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Sequence and Genetic Characterization of *etrA*, an *fnr* Analog That Regulates Anaerobic Respiration in *Shewanella putrefaciens* MR-1

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An electron transport regulatory gene, *etrA*, has been isolated and characterized from the obligate respiratory bacterium *Shewanella putrefaciens* MR-1. The deduced amino acid sequence of *etrA* (EtrA) shows a high degree of identity to both the Fnr of *Escherichia coli* (73.6%) and the analogous protein (ANR) of *Pseudomonas aeruginosa* (50.8%). The four active cysteine residues of Fnr are conserved in EtrA, and the amino acid sequence of the DNA-binding domains of the two proteins are identical. Further, *S. putrefaciens etrA* is able to complement an *fnr* mutant of *E. coli*. In contrast to *fnr*, there is no recognizable Fnr box upstream of the *etrA* sequence. Gene replacement *etrA* mutants of MR-1 were deficient in growth on nitrite, thiosulfate, sulfite, trimethylamine-*N*-oxide, dimethyl sulfoxide, Fe(III), and fumarate, suggesting that EtrA is involved in the regulation of the corresponding reductase genes. However, the mutants were all positive for reduction of and growth on nitrate and Mn(IV), indicating that EtrA is not involved in the regulation of these two systems. Southern blots of *S. putrefaciens* DNA with use of *etrA* as a probe revealed the expected *etrA* bands and a second set of hybridization signals whose genetic and functional properties remain to be determined.

Shewanella putrefaciens is a gram-negative bacterium capable of both aerobic or anaerobic respiration but not fermentation. Anaerobic growth can be coupled to a wide variety of electron acceptors, including Mn(IV), Fe(III), fumarate, nitrate, nitrite, trimethylamine-*N*-oxide (TMAO), dimethyl sulfoxide (DMSO), thiosulfate, and sulfite (22, 23, 26), as well as elemental sulfur (24, 27) and Mn(III) (23a). This extreme metabolic versatility makes of *S. putrefaciens* MR-1 unusual, perhaps unique, among respiratory organisms. To our knowledge, this is the only facultative aerobe capable of reduction of, and growth on, thiosulfate, sulfite, and elemental sulfur (27). The electron acceptors utilized by *S. putrefaciens* span the range of electron potentials from highly electropositive compounds like oxygen to more electronegative ones like sulfite. An interesting question thus arises as to how an organism of such respiratory versatility regulates the synthesis and activity of its complex electron transport networks. In an attempt to address this question, our laboratory initiated an investigation of the regulatory mechanisms by which the various terminal reductases are controlled.

In recent years, it has become apparent that the control of anaerobic respiration is quite complex and highly regulated (14, 17, 39). In *Escherichia coli*, a facultative anaerobe, at least two transcriptional regulators control gene expression in response to anoxia. One, the ArcB-A sensor-regulator system, represses the expression of aerobic genes such as the tricarboxylic acid cycle enzymes and the aerobic electron transport chain (14, 16, 17). Another, the *fnr* product (Fnr), positively regulates the terminal reductase genes such as the fumarate and nitrate reductase operons in the absence of oxygen (13, 14). The N terminus of Fnr contains a four-cysteine cluster which under anaerobic conditions is thought to bind reduced iron, thus activating the protein (12, 38). This cysteine cluster appears to be a property of enzymes that regulate gene expression in response to anoxia (34). The DNA-binding domain is located near the C terminus and has the characteristic helix-turn-helix motif. In genes regulated by Fnr, a palindromic consensus sequence, the Fnr box, is found upstream of the promoter region to which the protein binds and activates gene expression (9, 35). This consensus sequence is also found in the promoter region of *fnr*, which negatively regulates its own expression under anaerobic conditions (35).

Analogous genes have been identified in a variety of organisms, such as *oxrA* of *Salmonella typhimurium*, which is similar in function to *fnr* except that mutants in this gene retain their ability to use TMAO and fumarate as terminal electron acceptors (37). In *Pseudomonas aeruginosa*, the product of *anr* (ANR) regulates nitrate reduction, HCN production, and the arginine deiminase pathway (11, 40). *hlyX*, a gene very similar in sequence to *fnr*, has been isolated from *Actinobacillus pleuropneumoniae* and shown to be involved in hemolytic activity in this organism (21). Similar genes encoding regulatory proteins involved in nitrogen fixation have also been isolated from *Rhizobium meliloti* and *R. leguminosarum* (3, 4).

In *S. putrefaciens*, metabolic inhibitors and competition experiments have suggested that regulation occurs at the levels of both synthesis (7, 22, 23, 28) and activity (1, 2, 6, 8, 22–25) of various components, but no detailed studies of gene regulation have been reported. We now report the isolation and characterization of a similar regulatory gene, *etrA* (electron transport regulator A), the deduced amino acid sequence of which is 73.6% identical to that of Fnr. The phenotype of *etrA* mutants suggest that this gene is involved in the anaerobic regulation of some, but not all, of the terminal reductase genes in *S. putrefaciens*. In addition, our results suggest that MR-1 has a second *etrA* analog, the structure and function of which are still to be determined.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source and/or reference
E. coli		
RZ7350	MG1655 but *lacZ* and *narG* 234::Mud1734	P. Kiley (18)
RZ8480	RZ7350 but *fnr*::Sp^r Sm^r (omega interposon)	P. Kiley
S. putrefaciens		
MR-1	Lake Oneida isolate	22
METR-1	MR-1 but *etrA*::pACYC184 Tc^r	This work
Plasmids		
pACYC184	Cloning vector, Tc^r Cm^r	New England Biolabs
pSPORT1	Cloning and sequencing vector, Amp^r	BRL
pUC19	Cloning and sequencing vector, Amp^r	BRL
pRZ7301	pCU19 containing the functional *E. coli fnr*	P. Kiley
pSETR1	pSPORT1 with the *etrA*-containing 2.3-kb *Hin*dIII-*Sst*I fragment	This work
pSETR2	pSPORT1 with the *etrA*-containing 1.5-kb *Pst*I-*Sst*I fragment	This work
pSETR3	pACYC184 containing the 165-bp *Dra*I-*Nco*I fragment of *etrA* Tc^r	This work

