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Interruption of the Ferredoxin (Flavodoxin) NADP$^+$ Oxidoreductase Gene of *Escherichia coli* Does Not Affect Anaerobic Growth but Increases Sensitivity to Paraquat

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Ferredoxin (flavodoxin) NADP$^+$ oxidoreductase participates in methionine biosynthesis and in the function of two anaerobic enzymes, pyruvate formate-lyase and ribonucleotide reductase. We prepared insertion mutants of *Escherichia coli* lacking a functional enzyme. They do not require methionine and they grow well anaerobically, but they show increased sensitivity to paraquat.

Proteins with ferredoxin (flavodoxin) NADP$^+$ oxidoreductase (FNR) activity are present in microorganisms, plants, and animals. One speaks often of a ferredoxin reductase family to describe proteins or polypeptide sequences with FNR activity (1, 13). They participate in the electron transport between NADP(H) and reduced or oxidized ferredoxin (flavodoxin). The reduced redoxins subsequently participate in a variety of metabolic reactions, many of which involve reduction of an iron center.

In *Escherichia coli*, the gene for FNR (named *fpr*) was cloned only recently during a project concerning anaerobic deoxyribonucleotide synthesis (4). The protein was first discovered as an enzyme required for the activation of the cobalamine-dependent methionine synthase of *E. coli* (2, 10). It was subsequently shown also to be required for the activation of pyruvate formate-lyase (5), a reaction that generates a glycyl radical in the inactive enzyme. Finally, this enzyme was also found to participate in the generation of the glycyl radical of the anaerobic ribonucleotide reductase (9).

Thus, in *E. coli*, FNR participates in the synthesis of methionine, dissimilation of pyruvate, and synthesis of deoxyribonucleotides. The latter two reactions are anaerobic processes. In all cases, FNR functions together with flavodoxin, but not ferredoxin, in the transfer of electrons from NADPH to an acceptor.

An additional possible function for *E. coli* FNR arises from the recent demonstration (14) that synthesis of the enzyme is under control of the *soxRS* regulon, which is seen as an adaptive response against superoxide (8). FNR might therefore also be involved in the protection of the bacteria from damage by oxygen radicals.

Here, we describe the construction of insertion mutants of *E. coli* that have lost the ability to synthesize FNR and characterize their phenotype.

Construction of bacterial strains lacking flavodoxin reductase. The coding sequence of *fpr* carried by plasmid pEE1010 (4) was interrupted by inserting the 1.3-kb Kanamycin Resistance Gene Block (Pharmacia) in the unique *Afl*II site located at nucleotide 437 of *fpr*. The DNA methods used were those of Sambrook et al. (18). Restriction with *Xba*I and *Xho*I was used to assess the orientation of the Kan^r marker relative to *fpr*. Plasmid pEE1012, carrying *fpr* and the kanamycin resistance marker in the same orientation, was cleaved with *Sac*I and *Sph*I to obtain a 2.2-kb fragment encompassing the interrupted *fpr* gene. This fragment was ligated to plasmid pGP704 (12) cut with *Sac*I and *Sph*I to yield plasmid pEE1013 and transformed into *E. coli* UA 4856 (6). Plasmid pGP704 (12) is a plasmid with general suicide delivery properties carrying a conjugal transfer (Mob) sequence and *ori* R6K which confers *pir*-dependent replication. Minipreps of plasmids obtained from Kan^r Amp^r transformants were checked by cutting with *Bam*HI and with *Xho*I. Using the mobilizing strain *E. coli* S17-1(lambda *pir*) (7, 20), we transferred the recombinant suicide plasmid pEE1013 by conjugation into C-6006, a Rif^r strain isolated by us from the prototrophic *E. coli* C-1a (19) by selection with 100 μg of rifampin per ml. Kanamycin-resistant exconjugants were screened for loss of vector-mediated ampicillin resistance.

