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Flavodoxin Mutants of *Escherichia coli* K-12

PHILIPPE GAUDU AND BERNARD WEISS*

Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, Georgia 30322

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The flavodoxins are flavin mononucleotide-containing electron transferases. Flavodoxin I has been presumed to be the only flavodoxin of *Escherichia coli*, and its gene, *fldA*, is known to belong to the *soxRS* (superoxide response) oxidative stress regulon. An insertion mutation of *fldA* was constructed and was lethal under both aerobic and anaerobic conditions; only cells that also had an intact ($fldA^+$) allele could carry it. A second flavodoxin, flavodoxin II, was postulated, based on the sequence of its gene, *fldB*. Unlike the *fldA* mutant, an *fldB* insertion mutant is a viable prototroph in the presence or absence of oxygen. A high-copy-number $fldB^+$ plasmid did not complement the *fldA* mutation. Therefore, there must be a vital function for which FldB cannot substitute for flavodoxin I. An *fldB-lacZ* fusion was not induced by H_2O_2 and is therefore not a member of the *oxyR* regulon. However, it displayed a *soxS*-dependent induction by paraquat (methyl viologen), and the *fldB* gene is preceded by two overlapping regions that resemble known *soxS* binding sites. The *fldB* insertion mutant did not have an increased sensitivity to the effects of paraquat on either cellular viability or the expression of a *soxS-lacZ* fusion. Therefore, *fldB* is a new member of the *soxRS* (superoxide response) regulon, a group of genes that is induced primarily by univalent oxidants and redox cycling compounds. However, the reactions in which flavodoxin II participates and its role during oxidative stress are unknown.

Flavodoxins are small flavin mononucleotide (FMN)-containing electron transferases that are found in bacteria and algae. In *Escherichia coli*, a flavodoxin is required for the reductive activation of cobalamin-dependent methionine synthase (31), for biotin synthesis (34), and for the anaerobic activation of both ribonucleoside triphosphate reductase (8, 9) and pyruvate formate-lyase (36) through the formation of glycyl free radicals at their active centers. In nitrogen-fixing bacteria, a flavodoxin is the electron donor for the Fe-containing protein of the nitrogenase complex (16). A flavodoxin can often substitute for a ferredoxin, a small electron transfer protein with an iron-sulfur center (reviewed in reference 31). Thus, both a flavodoxin and a ferredoxin are substrates for the NADPH:ferredoxin oxidoreductases of cyanobacteria and of *E. coli*, for the pyruvate-ferredoxin oxidoreductase of *Clostridium pasteurianum*, and for the enzymes of dissimilatory sulfate reduction. In iron-poor media, where we should expect a ferredoxin deficiency, the flavodoxins of cyanobacteria and *Anacystis nidulans* are induced.

Most of the reactions of *E. coli* flavodoxin were demonstrated in vitro with purified flavodoxin I, the product of the cloned *fldA* gene, which was presumed to be the only flavodoxin of *E. coli*. However, a putative second flavodoxin gene, *fldB*, was discovered during the sequencing of the unrelated neighboring *xerD* gene (GenBank accession no. AE000373 [F. R. Blattner] and Z48060 [F. Hayes]), which encodes a site-specific recombinase. The deduced structure of flavodoxin II (FldB) has 43% identity with *E. coli* flavodoxin I (FldA), and it has the characteristic flavodoxin signature, an FMN binding motif near its N terminus.