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1.

Media and growth conditions. LB (29) was routinely used for aerobic growth of MR-1 and *E. coli* strains. Anaerobic cultures of MR-1 strains were grown in M1 defined medium described previously (22) containing 30 mM lactate but with pH adjusted to 7.9, which gives better growth yields and more reproducible results (6a). Terminal electron acceptors were added to a final concentration of 2 mM. Cultures were incubated in a Coy anaerobic chamber for up to 7 days. Streptomycin, chloramphenicol, tetracycline, and ampicillin were added as needed to final concentrations of 50, 30, 17, and 100 μg/ml, respectively. Mutants that have pACYC184 integrated into the chromosome were grown in the presence of 8 μg of tetracycline per ml.

DNA manipulations. Genomic and plasmid DNA isolation, restriction digestion, ligation, and Southern transfers were performed by using standard techniques (29).

Screening of genomic library with the *E. coli fnr*. Total MR-1 DNA was partially digested with *Sau*3A1, and the purified 15- to 20-kb fragments were used to construct a genomic library in λ-Gem11 (λ-Gem11 *Bam*HI arms; Promega). The library was screened by using the *E. coli fnr* as a probe. The probe was radiolabelled by using a random priming kit (Bethesda Research Laboratories [BRL]) and hybridized to the filters at 37°C. The filters were then washed in 0.2% sodium dodecyl sulfate–0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 40°C. Plaques that resulted in hybridization signals were rescreened twice, using the same probe. One clone, λfn-11, was chosen for further analyses. Phage DNA from λfn-11 was purified by using λ-Sorb (Promega), and a restriction map of the insert was obtained by using the λ-Map system (Promega) according to the manufacturer's instructions.

DNA sequencing. Southern transfer experiments using λfn-11 DNA digested with several enzymes and hybridization to radiolabelled *fnr* indicated that the *S. putrefaciens* analog is located on a 2.3-kb *Hin*dIII-*Sst*I fragment. This DNA fragment was purified from agarose gels and subcloned into pSPORT1 and M13mp19 (BRL). Both plasmid and phage DNA were used for sequencing. DNA sequencing was performed by the chain termination procedure (30), using the Klenow fragment of the *E. coli* polymerase (BRL). The sequencing reactions were run on either 6% acrylamide or 5% Hydrolink (AT Biochem, Malvern, Pa.) gels. Primers for DNA sequencing were synthesized on an Applied Biosystems 38A automated DNA synthesizer by using phosphoramidite chemistry as recommended by the manufacturers. Primers were purified on Poly-pak cartridges (Glen Research Corp.), lyophilized in a Savant Speed-Vac concentrator, and suspended in distilled water.

Primer extension reactions. MR-1 was grown aerobically in LB, and total RNA was extracted by previously described methods (15, 32). To determine the 5′ end of the mRNA, primer extension analysis was done by a modification of the method described by Sawers (31), using an oligonucleotide complementary to nucleotides 725 to 742 of the *etrA* coding region. The primer extension reaction was performed by annealing 4 ng of oligonucleotide to 7 μg of total RNA. The extension reaction was carried out at 37°C for 30 min following the addition of 5 U of avian myeloblastosis virus reverse transcriptase (Seikagaku America, Inc., Rockville, Md.), 10 μCi of [α-^{32}P]ATP, and 2 mM of each dCTP, dGTP, and dTTP. The resulting DNA-RNA hybrids were denatured at 90°C before being run on 6% acrylamide gels. The same oligonucleotide primer was used to generate a sequence ladder complementary to the mRNA sequence, using pSETR1 DNA.

Complementation of an *E. coli fnr* mutant. RZ8480, an *E. coli* Fnr$^-$ strain (Table 1) that contains a *narG-lacZ* transcriptional fusion, was transformed with pSETR1, pSETR2, and pSPORT1 containing a random 1.5-kb *Pst*I fragment of MR-1. Since *narG* is controlled by both Fnr and nitrate, only cells with a functional *fnr* or homolog exhibit β-galactosidase activity. *E. coli* RZ7350, which is similar to RZ8480 except that it has a functional *fnr*, was used as a positive control. The cultures were grown anaerobically in M9 medium with 20 mM glucose and 2 mM nitrate and assayed for β-galactosidase activity as described previously (18).