The genotype of the presumptive *fpr*-inactivated clones was assessed by PCR analysis (Fig. 1). The synthetic oligonucleotides used as primers were obtained from Scandinavian Gene Synthesis AB (Köping, Sweden). They were 5′-CGGAGAAC GAAGATAAGGC-3′ (primer 1 [4]) and 5′-GTGGATTGC CGCACAGCATC-3′ (primer 6), both complementary to *fpr*, and 5′-ATGGGCTCGCGATAATGTCG-3′ (primer F) and 5′-TCTGCGATTCCGACTCGTCC-3′ (primer R), both complementary to the kanamycin resistance marker from transposon Tn*903* (17) (Fig. 1A). PCR conditions were those described previously (4). Amplification with primers 1 and 6 produces a fragment 690 nucleotides long from the wild-type gene and 1,990 nucleotides long from the interrupted sequence. Only one short fragment was expected from the DNA of wild-type bacteria and only one long fragment was expected from mutant DNA, while fragments of both sizes would indicate that the bacteria contained both the wild-type and mutated forms of *fpr*. Of 69 clones analyzed, 2 clones, C-6007 and C-6008, gave rise to only one long amplification product (Fig. 1B), while the others produced two bands (not shown). The presence of the kanamycin resistance marker interrupting *fpr* in C-6007 is demonstrated by the PCR fragments obtained with the two combinations of *fpr* and kanamycin resistance marker primers, primers F-6 (1,240 nucleotides) and 1-R (1,294 nucleotides) (Fig. 1B, lanes 5 and 6). Moreover, Fig. 1B shows that amplification with primers F and 6 yielded the same product from the genomic DNA of C-6007 and from plasmid pEE1012, which carries the mutated *fpr* gene.

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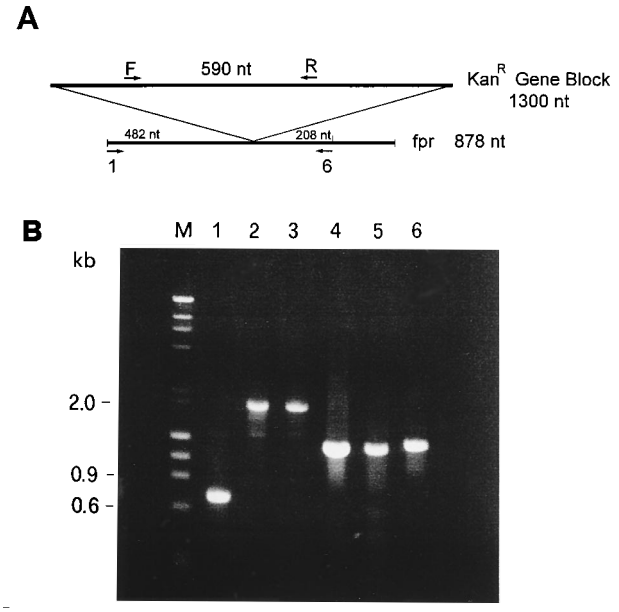

FIG. 1. PCR analysis of the structure of the *fpr* gene in the *fpr*-deficient mutants. (A) Scheme of the relative positions of primers 1 and 6, complementary to *fpr*, and primers F and R, complementary to the coding sequence of the enzyme aminoglycoside 3′-phosphotransferase (Kan^r marker) (17) inserted at the *Afl*II site of *fpr*. (B) Genomic DNAs of wild-type bacteria (lane 1) and clones C-6007 and C-6008 (lanes 2 and 3) were used as templates in PCRs with primers 1 and 6. Both mutants yielded only one amplification product about 2 kb long, while amplification of wild-type DNA yielded the expected 690-nucleotide fragment. Lanes 4 and 5, primers F and 6 yielded the same PCR product from plasmid pEE1012 and genomic DNA of C-6007. Lane 6, mutant C-6007 DNA amplified with primers 1 and R. M, markers; nt, nucleotides.