This study of the flavodoxins was prompted by our interest in the *soxRS* (superoxide response) regulon because it includes the genes for flavodoxin I (*fldA*) (49) and for NADPH:ferredoxin (flavodoxin) oxidoreductase (*fpr*) (27). The *soxRS* regulon (17, 44) responds to the oxidative stress produced by redox agents that engage mainly in one-electron transfers, agents such as superoxide, nitric oxide, and paraquat (methyl viologen). The sensor for the regulon resides in the [2Fe-2S] centers of the ferredoxin-like SoxR protein. When these centers are oxidized, SoxR becomes a transcriptional activator of *soxS*, and the newly synthesized SoxS protein (itself a transcriptional activator) then induces other genes of the regulon. In the uninduced cell, SoxR is mainly in its inactive, reduced form, and because it is auto-oxidizable, it must be continually reduced. Through separate NADPH-linked reductases, both SoxR (23) and flavodoxin (11) are in redox equilibrium with $NADP^+$/NADPH. Depletion of NADPH, e.g., during the production of superoxide by the redox cycling of paraquat, activates the regulon. FldA and Fpr may be induced to help restore the redox balance of the oxidatively stressed cell (27, 28).

In this study, we produce insertion mutations of *fldA* and *fldB* and we generate an *fldB-lacZ* gene fusion. These constructs are then used to approach the following questions. What are the phenotypes of the mutants? Is either gene essential? Do flavodoxins I and II have the same functions? Is *fldB* a member of the *soxRS* regulon? Does flavodoxin II have a discernible role in protection against oxidative stress?

MATERIALS AND METHODS

Nomenclature. *cat*, *tet*, and *bla* refer to plasmid- or transposon-derived DNA segments specifying resistance to chloramphenicol (Cam^r), tetracycline (Tet^r), and ampicillin or carbenicillin (Amp^r), respectively. λ*attP* and *att*λ are the preferred DNA sequences in phage λ and *E. coli*, respectively, at which site-specific integration of phage λ occurs.

Strains and plasmids. Bacterial strains and plasmids used are listed in Table 1. Some of the plasmid constructions are detailed in Fig. 1 and 2. Insertions into *att*λ were performed as previously described (18), except that a $recA^+$ strain was used because it was a better donor for subsequent transductions. The *att*λ::($fldA^+$ bla^+) element of BW1527 was prepared from plasmid pWB52 (Table 1), which had been cut with *Not*I to yield a bla^+-$fldA^+$-λ*attP* fragment. This DNA was then circularized by ligation and used to transform strain GC4468(pLDR8) to carbenicillin resistance at 42°C. The cells were grown in Luria-Bertani (LB) broth at 37°C for 1.5 h to allow gene expression before selection. pLDR8 specifies a thermoinducible λ integrase that mediated the insertion of the bla^+-$fldA^+$ segment into the *att*λ site. BW1527 was Kan^s, indicating that it had been cured of the temperature-sensitive helper plasmid. The *att*λ::(Φ[*soxS′*-*′lacZ*] bla^+) element of BW1157 was constructed similarly. λRZ5::*fldB* was produced by growing λRZ5 on a strain carrying plasmid pWB51; the lysate was used to infect Δ*lac* strain

* Corresponding author. Mailing address: Glenn Memorial Building, 69 Butler St., S.E., Atlanta, GA 30303. Phone: (404) 616-0602. Fax: (404) 616-7455. E-mail: bweiss2@emory.edu.

