, and λJ115 phages were then introduced into the chromosome of the *E. coli* strain MC4100 by lysogeny (44). Additionally, the λJ100 and λJ115 phages were introduced into the chromosome of the Fnr^- strain RG100 (this work; 48), and the λJ100 phage was introduced into the $NarK^-$ strain RK5266 (49) and the isogenic $NarK^+$ strain RK4353 (49). Spontaneous phage induction frequencies and the cotransduction of the Lac^+ Ap^r phage phenotype with the Gal^+ chromosomal phenotype indicated that the lambda phages were integrated in each of the strains at the bacterial lambda attachment site (*attλ*, which is adjacent to *gal* at 17 min) and not at the *frd* locus (94 min) or the *fnr* locus (29 min). For each strain construction, approximately 12 independent lysogens were grown and assayed for β-galactosidase expression to identify single versus multiple lambda lysogens. Lysogens containing the appropriate phage in single copy at the lambda attachment site on the chromosome were used for the *frdA'-'lacZ* and *fnr'-'lacZ* expression analyses.

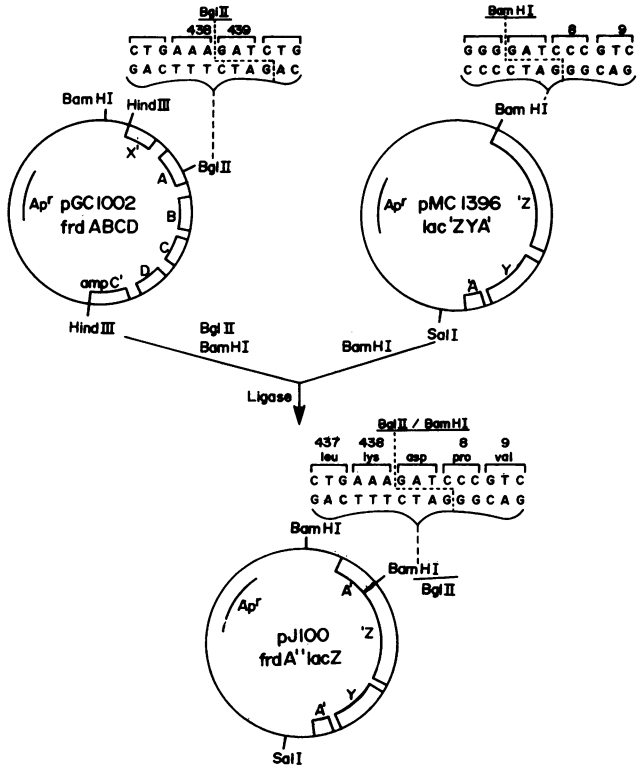

FIG. 1. Construction of the *frdA'-'lacZ* protein fusion plasmid. Amino acids and their respective codons are indicated at the *frdA'-'lacZ* fusion junction. DNA sequence analysis predicted that the fusion between the *frdA* and *lacZ* genes would result in the production of a hybrid FrdA–β-galactosidase protein of approximately 160 kDa.

Cell growth. For strain, phage, and plasmid constructions, cells were grown on Luria-Bertani (LB) liquid or solid medium (33, 44). When required, X-Gal was spread on LB plates (0.1 ml of X-Gal solution [20 mg/ml] in *N,N*-dimethyl formamide). The antibiotics ampicillin, tetracyline, and chloramphenicol were used at 40, 15, and 20 μg/ml, respectively.

For β-galactosidase assays, cells were grown in 50-ml volumes of glucose (40 mM) minimal medium (pH 7.0) (46) supplemented with 0.1 mg of thiamine per ml and, when indicated, 40 mM potassium fumarate (pH 7.0), 40 mM sodium nitrate (pH 7.0), or 20 mM trimethylamine-*N*-oxide (pH 7.0). Aerobic cell growth was perfomed in 2-liter baffled flasks shaken vigorously (380 rpm) at 37°C in an air shaker (New Brunswick Scientific Co., Inc., Edison, N.J.) Anaerobic cell growth was performed in 100-ml serum vials fitted with butyl rubber stoppers as previously described (27). Flasks of the indicated medium were inoculated from overnight cultures grown under identical conditions, and the cultures were allowed to double four to five times prior to harvesting of the cells at mid-exponential phase (optical density at 600 nm of 0.4 to 0.425 for aerobic cultures and 0.375 to 0.40 for anaerobic cultures). Cell density measurements were made by using a Uvikord 210 spectrophotometer (Kontron AG, Zurich, Switzerland). Culture aeration described above consistently gave the fastest cell doubling time, the lowest *frd*-directed β-galactosidase activity, and no detectable nitrite in the spent culture medium (which if present would be indicative of the anaerobic nitrate reductase enzyme activity) (37). To ensure that the anaerobic cultures were truly anaerobic, the oxygen content of the anaerobic serum vial headspace was monitored before and after cell growth by gas chromatography as previously described (27). For Western immunoblotting experiments, cultures were grown anaerobically in 20-ml septum tubes that contained 5 ml of minimal medium (pH 7.0) (46) supplemented with 0.1 mg of thiamine per ml, 40 mM potassium fumarate (pH 7.0), and either 40 mM glucose or 40 mM lactose as indicated.

β-Galactosidase assays. Cell extracts were prepared from 30 ml of mid-exponential-phase cultures harvested by centrifugation. The cell pellet was suspended in 2 ml of PM2 buffer (0.1 M $NaPO_4$ [pH 7.0], 0.001 M $MgSO_4$, 2×10^{-4} M $MnSO_4$), sonicated, and then centrifuged at 20,000 × *g* for 20 min to remove cell debris. Less than 5% of the FrdA–β-galactosidase hybrid protein activity or the wild-type β-galactosidase activity was lost in the centrifugation step. The β-galactosidase assays were performed in duplicate on appropriately diluted cell extracts by previously described methods (19, 22). Protein concentration was determined by using the Bradford reagent (2) with bovine serum albumin as a standard. One unit of β-galactosidase activity is given as 1 nmol of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per min per mg of protein. All the data represent the results of six to seven experiments.

