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Transcription of the *Escherichia coli* Fumarate Reductase Genes (*frdABCD*) and Their Coordinate Regulation by Oxygen, Nitrate, and Fumarate

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The fumarate reductase enzyme complex allows *Escherichia coli* to grow anaerobically with fumarate as a terminal electron acceptor for oxidative phosphorylation when the preferred compounds oxygen and nitrate are not available. We used the pKO promoter test vectors to identify a single promoter for the *frdABCD* genes which encode fumarate reductase. Expression of galactokinase from the *frd* promoter-*galK* operon fusion plasmid was repressed by oxygen and by nitrate and was induced by fumarate, indicating that *frd* gene expression is regulated at the transcriptional level by these terminal electron acceptors. S1 nuclease analysis, using a single-stranded DNA probe from the *frd* promoter region and mRNA isolated from a fumarate reductase-induced culture, revealed that the *frd* mRNA transcript initiates with an adenine residue 93 bases prior to the start of *frdA* translation. No promoters internal to the *frd* genes were revealed with the plasmid promoter screening system. S1 nuclease analysis revealed that the *frd* mRNA terminates in a uridine-rich region centered at 46 bases after the last codon of *frdD*. A stem and loop structure previously described as the growth rate-dependent attenuator for the linked *ampC* gene precedes the *frd* mRNA terminus. This result confirms the proposal that the stem and loop structure serves the dual role of a *frd* terminator anaerobically and an *ampC* attenuator aerobically. The four *frd* genes encoding the subunits of the fumarate reductase complex thus comprise an operon which is regulated at the transcriptional level in response to the cellular availability of the alternate electron acceptors oxygen, nitrate, and fumarate.

As a facultative organism, *Escherichia coli* can obtain energy for growth by substrate level phosphorylation and oxidative phosphorylation (17). Aerobically, oxygen serves as a terminal electron acceptor for the high-energy-yielding reactions of oxidative phosphorylation. Anaerobically, *E. coli* can also support oxidative phosphorylation by utilizing a number of alternate terminal electron acceptors. The utilizable terminal oxidants, in the order of decreasing redox potential (hence, less energetically favorable), include nitrate, trimethylamine-*N*-oxide, fumarate, and dimethyl sulfoxide (3, 17). The enzyme fumarate reductase catalyzes the reversible conversion of fumarate to succinate in the final step of the anaerobic electron transport pathway to fumarate.

Fumarate reductase is a distinct enzyme from succinate dehydrogenase. The two enzymes reversibly interconvert fumarate and succinate but do so with different substrate affinities and reaction rates (16). The two enzymes are encoded at different genetic loci: fumarate reductase (*frd*) at 94 min and succinate dehydrogenase (*sdh*) at 17 min (2). Enzyme activity measurements in *frd* and *sdh* mutant strains indicate that fumarate reductase is active almost exclusively in anaerobic cultures, whereas succinate dehydrogenase is active only in aerobic cultures (16, 32).

The *E. coli* fumarate reductase is a membrane-bound complex composed of four nonidentical polypeptides designated A, B, C, and D. The A subunit is a 69 kilodalton protein which contains a covalently bound flavin adenine dinucleotide (10, 34). The 27-kilodalton B protein appears to contain the iron-sulfur centers of the enzyme (9; J. E. Morningstar, M. K. Johnson, G. Cecchini, and B. A. C. Ackrell, J. Biol. Chem., in press). The C and D polypeptides of 15 and 13 kilodaltons, respectively, are membrane proteins which bind the catalytic AB subunits to the inner side of the cytoplasmic membrane (5, 14, 23).

The genes encoding the individual fumarate reductase polypeptides have been located at 94 min on the *E. coli* genetic map (22), they have been cloned onto phage and plasmid vectors (5, 11, 12, 15, 24), and their DNA sequence has been determined (9, 10, 14). The *frdABCD* genes are contiguously arranged on the DNA and encode the A, B, C, and D polypeptides, respectively. Upstream of *frdA* is an open reading frame designated gene X, and downstream of *frdD* is the *ampC* gene conferring a chromosomally encoded ampicillin resistance (14). The close proximity of the four *frd* genes has led to the assumption that the genes are in an operon. Attempts to identify promoters for any of the four *frd* genes by DNA sequence comparisons with the consensus bacterial promoter sequence have been unsuccessful (9, 10, 14). Similarly, in vitro transcription experiments performed with *frdA* DNA failed to reveal a *frd* promoter (10). A DNA sequence after *frdD* has been identified which has the characteristics of a consensus bacterial *rho*-independent terminator (14). This presumed stem and loop structure also has been described as a growth rate-dependent attenuator for the linked *ampC* gene (19). A dual role for the stem and loop structure as a *frd* terminator and an *ampC* attenuator has been proposed from in vitro transcription-translation experiments performed with aerobically grown cells (14), a

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condition which, in vivo, results in virtually no fumarate reductase enzyme activity. Whether the stem and loop structure functions as a *frd* terminator under anaerobic conditions has not been reported.

Fumarate reductase enzyme activities have been reported to be increased from 4- to 60-fold by anaerobiosis (18, 23, 32), increased 2-fold by anaerobic growth with fumarate (8, 18), and decreased 20- to 40-fold during anaerobic growth with nitrate (8, 18). Ruch et al. initially reported that the expression of a *frd-lacZ* operon fusion was regulated by oxygen, fumarate, and nitrate (30), but later the fusion was mapped to the *trp* locus at 28 min and not to the *frd* locus at 94 min (18). It is therefore still unclear whether the regulation of fumarate reductase by oxygen, fumarate, and nitrate is occurring at the enzymatic or gene expression level.

A *frd*-linked mutation which renders fumarate reductase insensitive to regulation by oxygen and fumarate has been identified (18), but it is not known whether this mutation affects a *frd* DNA regulatory site, the gene for a closely linked regulatory protein, or a regulatory region in the *frd* protein products. No mutants have been identified which help elucidate the mechanism of nitrate repression of fumarate reductase. Studies of a class of *trans*-acting mutations which affect fumarate utilization suggest that the regulation by oxygen is mediated through the product of the *fnr* gene located at 29 min on the *E. coli* genetic map (2). Mutants in *fnr* are defective in fumarate reductase enzyme activity and in the activities of a number of other enzymes involved in anaerobic respiration, including nitrate reductase (*chl nar*) and *sn*-glycerol-3-phosphate dehydrogenase (*glp*) (6, 7, 21, 22, 28, 33). It is clear from expression studies with *chlI-lacZ* (6) and *glpA-lacZ* (21) fusions in *fnr*$^+$ and *fnr* strains that the Fnr protein positively activates anaerobic gene transcription at these loci. It is likely, although untested, that Fnr similarly acts to positively activate *frd* transcription in response to anaerobiosis. No *trans*-acting regulatory loci have yet been identified which affect either the nitrate repression or fumarate induction of fumarate reductase enzyme activity.

In this study, we report the presence of a strong promoter upstream of *frdA* and the lack of promoters internal to the *frd* genes, thus establishing that the *frdABCD* genes comprise an operon. We identify the 5′ and 3′ termini of the in vivo *frdABCD* mRNA and establish that the *ampC* attenuator structure functions anaerobically as the *frd* terminator. Finally, we report that the differential expression of fumarate reductase in the presence of oxygen, nitrate, and fumarate is mediated at the level of *frd* gene transcription.