TABLE 1. Bacterial strains, phages, and plasmids used

Strain or plasmid	Description[a]	Reference or source[b]
E. coli strains		
BW831	GC4468 *soxS3*::Tn*10*	48
BW1157	GC4468 *att*λ::(Φ[*soxS′*-*′lacZY*] *bla*$^{+}$); circularized *Not*I fragment of pWB53 integrated with the help of pLDR8	This study
BW1485	JC7623 *fldB*::*tet*	pPG5 × JC7623
BW1526	KL16 *att*λ::(*fldA*$^{+}$ *bla*$^{+}$)	P1(BW1527) × KL16
BW1527	GC4468 *att*λ::(*fldA*$^{+}$ *bla*$^{+}$)	See Materials and Methods
BW1528	JC7623 *att*λ::(*fldA*$^{+}$ *bla*$^{+}$)	P1(BW1527) × GC4468
BW1529	JC7623 *fldA*::*cat att*λ::(*fldA*$^{+}$ *bla*$^{+}$)	pPG1::*cat* × BW1524
BW1530	JC7623 *fldA*::*cat zbf-3057*::Tn*10 att*λ::(*fldA*$^{+}$ *bla*$^{+}$)	P1(CAG18433) × BW1529
BW1531	W3110 *fldB*::*tet*	P1(BW1485) × W3110
BW1534	BW1157 *fldB*::*tet*	P1(BW1485) × BW1157
BW1535	GC4468(λRZ5::*fldB*)*soxS3*::Tn*10*	P1(BW831) × GC4468(λRZ5::*fldB*)
CAG18433	*zbf-3057*::Tn*10*	40
GC4468	Δ(*argF-lac*)*169 rpsL179* IN(*rrnD-rrnE*)	12
JC7623[c]	*recB21 recC22 sbcB15 sbcC201*	25
KL16	Hfr PO-45 *spoT1 rel-1 thi-1*	4
W3110	Prototroph; IN(*rrnD-rrnE*)	4
Plasmids		
pBR322	ori_{ColE1} *bla tet*; cloning vector	GenBank accession no. J01749
pLDR8	pSC101(Ts) derivative containing λp_R *int*$^{+}$ *c*I857 segment; Kan^{r}	18
pLDR10	Cloning vector containing *att*λ; Amp^{r} Cam^{r}	18
pMOB02	pBR322 *bla*::Tn*9*; Cam^{r} Tet^{r} Amp^{s}	10
pPG1	*fldA*$^{+}$; Amp^{r}; partial deletion of pRM*Eco*RI	Fig. 1
pPG1::*cat*	*fldA*::*cat*; Amp^{r}	Fig. 1
pPG2	*fldB*$^{+}$; Amp^{r}; high copy number	Fig. 2
pPG5	pPG2 *fldB*::*tet*	Fig. 2
pRM*Eco*RI	*fldA* gene on 5.8-kb insert in phagemid pBluescript II SK^{+}; Amp^{r}	31; Fig. 1
pRS414	Protein fusion vector containing, in sequence, *bla*$^{+}$, $T1_4$, *Eco*RI site, *Sma*I site, and *′lacZ*	39
pWB51	pRS414 containing an *fldB′*-*′lacZ* protein fusion; *Eco*RI-*Msc*I piece of pPG2 (Fig. 2) replacing *Eco*RI-*Sma*I segment of pRS414.	This study
pWB52	pLDR10::*fldA*; 1-kb *Dra*I fragment of pRMEcoRI (Fig. 1) cloned into *Sma*I site of pLDR10	This study
pWB53	pLDR10::Φ(*soxS′*-*′lacZ*); λJW2 piece from *Sca*I (in *J*) to *Hin*dIII (in *kan*) replacing *Sma*I-*Hin*dIII segment of pLDR10	This study
Phages		
λ469	Kohara phage containing the *fldB* region	24
λJW2	Φ(*soxS′*-*′lacZ*) *bla*$^{+}$ *kan*$^{+}$	48
λRZ5	*c*$^{+}$ *′bla ′lacZ lacY*$^{+}$; prophage vector for acquiring *lacZ* fusions from plasmids by recombination	32; from R. Zagursky
λRZ5::*fldB*	λRZ5 Φ(*fldB′*-*′lacZ*) *bla*$^{+}$	λRZ5 × pWB51

[a] Bacterial strains are derivatives of *E. coli* K-12 λ^{-}. Unless otherwise noted, the strains are F^{-}, and genetic descriptions are complete. $T1_4$ is a set of four tandem transcriptional terminators (39).
[b] P1 transductional crosses are described as follows: P1(donor) × recipient.
[c] For a complete listing of markers, see reference 25.

GC4468, and the desired lysogens were selected as red colonies on MacConkey agar (Difco) containing ampicillin.