Construction of an *etrA* mutant by gene replacement. Mutagenesis of wild-type *etrA* by gene replacement was essentially performed as described by Zimmerman et al. (40). A 165-bp *Dra*I-*Nco*I fragment at the 5′ end of the *etrA* gene was cloned into the Tc^r gene of pACYC184 (New England Biolabs). The resulting plasmid DNA was introduced into MR-1 cells by electroporation, using a Bio-Rad Gene Pulser apparatus at 5,500 V/cm as instructed by the manufacturer. The cells were plated on LB agar supplemented with tetracycline at 8 μg/ml. Six colonies were obtained and tested for the ability to use the various terminal electron acceptors anaerobically on M1 agar plates (pH 7.9) containing 2 mM the terminal electron acceptors, 30 mM lactate, and 8 μg of tetracycline per ml to

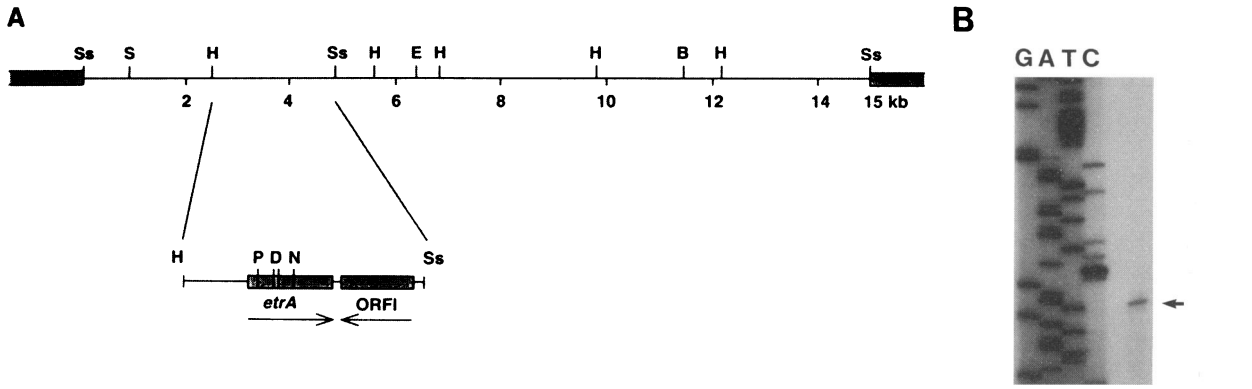

FIG. 1. (A) Restriction map of λfn-11. Abbreviations: B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; Ss, *Sst*I. The positions of *etrA* and ORF1 and the direction of transcription of each are shown. (B) Primer extension analysis of the transcription initiation site of *etrA*. The order of sequencing lanes and extension product (arrow) are indicated.

maintain the presence of pACYC184. To determine whether pACYC184 was inserted into *etrA*, chromosomal DNA was isolated from one of the mutants, and Southern transfers of the digested DNA were hybridized with the 1.5-kb *Pst*I-*Sst*I fragment.

Chemical measurements. Both wild-type and mutant strains were grown in M1 (pH 7.9) containing 2 mM ferric citrate as the source of Fe(III). Tetracycline was added to the cultures of the mutant strains. Samples were taken every 24 h and filtered through Nuclepore filters (0.2-μm pore size), and the amount of Fe(II) present was measured by the ferrozine assay as described previously (23). Nitrate reduction was assayed by testing for the production of nitrite, using sulfanilamide and *N*-1 (1-naphthyl) ethelyenediamine hydrochloride as described previously (19). The presence of a red color, which indicates nitrate reduction, also means that nitrite was not reduced by the mutant strains. Wild-type MR-1, when grown on nitrate broth, reduces nitrate and nitrite, and the red color is not detected. Growth and the characteristic odor of the end products (trimethylamine, dimethyl sulfate, and H_2S) were used to score the ability of the culture to utilize TMAO, DMSO, and thiosulfate, respectively, as terminal electron acceptors in a defined medium.

Sequence analysis. Sequences were analyzed by a computer-assisted search of sequences on deposit in GenBank/EMBL, using the software package (version 7.2, October 1992) from the Genetics Computer Group (5) and using LaserGene by DNASTAR (GenBank release 74, EMBL release 33, and NBRF-PIR release 34, January 1993). Amino acid sequence alignments were obtained with the progressive alignment algorithm of Feng and Doolittle (10).

Nucleotide sequence accession number. The sequence shown in Fig. 2 will appear in GenBank under accession number L13868.

RESULTS

Isolation of the *S. putrefaciens fnr* analog. A genomic clone, λfn-11, was isolated by using the *E. coli fnr* as a probe at moderate stringency. The purified recombinant phage DNA was analyzed by restriction enzyme digestion and was found to contain a 15-kb insert, the restriction map of which is shown in Fig. 1A. The *fnr* analog was localized to the 2.3-kb *Hin*dIII-*Sst*I fragment (Fig. 1A). This fragment was cloned into plasmid pSPORT1, and the resulting plasmid, pSETR1, was used for sequencing.