MATERIALS AND METHODS

Materials. D-[^{14}C]galactose (0.04 to 0.06 Ci/mmol), [γ-^{32}P]ATP (3 Ci/mol), and [α-^{32}P]dATP (0.4 Ci/mol) were purchased from Amersham Corp., Arlington Heights, Ill. T4 polynucleotide kinase was obtained from P-L Biochemicals, Inc., Milwaukee, Wis. DNA polymerase I large fragment was obtained from New England Biolabs, Inc., Beverly, Mass. S1 nuclease was obtained from Miles Laboratories, Inc., Elkhart, Ind. All other enzymes and reagents were of the highest purity commercially available.

Bacterial strains and plasmids. *E. coli* N100 (*galK recA pro*) was used for plasmid transformations, plasmid preparations, and galactokinase assays (27). *E. coli* A19 [*rns* Δ(*tonB-trpAE*)*1 trpR* X$^-$, where X represents an unknown growth requirement satisfied by 0.05% acid-hydrolyzed casein] (37) was used for mRNA isolation. The plasmid pGC1002 which contains the *frdABCD* genes on a 4.9-kilobase (kb) *Hin*dIII chromosomal DNA fragment inserted into pBR322 was used for all *frd* plasmid constructions (5). Plasmids pKO-4, pKO-5, pKO-6, pKO-11, pKG1800 (27), and pUC9 (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) were used as indicated.

Construction of plasmids. Restriction endonuclease digestions were performed as indicated by the supplier. Phosphatase treatments, ligations, cell transformations, and DNA preparations were performed as described previously (25).

Cell growth. For galactokinase assays and mRNA isolations, cells were grown in 50-ml volumes of glucose minimal medium (32) supplemented with the indicated electron acceptors. Aerobic cell growth was performed in 500-ml Erlenmyer flasks shaken vigorously in a New Brunswick 37°C water bath. Anaerobic cell growth was performed in 100-ml serum vials (Wheaton Scientific, Millville, N.J.) fitted with anaerobic tube stoppers and sealed with aluminum seals using a seal crimper. The anaerobic tube stoppers, aluminum seals, and seal crimper were purchased from Bellco Glass, Inc., Vineland, N.J. The vials were evacuated and flushed with N_2 at 20 lb/in^2 three times and then vented to a final N_2 pressure of 5 lb/in^2. Cultures were shaken in a New Brunswick 37°C water bath. Cell samples were removed with a 1-ml sterile syringe and needle for optical density measurements. The oxygen content of the atmosphere was measured before and after cell growth by gas chromatography (Carle gas chromatograph with a molecular sieve 5A column). The O_2 content was at all times less than 0.03%.

Media. Glucose (40 mM) minimal medium (32) supplemented with 40 mM potassium fumarate (pH 7.0) and 40 mM sodium nitrate (pH 7.0) as indicated was used for the growth of cells for galactokinase assays and mRNA preparations. Thiamine (0.1 mg/ml) was added to all liquid cultures, and proline (0.23 mg/ml) was added to *E. coli* N100 liquid cultures. Luria broth plates (25) and MacConkey-galactose (1%) plates (Difco Laboratories, Detroit, Mich.) were used when indicated. Ampicillin (Sigma Chemical Co., St. Louis, Mo.) was used at 40 μg/ml.

Galactokinase assay of extracts from promoter test plasmid-bearing cells. Galactokinase assays were performed as described previously (20) with the following modifications. Cell extracts were prepared from 30 ml of mid-exponential growth phase cultures (optical density at 600 nm of 0.4). Time course assays were performed in duplicate. Fractions from the reaction mixture were removed at 1, 2, 3, and 5 min to Whatman DE81 filter disks which were resting on dry ice. The filters were washed, with 2.4-cm Whatman GF/C filters used for support. The galactokinase activities reported in Tables 2 and 3 are average values obtained from three to six experiments.

Preparation of in vivo mRNA. mRNA was prepared as described previously (31) from *E. coli* A19 grown anaerobically in glucose minimal medium supplemented with 40 mM potassium fumarate to induce fumarate reductase.

S1 protection experiments and DNA sequencing analysis. The phosphatase-treated $Acc\text{I}_{405}$ (throughout this report, subscripts are DNA fragment sizes in base pairs) fragment of pfrdPr1 (Fig. 1) was end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP as described previously (20). The $Sau\text{3A}_{277}$ fragment of pfrdPr6 (Fig. 1) was labeled by end filling with DNA polymerase I large fragment and [α-^{32}P]dATP. Strand separation and DNA sequencing of the labeled fragments were performed as described previously (26). mRNA-DNA hybridizations and S1 nuclease treatments

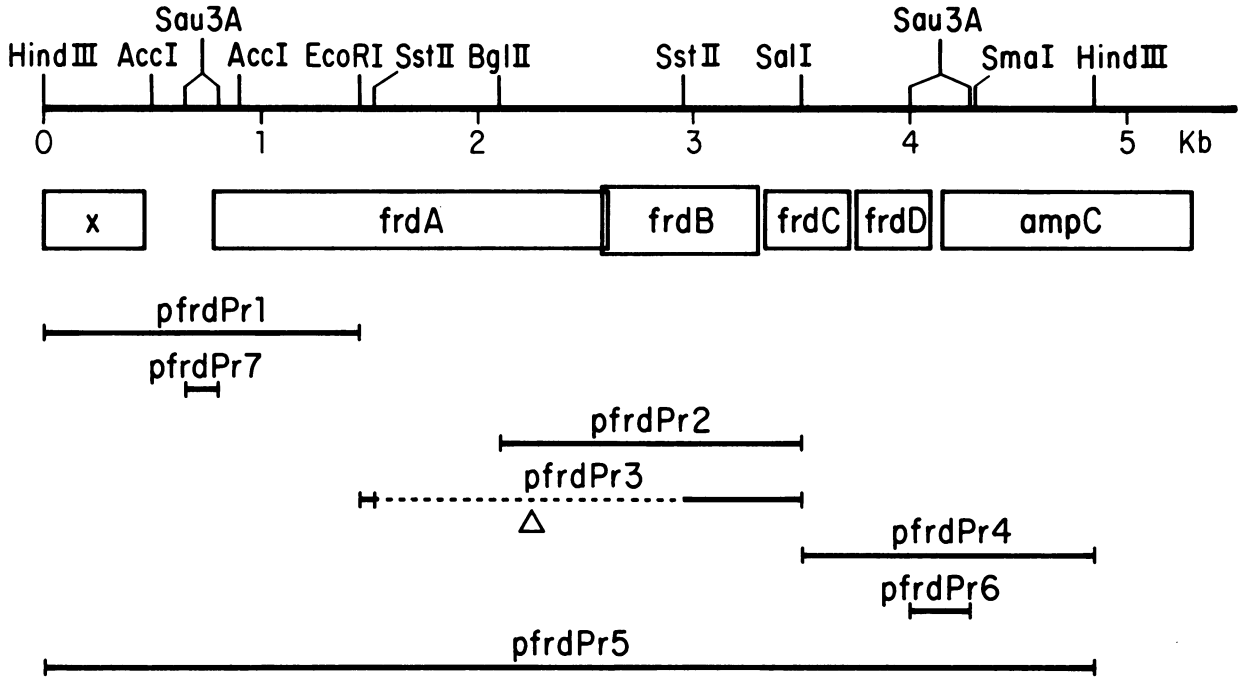

FIG. 1. *frd* promoter test plasmids. A map of the *frd* locus is shown with distances given in kilobases. Pertinent restriction endonuclease recognition sites are labeled. The genes within the *frd* region are boxed. The *frdABCD* and *ampC* genes are transcribed from left to right as drawn. The unidentified open reading frame designated gene X would be transcribed from right to left. The lines indicate the location and size of individual restriction fragments which were cloned into the appropriate promoter screening vectors. The resulting plasmid designations are given above the lines. The vector used for each plasmid, the inserted restriction fragment and size, and the plasmid size in kilobases are shown in Table 1. All plasmids confer ampicillin resistance. Plasmids were constructed by standard methods (25). Plasmids pfrdPr2, pfrdPr3, and pfrdPr4 were constructed through an intermediate vector, pUC9, which provided the restriction sites necessary for cloning into the final promoter test vector.

were as described previously (20), except that the S1 nuclease incubations were carried out at 0°C for 4 h. DNA fragments from the sequencing and S1 nuclease protection experiments were suspended in formamide buffer and separated electrophoretically on an 8% polyacrylamide–8M urea sequencing gel as described previously (26).