Media and growth conditions. LB media (29) were used for the routine growth of *E. coli*. The minimal medium used for aerobic growth (VB medium) was medium E described by Vogel and Bonner (43) that was supplemented with 0.4% glucose and 1 μg of thiamine/ml. The minimal medium used under anaerobic conditions was a glycerol-fumarate medium without Casamino Acids (41). For anaerobic growth in rich solid media, the cells were suspended in 15 ml of nutrient agar (Difco) containing an *E. coli* membrane preparation (EC Oxyrase; Oxyrase, Inc.), overlaid with a barrier of 2% agar (in H_2O), and incubated in air (1). Alternatively, the cells were grown on the surfaces of agar plates in an AnaeroPak chamber (Mitsubishi Gas Chemical America, Inc.) under 80% N_2–20% CO_2. Growth in broth under stringent anaerobic conditions was performed in Na_2S-supplemented media (20). Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 25 μg/ml; tetracycline, 5 or 15 μg/ml. The lower concentration of tetracycline was required for the selection of single copies of *fldB*::*tet*. Carbenicillin (50 μg/ml) was used for the selection of single copies of *bla*.

Gene transfers. Generalized transductions were performed with bacteriophage P1 *dam rev6* (42). Bacterial transformations were performed as previously described (14, 22). Transfers of *fldA* and *fldB* insertion mutations from plasmid to chromosome (by double crossovers) were accomplished as previously described (30) with a *recBC sbcBC* strain (JC7623), which does not support the growth of ColE1-derived replicons (6).

Computer analysis. Sequence similarity searches were performed with the BLAST, version 2.0, program at the National Center for Biotechnology Information website. FMN binding sites were detected with the MOTIFS program of the Wisconsin Package, version 9.1 (Genetics Computer Group, Madison, Wis.).

Other methods. PCR and general cloning methods were as described previously (3). DNA fragments with incompatible ends were blunted with bacteriophage T4 DNA polymerase before being joined. Curing of the *att*λ::*fldA* element was accomplished by infection with λ*c*$^{+}$ phage at a multiplicity of 10 (38). PCR amplifications of the *fldA* and *fldB* regions were performed with *Taq* DNA polymerase and the following primers: 5′-GAAGAAGTCATCCCAGTCACA-3′ and 5′-ACCCCCATTTCAATAAGTTTC-3′ for *fldA* and 5′-TTAGTTTCATCCAGCGCC-3′ and 5′-CCATTATGCCTTATTGTGCC-3′ for *fldB*. Treatments of growing cells with paraquat or H_2O_2 were as previously described (13). After 1 h, the cultures were chilled, and 0.1 volume of ethanol (37) was added to each. Assays for β-galactosidase were performed by the method of Miller (29) on cells that were permeabilized with polymyxin B (37); specific activity is reported in Miller units. Catalase assays were performed as previously described (13).