Sequence analysis of *etrA*. The DNA sequence of the 2.3-kb *Hin*dIII-*Sst*I fragment is shown in Fig. 2. It contains a 750-bp open reading frame, designated *etrA*, the deduced amino acid sequence of which shares a high degree of similarity with the sequence of Fnr. The protein encoded by *etrA* has a calculated molecular weight of 27,864. The transcription initiation site was determined by primer extension to lie 114 bp upstream of the AUG codon (Fig. 1B). The −10 element of the promoter (575-GATTAT) matches the *E. coli* consensus sequence (TATAAT) at four of six positions, while the −35 element (552-TTGGGT) matches the consensus (TTGACA) at three of six positions (19a). The −10 and −35 regions have a 17-bp spacing and are in the right position relative to the transcription start. The similarity between the *E. coli* and *S. putrefaciens* promoters is not surprising given the close phylogenetic grouping of the two organisms. The 3′ end of *etrA* contains a region of dyad symmetry that may act as a termination signal. In contrast to the *fnr* sequence, the *etrA* upstream region does not contain an Fnr-binding site. This is similar to the finding that the *anr* upstream region also lacks this sequence (11). Another open reading frame, ORF1, located downstream of *etrA*, is 615 bp in length and is transcribed from the complementary DNA strand. Comparison of both the nucleotide and inferred amino acid sequences of ORF1 with sequences in GenBank and protein data bases yielded no significant similarities. Another ORF was also found upstream of *etrA* (nucleotides 1 to 645); this ORF also lacked similarity with known sequences.

Comparison of EtrA with other regulatory proteins. Comparison of the inferred amino acid sequence of EtrA to the others revealed 73.6, 50.8, and 67% identity and 84.4, 71, and 82.5% similarity to the sequences of Fnr, ANR, and HlyX, respectively. However, the nucleotide sequences of *fnr* and *etrA* are only 69% similar, which may be due to the difference in GC content of *E. coli* (49.3%) and *S. putrefaciens* (43%) (20). The five cysteine residues found in Fnr, four of which are essential for protein activity, are also found in EtrA (Fig. 3). In addition, the amino acid sequence of the helix-turn-helix motif was seen to be identical in Fnr and EtrA, suggesting that the two proteins bind to similar DNA sequences.

Complementation of an *fnr* mutant. To demonstrate a functional connection between the *Hin*dIII-*Sst*I fragment and the *fnr* probe, two strains of *E. coli*, RZ7350 and RZ8480, were used. Both strains contain *lacZ* fused to the *narG* promoter, which requires both functional Fnr and nitrate for expression (18). Therefore, RZ7350, which contains a functional *fnr*,

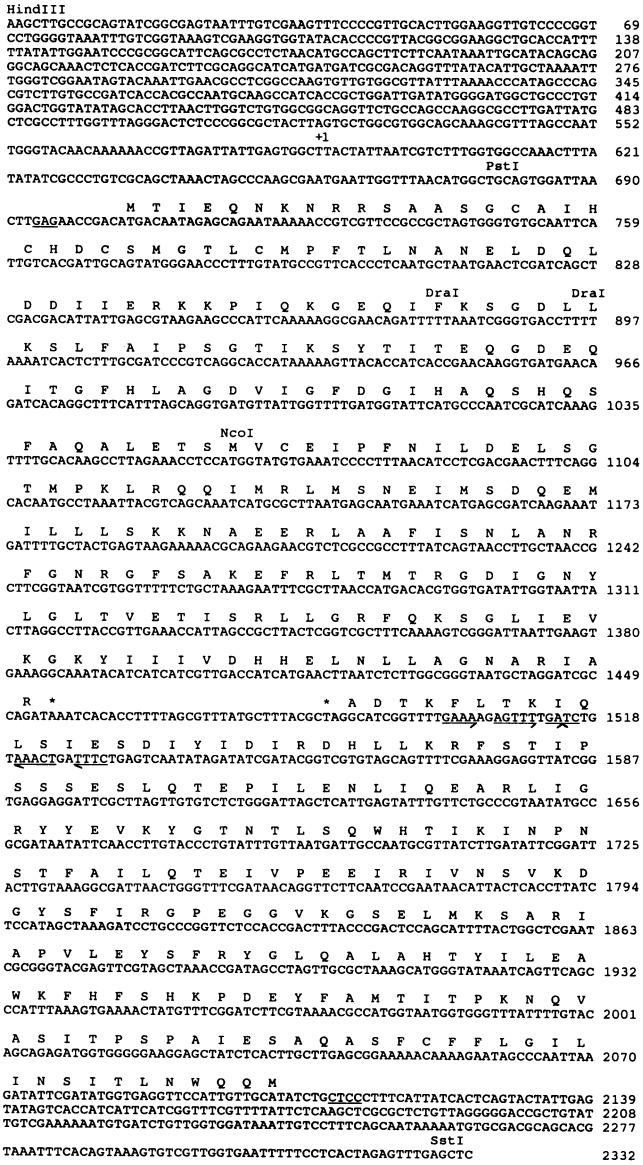

FIG. 2. Nucleotide sequences of *etrA* and ORF1. The inferred amino acid sequences are indicated. Putative ribosome binding sites are underlined, and the arrows indicate a region of dyad symmetry that may act as a termination signal.

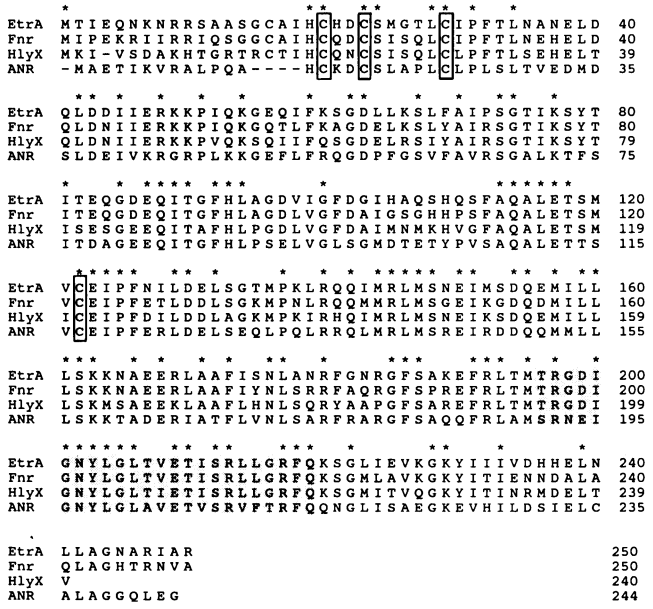

FIG. 3. Alignment of EtrA, Fnr, ANR, and HlyX amino acid sequences. The four conserved cysteines are boxed, and the DNA-binding domain is shaded. Asterisks indicate amino acids that are conserved in all four sequences.