Purification of FNR. The flavodoxin reductase used in our experiments was prepared from *E. coli* C-1a carrying plasmid pEE1010 (4). The bacteria were grown in 20 liters of Luria-Bertani medium containing 0.2% glucose and ampicillin (50 μg/ml) overnight, and 110 g of bacteria pelleted from the culture medium was collected by centrifugation. A portion (44 g) was extracted with lysozyme (9) and centrifuged. The clear supernatant (67 ml containing 1.71 g of protein) was passed through an 800-ml column of Sephadex G-25 equilibrated with 20 mM Tris-HCl (pH 7.5)–2 mM dithiothreitol–1 mM phenylmethylsulfonyl fluoride. When assayed by complementation of ribonucleotide reductase activity, the solution contained a total of 97,000 U of FNR activity, with a specific enzyme activity of 55. The material was then adsorbed to a 130-ml column of DEAE-Sepharose C1-6B (Pharmacia) equilibrated with 20 mM Tris-HCl, pH 7.5. Elution was made with a linear gradient of 1,600 ml each of 20 mM Tris-HCl, pH 7.5, and 0.2 mM KCl in 20 mM Tris-HCl, pH 7.5. FNR was eluted as a sharp yellow peak at 0.1 M KCl. The material (120 mg of protein; specific activity, 1,200) was concentrated to 2 ml in Centricon 10 tubes and chromatographed on a 320-ml Superdex-75 column which was equilibrated with 50 mM Tris-HCl (pH 7.5)–0.2 M KCl and attached to fast protein liquid chromatography equipment (Pharmacia). The strongly yellow-colored FNR peak eluted after 182 ml. After concentration in a Centricon 10 tube, 45 mg of FNR with an estimated purity of at least 80% was recovered. This material had a specific activity of 4,700 and was used for most experiments. Part of the material was further purified for antibody preparation. Solid ammonium sulfate (7.9 g) was dissolved in 1.8 ml of a solution containing 15 mg of FNR. The material was adsorbed to a 15-ml phenyl-Sepharose column. The column was first washed with 1.0 M ammonium sulfate, and FNR (9.9 mg) was subsequently eluted with 0.5 M ammonium sulfate. The solution was concentrated in a Centricon 10 tube and freed of salt by washing with 50 mM Tris-HCl, pH 7.5. This preparation of FNR appeared completely homogeneous by sodium dodecyl sulfate gel electrophoresis and was used to produce rabbit immune serum according to standard procedures.

Immunoreactive FNR is not present in extracts from bacterial mutants. Extracts from the two bacterial mutants C-6007 and C-6008 that contained only the interrupted *fpr* gene were analyzed by Western blotting (immunoblotting), together with extracts from the parent C-1a strain and from C-6009, a strain containing both the normal and the interrupted gene (data not shown). The blots clearly showed that the FNR band at 29 kDa present in the C-1a extract and in C-6009 was absent from the two mutant extracts. Extracts from bacteria harboring plasmid pEE1010 which contains the intact FNR gene gave a very strong 29-kDa band.

Growth characteristics of mutant bacterial strains. A comparison of the ability to form colonies on Luria-Bertani plates after serial dilution showed no difference between the parent C-1a strain and the two mutant strains, under both aerobic and anaerobic conditions. Also, during growth in liquid Luria-Bertani medium containing 0.2% glucose, the three strains showed identical growth rates at 37°C in air or during continuous nitrogen bubbling. These results show that the loss of *fpr* had no effect on anaerobic growth. Finally, mutants and parent strains grew equally well on plates prepared with minimal medium, demonstrating that the mutant strains did not require methionine for growth.