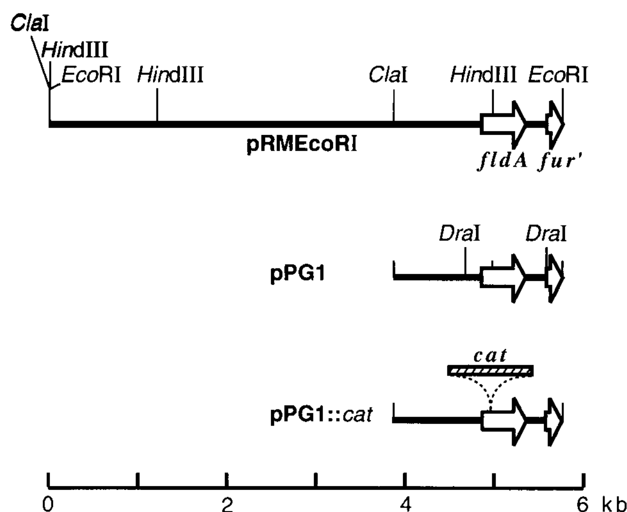

FIG. 1. *fldA* plasmids. Only the inserted *E. coli* DNA is shown, together with part of the multiple cloning site. pRMEcoRI was digested with *Cla*I and religated, thereby removing all but one *Hin*dIII site and producing plasmid pPG1. A chloramphenicol resistance (*cat*) element from Tn*9* was excised from plasmid pMOB02 as a 1.9-kb *Fsp*I fragment and ligated into the *Hin*dIII site of pPG1 to produce pPG1::*cat*. For clarity, the *Dra*I sites that were used for subcloning *fldA* in pWB52 are shown on only one of the plasmids.

TABLE 2. P1-mediated transduction of *fldA*::*cat* and a nearby Tn*10* into strains carrying either one or two copies of $fldA^+$

Recipient	Genotype	No. of transductants[a]			
		Aerobic		Anaerobic[b]	
		Cam^r	Tet^r	Cam^r	Tet^r
KL16	$fldA^+$	4	252	0	33
BW1526	$fldA^+$ *att*λ::($fldA^+$)	1,331	866	176	63

[a] Transductions were each performed with equal amounts a lysate of the donor strain, BW1530 [*fldA*::*cat zbf-3507*::Tn*10 att*λ::($fldA^+$ bla^+)]. After 1 h of incubation to permit gene expression, each cell suspension was divided into four equal portions and plated.

[b] Anaerobic growth was for 3 days at 37°C in nutrient agar (Difco) containing Oxyrase.

RESULTS

***fldA* insertion mutation.** The *fldA*::*cat* mutation was constructed on a plasmid (Fig. 1). However, we were unable to transfer the mutation to the chromosome by common methods of transformation with linear DNA (33, 45). This result suggested that the mutation might be lethal, in which case the host chromosome should accept *fldA*::*cat* by substitutive recombination only if the cell has a second copy of $fldA^+$. We introduced a second copy of $fldA^+$ as part of a nonreplicating circular element that integrated into *att*λ (see Materials and Methods). The cloned DNA was a *Dra*I segment of pRMEcoR1 (Fig. 1) in which *fldA* was the only open reading frame (GenBank accession no. AE000372 [F. R. Blattner]). The *att*λ::($fldA^+$ *bla*) element was then transduced into JC7623, a *recBC sbcBC* strain that can undergo chromosomal transformation. The resulting $fldA^+$ diploid, BW1528, could now be transformed to Cam^r by a double crossover with plasmid pPG1::*cat* (containing *fldA*::*cat*) to yield strain BW1529. The Cam^r trait of BW1529 could be crossed out in transductions with strain CAG18433, which contains a Tn*10* near *fldA*; the linkage was 50% (24 of 48). Therefore, in strain BW1529, the insertion mutation is in the normal chromosomal *fldA* locus; it is not on a plasmid or in the inserted *att*λ::$fldA^+$ element.

***fldA* is a vital gene.** An *fldA*::*cat* insertion mutation was readily transduced into a strain carrying two copies of $fldA^+$, but it could not be efficiently transferred to a wild-type strain under aerobic or anaerobic conditions (Table 2). This result suggested that the insertion mutation is lethal. A nearby Tn*10* served as a control for the efficiency of transduction. It was easily transferred to strains carrying either one or two intact copies of *fldA*. If the *fldA*::*cat* mutation is lethal, the number of Tet^r recombinants of the wild-type strain should be reduced by the number that would have coinherited *fldA*::*cat*. Compared to the $fldA^+$ diploid, the wild-type strain had 71% fewer Tet^r recombinants aerobically and 48% fewer anaerobically (Table 2). These results, too, are consistent with the inviability of the Cam^r cotransductants. *fldA* is the only open reading frame from its region that is contained within the *Dra*I fragment that was cloned in the *att*λ element (Fig. 1) (GenBank accession no. AE000372). The fact that this element complements the lethality of *fldA*::*cat* indicates that the defect is due solely to the loss of *fldA* function and not to a polar effect of the insertion mutation.