exhibits β-galactosidase activity, while RZ8480, which is *fnr* mutant, does not (18). We used two plasmids to complement the *fnr* in strain RZ8480. Plasmid pSETR1 contains the complete *etrA* sequence, including the promoter region. Plasmid pSETR2 contains the 1.5-kb *Sst*I-*Pst*I fragment which includes the complete coding region of *etrA* but lacks the promoter region. Transcription of the gene in this plasmid is initiated by using the T7 polymerase promoter. Since the plasmid without an insert could not be used as a negative control because of the presence of a functional *lacZ*, a plasmid that contains a 1.5-kb *Pst*I fragment of MR-1 DNA was used to transform RZ8480 to serve as a negative control. This fragment did not show any cross-hybridization with *etrA* in Southern transfer experiments (data not shown). RZ8480 with or without the negative control plasmid did not result in any detectable β-galactosidase activity when the cells were grown anaerobically with nitrate. In contrast, RZ7350 and RZ8480 containing either pSETR1 or pSETR2 exhibited β-galactosidase activity, indicating the ability of *etrA* to functionally complement an *E. coli fnr* mutant.

Construction and complementation of an *etrA* mutant. We have recently reported the isolation and complementation of two *S. putrefaciens* pleiotropic mutants deficient in anaerobic respiration (28). The complementing DNA fragments were shown not to contain *fnr* analogs. Therefore, we constructed an *etrA* mutant to identify the function of this gene in MR-1. Plasmid pACYC184 was used in gene replacement experiments (Fig. 4A), since this plasmid is not replicated in MR-1. Six colonies that have pACYC184 integrated into the chromosome by a single crossover event (tetracycline resistant) were obtained. To confirm the interruption of *etrA* by the plasmid, the DNA of one mutant, METR-1, was isolated and analyzed. Figure 4B shows a Southern transfer of METR-1 DNA following hybridization to pACYC184 DNA and to the 1.5-kb *Pst*I-*Sst*I *etrA*-containing fragment. In both cases, two bands that correspond to 3.8 and 3.5 kb in length resulted in hybridization, confirming the interruption of *etrA* by the plasmid. A third band was seen when the *etrA* probe was used, which suggests there is another *etrA* analog that was not affected by the mutagenesis (see below).

Phenotype of *etrA* mutants. All six mutants that were obtained from the gene replacement experiment were tested for the ability to grow on the various terminal electron acceptors used by the wild type. The mutants were grown anaerobically both in liquid medium and on agar plates. All six mutants were able to couple anaerobic growth to the reduction of Mn(IV) or nitrate. Mn(IV) reduction is detected by a change in color from brown (insoluble manganese) to clear (soluble manganese). When grown on nitrate broth, the mutants were able to reduce nitrate, but not nitrite, which accumulated in the medium. None of the mutants showed any

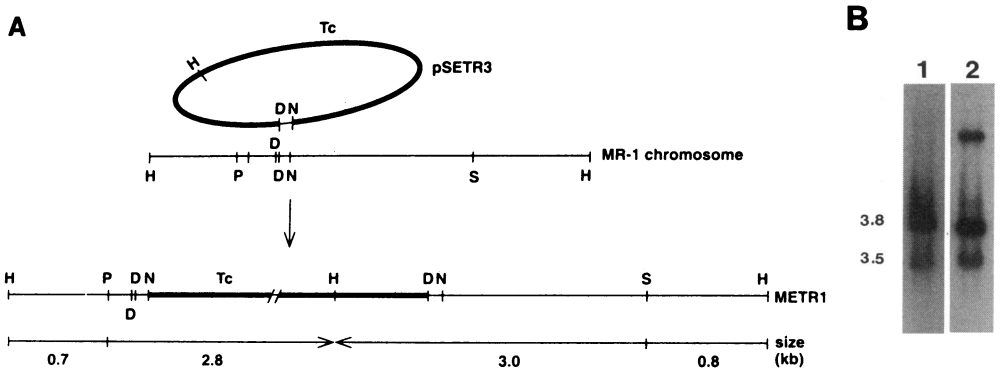

FIG. 4. (A) Mutagenesis of MR-1 by gene replacement. Plasmid pSETR3 was integrated into the MR-1 chromosome by a single crossover event. Both a map of *etrA*, which is interrupted by pSETR3, and the sizes of the expected fragments are shown. (B) Southern transfer of METR-1 DNA digested with *Hin*dIII and hybridized to pACYC184 DNA (lane 1) and the 1.5-kb *Pst*I-*Sst*I *etrA*-containing fragment (lane 2). Two bands that correspond to the expected sizes in both lanes are observed. A third band, seen in lane 2, which corresponds to another *etrA* analog was not affected by the mutagenesis (see Results).

growth on defined media supplemented with fumarate, nitrite, sulfite, thiosulfate, TMAO, or DMSO. Even though the mutants were incapable of growth with Fe(III) as a terminal electron acceptor, they were capable of iron reduction. The amount of reduced iron was measured by using ferrozine (23) and was found to be very similar in both the mutant and the wild-type cultures. A summary of the EtrA$^-$ phenotype is shown in Table 2.