Western immunoblotting. Cell cultures were harvested, suspended in 2× loading buffer, boiled, and centrifuged to remove cell debris as previously described (44). Approximately 20 μg of the resulting protein extracts was electrophoresed on a 7.5% sodium dodecyl sulfate-polyacrylamide gel (29). Proteins were electrophoretically transferred to nitrocellulose paper (2-μm pore size; Schleicher & Schuell, Inc., Keene, N.H.) overnight at 35 mA. Subsequent procedures were as described in the Immuno-Blot (GAR-HRP) assay kit manual (Bio-Rad), except that Carnation nonfat dry milk was substituted for gelatin. Anti-β-galactosidase anti-

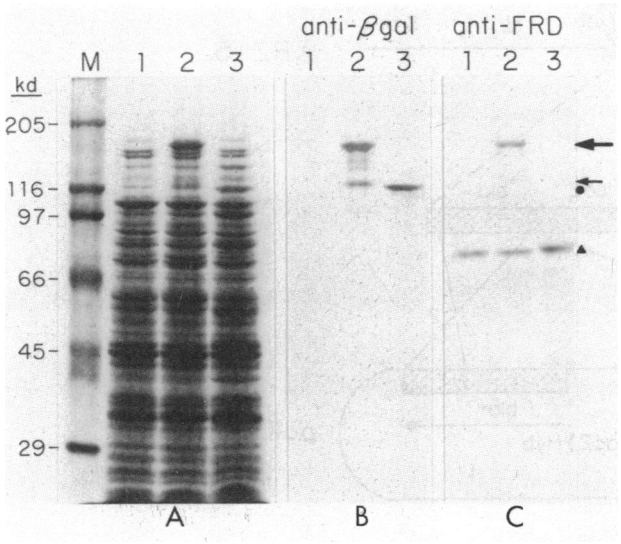

FIG. 2. Western analysis of the FrdA–β-galactosidase hybrid protein. Cell extracts of the strains indicated below were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and one gel portion was stained with Coomassie blue stain (A), while the remaining gel portion was transferred to nitrocellulose and probed with antibody to either β-galactosidase (βgal) (B) or fumarate reductase (FRD) (C) as described in Materials and Methods. Extracts were from the following strains: lane 1, MC4100; lane 2, MC4100(pJ100); and lane 3, W3110 (*lac*$^+$ control). Lane M contains molecular-size marker proteins. The large arrow indicates a 168-kDa (Kd) hybrid FrdA–β-galactosidase protein. The small arrow indicates a 121-kDa hybrid protein degradation product. The FrdA polypeptide of approximately 69 kDa is indicated by the triangle. The 116-kDa wild-type β-galactosidase protein is indicated by the solid circle.

serum was used at a 1:1,000 dilution, and anti-fumarate reductase antiserum was used at a 1:500 dilution.

RESULTS

Construction of *frdA'-lacZ* protein and operon fusions. Protein and operon fusions between the *frdA* gene and the *lacZ* gene encoding β-galactosidase were constructed to study the regulation of fumarate redutase operon (*frdABCD*) expression at the transcriptional and translational levels. The *frdA'-'lacZ* protein fusion plasmid pJ100 was constructed by inserting a 2.4-kilobase *Bam*HI-*Bgl*II DNA fragment containing the *frd* operon promoter and approximately two-thirds of the *frdA* coding region into the unique *Bam*HI site of the *lacZ* protein fusion vector pMC1396 (3) (Fig. 1). The analogous *frdA'-lacZ*$^+$ operon fusion plasmid pJ110 was constructed by inserting the identical *frd* DNA fragment into the *Bam*HI site of the *lacZ*$^+$ operon fusion vector pRS415 (45). *E. coli* MC4100 transformants containing the respective *frdA'-lacZ* fusion plasmids were identified phenotypically as Lac$^+$ Ap^r colonies on LB–ampicillin–X-Gal indicator medium. Restriction endonuclease analysis confirmed that the pJ100 and pJ110 plasmids were constructed as intended.

Western analysis, using fumarate reductase- and β-galactosidase-specific antibodies, was performed to determine whether the plasmids pJ100 and pJ110 directed the synthesis of the expected 160-kDa FrdA–β-galactosidase hybrid protein and the wild-type 116-kDa β-galactosidase protein, respectively. The anaerobically grown MC4100 cultures containing pJ100 produced a protein of approximately 168 kDa which reacted with both the fumarate reductase and β-galactosidase antibodies (Fig. 2, large arrow). An additional 121-kDa protein was observed in the MC4100(pJ100) extract treated with the β-galactosidase antibody (Fig. 2). In vitro transcription-translation experiments suggested that the 121-kDa protein resulted from an amino-terminal degradation of the 168-kDa hybrid protein rather than from translation initiation within the *frdA'-'lacZ* coding region (data not shown). Analogous Western blot experiments performed with MC4100 cultures containing the *frdA'-lacZ*$^+$ operon fusion plasmid confirmed that pJ110 directs the synthesis of the 116-kDa β-galactosidase protein as expected (data not shown).

Transfer of *frdA'-lacZ* fusions to the chromosome of *E. coli* strains. The *frdA'-'lacZ* protein and operon fusions were transferred to a lambda phage, λRZ5, so that their expression could be analyzed in single copy on the bacterial chromosome. λRZ5 contains 300 base pairs of the 3′ terminal region of the β-lactamase gene (*bla*) as well as the last 53 base pairs of *lacZ* and all of *lacY* (Fig. 3; R. Zagursky, unpulished data). Homologous recombination between the phage λRZ5 and the fusion plasmids pJ100 (Fig. 3) and pJ110 (not shown) at the common *'bla* and *'lacZY* sequences resulted in the transfer of the *frdA'-lacZ* fusions to the lambda phage. The resulting protein fusion phage λJ100 and operon fusion phage λJ110 were identified by a Lac$^+$ Ap^r phenotype conferred to their respective host strains. Restriction endonuclease analysis of DNA prepared from the purified λJ100 and λJ110 phages confirmed that the phage contained the respective *frdA'-'lacZ* protein and *frdA'-lacZ*$^+$ operon fusions.

The λJ100 and λJ110 phages were introduced into the chromosome of wild-type *E. coli* MC4100 by lysogeny (44). Additionally, the λJ100 protein fusion phage was introduced into the chromosome of the Fnr$^-$ strain RG100 (this work; 48), the NarK$^-$ strain RK5266 (49), and the NarK$^+$ strain RK4353 (49). Lysogens containing the appropriate phage in single copy at the lambda attachment site on the bacterial chromosome (*attB*, 17 min) were used for all subsequent experiments.