Four *fldA*::*cat* recombinants in an apparently haploid strain were observed (Table 2). Although this result might seem to argue against the lethality of the mutation, it was expected because *E. coli* acquires tandem duplications of any chromosomal gene at a frequency of about 1% (2); substitution of one tandem $fldA^+$ allele by *fldA*::*cat* would leave the second copy of *fldA* functional. The presence of intact *fldA* genes was confirmed by PCR. Aerobic transduction of the haploid strain was repeated on a larger scale, and eight Cam^r recombinants were analyzed. The PCR primers were complementary to sequences that flank the *cat* insertion site. All eight transductants yielded the 248-bp products that were expected from templates containing uninterrupted *fldA* genes.

The following experiment produced independent evidence for the lethality of *fldA*::*cat*. If *fldA* is vital, we should not be able to eliminate the *att*λ::$fldA^+$ element from a cell bearing the *fldA*::*cat* mutation. To cure strains of this element, they were infected with λc^+ bacteriophage at a high multiplicity. During lysogenization, the transient production of the phage-encoded Int and Xis proteins should lead to the excision and subsequent loss of the nonreplicating *att*λ::($fldA^+$ bla^+) element. The method was the same as that for the curing of λ prophages by superinfection (38). After infection of BW1528 [$fldA^+$ *att*λ::($fldA^+$ bla$^+$), 24% (23 of 96) of the tested colonies lost the *att*λ element (i.e., became Amp^s). However, none (0 of 89) of the tested colonies of BW1529 [*fldA*::*cat att*λ::($fldA^+$ bla^+)] were cured, indicating that a functional *fldA* gene is essential for viability.

An *fldB* insertion mutant is viable. In plasmid pPG5 (Fig. 2), a tetracycline resistance element replaced most of the *fldB* gene. The mutation was transferred to the chromosome of strain JC7623 (see Materials and Methods). All twenty of the Tet^r recombinants examined were Amp^s, i.e., plasmid free. Four of the transformants were used as PCR templates in reactions with primers that bracketed the insertion site (see Materials and Methods). The mutant DNAs generated the 1.6-kb products expected for the interrupted gene, and they failed to yield the 0.6-kb product that was obtained from $fldB^+$ chromosomal DNA (results not shown). Although the recombinants no longer had an intact copy of *fldB*, their colony size on LB agar was observed to be equal to that of their $fldB^+$ parents under both aerobic and anaerobic conditions. Their growth rate and viability in LB broth under stringent anaerobic conditions (with Na_2S) were also the same as those of their $fldB^+$ parent. Therefore, unlike the *fldA* insertion mutant, the *fldB* mutant is viable.