RESULTS

Construction of *frd-galK* promoter test plasmids. To identify the promoters for the *frdABCD* genes, we cloned various regions of the *frd* DNA into the pKO promoter test vectors (27). The insertion of a promoter sequence into the pKO vectors leads to the expression of the downstream *galK* gene in transformed cells. The levels of galactokinase, the *galK* gene product, present in cell extracts prepared from the promoter test plasmid-transformed strains then indicate both the presence and strength of a promoter in the newly inserted sequence. The *frd* DNA fragments which were inserted into the promoter test vectors are shown in Fig. 1. Restriction enzyme fragments were chosen such that promoters for each of the four *frd* genes would be identified. A promoter for *frdABCD* or *frdA* would be present on pfrdPr1 and pfrdPr5, while a promoter for *frdB* would be present on pfrdPr2 and pfrdPr5. A *frdC* promoter would be found on pfrdPr2, pfrdPr3, and pfrdPr5; and a promoter for *frdD* would be on pfrdPr4 and pfrdPr5. The *ampC* promoter would be present on the cloned restriction fragments of pfrdPr4, pfrdPr5, and pfrdPr6. The DNA restriction fragments shown in Fig. 1 were ligated into the indicated *galK* promoter test vectors (Table 1), and the resulting plasmids were transformed into *E. coli* N100 and plated on Luria agar-ampicillin plates. Plasmid DNA was prepared from colonies picked from the Luria agar-ampicillin plates. Restriction enzyme analysis was performed on the plasmid DNA to confirm the presence of the *frd* inserts and their respective orientations within the newly constructed promoter test plasmids. The cloned *frd* DNA fragment sizes, as determined by agarose and polyacrylamide gel electrophoresis, are given in Table 1 and are consistent with those expected from previously published DNA sequence data (9, 10, 14). The inserted sequences of pfrdPr1 and pfrdPr6 were confirmed by DNA sequence analysis (see below).

Colonies containing each of the constructed plasmids were screened for *galK* expression by streaking them onto MacConkey-galactose ampicillin indicator plates. Colonies containing the *frd* promoter plasmid pfrdPr1 and the control *gal* promoter plasmid pKG1800 appeared red on aerobically incubated indicator plates. The red color is due to acid production resulting from galactose utilization, the first step of which is catalyzed by galactokinase. The indicator plates were incubated aerobically because both *galK*$^+$ and *galK* cells appeared red on the MacConkey medium under anaerobic cell culture conditions. Colonies containing pfrdPr2, pfrdPr3, pfrdPr4, pfrdPr5, pfrdPr6, and pfrdPr7 appeared white on the aerobically incubated indicator plates, as did the promoter minus vectors pKO-4, pKO-5, pKO-6, and pKO-11. The indicator plate colony colors suggest that there is a single promoter for the four *frd* genes which is contained on pfrdPr1.

Galactokinase activities in promoter test plasmid-bearing strains indicate the existence and strength of an *frd* promoter.

TABLE 1. *frd* promoter test plasmids used for in vivo galactokinase assays

Plasmid	*frd* fragment[a]	Genes encoded[b]	Plasmid vector	Total size (kb)
pfrdPr1	*Hin*dIII-*Eco*RI$_{1,463}$	*X'frdA'*	pKO-6	5.4
pfrdPr2	*Bgl*II-*Sal*I$_{1,323}$	*frdA'BC'*	pKO-4	5.3
pfrdPr3	*Eco*RI-*Sst*II *Sst*II-*Sal*I$_{610}$	*frdA'B'C'*	pKO-4	4.6
pfrdPr4	*Sal*I-*Hin*dIII$_{1,351}$	*frdC'D ampC'*	pKO-5	5.0
pfrdPr5	*Hin*dIII$_{4,859}$	*X'frdABCD ampC'*	pKO-4	8.8
pfrdPr6	*Sau*3A$_{277}$	*frdD' ampC'*	pKO-4	4.3
pfrdPr7	*Sau*3A$_{162}$	*frdA'*	pKO-11	4.1

[a] Subscripts are DNA fragment sizes (in base pairs).
[b] A prime indicates a partial gene.

Promoter-dependent *galK* expression was determined by measuring the galactokinase activities in cell extracts prepared from each of the promoter test plasmid-bearing strains. The strains were grown anaerobically in glucose minimal medium containing fumarate to induce fumarate reductase. Mid-exponential growth phase cells were harvested by centrifugation, and cell extracts were prepared. The galactokinase levels in extracts from the *frd* promoter test plasmid-containing strains as well as control plasmid-bearing strains are shown in Table 2.

Cells bearing the *frd-galK* plasmid pfrdPr1 express galactokinase at high levels (1,235 U) relative to cells containing the promoter minus vector pKO-4 (9 U) and the *gal* promoter-*galK* plasmid pKG1800 (762 U) when grown under anaerobic fumarate reductase-inducing conditions. The high galactokinase values in pfrdPr1 cell extracts indicate that a very strong *frd* promoter is located on the 1.46-kb *Hin*dIII-*Eco*RI fragment of pfrdPr1. In an attempt to better localize the *frd* promoter present on pfrdPr1, a 162-base-pair (bp) *Sau*3A fragment containing 144 bp of DNA 5' of the *frdA* translation start site was cloned into pKO-11, creating pfrdPr7 (Fig. 1 and Table 1). Galactokinase levels in cell extracts from pfrdPr7-bearing cells were very low (13 U), indicating that a functional *frd* promoter is not present on this *Sau*3A fragment. Cell extracts from the pfrdPr2, pfrdPr3, and pfrdPr4 had very low levels of galactokinase (21 U, 17 U, and 19 U, respectively), suggesting that there are no promoters internal to the *frdABCD* genes and hence that the *frdABCD* genes comprise an operon. Extracts of the pfrdPr5-bearing cells contained little galactokinase (29 U), even though the pfrdPr5 *frd* fragment contained the *frd* promoter located on pfrdPr1. These results suggest that the expression from the strong *frd* promoter is being efficiently terminated within the 4.86-kb *Hin*dIII fragment of pfrdPr5, reducing the 1,235 U of galactokinase expression to 29 U. Extracts of the pfrdPr6-containing cells have very low levels of galactokinase (20 U), indicating that the *ampC* promoter which is located on this plasmid is not expressed significantly under anaerobic growth conditions in glucose fumarate minimal medium.