Oxygen, fumarate, and nitrate regulated fumarate reductase expression by altering the transcription of the *frdABCD* operon. The effect of oxygen, fumarate, and nitrate on *frd* transcription and translation was determined by measuring the expression of β-galactosidase in MC4100 (λJ110) and MC4100 (λJ100) cell extracts. The β-galactosidase activities in the operon and protein fusion strains varied similarly in response to the electron acceptors: oxygen caused an approximately 11-fold decrease in β-galactosidase activity; fumarate caused β-galactosidase activities to increase about 1.6-fold anaerobically and 1.2-fold aerobically; and nitrate caused a decrease in β-galactosidase activities of approximately 23-fold anaerobically and 3-fold aerobically (Tables 2 and 3). Expression of the *frd* operon was therefore regulated at the transcriptional level in response to the availability of the alternate electron acceptors oxygen, fumarate, and nitrate.

Requirement of the *fnr* gene for anaerobic induction of *frdA'-'lacZ* expression. The effect of the *fnr-250* mutant allele (48) on *frdA'-'lacZ* expression was analyzed in the MC4100 strain background. The anaerobic expression of the *frdA'-'lacZ* fusion in RG100 (λJ100) was reduced to approximately one-ninth of that in the isogenic *fnr*$^+$ MC4100 (λJ100) strain (Table 2). Anaerobic induction, although still evident in the *fnr* mutant strain, was reduced from approximately 10- to

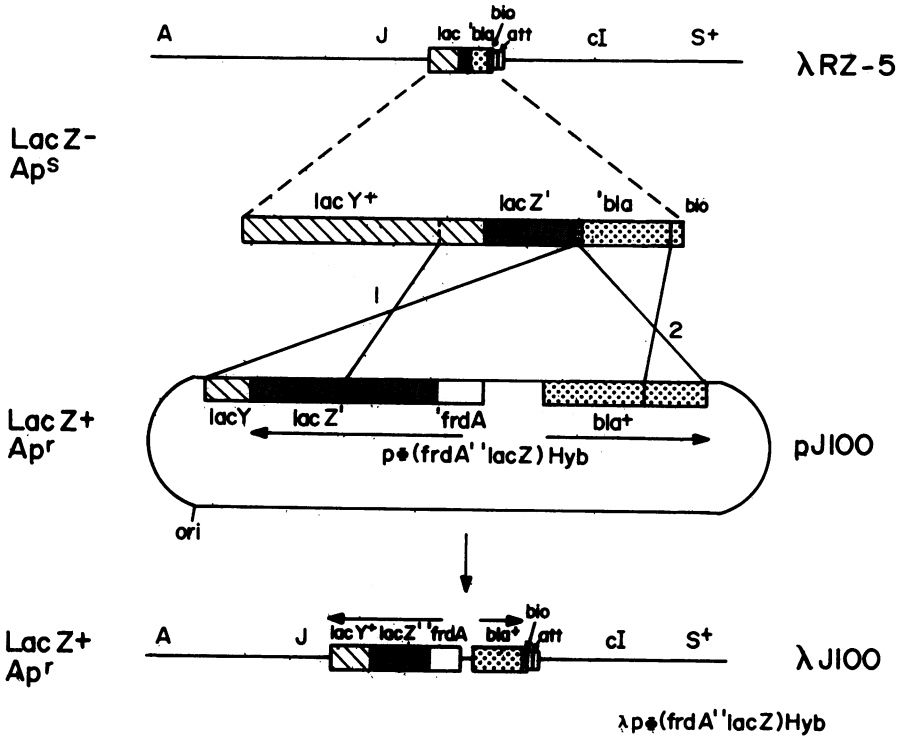

FIG. 3. Transfer of the *frdA'-'lacZ* protein fusion from pJ100 to λRZ5 by homologous recombination to create λJ100. The phage λRZ5 and the plasmid pJ100 contain identical *'bla* (▩) and *'lacZY* (■ and ▨, respectively) sequences. The *frdA'* sequence is indicated (□). Recombination of the homologous 3' regions on the phage and plasmid resulted in transfer of the *frdA'-'lacZ* fusion to give λJ100. The thin arrows indicate and direction of transcription. The phenotype of the plasmid- or phage-containing cells is indicated at the left.

3-fold (Table 3). The addition of the *fnr*$^+$ gene in *trans* restored the anaerobic induction of *frdA'-'lacZ* expression (Tables 2 and 3, RG100 [λJ100, pfnr3]). These results demonstrate that the *fnr* gene product, Fnr, was required for the activation of *frd* operon expression under anaerobic conditions.

The regulation of *frdA'-'lacZ* expression by nitrate was still observed in the *fnr* mutant with or without the *fnr*$^+$ plasmid (Tables 2 and 3). These results, as well as the observation of nitrate regulation under aerobic growth conditions, demonstrate that the mechanism of nitrate control was independent of anaerobic control by Fnr and was independent of nitrate respiration. The regulation of *frdA'-'lacZ* expression by fumarate, in contrast, appears to be mildly affected by the *fnr* mutation. The fumarate induction under anaerobic conditions was reduced from 1.5-fold in MC4100 (λJ100) to 1.1-fold in RG100 (λJ100), and addition of the *fnr*$^+$ gene plasmid caused the anaerobic fumarate induction to increase to 1.3-fold. These results suggest that the mechanism of fumarate induction involves Fnr directly or indirectly.

Expression of a *fnr'-'lacZ* protein fusion indicates that *fnr* transcription did not vary in response to oxygen. The mechanism for Fnr stimulation of *frd* gene expression during

TABLE 2. Effect of alternate electron acceptors and *E. coli* mutations on *frdA'-lacZ* expression

Growth condition[a]			β-Galactosidase activity[b] in bacterial strain[c] (phage):						
O_2	Fum	NO_3^-	MC4100 (λJ110)	MC4100 (λJ100)	RG100 (λJ100)	RG100 (λJ100, pfnr3)	RK5266 (λJ100)	RK4353 (λJ100)	MC4100 (λJ100 + TMAO)
+	−	−	381	91	46	125	ND	ND	101
+	+	−	480	109	58	167	108	139	113
+	+	+	197	30	17	38	23	34	ND
+	−	+	187	27	14	36	ND	ND	ND
−	−	−	4,657	936	156	2,464	ND	ND	1,219
−	+	−	8,107	1,447	167	3,193	928	757	1,147
−	+	+	365	62	16	266	23	32	60
−	−	+	369	63	14	235	ND	ND	63

[a] Cells were grown on glucose minimal medium in the presence (+) or absence (−) of the electron acceptors oxygen (O_2), fumarate (Fum), nitrate (NO_3^-), and trimethylamine-*N*-oxide (TMAO) as described in the text.