MR-1 contains a second *etrA* analog. Our finding that *etrA* is not involved in nitrate or Mn(IV) reduction implies the presence of yet another gene that controls the expression of these two reductases. MR-1 DNA was digested with several enzymes and hybridized to the 1.5-kb *Pst*I-*Sst*I fragment that contains *etrA*. Two signals were obtained in each enzyme digestion, suggesting the presence of another *etrA* analog (Fig. 5); its function, structure, and involvement in regulation of anaerobic respiration are yet to be determined. Similar experiments using the internal 165-bp *Nco*I-*Dra*I fragment of *etrA* as a probe yielded identical results. The expected size bands containing *etrA* were obtained in each case except when the DNA was digested with *Hin*dIII. The *Hin*dIII fragment that has *etrA* is 3 kb in length (Fig. 1A), but the chromosomal band that hybridizes corresponds to 4 kb in length. One possible explanation for this apparent inconsistency is the inability of the enzyme to recognize all of the *Hin*dIII sites because of DNA modification. It should be noted that there is another *Hin*dIII site 1.2 kb farther downstream which would result in a 4.2-kb fragment if the preceding site was not recognized.

TABLE 2. Growth and electron acceptor reduction by METR-1

Electron acceptor	Growth/reduction
Oxygen	+/+
Nitrate	+/+
Manganese	+/+
Iron	−/+
Nitrite	−/−
Fumarate	−/ND[a]
Thiosulfate	−/−
TMAO	−/−
DMSO	−/−
Sulfite	−/ND

[a] ND, not determined.

DISCUSSION

S. putrefaciens can couple its respiratory growth to more than 10 different terminal electron acceptors. The isolation of pleiotropic mutants deficient in anaerobic growth (7, 28) led us to believe that MR-1 must have a regulator similar in function to the genes identified in *E. coli* (*fnr*) and other organisms, including *Pseudomonas* (11), *Actinobacillus* (21), and *Rhizobium* (3, 4) species. Using the *E. coli fnr* as a probe, we were able to isolate a gene, *etrA*, that shares a high degree of similarity with *fnr*. The existence of the gene in *S. putrefaciens* is not particularly surprising, since members of the *S. putrefaciens* group are phylogenetically related to *E. coli*, being members of the gamma group of the proteobacteria (28a). The

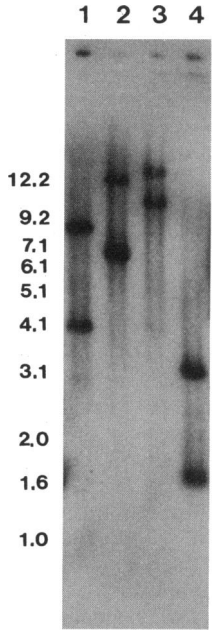

FIG. 5. Autoradiograph of MR-1 DNA digested with *Hin*dIII, *Bam*HI, *Eco*RI, and *Pst*I (lanes 1 to 4, respectively), hybridized to the 1.5-kb *Pst*I-*Sst*I *etrA*-containing fragment. In each case, two bands with strong hybridization signals are seen.

inferred amino acid sequence, designated EtrA, is 73.6, 50.8, and 67% identical to the sequences of Fnr, ANR, and HlyX, respectively. Given this high degree of structural similarity to Fnr, it is perhaps not surprising that *etrA* complemented an *E. coli fnr* mutant strain. The DNA-binding domains of the two proteins are virtually identical, suggesting that they recognize similar, if not identical sequences, consistent again with the ability of *etrA* to complement the *fnr* mutant of *E. coli*. Unlike the case for *fnr*, however, the upstream *etrA* sequence lacks the Fnr consensus sequence, or Fnr box, to which the protein binds and negatively regulates its own synthesis. The absence of such a sequence suggests that EtrA is not involved in autoregulation.

To assess the function of EtrA, *etrA* mutants were generated by gene replacement and tested for the ability to grow anaerobically with the various terminal electron acceptors. The *etrA* mutant strain, METR-1, was unable to grow anaerobically with Fe(III), thiosulfate, or sulfite. Further, METR-1 is not capable of anaerobic growth on TMAO, DMSO, nitrite, or fumarate, the reduction of which is also regulated by Fnr in *E. coli.*

Although METR-1 was incapable of anaerobic growth with Fe(III) as the terminal electron acceptor, it was able to reduce it at levels similar to those found for the wild type. Studies of *S. putrefaciens* sp200 indicated the presence of two different iron reductases, one constitutive and the other induced by growth under oxygen-limiting conditions (1, 2). The presence of two iron reductase systems in *S. putrefaciens* may explain the phenotype of METR-1 with regard to Fe(III) reduction. One possibility is that the induced Fe(III) reductase is controlled by EtrA. Since the constitutive Fe(III) reductase activity is located farther downstream along the electron transport chain than the inducible enzyme is (2), cells containing the constitutive, but not the induced Fe(III) reductase may be incapable of anaerobic growth with Fe(III) even though they can reduce it. The situation with iron may be more complex, and the dissimilatory (inducible) iron reductase may be under dual control of *etrA* and another, so far unidentified gene, as is known for other anaerobic genes such as the pyruvate formate lyase (32, 33) or formate hydrogen lyase (36) gene, both of which are controlled by more than one global regulator.