Extracts from mutant bacteria complement the enzymatic activation of the anaerobic ribonucleotide reductase. Reduced flavodoxin is required for the activation of both the anaerobic ribonucleotide reductase and pyruvate formate-lyase. Apparently, an enzyme activity different from FNR can reduce flavodoxin in the mutant strains, since they grew well under anaerobic conditions. We tested the ability of the crude extract of one mutant to substitute for FNR in the ribonucleotide reductase assay (3, 9). With the purified system used here, CTP reduction measures FNR activity. Reactions were run in small tubes connected to an anaerobic manifold, continuously flushed with moist argon. In a first step, ribonucleotide reductase was activated anaerobically with the activating system for 60 min; in the second step, the substrate mixture was added anaerobically, CTP reduction proceeded during 20 min (9), and the amount of dCTP formed was determined. One unit of activity is 1 nmol of dCTP formed per min. Specific activity is expressed as units per milligram of protein. The activating system contained, in a volume of 40 μl, 24 mM Tris-HCl (pH 8.0), 24 mM KCl, 0.3 mM *S*-adenosylmethionine, 1.0 mM NADPH, 4 mM dithiothreitol, 1 μg of ribonucleotide reductase, 1 μg of flavodoxin, 0.4 μg of activase (21), and the unknown amount of FNR. The substrate mixture contained, in a volume of 10 μl, 5 mM [^{3}H]CTP, 1 mM ATP, and 50 mM $MgCl_2$.

We found that an extract from *fpr* mutant bacteria was approximately one third as active as a wild-type extract or an extract from the overproducing strain carrying pEE1010 (Fig. 2A). When the bacterial extracts were dialyzed before testing, the mutant extract became inactive, whereas the other two extracts retained a good part of their activity (Fig. 2B). In the mutant extract, the ability to replace FNR was lost after dialysis, suggesting an involvement of low-molecular-weight components.

We have not attempted to identify the nature of the second

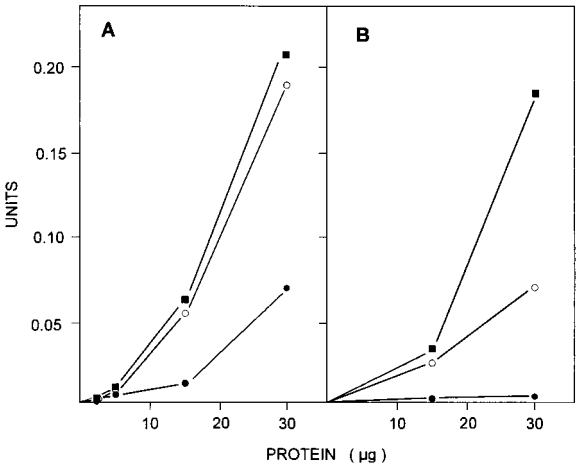

FIG. 2. Complementation of ribonucleotide reduction with bacterial extracts before (A) and after (B) dialysis. ○, wild-type C-1a; ■, C-1a(pEE1010); ●, C-6007. Various amounts of the different extracts were substituted for FNR in the assay for CTP reduction by pure anaerobic ribonucleotide reductase.

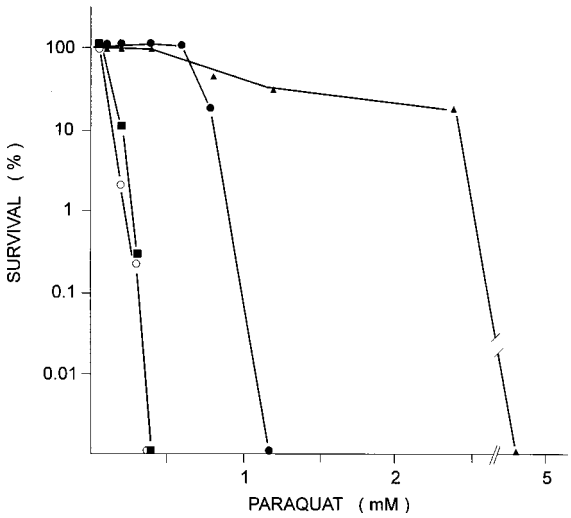

FIG. 3. Paraquat survival curves of different bacterial strains. Cells were spread on Luria-Bertani agar medium containing the indicated concentrations of paraquat, plus 50 μg of kanamycin per ml in the case of the *fpr*-deficient Kan^r mutants or 50 μg of ampicillin per ml in the case of the overproducer strain C-1a carrying pEE1010. ●, wild-type C-1a; ■, C-6007; ○, C-6008; ▲, overproducer strain.