Precise location of the *frdABCD* promoter. The location of the *frd* promoter on pfrdPr1 was determined by S1 nuclease mapping of the 5' end of the *frd* mRNA. The *Acc*I$_{405}$ restriction fragment of pfrdPr1 (Fig. 1) was 5' end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP, the two strands were separated, and each strand was subjected to DNA sequencing to identify the strand complementary to the *frd* mRNA. The appropriate strand was hybridized to excess mRNA prepared from *E. coli* A19 grown under fumarate reductase-inducing conditions (anaerobic growth in glucose minimal medium containing fumarate). The resulting hybrids were treated with S1 nuclease and electrophoresed adjacent to a DNA sequencing ladder for this strand. Autoradiography of the resulting gel revealed a major protected species (Fig. 2A, lane b), indicating that the *frd* mRNA initiates in a single region within the *Acc*I$_{405}$ DNA restriction fragment. When the samples were run further on the gel to resolve species in the high-molecular-weight region (Fig. 2B) it became clear that there were two strongly and one weakly protected DNA species. The position of the *frd* mRNA 5' end was deduced by comparing the position of these S1 nuclease-protected DNA species with the sequencing ladder for the corresponding DNA strand. The S1 nuclease-protected DNA fragments run approximately one and a half bases slower than the corresponding bands in the sequencing lane since S1 cleavage yields a 3' hydroxyl group on the protected terminal residue, whereas the chemical degradation in the Maxam and Gilbert (26) sequencing reactions destroy the terminal residue leaving a 3' phosphate group (1). The presence of two adjacent strongly protected species indicate that the *frd* mRNA initiates with either of two adenine residues at 92 or 93 bases before the start of *frdA* translation. The presence of a weakly protected species may indicate that there is a minor *frd* mRNA start site at 95 bases before the *frdA* translation initiation site. We have indicated that the adenine residue at 93 bases prior to *frdA* translation is the major initiation site for the *frd* mRNA (shown as +1 in Fig. 2) since it represents the major S1 nuclease-protected species and since the band representing a 5' end at −92 relative to the *frdA* cistron may be an artifact of S1 nuclease nibbling at the AT-rich termini of the *frd* mRNA-DNA hybrid. It should be noted that a small percentage of the DNA fragments were insensitive to S1 nuclease digestion and ran as fully protected species in lanes a and b (Fig. 2B). The presence of the fully protected species in lane a (Fig. 2B), in which DNA but not mRNA was included in the reaction mixture, indicates that the bands are not due to S1 nuclease protection through DNA-*frd* mRNA hybridization but rather through DNA-DNA hybridization resulting from a slight contamination of the single-stranded DNA mixture with the opposing strand.

Determination of the in vivo *frd* mRNA 3' end by S1 nuclease analysis. It has been proposed from DNA sequence analysis and from in vitro transcription-translation experiments that the stem and loop structure described as the

TABLE 2. Galactokinase activities of *frd* promoter test plasmid-bearing strains grown under *frd*-inducing conditions.

Plasmid type	Plasmid designation	Galactokinase units[a]
Promoter	pfrdPr1	1,235
	pfrdPr2	21
	pfrdPr3	17
	pfrdPr4	19
	pfrdPr5	29
	pfrdPr6	20
	pfrdPr7	13
Control[b]	pKO-4	9
	pKG1800	762

[a] Units given in nanomoles of D-galactose-1-phosphate formed per milligram of protein per minute.
[b] The control plasmids pKO-4 and pKG1800 are a promoter minus the *galK* vector and a *gal* promoter-containing *galK* plasmid, respectively.

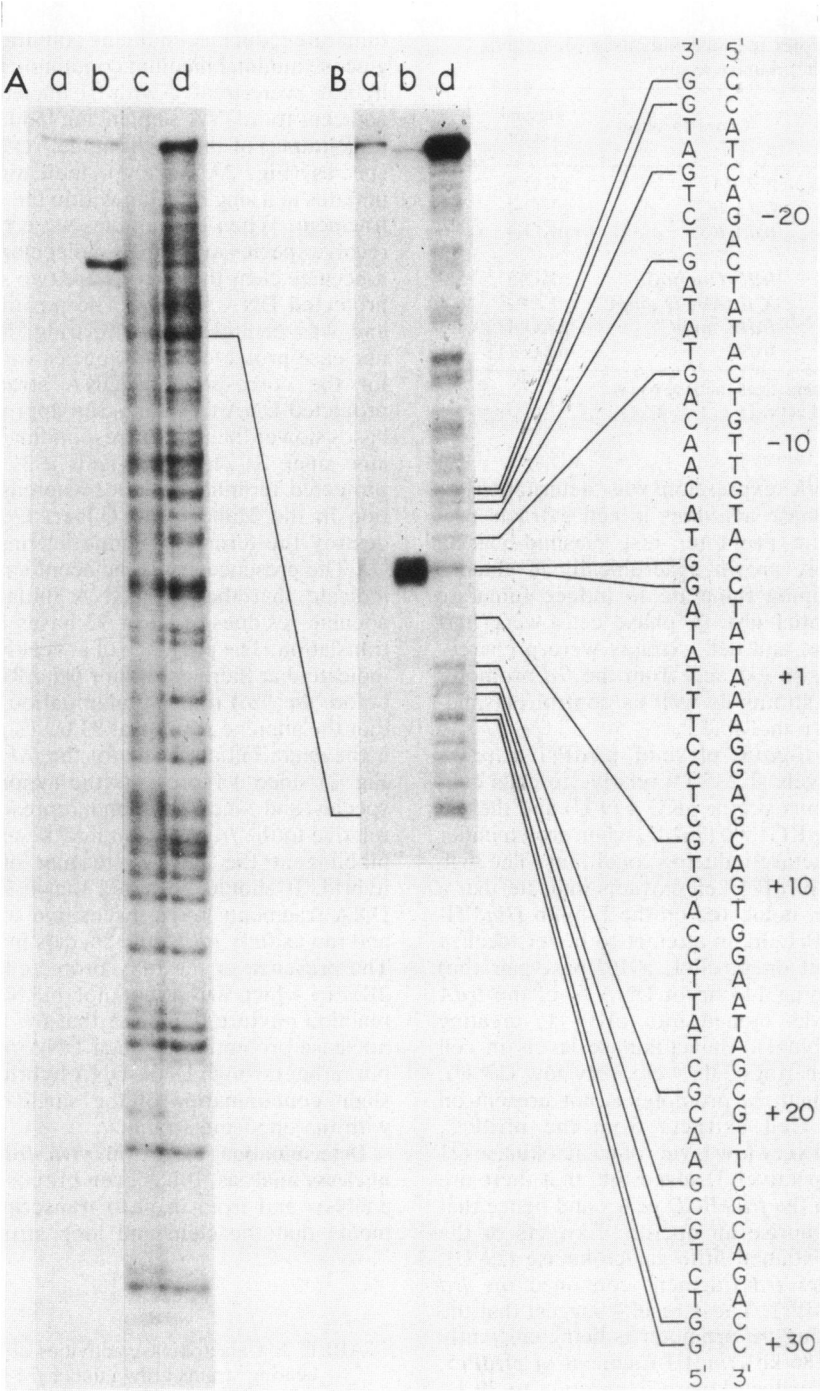

FIG. 2. Location of the in vivo *frd* mRNA 5′ terminus. The single-stranded $AccI_{405}$ fragment of pfrdPr1 labeled at the 5′ end was hybridized to mRNA and treated with S1 nuclease. Lanes a represent treatment in the absence of mRNA. Lanes b represent treatment with mRNA prepared from *E. coli* A19 grown under fumarate reductase-inducing conditions. In lane c and lanes d are the AG and G sequencing reactions, respectively, from the $AccI_{405}$ fragment. (A) S1 nuclease and sequencing analysis for the entire $AccI_{405}$ fragment. (B) Samples run further on the sequencing gel to better resolve fragments in the high-molecular-weight region. Numbering is relative to the deduced 5′ start of *frd* transcription.