EtrA does not appear to be involved in the regulation of either nitrate or manganese reduction, as evidenced by the ability of the mutants to both reduce the electron acceptors and exhibit growth. Manganese and nitrate have previously been shown to compete with each other as electron acceptors (22), while no such competition was observed with regard to manganese and fumarate. These data are consistent with the results presented here and may suggest that a separate regulatory system exists for the control of nitrate and manganese reductases. We cannot rule out the possibility, however, that *etrA* controls a respiratory chain component, such as a menaquinone, in common to most respiratory systems, but not manganese or nitrate. Further experiments are needed to determine the level at which *etrA* regulates anaerobic respiration in *S. putrefaciens.*

Southern hybridization experiments revealed the presence of another *etrA* analog in MR-1. This second gene gives a strong hybridization signal, indicating that it is quite similar to *etrA*. The gene replacement experiments described here, however, did not appear to affect this second gene, and the phenotypes obtained were solely due to the inactivation of *etrA*. The function of this *etrA* analog is not yet known; one possibility is a role in regulating both nitrate and Mn(IV) reduction. Having two very similar regulators may present the cell with unusual problems, and it is of great interest and importance to elucidate the structure and function of this gene, which is the focus of ongoing research in our laboratory.

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REFERENCES

1. **Arnold, R., T. DiChristina, and M. Hoffman.** 1986. Inhibitor studies of dissimilative Fe(III) reduction by *Pseudomonas* sp. 200 ("*Pseudomonas ferrireductans*"). Appl. Environ. Microbiol. **52:** 281–289.
2. **Arnold, R., M. Hoffman, T. DiChristina, and F. Picardal.** 1990. Regulation of dissimilative Fe(III) reduction in *Shewanella putrefaciens*. Appl. Environ. Microbiol. **56:**2811–2817.
3. **Batut, J., M. L. Daveran-Mignot, M. David, A. M. Garnerone, and D. Kahn.** 1989. *fixK*, a gene homologous with *fnr* and *crp* from *Escherichia coli*, regulates nitrogen fixation genes both positively and negatively in *Rhizobium meliloti*. EMBO J. **8:**1279–1286.
4. **Colonna-Romano, S., W. Arnold, A. Schluter, P. Boistard, A. Puhler, and U. Priefer.** 1990. An Fnr-like protein encoded in *Rhizobium leguminosarum* biovar viciae shows structural and functional homology to *Rhizobium meliloti* FixK. Mol. Gen. Genet. **223:**138–147.
5. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
6. **DiChristina, T.** 1992. Effects of nitrate and nitrite on dissimilatory iron reduction by *Shewanella putrefaciens* 200. J. Bacteriol. **174:** 1891–1896.

6a. **DiChristina, T.** Personal communication.

7. **DiChristina, T., and E. DeLong.** 1992. Isolation and characterization of iron-reduction mutants of *Shewanella putrefaciens* strain 200, abstr. K-138, p. 279. Abstr. 92nd Gen. Meet. Am. Soc. Microbiol. 1992.
8. **DiChristina, T., M. Hoffman, R. Arnold, and M. Lidstrom.** 1988. Dissimilative Fe(III) reduction by the marine eubacterium *Alteromonas putrefaciens* 200. Water Sci. Technol. **20:**69–79.
9. **Eiglmeier, P., K. Nallonore, S. Iuchi, E. C. C. Lin, and S. T. Cole.** 1989. Molecular genetic analysis of FNR-dependent promoters. Mol. Microbiol. **3:**869–878.
10. **Feng, D. F., and R. F. Doolittle.** 1984. Progressive sequence alignment as a prerequisite to correct phylogenetic trees. J. Mol. Evol. **25:**351–360.
11. **Galimand, M., M. Gamper, A. Zimmermann, and D. Haas.** 1991. Positive Fnr-Like control of anaerobic arginine degradation and nitrate respiration in *Pseudomonas aeruginosa*. J. Bacteriol. **173:** 1598–1606.
12. **Green, J., M. Trageser, S. Six, G. Unden, and J. R. Guest.** 1991. Characterization of the Fnr protein of *Escherichia coli*, and iron-binding transcriptional regulator. Proc. R. Soc. London Ser. B **244:**137–144.
13. **Guest, J. R.** 1992. Oxygen-regulated gene expression in *Escherichia coli*. J. Gen. Microbiol. **138:**2253–2263.
14. **Gunsalus, R. P.** 1992. Control of electron flow in *Escherichia coli*: coordinated transcription of respiratory pathway genes. J. Bacteriol. **174:**7069–7074.
15. **Ikamura, T., and J. E. Dahlberg.** 1973. Small ribonucleic acids of *Escherichia coli*. I. Characterization by polyacrylamide gel electrophoresis and fingerprint analysis. J. Biol. Chem. **248:**5024–5033.
16. **Iuchi, S., D. C. Cameron, and E. C. C. Lin.** 1989. A second global regulator gene (*arcB*) mediating repression of enzymes in aerobic pathways in *Escherichia coli*. J. Bacteriol. **171:**868–873.
17. **Iuchi, S., and E. C. C. Lin.** 1988. arcA (dye), a global regulatory gene in *Escherichia coli* mediating repression of enzymes in aerobic pathways. Proc. Natl. Acad. Sci. USA **85:**1888–1892.
18. **Kiley, P. J., and W. S. Reznikoff.** 1991. *fnr* mutants that activate gene expression in the presence of oxygen. J. Bacteriol. **173:**16–22.
19. **Koneman, E., S. D. Allen, V. R. Dowell, Jr., and H. M. Sommers.**

1983. Diagnostic microbiology, 2nd ed. J. B. Lippincott Co., New York.

19a. **Lisser, S., and H. Margalit.** 1993. Compilation of *E. coli* mRNA promoter sequences. Nucleic Acids Res. **21:**1507–1516.