[b] β-Galactosidase activity units are given as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per milligram of protein per minute. Values reported are the averages of at least six determinations. ND, Not determined.

[c] Relevent phenotypes or features of the indicated strains and phage are: MC4100, wild type; RG100, Fnr$^-$; RG100, pfnr3, Fnr$^+$; RK5266, NarK$^-$; RK4353, NarK$^+$; λJ110, *frdA'-lacZ*$^+$ operon fusion; and λJ100, *frdA'-'lacZ* protein fusion.

TABLE 3. Fold levels of *frdA'-lacZ* regulation imparted by the electron acceptors in the indicated bacterial strains[a]

Electron acceptor	*frdA'-lacZ* regulation (fold) with strain (phage):					
	MC4100 (λJ110)	MC4100 (λJ100)	RG100 (λJ100)	RG100 (λJ100, pfnr3)	RK5266 (λJ100)	RK4353 (λJ100)
Oxygen	12 ↓	10 ↓	3 ↓	20 ↓	9 ↓	6 ↓
Fumarate	1.7 ↑	1.5 ↑	1.1 ↑	1.3 ↑	ND	ND
Nitrate	22 ↓	24 ↓	11 ↓	12 ↓	42 ↓	24 ↓

[a] Values were determined by dividing β-galactosidase activity values presented in Table 2 as follows: oxygen, anaerobic/aerobic; fumarate, anaerobic plus fumarate/anaerobic minus fumarate; nitrate, anaerobic plus nitrate and fumarate/anaerobic plus fumarate. Arrows indicate whether the electron acceptor increases (↑) or decreases (↓) *frdA'-lacZ* expression. ND, Not determined.

anaerobiosis is unknown. The observed increase in *frd* transcription may be explained by an increased production (synthesis) of Fnr protein during anaerobic cell growth relative to aerobic growth. Alternatively, the cellular levels of Fnr protein may remain relatively constant during aerobic and anaerobic culture conditions, and stimulation of *frd* gene transcription may be dependent on the proportion of Fnr protein in an active form able to bind the *frd* regulatory region. To test these possibilities, we constructed a *fnr'-'lacZ* protein fusion (Fig. 4) which could be used to monitor the levels of *fnr* transcription and translation during aerobic and anaerobic cell growth. The *fnr'-'lacZ* protein fusion contained in pJ115 was transferred to λRZ5, and the resulting phage λJ115 was subsequently transferred to the chromosome of the isogenic *E. coli* MC4100 (*fnr*$^+$) and RG100 (*fnr*). Production of the expected 116-kDa hybrid Fnr–β-galactosidase protein was demonstrated by protein analysis of MC4100 (λJ115) cultures and by in vitro protein synthesis procedures (data not shown). Levels of hybrid Fnr–β-galactosidase remained relatively constant in MC4100 cells grown either aerobically or anaerobically (i.e., the level with anaerobic growth was 83% of that with aerobic growth). The expression of the *fnr'-'lacZ* fusion in the *fnr-250* mutant strain RG100 was not significantly different than it was in the isogenic *fnr*$^+$ MC4100 strain (i.e., 89 and 100%, respectively, with aerobic growth and 129 and 83%, respectively, with anaerobic growth). These results indicate that Fnr production remained relatively constant within the cell under aerobic and anerobic growth. A similar observation has been reported by Pascal et al. (38).

***narK* locus did not encode a nitrate repressor for fumarate reductase expression.** The regulation of fumarate reductase expression by nitrate has been hypothesized to be mediated through a nitrate repressor protein encoded by the *narK* locus (49). To test this hypothesis, we assayed the expression of the *frdA'-'lacZ* fusion in the NarK$^-$ strain RK5266 and its parent NarK$^+$ strain RK4353 (49). The repression of *frdA'-'lacZ* expression by nitrate occurred efficiently in both the NarK$^-$ (RK5266 [λJ100]) and NarK$^+$ (RK4353 [λJ100]) strains (Tables 2 and 3). These results demonstrate that the *narK* locus was not responsible for the nitrate repression of *frdA'-'lacZ* expression.

Although the *narK* mutation did not appear to affect *frdA'-'lacZ* expression, it did appear in our hands to be somewhat defective in nitrate respiration (data not shown). The *narK* mutant RK5266 (λJ100) grew poorly in anaerobic glycerol nitrate minimal medium compared with growth of the parent strain RK4353 (λJ100). Additionally, the spent culture medium contained little detectable nitrite compared with that of strain RK4353 (λJ100). The explanation for this discrepancy with reported data is unclear (48, 49).

Effect of trimethylamine-*N*-oxide on *frdA'-'lacZ* expression. Trimethylamine-*N*-oxide can serve as a terminal electron acceptor in *E. coli* and, like oxygen and nitrate, it has a higher redox potential than fumarate does. We therefore assayed whether trimethylamine-*N*-oxide can alter the expression of the *frdA'-'lacZ* fusion. Trimethylamine-*N*-oxide did not affect the expression of the *frdA'-'lacZ* fusion in the presence of oxygen and nitrate (Table 2). However, trimethylamine-*N*-oxide did stimulate *frdA'-'lacZ* expres-