ampC growth rate-dependent attenuator also serves as a *rho*-independent *frd* transcription terminator (14). To test whether the stem and loop structure serves in vivo as the *frd* terminator anaerobically, we performed S1 nuclease analysis to locate the 3′ end of the *frd* mRNA. The $Sau3A_{277}$ fragment of pfrdPr6 (Fig. 1) was 3′ end labeled by filling in the overhanging *Sau*3A ends using DNA polymerase I large fragment and [α-^{32}P]dATP. The strands were separated, and each strand was subjected to DNA sequencing to identify the strand complementary to the *frd* mRNA. The appropriate strand was hybridized to excess mRNA prepared from *E. coli* A19 grown under fumarate reductase-inducing conditions (anaerobic growth in glucose minimal medium containing fumarate). The resulting hybrids were treated with S1

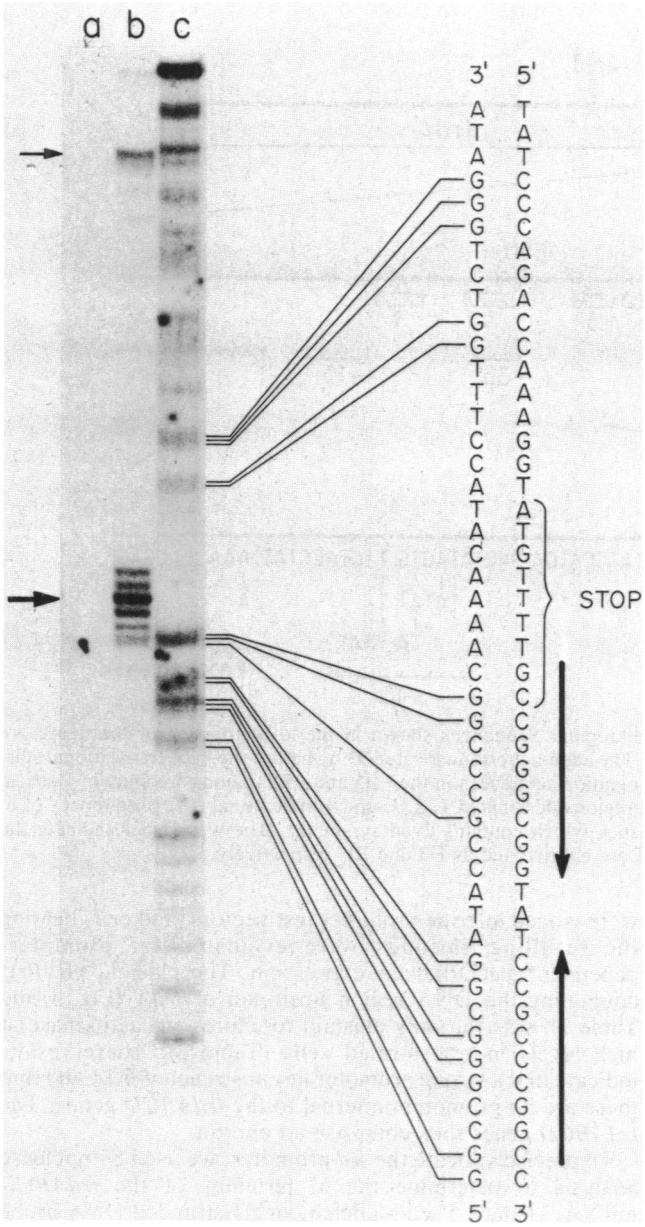

FIG. 3. Location of the in vivo *frd* mRNA 3' terminus. The single-stranded $Sau3A_{277}$ fragment of pfrdPr6 labeled at the 3' end was hybridized to mRNA and treated with S1 nuclease. Lane a, treatment in the absence of mRNA; lane b, treatment with mRNA prepared from *E. coli* A19 grown under fumarate reductase-inducing conditions; lane c, the G sequencing reaction from the $Sau3A_{277}$ fragment. The vertical opposing arrows show the location of the dyad symmetrical sequences corresponding to the *ampC* attenuator structure and the deduced *frd* terminator structure.

nuclease and electrophoresed adjacent to a DNA sequencing ladder for this strand. The majority of the radioactivity (88%) was found in a cluster of protected species (Fig. 3, lane b, large arrow), indicating that there is a major *frd* mRNA 3' end which terminates over several adjacent residues in a uridine-rich region. This region is centered at 46 bases after the last codon for *frdD* translation and 13 bases after the center of the *ampC* attenuator stem and loop structure. These protected bands in Fig.3, lane b (large arrow), are not due to S1 nuclease nicking within the loop of the stem and loop structure because the same protected bands are not present in lane a (Fig. 3) in which DNA but not mRNA was present in the S1 nuclease reaction. The remainder of the radioactivity (12%) was found in a second region indicated by the smaller arrow (Fig. 3, lane b). The presence of this protected species may indicate that a small percentage of *frd* mRNAs terminates at 158 bases after the last codon of *frdD*. Inspection of the DNA in this region revealed no sequences which resembled a *rho*-independent terminator. In longer autoradiographic exposures, this minor species was visualized in lane a (Fig.3) in which DNA but not mRNA was included in the S1 nuclease reaction, and this may indicate that the band is an artifact. However, we cannot eliminate the possibility that this species represents a minor *frd* transcriptional stop site or that it represents the major stop site and that the majority of *frd* mRNAs are digested back from this 3' end to the stem and loop structure. Because no fully protected $Sau3A_{277}$ species was observed (Fig. 3, lane b), it is clear that *frd* mRNA termination is not occurring downstream of the *Sau*3A site 185 bp after the last codon of *frdD*. It should be noted that the single-stranded $Sau3A_{277}$ DNA fragment may hybridize to *ampC* mRNA as well as *frd* mRNA; however, the *ampC*-protected hybrids cannot be visualized by autoradiography since the label at the single-stranded fragments 3' end are not protected by the *ampC* mRNA against S1 nuclease digestion.

Anaerobiosis, fumarate, and nitrate affect fumarate reductase enzyme activities by regulating *frdABCD* gene transcription. Results of previous work have shown that fumarate reductase enzyme activities are maximal in cells grown anaerobically with fumarate and without nitrate (8, 18, 23, 32). It is not clear whether the effect of anaerobiosis, nitrate, or fumarate was exerted at the fumarate reductase enzymatic level or at the *frd* gene expression level. The *frd* promoter-*galK* operon fusion plasmid described above provided a convenient way to test whether *frd* gene transcription was altered by the presence of the potential electron acceptors in the growth medium. The N100 strain containing the plasmid pfrdPr1 was grown on glucose with the various electron acceptors, and the *frd* promoter-dependent galactokinase expression in the cells was measured (Table 3). Under fumarate reductase-inducing conditions (minus oxygen and nitrate, plus fumarate) the cell extracts contained 1,235 U of galactokinase (Tables 2 and 3). When grown in the presence of the preferred electron acceptor

TABLE 3. Effect of oxygen, nitrate, and fumarate on *frd* transcription

Electron acceptor present[a]			Galactokinase activity in cells bearing the following plasmids[b]:			% maximal *frd* expression[c]
Oxygen	Nitrate	Fumarate	pKO-4	pKG1800	pfrdPr1	
−	−	+	9	762	1,235	100
+	−	−	7	700	59	4
−	−	−	12	734	666	53
−	+	+	7	666	176	14

[a] Cells were grown in glucose minimal medium with the indicated electron acceptors present.