flavodoxin reductase but consider it highly likely that the activity depends on the presence of pyruvate:ferredoxin (flavodoxin) oxidoreductase in the *E. coli* extract. The presence of this enzyme in *E. coli* and its ability to substitute for FNR during activation of pyruvate formate-lyase was discovered by Blaschkowski et al. (5). For its activity the enzyme requires pyruvate, acetyl coenzyme A, and thiamine diphosphate, cofactors that are lost during dialysis.

***E. coli* lacking FNR shows increased sensitivity to paraquat.** The demonstration that FNR is under the control of the *soxRS* regulon (14) suggested the possibility that FNR participates in the bacterial defense against superoxide radicals. We therefore compared the killing action of paraquat with the two mutant strains, the parent strain and a strain overproducing FNR. Paraquat is highly toxic to cells, since it increases the intracellular superoxide concentration (11, 14). In preliminary experiments with paraquat concentrations ranging between 0.1 and 5.4 mM, we found that the inactivation of *fpr* increased the sensitivity of the cells to paraquat about threefold after overnight incubation at 30°C in liquid culture. The results of the plating experiments are shown in Fig. 3. Appropriate dilutions of saturated cultures were inoculated on LA (Luria-Bertani medium plus agar) plates containing 0.1 to 4.8 mM paraquat and 50 μg of kanamycin per ml, in the case of mutant strains C-6007 and C-6008, or 50 μg of ampicillin per ml in the case of the flavodoxin reductase overproducer C-1a strain carrying pEE1010. The number of colonies recovered was corrected for the plating efficiencies in the absence of paraquat. The curves in Fig. 3 look like multihit survival curves (15), which indicates that killing was produced by the joint action of one or more events. The shift of the toxicity threshold indicates that the sensitivity of the two mutant strains was increased approximately fivefold, whereas the overproducer was considerably more resistant than the wild-type strain.

The only phenotypic change in the mutants observed by us was their increased sensitivity to paraquat. FNR is one of the three known paraquat diaphorases (11, 14), the other two being thioredoxin reductase (14) and sulfite reductase (11). The diaphorases have different implications for paraquat toxicity. For sulfite reductase, it was recently shown (11) that paraquat sensitivity was decreased in a bacterial strain devoid of this enzyme. In that case, it appears that the diaphorase increases the toxicity of paraquat by generating oxygen radicals. The reverse is true for FNR: the enzyme seems to protect cells from oxidative stress, and bacteria lacking the enzyme show increased sensitivity to the drug.

A paraquat-sensitive phenotype was described previously for *E. coli* and attributed to a mutant *mvrA* gene (16). However, two independent lines of evidence disprove the original claim for the existence of such a gene. The DNA sequence attributed to *mvrA* was found to belong to two consecutive genes located at 88 min on the *E. coli* chromosome (4, 22). The putative 5′ 410 nucleotides of *mvrA* coincide with the end of *glpX* (22), while the 5′ portion of the *fpr* gene covers the 3′ region of *mvrA* (4). Several mistakes in the sequence attributed to *mvrA* caused the misreading of the stop codon of *glpX* (22) and the premature stop within the *fpr* open reading frame (4). This situation already created some confusion in the literature when the deduced amino acid sequence of *mvrA* and the N-terminal amino acid sequence of *E. coli* flavodoxin reductase were compared as separate entities with the N-terminal end of *E. coli* paraquat:NADP diaphorase (14). We suggest that the database be modified.

The increased sensitivity of the *fpr* mutants to paraquat agrees with the finding that paraquat induces the synthesis of FNR activity and that this process belongs to the *soxRS* regulon (14). The mechanism for the protective effect of FNR remains to be elucidated.

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