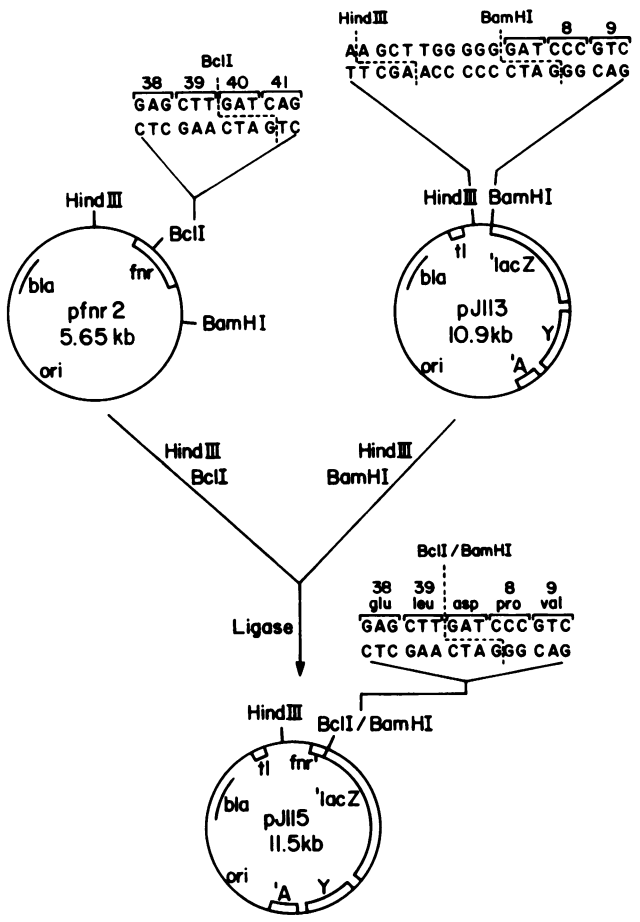

FIG. 4. Construction of the *fnr'-'lacZ* protein fusion. The 0.64-kilobase *Hin*dIII-*Bcl*I DNA fragment of pfnr2 was combined with the large *Hin*dIII-*Bam*HI fragment of pJ113 to create the plasmid pJ115. The fusion between the *fnr* and *lacZ* genes results in the production of a 116-kDa hybrid Fnr–β-galactosidase protein. Amino acids and their respective codons are indicated. The plasmid pJ113 was constructed by insertion of a *Hin*dIII octamer linker into the unique *Sma*I site of pRS414. Transcription of *lacZ* from vector promoter(s) is prevented by the presence of four tandem transcription terminators originating from pRS414 (45), which are positioned upstream of the *fnr* DNA fragment.

sion 1.4-fold under anaerobic growth conditions and appeared to inhibit the further stimulation of anaerobic *frd* expression by fumarate.

DISCUSSION

Fumarate reductase enzyme activities are very low in *E. coli* cultures grown in the presence of the preferred electron acceptors oxygen and nitrate, whereas the enzyme activities are high in cultures grown anaerobically with fumarate (10, 25, 31, 46). Previously, we have used *frd* promoter-*galK* operon fusion plasmids to establish that transcription from the *frd* promoter is decreased by oxygen and nitrate and is increased by fumarate (27). To more rigorously examine *frd* operon expression in response to the alternate electron acceptors, we constructed *frdA'-'lacZ* protein and *frdA'-lacZ*$^+$ operon fusions and placed them in single copy at the lambda attachment site on the *E. coli* chromosome. This chromosomal placement of the fusion at *attB* preserved an intact copy of the *frd* operon at 94 min, which was necessary for wild-type expression of the fumarate reductase complex. The *frdA'-'lacZ* fusion strains could thus be grown under conditions in which production of a fumarate reductase is essential for cell survival. This is not the case for the Mu d1(Ap^r *lac*)-generated gene fusions, which cause insertional inactivation of the target gene under investigation. Additionally, since the resulting *frdA'-lacZ* fusions are situated on a λ phage, they can be readily introduced into and rescued from various *E. coli* strains, thus facilitating mutant isolation and analysis.

The *frdA'-lacZ* protein and operon fusions were expressed at a low basal level in aerobically grown cells (Table 2). Under anaerobic growth conditions, expression was increased 10- and 12-fold in the protein and operon fusion strains, respectively. From these findings, we conclude that anaerobiosis regulates fumarate reductase operon expression predominantly at the transcriptional level. The 10-fold induction of *frdA'-'lacZ* expression by anaerobisis is within the 4- to 60-fold range previously determined by fumarate reductase enzyme assays (25, 31, 46). The variation in enzyme activities reflects the difficulty in performing the fumarate reductase assay owing to interference by succinate dehydrogenase activity, which, like fumarate reductase, reversibly interconverts fumarate and succinate. The methods used to grow cultures under either fully aerobic or fully anaerobic conditions also varied in these studies. The use of well-defined anaerobic cell culture techniques coupled with *frdA'-lacZ* fusion methods provides an accurate basis for evaluating anaerobic induction of *frd* expression.

Levels of β-galactosidase were consistently fivefold higher in the operon fusion strain than in the protein fusion strain (Table 2) when compared under similar cell growth conditions. This difference was not due to readthrough from an adjacent vector promoter, since the control phage λJ415 (which contains the promoterless *lacZ*$^+$ gene in the operon fusion vector pRS415) allowed only 3 to 5 U of β-galactosidaseexpression under analogous growth conditions. The difference in the levels of operon fusion and protein fusion expression, however, may reflect either an instability of the FrdA–β-galactosidase hybrid protein or a greater efficiency for translation of the *lacZ*$^+$ gene than for the *frdA'-'lacZ* gene fusion. Consistent with the latter interpretation, *lacZ*$^+$ begins with an AUG codon, while *frdA* begins with a less common GUG initiation codon (12). Recently Reddy et al. (39) have shown that a GUG codon is less efficient (ca. threefold) than an AUG codon is in initiating translation of the *E. coli* adenylate cyclase gene.

Mutations in a locus designated *fnr*, located at 29 min on the *E. coli* genetic map, resulted in defective expression of the anaerobic respiratory enzymes nitrate reductase, fumarate reductase, formate dehydrogenase, and *sn*-glycerol-3-phosphate dehydrogenase (9, 36). Results of *lac* operon fusion studies with the genes for nitrate reductase (8, 48) and *sn*-glycerol-3-phosphate dehydrogenase (28) showed that the anaerobic transcription of these genes is impaired in the *fnr* mutants. Anaerobic transcription of the *frd* operon was similarly impaired in an *fnr* mutant (Tables 2 and 3). Under anaerobic growth conditions, the expression of the *frdA'-'lacZ* protein fusion was reduced 10-fold relative to that in the isogenic *fnr*$^+$ strain (Table 2). The *fnr* mutant was thus deficient in the anaerobic induction of fumarate reductase expression. These data support the proposal that the *fnr* gene product, Fnr, is a positive regulatory protein essential for the transcription of genes encoding anaerobic respiratory proteins. The anaerobic expression of the *frdA'-'lacZ* fusion in the *fnr* mutant was, however, not as low as that in the aerobic cultures (Table 2). Anaerobiosis still caused approximately a threefold induction in *frd* expression in the *fnr* mutant. The *fnr-250* mutant has not been characterized at the protein level or at the DNA sequence level and perhaps may contain a partially active Fnr protein which is capable of stimulating *frdA'-'lacZ* expression at a reduced level. However, by using an independently isolated *fnr* mutation, the expression of a *glpA'-lacZ*$^+$ operon fusion (*glpA* encodes the anaerobic *sn*-glycerol-3-phosphate dehydrogenase) was induced similarly by a 3-fold level anaerobically, whereas in the *fnr*$^+$ strains anaerobic induction was 10-fold (28). These results pose the question of whether additional anaerobic activator elements exist in *E. coli*. In *Salmonella typhimurium*, the anaerobic induction of *pepT* genes involves two loci designated *oxrA* and *oxrB* (50). The *oxrA* locus appears to be analogous to the *fnr* locus of *E. coli*. It is not clear whether a putative *oxrB* locus in *E. coli* may contribute to the additional threefold anaerobic induction seen in the *fnr* mutant.