[b] Units given in nanomoles of D-galactose-1-phosphate formed per milligram of protein per minute.

[c] Percentages were determined by subtracting the pKO-4 galactokinase units from the pfrdPr1 units for each growth condition, multiplying by 100, and dividing the resulting value by that for the optimal fumarate reductase-inducing growth condition (minus oxygen and nitrate and plus fumarate).

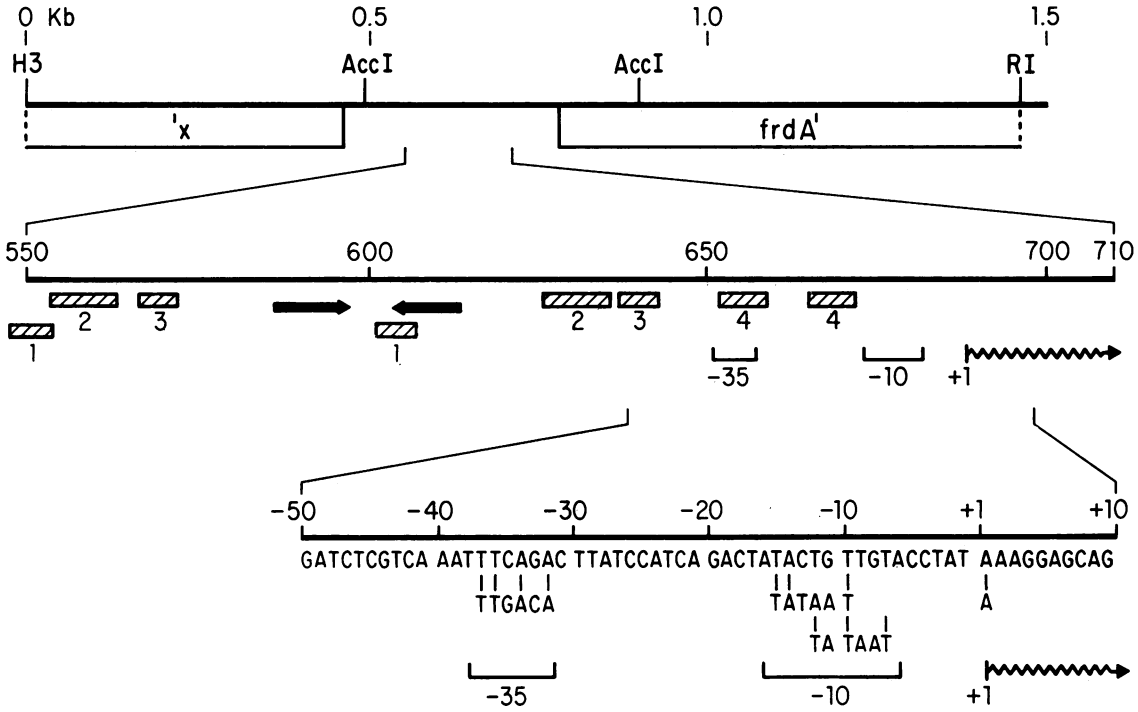

FIG. 4. Restriction map and nucleotide sequence in the *frd* promoter region. Sequences shown in the lower portion of the figure are numbered relative to the deduced 5′ terminus of the *frd* mRNA (see text). The adenine residue located 93 bp before the *frdA* translational start site is given as +1. The consensus sequence of the RNA polymerase recognition sequences in the −35 and −10 regions are shown. Vertical dashes indicate homology with the consensus sequence (29). The boxed regions designated 1, 2, 3, and 4 indicate repeated sequences of 6, 10, 5, and 7 bp, respectively. The heavy arrows indicate a 10-bp sequence which contains dyad symmetry. The wavy lines indicate the *frdABCD* mRNA. The restriction endonuclease sites *Hin*dIII and *Eco*RI are abbreviated as H3 and RI, respectively.

oxygen, the *frd* promoter-dependent galactokinase expression was reduced 25-fold in extracts to 59 U (Table 3), giving a net expression of galactokinase which was 4% of that in optimally induced cells. When grown in the absence of oxygen, nitrate, and fumarate, the *frd* promoter-dependent galactokinase expression was reduced twofold to 666 U, giving a net expression which was 53% of that in optimally induced cells. When grown anaerobically in the presence of both nitrate and fumarate, the galactokinase levels in the cell extracts were reduced sevenfold to 176 U, which was 14% of that observed in fully induced cells. Under all growth conditions, cells containing the control plasmids pKO-4 and pKG1800 expressed comparable levels of galactokinase, indicating that the plasmid gene dose remains constant under all the growth conditions employed. The *frd* promoter-*galK* plasmid data indicate that expression of fumarate reductase is regulated by the presence of the alternate electron acceptors, oxygen, nitrate, and fumarate at the level of *frd* gene transcription.

DISCUSSION

The *E. coli* fumarate reductase, encoded by the *frdABCD* genes, allows fumarate to be used as a terminal oxidant for anaerobic respiration. The close proximity of the four genes and the existence of a single sequence after *frdD* which has the characteristics of a *rho*-independent terminator, led to the proposal that the *frdABCD* genes comprise an operon. To experimentally test this proposal, we examined the transcription of the *frd* genes.

We utilized the pKO promoter test plasmid system (27) to locate *frd* promoters. DNA fragments which would contain promoters for each of the four *frd* genes, if they existed, were cloned into the promoter test vectors, and cells bearing the resulting plasmids were evaluated for promoter-dependent galactokinase expression. The plasmid pfrdPr1, containing the DNA region upstream of *frdA* (Fig. 1 and Table 1), was the only plasmid to express galactokinase at high levels in transformed cells (Table 2). These results indicate that a strong promoter lies upstream of *frdA* and that there are no promoters internal to the *frdABCD* genes. The *frdABCD* genes thus comprise an operon.

To precisely locate the *frd* promoter, we used S1 nuclease analysis to determine the 5′ terminus of the *frdABCD* mRNA. Using a 5′-end-labeled, single-stranded DNA probe from the *frd* promoter region and mRNA isolated from a *frd*-induced cell culture, we determined that the *frd* mRNA begins with an adenine residue 93 bases before the *frdA* initiation codon (Fig. 2). Inspection of the DNA within the 93-base *frd* leader region did not reveal sequences which may produce possible secondary structures characteristic of translationally controlled genes (36). However, we are currently testing whether additional control of fumarate reductase expression is exerted at the translational level.