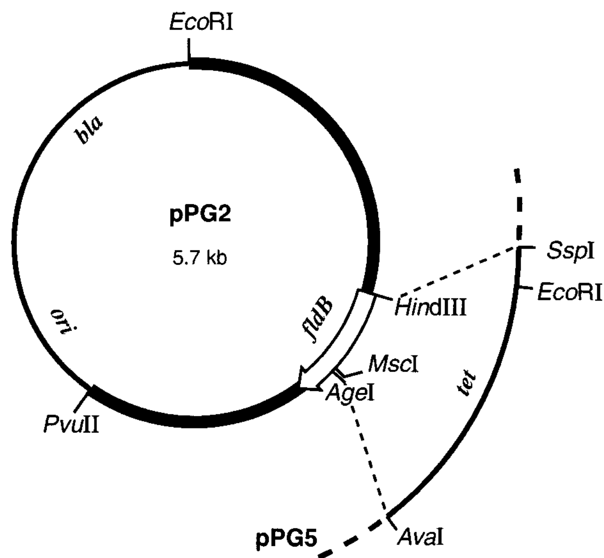

FIG. 2. *fldB* plasmids. Plasmid pPG2, an $fldB^+$ plasmid with a high copy number (due to a partial *rop* deletion), was produced by replacing the *Eco*RI-*Pvu*II region of pBR322 with a 3.4-kb *Eco*RI-*Pvu*II fragment of Kohara phage λ469. Plasmid pPG5 is a derivative of pPG2 in which most of the *fldB* gene was replaced by a 1.6-kb segment of pBR322 containing the *tet* gene. The *Hin*dIII site is in the ribosome-binding site for *fldB*. The thin lines represent pBR322 DNA.

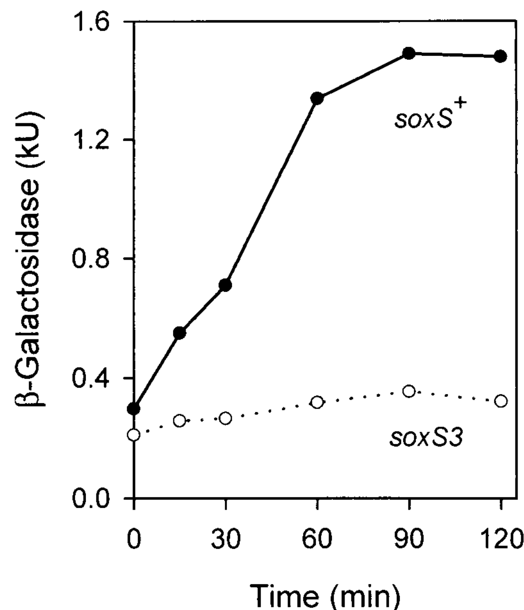

FIG. 3. *SoxS*-dependent induction of an *fldB'-lacZ* fusion by paraquat. At zero time, paraquat was added to aerated log-phase cultures to a final concentration of 0.2 mg/ml. Samples were removed periodically, and the specific activity of β-galactosidase was measured in Miller units (29). The strains used were GC4468(λRZ5::*fldB*) and its *soxS3*::Tn*10* derivative, BW1535.

***fldB* mutants are still prototrophs.** The *fldB*::*tet* mutation was transduced from strain BW1485 into strain W3110, an ancestral K-12 ($F^- \lambda^-$) prototroph. Three transductants were plated on a glycerol-fumarate minimal medium under both aerobic and anaerobic (80% N_2–20% CO_2) conditions and on VB (glucose) minimal medium under aerobic conditions. Their growth was indistinguishable from that of the $fldB^+$ parental strain grown on the same media.

Multiple copies of *fldB* cannot substitute for *fldA*. Why is an *fldB* mutation not lethal whereas an *fldA* mutation is lethal? Either flavodoxins I and II are needed for different reactions or, if they participate in the same reactions, the activity of FldB may be too low to permit an *fldA* mutant to survive. To see if overproduced FldB could substitute for FldA, we attempted to transduce an *fldA*::*cat* mutation into a strain carrying pPG2, a high-copy-number *fldB* plasmid. The plasmid contains a functional promoter for *fldB*, which was used to construct an *fldB'-'lacZ* gene fusion, as described in the next section. The results (Table 3) were similar to those obtained with a wild-type plasmid-free strain (Table 2): whereas a Tn*10* marker was readily transferred, the closely linked *fldA*::*cat* mutation was not. In the control experiment, both markers were transferred efficiently to a strain carrying two copies of $fldA^+$. Therefore, even a high gene dose of *fldB* cannot prevent the lethality of an *fldA* mutation. These results suggest that in *E. coli* there is at least one vital reaction that specifically requires FldA.