To confirm that the *fnr* gene product is responsible for the anaerobic induction of *frd* gene expression, we introduced a *fnr*$^+$ gene plasmid into the *fnr-250* mutant strain RG100 containing the *frdA'-'lacZ* fusion phage. Anaerobic expression of the *frdA'-'lacZ* fusion increased about 16-fold and restored anaerobic induction of *frdA'-'lacZ* expression. Although the *fnr*$^+$ plasmid-bearing *fnr* mutant strain contains approximately 20 more copies of the *fnr*$^+$ gene than the wild-type *fnr*$^+$ strain does, anaerobic *frdA'-'lacZ* expression was only increased two- to threefold. This suggests that Fnr is limiting. These data also suggest that aerobic cultures contain partially active Fnr, as evidenced by a twofold stimulation of *frdA'-'lacZ* expression in the *fnr*$^+$ plasmid-containing strain relative to the wild-type *fnr*$^+$ strain.

The presence of fumarate, the substrate for fumarate reductase, increased *frd* gene expression by a slight but reproducible amount, both anaerobically and aerobically. Anaerobically, there was a 1.5- and 1.7-fold increase in the protein and operon fusion strains, respectively. Aerobically, the effect was less (Table 3). Since fumarate caused the expression of both the protein and operon fusions to increase by approximately the same amount, we conclude that fumarate regulates *frd* operon expression predominantly at the level of transcription. Fumarate induction of approximately twofold was also observed by fumarate reductase enzyme assays (10, 25) and by the *frd'-galK*$^+$ operon fusion plasmid system (27). The loss of fumarate induction in the *fnr* mutant (1.1-fold; Table 2) was restored when the *fnr*$^+$ gene was

provided in *trans* (1.3-fold). These data suggest that fumarate induction is mediated through Fnr. Iuchi et al. (25) have identified a mutant which is relieved for regulation by both anaerobiosis and fumarate. This mutant, carrying a mutation designated *frd*(Con), also supports the hypothesis that fumarate induction is linked with anaerobic induction. The basis for the observed induction of fumarate reductase expression by its substrate is unclear but may reflect the cellular requirement for fumarate as an intermediary metabolite in addition to its role as a terminal electron acceptor.

The presence of nitrate in the medium reduced expression of the *frd* operon in both aerobic and anaerobic cultures (Tables 2 and 3). Aerobically, a 2- to 3-fold effect was seen, whereas anaerobically, the nitrate repression was more pronounced (23-fold). These results indicate that nitrate acts aerobically to repress fumarate reductase expression. This observation has not been previously reported. The 23-fold anaerobic regulation of *frd* transcription by nitrate is consistent with the previously reported 20- to 40-fold anaerobic regulation of fumarate reductase enzyme activities by nitrate (10, 25). As nitrate exerted similar levels of control in both the protein and operon fusion strains, we conclude that nitrate regulates fumarate reductase expression predominantly at the level of *frd* operon transcription. We showed that nitrate repression of *frdA'-lacZ* expression was detected both aerobically and anaerobically in the *fnr* mutant and was independent of Fnr. Supplementing the *fnr* mutant with multiple copies of the fnr^+ gene did not affect the ability of nitrate to repress *frd* expression in a corresponding fashion. A *frd* mutation, designated *frd*(Con), which is relieved for anaerobic and fumarate control but which is unaffected for nitrate regulation (25, 26) also indicates that the mechanisms of nitrate repression and anaerobic induction of *frd* are independent. Because nitrate repression occurred in aerobic cells and in the *fnr* mutant, we conclude that nitrate repression of *frd* transcription does not require nitrate respiration, as had been previously suggested, and that repression of *frd* gene expression by nitrate is not mediated by the *narK* gene product (49), since nitrate repression was similar in *narK* and $narK^+$ strains (Tables 2 and 3).

The presence of another electron acceptor used by *E. coli*, trimethylamine-*N*-oxide, did not appear to exert significant regulatory control over fumarate reductase gene expression at the transcriptional or translational level (Table 2). It is of interest that the oxidation-reduction potential of this compound (130 mV) is more positive than that of fumarate (50 mV) but less than that of either nitrate (400 mV) or oxygen (800 mV) (4, 24). Regulatory elements for the preferential use of this alternative electron acceptor over fumarate apparently have not evolved in *E. coli*.

On the basis of the results presented above, we propose a working model for fumarate reductase gene regulation (Fig. 5). Under anaerobic fumarate reductase-inducing conditions, we propose that the product of the *fnr* gene, Fnr, binds upstream of the *frd* RNA polymerase-binding site and acts to enhance RNA polymerase binding and transcription of the *frd* operon. This model for Fnr function would be analogous to the role of catabolite gene activator protein in the activation of the sugar operons in *E. coli* (15). The role of Fnr as a classical positive-activator protein rather than as a sigma factor specific for anaerobic gene expression is based on three lines of evidence. (i) A 162-base-pair *frd* DNA fragment containing sequences 50 nucleotides 5′ to the start of *frd* transcription is incapable of promoting *frd* transcription in a *galK* plasmid promoter test system (27), which demonstrates the requirement for a Fnr-dependent regulatory site further upstream of the RNA polymerase recognition element. (ii) The −35 and −10 regions of the *frd* promoter contain DNA sequences homologous to the consensus −35 and −10 RNA polymerase recognition sequences (27), in contrast to that seen for the alternative sigma-factor-dependent promoters (e.g., heat shock gene promoters [14] and nitrogen-regulated gene promoters [1, 16, 23]). (iii) The Fnr protein has significant amino acid sequence homology to the catabolite gene activator protein in the DNA-binding and the nucleotide-binding domains (43), whereas it does not have significant homology to the known *E. coli* and *Bacillus subtilis* sigma factors in the regions thought to be involved in RNA polymerase binding (unpublished observation; 34, 47). We propose that the majority of the Fnr protein under aerobic or noninducing conditions is in an inactive DNA-binding form and is unable to stimulate *frd* gene transcription by RNA polymerase. Because *frdA'-'lacZ* expression was reduced by one-half in the *fnr* mutant strain, we believe that a small portion of Fnr is maintained in an active form in wild-type aerobic cultures. Because *fnr* gene expression proceded at roughly similar levels during both aerobic and anaerobic cell growth, we conclude that Fnr does not activate *frd* gene expression via a cascade mechanism similar to nitrogen-controlled genes (23). However, these results do not rule out a model whereby Fnr is irreversibly inactivated during aerobiosis.