The nucleotide sequence in the *frd* promoter region is shown in Fig. 4. The numbering is relative to the deduced 5′ terminus of the *frd* mRNA. Homology between the *frd* promoter sequences and the RNA polymerase consensus recognition sequences in the −35 and −10 regions is shown in Fig. 4 (29). Of the 6 bp in the *frd* −35 region, 4 were homologous with the consensus −35 sequence. Two possible *frd* −10 regions can be assigned, and each contained 3 of 6 bp homologous to the −10 consensus sequence. Both −10 regions met the spacing requirements for the 1 to −10 and the −10 to −35 regions for RNA polymerase recognition at

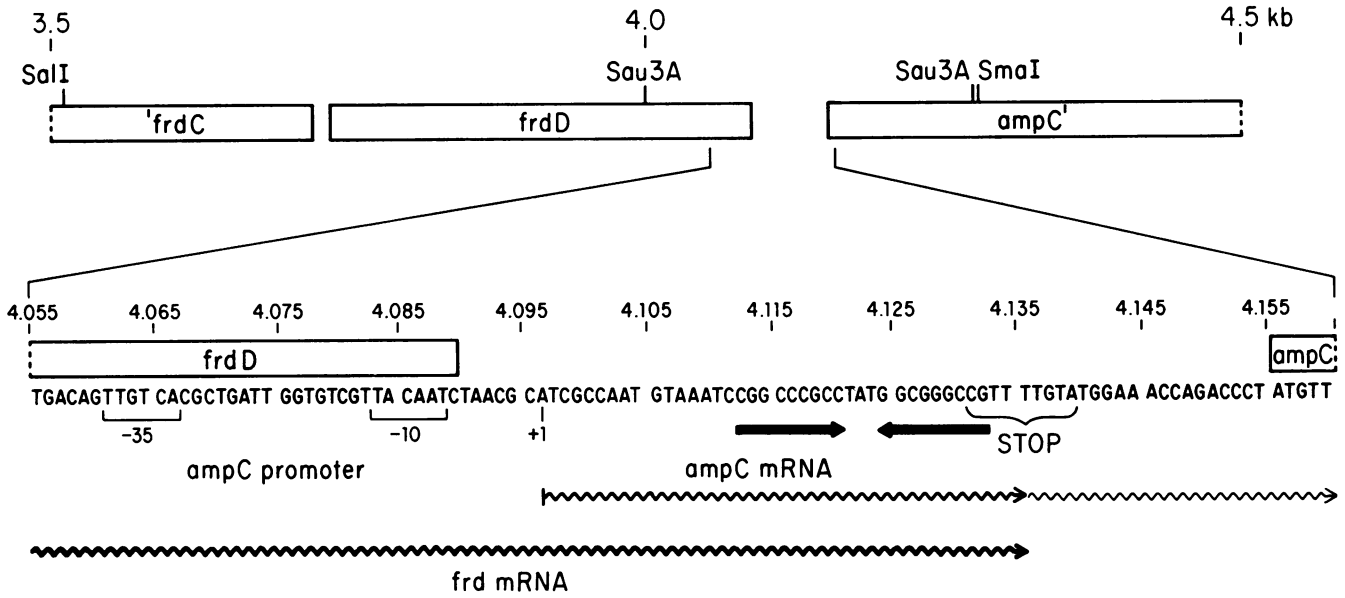

FIG. 5. Restriction map and nucleotide sequence in the *frd* terminator and *ampC* promoter region. Sequences are numbered relative to the *Hin*dIII site, at which DNA sequencing of the *frd* locus was initiated. The 3′ end of the *frd* mRNA is centered at the uridine residue 46 bases after the last codon for *frdD* translation and 13 bases after the center of the stem and loop structure. The heavy arrows indicate the sequences with dyad symmetry which can form the stem and loop structure described as the *ampC* growth rate-dependent attenuator and which functions as the *frdABCD* terminator anaerobically. The wavy lines indicate the *frdABCD* and *ampC* mRNA species. The *ampC* promoter −35, −10, and +1 sequences are indicated.

known *E. coli* promoter sites (29). The precise determination of which −10 region is recognized by RNA polymerase must await further studies. It is interesting that cells bearing the pfrdPr7 plasmid, which contains *frd* DNA to −50 bp relative to the start of *frd* transcription, express very little galactokinase, even though the plasmid contains the RNA polymerase recognition signals (Fig. 1 and Table 2). This suggests that sequences upstream from the position −50 are necessary for fumarate reductase transcription under anaerobic growth conditions. A number of tandemly repeated sequences and a sequence with dyad symmetry were found upstream of the *frd* promoter. Such structures are often correlated with procaryotic regulatory sequences. Because the sequence with dyad symmetry and the tandemly repeated sequences labeled 1, 2, and 3 (Fig. 4) lie within the *frd* fragment of pfrdPr1 (which is regulated by oxygen, fumarate, and nitrate) and lie upstream of the nonfunctional promoter-containing *frd* fragment of pfrdPr7, it is possible that these structures play a role in the regulation of fumarate reductase gene expression. Another tandemly repeated sequence of 7 bp in length (labeled 4, Fig. 4) is present within the RNA polymerase recognition sequences. It will be interesting to determine whether the *frd* mutant identified recently (18) which is relieved for both oxygen and fumarate regulation of fumarate reductase activity contains a lesion within any of these tandemly repeated or symmetrically opposed sequences. We are currently isolating *frd* regulatory mutants to determine whether these sequences are important for fumarate reductase control by oxygen, nitrate, and fumarate.

The low level of galactokinase expression in the pfrdPr5-transformed strains, even though pfrdPr5 contains the *frd* promoter, suggests that strong termination of *frd* gene transcription is occurring within the 4.9-kb *Hin*dIII insert (Fig. 1 and Table 2). The efficiency of this termination is indicated by the large decrease (62-fold) of the *frd* promoter-directed galactokinase expression (pfrdPr1 versus pfrdPr5) from 1,226 U to 20 U (units corrected for pKO-4 background activity).

We performed S1 nuclease analysis to locate the in vivo 3′ terminus of the *frdABCD* mRNA. A single-stranded, 3′-end-labeled DNA fragment which contained the *frdD-ampC* gene junction and in vivo mRNA isolated from a fumarate reductase-induced culture were used for this analysis. The *frd* mRNA terminated over several adjacent residues in a uridine-rich region that was centered at 46 bases after the last codon of *frdD* and 13 bases after the center of the *ampC* attenuator stem and loop structure (Fig. 3). The nucleotide sequence in the *frd* terminator region as well as the position of the *ampC* promoter is shown in Fig. 5. The *frdABCD* termination site contains an inverted repeat sequence which has the ability to form a stable base-paired structure within the same RNA or DNA molecule, producing a stem and loop structure with a ΔG of −29.4 kcal (19). The stem is composed of 9 GC bp, the loop contains three residues, and the entire structure is followed by an eight-base uridine-rich region. The *frdABCD* terminator thus exhibits all the DNA sequence characteristics of a *rho*-independent terminator (29). In vitro analysis of transcripts from the *ampC* promoter demonstrated that *ampC* attenuation at this site is *rho* independent (19). It remains to be determined whether anaerobic *frdABCD* transcription termination is *rho* independent. These analyses establish that the stem and loop structure described as the *ampC* attenuator does function anaerobically as a *frdABCD* terminator in vivo.

A reason for the *frd-ampC* overlap and the consequent dual role of the stem and loop structure is unclear. In *Citrobacter freundii*, a close relative of *E. coli*, there is a 1,100-bp DNA segment separating the *frd* and *ampC* genes (35). The *frd-ampC* overlap in *E. coli* then may have arisen from a DNA deletion event. This gene overlap may be tolerable since the *frd* and *ampC* genes appear to be maxi-

mally expressed under opposing cellular growth conditions. The *frdABCD* genes are highly expressed anaerobically and very slightly expressed aerobically (this study; Table 3). The *ampC* gene is subject to growth rate attenuation and, hence, is maximally expressed in rapidly growing aerobic cultures (19) but barely expressed in the slowly growing anaerobic cultures (this study; Table 2, pfrdPr6 data). Under anaerobic growth conditions the stem and loop structure may act solely to efficiently terminate *frd* transcription, whereas under aerobic growth conditions the structure may act solely to regulate the growth rate-dependent expression of the *ampC* gene.