20. **MacDonell, M. T., and R. R. Colwell.** 1985. Phylogeny of the Vibrionaceae, and recommendation for two new genera, *Listonella* and *Shewanella*. Syst. Appl. Microbiol. **6:**171–182.

21. **MacInnes, J. I., T. E. Kim, C.-J. Lian, and G. A. Soltes.** 1990. *Actinobacillus pleuropneumoniae hlyX* gene homology with the *fnr* gene of *Escherichia coli*. J. Bacteriol. **172:**4587–4592.

22. **Myers, C., and K. Nealson.** 1988. Bacterial manganese reduction and growth with manganese oxide as the sole electron acceptor. Science **240:**1319–1321.

23. **Myers, C., and K. Nealson.** 1990. Respiration-linked proton translocation coupled to anaerobic reduction of Mn(IV) and Fe(III) in *S. putrefaciens* MR-1. J. Bacteriol. **172:**6232–6238.

23a. **Nealson, K.** Unpublished data.

24. **Nealson, K., D. Saffarini, and D. Moser.** Anaerobic respiration of *Shewanella putrefaciens*: potential use of solid electron acceptors for pollutant removal and bioremediation in anoxic environments. *In* M. Deluca (ed.), Bioremediation of contaminants in soils and sediments, in press. Rutgers University Press, New Brunswick, N.J.

25. **Obuekwe, C., and W. Westlake.** 1982. Effect of reducible compounds (potential electron acceptors) on reduction of ferric iron by *Pseudomonas species*. Microbios Lett. **19:**57–62.

26. **Obuekwe, C., W. Westlake, and F. Cook.** 1981. Effect of nitrate on reduction of ferric iron by a bacterium isolated from crude oil. Can. J. Microbiol. **27:**692–697.

27. **Perry, K., J. Kostka, G. W. Luther III, and K. H. Nealson.** 1993. Mediation of sulfur speciation by a Black Sea facultative anaerobe. Science **259:**801–803.

28. **Saffarini, D., D. Bermudes, and K. Nealson.** 1992. Isolation and localization of anaerobic regulatory gene(s) of *Shewanella putrefaciens* MR-1, abstr. K-137, p. 279. Abstr. 92nd Gen. Meet. Am. Soc. Microbiol. 1992.

28a. **Saffarini, D., E. DeLong, and K. Nealson.** Unpublished data.

29. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

30. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.

31. **Sawers, R. G.** 1991. Identification and molecular characterization of a transcriptional regulator from *Pseudomonas aeruginosa* PAO1 exhibiting structural and functional similarity to the Fnr protein of *Escherichia coli*. Mol. Microbiol. **5:**1469–1481.

32. **Sawers, R. G., and A. Bröck.** 1989. Novel transcriptional control of the pyruvate formate lyase gene: upstream regulatory sequences and multiple promoters regulate anaerobic expression. J. Bacteriol. **171:**2485–2492.

33. **Sawers, R. G., and B. Suppmann.** 1992. Anaerobic induction of pyruvate formate-lyase gene expression is mediated by the ArcA and Fnr proteins. J. Bacteriol. **174:**3474–3478.

34. **Spiro, S., and J. R. Guest.** 1987. Regulation and over-expression of the fnr gene of *Escherichia coli*. J. Gen. Microbiol. **133:**3279–3288.

35. **Spiro, S., and J. R. Guest.** 1990. FNR and its role in oxygen regulated gene expression in *Escherichia coli*. FEMS Microbiol. Rev. **75:**399–428.

36. **Stewart, V., and B. L. Berg.** 1988. Influence of *nar* genes on nitrate inhibition of formate-hydrogen lyase and fumarate reductase gene expression in *Escherichia coli* K-12. J. Bacteriol. **170:**4437–4444.

37. **Strauch, K. L., J. B. Lenk, B. L. Gamble, and C. G. Miller.** 1985. Oxygen regulation in *Salmonella typhimurium*. J. Bacteriol. **161:** 673–680.

38. **Trageser, M., and G. Unden.** 1989. Role of cysteine residues and of metal ions in the regulatory functioning of Fnr, the transcriptional regulator of anaerobic respiration in *Escherichia coli*. Mol. Microbiol. **3:**593–599.

39. **Winkelman, J. W., and D. P. Clark.** 1986. Anaerobically induced genes of *Escherichia coli*. J. Bacteriol. **167:**362–367.

40. **Zimmerman, A., C. Reimmann, and D. Haas.** 1991. Anaerobic growth and cyanide synthesis of *Pseudomonas aeruginosa* depend on *anr*, a regulatory gene homologous with *fnr* of *Escherichia coli*. Mol. Microbiol. **5:**1483–1490.