We also propose the existence of a nitrate-sensing regulatory protein (which we designate as NR) which binds tightly to the *frd* regulatory DNA in the presence of nitrate, thus prohibiting *frd* gene transcription. Because nitrate repression still occurred efficiently in the *fnr* mutant strain, we conclude that the nitrate repressor does not block Fnr binding but rather must bind elsewhere in the *frd*-regulatory region to prevent RNA polymerase binding or transcription

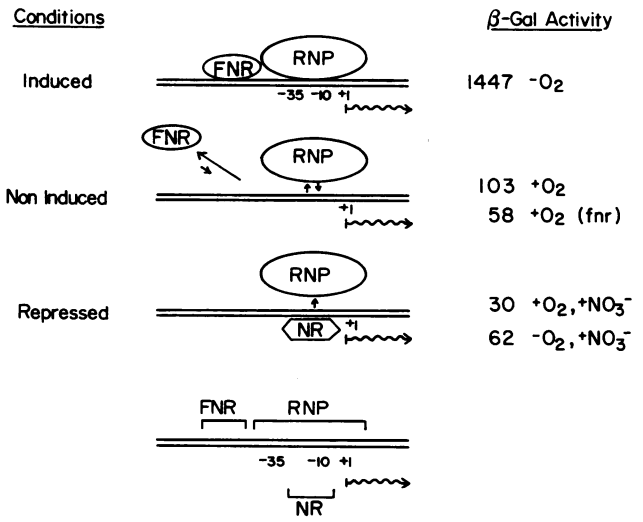

FIG. 5. Proposed model for the regulation of *frd* gene expression. *frd* DNA is indicated by the parallel horizontal lines. The start of *frd* transcription is indicated by +1, and the *frdABCD* mRNA is represented by the wavy line. The predicted *frd* RNA polymerase (RNP)-binding site is indicated by −35 and −10. The proposed interaction of FNR, the product of the *fnr* gene, and RNA polymerase (RNP) under conditions in which *frd* transcription is induced or not induced is shown. The proposed inhibition of RNA polymerase binding by a nitrate repressor (NR) is shown under *frd* transcription-repressing conditions. Relevant FrdA–β-galactosidase (β-Gal) hybrid protein activities are shown to the right of the indicated induced, noninduced, and repressed *frd* expression conditions.

initiation. The nitrate repressor-binding site in the −10 region of the *frd* promoter is shown in Fig. 5. However, the binding site could be at any position that would interfere with RNA polymerase binding at its recognition sequences. The *narK* gene product is not a candidate for this nitrate repressor as demonstrated by the results presented above (Tables 2 and 3). We have recently obtained genetic evidence for such a nitrate repressor protein (L. V. Kalman and R. P. Gunsalus, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, H165, p. 167) that supports the mechanism of nitrate control as proposed in our model.

Our working model for *frd* gene regulation predicts the location of the DNA-binding sites for Fnr, RNA polymerase, and the nitrate repressor as shown in Fig. 5. We are currently isolating *cis*-acting *frd* regulatory mutations to test the predictions of our model and to establish the precise location of each of the protein-binding sites. Additional genetic studies are required to determine the mechanism for fumarate induction of fumarate reductase gene regulation.

ACKNOWLEDGMENTS

We thank Gary Cecchini for the *E. coli* fumarate reductase antibodies, Bob Simons for pRS414 and pRS415, and Valley Stewart and Robert Kadner for strains RK4353, RK5288, and RK5266. We are also grateful to Bob Zagursky and Mike Berman for λRZ5 as well as for their advice and encouragement. Finally, we thank Ann Tsukamoto for her assistance with the Western blotting experiments.

This work was supported by grant PCM-8402974 from the National Science Foundation and Public Health Service grant AI-21678 from the National Institutes of Health. H.M.J. was supported by the Public Health Service Cellular and Molecular Biology Training Grant GM-07185 from the National Institutes of Health.