Fumarate reductase enzyme activities previously have been shown to vary dramatically in response to the cellular availability of potential terminal electron acceptors. Fumarate reductase activities were elevated 4- to 60-fold by anaerobiosis (8, 23, 32), elevated 2-fold by anaerobic growth with fumarate (8, 18), and decreased 20- to 40-fold by anaerobic growth with nitrate (8, 18). To determine whether the differential expression of fumarate reductase is mediated at the level of *frd* gene transcription, we assayed whether the presence of oxygen, nitrate, and fumarate during cell growth could affect the expression of galactokinase from the *frd* promoter-*galK* operon fusion plasmid pfrdPr1. Galactokinase activities were reduced 24-fold by oxygen, reduced approximately 7-fold by nitrate, and elevated 2-fold by fumarate (Table 3). The presence of oxygen, nitrate, and fumarate affect fumarate reductase expression at the level of *frd* gene transcription. Interestingly, the fold levels of regulation by oxygen, nitrate, and fumarate determined with the multicopy *frd* promoter-*galK* system were similar to those obtained by assaying fumarate reductase enzyme activities in frd^+ cells (8, 18, 23, 32). Anaerobic induction was 25-fold in the galactokinase assay, whereas it was from 4- to 60-fold in the fumarate reductase assays. Fumarate induction was approximately twofold by both assay methods. Nitrate repression, however, was only about 7-fold, as determined by the galactokinase assay, whereas it was 20- to 40-fold in fumarate reductase assays. This difference in fold regulation by nitrate could be explained by the presence of a *frd*-specific nitrate repressor which is titrated by multiple copies of the *frd* promoter-operator region on the pfrdPr1 plasmid.

The *E. coli* fumarate reductase genes thus comprise an operon which is coordinately regulated by the presence of alternate electron acceptors in the growth medium. The presence of the preferred terminal electron acceptor, molecular oxygen, prevents use of the less-efficient electron transport pathways to nitrate and fumarate by not allowing transcription of the genes encoding nitrate reductase (6, 13, 33) and fumarate reductase (this study; Table 3), respectively. Anaerobically, the presence of the alternate electron acceptor nitrate induces the transcription of the *chl* (*nar*) genes encoding nitrate reductase (6, 13, 33) and represses the transcription of the *frd* genes (this study; Table 3), thus ensuring the cellular use of the more energy efficient electron transport pathway to nitrate. When neither oxygen nor nitrate is available as electron acceptor, the fumarate reductase genes are actively transcribed, and this transcription is further induced by the presence of fumarate in the growth medium (this study; Table 3). It is possible that under these conditions the presence of fumarate represses the synthesis of enzymes necessary for the pathways of fermentation, thereby allowing the preferential use of the more energy efficient electron transport pathway to fumarate. Thus, *E. coli* may ensure optimal growth by the hierarchical regulation of enzymes involved in energy generation.

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LITERATURE CITED

1. **Aiba, H.** 1983. Autoregulation of the *Escherichia coli crp* gene: CRP is a transcriptional repressor for its own gene. Cell **32**:141–149.
2. **Bachmann, B. J.** 1983. Linkage map of *Escherichia coli* K-12, Edition 7. Microbiol. Rev. **47**:180–230.
3. **Bilous, P. T., and J. H. Weiner.** 1985. Dimethyl sulfoxide reductase activity by anaerobically grown *Escherichia coli* HB101. J. Bacteriol. **162**:1151–1155.
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, 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, New York.
6. **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.
7. **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.
8. **Cole, J. A., and J. W. T. Wimpenny.** 1968. Metabolic pathways for nitrate reduction in *Escherichia coli*. Biochim. Biophys. Acta **162**:39–48.
9. **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.
10. **Cole, S. T.** 1982. Nucleotide sequence coding for the flavoprotein subunit of the fumarate reductase of *Escherichia coli*. Eur. J. Biochem. **122**:479–484.
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. **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.
13. **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.
14. **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.
15. **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.
16. **Hirsch, C. A., M. Rasminsky, B. D. Davis, and E. C. C. Lin.** 1963. A fumarate reductase in *Escherichia coli* distinct from succinate dehydrogenase. J. Biol. Chem. **238**:3770–3774.
17. **Ingledew, W. J., and R. K. Poole.** 1984. The respiratory chains of *Escherichia coli*. Microbiol. Rev. **48**:222–271.
18. **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.
19. **Jaurin, B., T. Grundstrom, T. Edlund, and S. Normark.** 1981. The *Escherichia coli* β-lactamase attenuator mediates growth rate dependent regulation. Nature (London) **290**:221–225.
20. **Jones, H. J., C. M. Brajkovich, and R. P. Gunsalus.** 1983. In

vivo 5′ terminus and length of the mRNA for the proton translocating ATPase (*unc*) operon of *Escherichia coli*. J. Bacteriol. **155**:1279–1287.

21. **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.
22. **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.
23. **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.
24. **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.
25. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base specific chemical cleavages. Methods Enzymol. **65**:499–560.
27. **McKenney, K., H. Shimatake, D. Court, U. Schmeissner, C. Brady, and M. Rosenberg.** 1981. A system to study promoter and terminator signals recognized by *Escherichia coli* RNA polymerase, p. 383–415. *In* J. C. Chirikjian and T. S. Papas (ed.), Gene amplification and analysis, vol. II: Analysis of nucleic acids by enzymatic methods. Elsevier/North-Holland Publishing Co., Amsterdam.
28. **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.
29. **Rosenberg, M., and D. Court.** 1980. Regulatory sequences involved in the promotion and termination of RNA transcription. Annu. Rev. Genet. **13**:319–359.
30. **Ruch, F. E., D. R. Kuritzkes, and E. C. C. Lin.** 1979. Use of *lac* operon fusions to isolate *Escherichia coli* mutants with altered expression of the fumarate reductase system in response to substrate and respiratory controls. Biochem. Biophys. Res. Comm. **91**:1365–1370.
31. **Salser, W., R. F. Gesteland, and A. Bolle.** 1967. In vitro synthesis of bacteriophage lysozyme. Nature (London) **215**:588–591.
32. **Spencer, M. E., and J. R. Guest.** 1973. Isolation and properties of fumarate reductase mutants of *Escherichia coli*. J. Bacteriol. **114**:563–570.
33. **Stewart, V.** 1982. Requirement of Fnr and NarL functions for nitrate reductase expression in *Escherichia coli* K-12. J. Bacteriol. **151**:1320–1325.
34. **Weiner, J. H., and P. Dickie.** 1979. Fumarate reductase of *Escherichia coli*: elucidation of the covalent-flavin component. J. Biol. Chem. **254**:8590–8593.
35. **Yamamoto, T., S. Y. Murayama, and T. Sawai.** 1983. Cloning and expression of the gene(s) for cephalosporinase production of *Citrobacter freundii*. Mol. Gen. Genet. **190**:85–91.
36. **Yanofsky, C.** 1981. Attenuation in the control of expression of bacterial operons. Nature (London) **289**:751–758.
37. **Zalkin, H., C. Yanofsky, and C. L. Squires.** 1974. Regulated in vitro synthesis of *Escherichia coli* tryptophan operon messenger ribonucleic acid and enzymes. J. Biol. Chem. **249**:465–475.