LITERATURE CITED

1. **Ausubel, F. M.** 1984. Regulation of nitrogen fixation genes. Cell **37**:5–6.
2. **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.
3. **Casadaban, M. J., J. Chou, and S. N. Cohen.** 1980. In vitro gene fusions that join an enzymatically active β-galactosidase segment to amino-terminal fragments of exogenous proteins: *Escherichia coli* plasmid vectors for the detection and cloning of translational initiation signals. J. Bacteriol. **143**:971–980.
4. **Castell, C. H.** 1950. The influence of trimethylamine oxide on the bacterial reduction of redox indicators. J. Fish. Res. Board Can. **7**:567–575.
5. **Cecchini, G., B. A. C. Ackrell, J. O. Deshler, and R. P. Gunsalus.** 1986. Reconsititution 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. Walter de Gruyter, Inc., New York.
7. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134**:1141–1156.
8. **Chippaux, M., V. Bonnefoy-Orth, J. Ratouchniak, and M. C. Pascal.** 1981. Operon fusions in the nitrate reductase operon and study of the control gene *nirR* in *Escherichia coli*. Mol. Gen. Genet. **182**:477–479.
9. **Chippaux, M., D. Giudici, A. Abou-Jaoude', F. Casse, and M. C. Pascal.** 1978. A mutation leading to the total lack of nitrite reductase activity in *Escherichia coli* K-12. Mol. Gen. Genet. **160**:225–229.
10. **Cole, J. A., and J. W. T. Wimpenny.** 1968. Metabolic pathways for nitrate reduction in *Escherichia coli*. Biochim. Biophys. Acta **162**:39–48.
11. **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.
12. **Cole., S. T.** 1982. Nucleotide sequence coding for the flavoprotein subunit of the fumarate reductase of *Escherichia coli*. Eur. J. Biochem. **122**:479–484.
13. **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.
14. **Cowing, D. W., J. C. A. Bardwell, E. A. Craig, C. Woolford, R. W. Hendrix, and C. A. Gross.** 1985. Consensus sequence for *Escherichia coli* heat shock gene promoters. Proc. Natl. Acad. Sci. USA **82**:2679–2683.
15. **de Crombrugghe, B., S. Busby, and H. Buc.** 1984. Activation of transcription by the cyclic AMP receptor protein. Science **224**:831–837.
16. **Dixon, D. A.** 1984. The genetic complexity of nitrogen fixation. J. Gen. Microbiol. **130**:2745–2755.
17. **Edlund, T., T. Grundstrom, and S. Normark.** 1979. Isolation and characterization of DNA repetitions carrying the chromosomal β-lactamase gene of *Escherichia coli* K-12. Mol. Gen. Genet. **173**:115–125.
18. **Fimmel, A. L., and B. A. Haddock.** 1979. Use of *chlC-lac* fusions to determine regulation of gene *chlC* in *Escherichia coli* K-12. J. Bacteriol. **138**:726–730.
19. **Fowler, A. V., and I. Zabin.** 1983. Purification, structure, and properties of hybrid β-galactosidase proteins. J. Biol. Chem. **258**:14354–14358.
20. **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.
21. **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.
22. **Horiuchi, T., J. Tomizawa, and A. Norick.** 1962. Isolation and properties of bacteria capable of high rates of β-galactosidase synthesis. Biochim. Biophys. Acta **55**:152–163.
23. **Hunt, T. P., and B. Magasnik.** 1985. Transcription of *glnA* by purified *Escherichia coli* components: core RNA polymerase and the products of *glnF*, *glnG*, and *glnL*. Proc. Natl. Acad. Sci. USA **82**:8453–8457.
24. **Ingledew, W. J., and R. K. Poole.** 1984. The respiratory chains of *Escherichia coli*. Microbiol. Rev. **48**:222–271.
25. **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.
26. **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.

26a. **Jamieson, D. J., R. G. Sawers, P. A. Rugman, D. H. Boxer, and C. F. Higgins.** 1986. Effects of anaerobic regulatory mutations and catabolite repression on regulation of hydrogen metabolism and hydrogenase isoenzyme composition in *Salmonella typhimurium*. J. Bacteriol. **168**:405–411.

27. **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.
28. **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.
29. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
30. **Lambden, P. R., and J. R. Guest.** 1976. Mutants of *Escherichia coli* K-12 unable to use fumarate as an anaerobic electron

acceptor. J. Gen. Microbiol. **97**:145–160.
31. **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.
32. **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.
33. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Merrick, M. J., and J. R. Gibbons.** 1985. The nucleotide sequence of the nitrogen-regulation gene *ntrA* of *Klebsiella pneumoniae* and comparison with conserved features in bacterial RNA polymerase sigma factors. Nucleic Acids Res. **13**:7607–7620.
35. **Morningstar, J. E., M. K. Johnson, G. Cecchini, B. A. C. Akrell, and E. B. Kearney.** 1985. The high potential iron-sulfur center in *Escherichia coli* fumarate reductase is a three-iron cluster. J. Biol. Chem. **260**:13631–13638.
36. **Newman, B. M., and J. A. Cole.** 1978. The chromosomal location and pleiotropic effects of mutations of the *nirA*$^+$ gene of *Escherichia coli* K-12: the essential role of *nirA*$^+$ in nitrite reduction and in other anaerobic redox reactions. J. Gen. Microbiol. **106**:1–12.
37. **Nicholas, D. J. D., and A. Nason.** 1957. Determination of nitrate and nitrite. Methods Enzymol. **3**:981–984.
38. **Pascal, M. C., V. Bonnefoy, M. Fons, and M. Chippaux.** 1986. Use of gene fusions to study the expression of *fnr*, the regulatory gene of anaerobic electron transfer in *Escherichia coli*. FEMS Microbiol. Lett. **36**:35–39.
39. **Reddy, P., A. Peterkofsky, and K. McKenney.** 1985. Translational efficiency of the *Escherichia coli* adenylate cyclase gene: mutating the UUG initiation codon to GUG or AUG results in increased gene expression. Proc. Natl. Acad. Sci. USA **82**:5656–5660.
40. **Shaw, D. J., and J. R. Guest.** 1981. Molecular cloning of the *fnr* gene of *Escherichia coli* K12. Mol. Gen. Genet. **181**:95–100.
41. **Shaw, D. J., and J. R. Guest.** 1982. Amplification and product identification of the *fnr* gene of *Escherichia coli*. J. Gen. Microbiol. **128**:2221–2228.
42. **Shaw, D. J., and J. R. Guest.** 1982. Nucleotide sequence of the *fnr* gene and primary structure of the Fnr protein of *Escherichia coli*. Nucleic Acids Res. **10**:6119–6130.
43. **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.
44. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
45. **Simons, R. W., F. Houman, and N. Klekner.** 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene **53**:85–96.
46. **Spencer, M. E., and J. R. Guest.** 1973. Isolation and properties of fumarate reductase mutants of *Escherichia coli*. J. Bacteriol. **114**:563–570.
47. **Stagier, P., C. Parsot, and J. Bouvier.** 1985. Two functional domains conserved in major and alternate bacterial sigma factors. FEBS Lett. **187**:11–15.
48. **Stewart, V.** 1982. Requirement of Fnr and NarL functions for nitrate reductase expression in *Escherichia coli* K-12. J. Bacteriol. **151**:1320–1325.
49. **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.
50. **Strauch, K. L., J. B. Lenk, B. L. Gamble, and C. G. Miller.** 1985. Oxygen regulation in *Salmonella typhimurium*. J. Bacteriol. **161**:673–680.
51. **Weiner, J. H., and P. Dickie.** 1979. Fumarate reductase of *Escherichia coli*: elucidation of the covalent-flavin component. J. Biol. Chem. **254**:8590–8593.