TABLE 3. A high gene dose of *fldB* does not prevent the lethality of an *fldA* mutation

Recipient	Genotype	No. of transductants[a]			
		Aerobic		Anaerobic[b]	
		Cam^r	Tet^r	Cam^r	Tet^r
BW1527	$fldA^+$ *att*λ::($fldA^+$)	288	179	159	145
BW1527(pPG2)	pPG2 ($fldB^+$)/$fldA^+$	0	155	1	70

[a] Transductions were performed with equal amounts of a lysate of the donor strain, BW1530 [*att*λ::($fldA^+$ bla^+) *fldA*::*cat zbf-3507*::Tn*10*]. The selective media contained carbenicillin in addition to chloramphenicol or tetracycline.

[b] Anaerobic growth was for 3 days at 37°C under 80% N_2–20% CO_2 on LB agar containing 40 mM glycerol and 40 mM sodium lactate.

***fldB* belongs to the *soxRS* regulon.** An *fldB'-'lacZ* protein fusion was constructed on a plasmid (pWB51; Table 1) and transferred by recombination to λRZ5. The λRZ5::*fldB'* prophage contained most of *fldB* and 1.7 kb of the upstream chromosomal region including the divergently transcribed *xerD* gene. The cloned sequence was preceded by transcriptional terminators from vector pRS414. The construction fused the promoter, ribosome binding site, and first 356 nucleotides (nt) of *fldB* to a 5'-truncated *lacZ* gene. It is unlikely that the cloned portion of *fldB* contained a promoter for a downstream gene because in the chromosome the next two open reading frames are transcribed in the opposite direction (GenBank accession no. AE000373). The expression of the *fldB'-'lacZ* fusion was determined by measuring the β-galactosidase activity in a *lac* deletion mutant (GC4468) containing the prophage. It was not significantly affected (≤20%) by anaerobic growth in liquid media. Treatment with 1 mM H_2O_2 resulted in an induction of catalase activity (7.5-fold) but not of *fldB* expression (≤20%). Therefore, *fldB* does not belong to the OxyR regulon. However, *fldB* expression was induced fivefold by paraquat (methyl viologen), and the induction was blocked by a mutation in the *soxS* gene (Fig. 3). The unresponsiveness of the *soxS* mutant was not caused by a general inhibition of protein synthesis: the cell mass increased 7.5-fold during the 2-h treatment. These results indicate that *fldB* is a member of the *soxRS* regulon.

***soxS* expression is not affected by *fldB*.** If FldB is required to maintain the normal redox balance of the cell, then an *fldB* mutant might constitutively overexpress *soxS*, the transcription of which is activated by the oxidized form of SoxR (19, 21). It might at least sensitize *soxS* to induction by a redox cycling agent such as paraquat, which depletes the cell of reducing

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                                                                    -35
1   ATCCTGTTTCACTTGCGCCCCTTATGGTCACTCATTTGATCCATTATGCCTTATTGTGCCGTGACTAAAG  70
    TAGGACAAAGTGAACGCGGGGAATACCAGTGAGTAAACTAGGTAATACGGAATAACACGGCACTGATTTC
     xerD                                          soxboxes

      -10        +1                         HindIII           fldB
                                               SD
71  CGATTCTGATACACTAGCCGCAAAAGCCACAGCAGAATCGAGAAGCTTACGTTATGAATATGGGTCTTTT  140
    GCTAAGACTATGTGATCGGCGTTTTCGGTGTCGTCTTAGCTCTTCGAATGCAATACTTATACCCAGAAAA
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FIG. 4. Putative regulatory sequences upstream of *fldB*. The sequence shown begins at nt 3037752 of the *E. coli* genome (GenBank accession no. AE000373). The −10 hexamer of the putative σ^{70} promoter of *fldB* is lightly underlined, as are the bases complementary to the end of 16S rRNA in the putative Shine-Dalgarno sequence (SD). The solid bars underline nucleotides that match the consensus soxbox (SoxS binding site) sequence, 5′-AX$_2$GCA(C/T)X$_2$(T/A)$_2$(G/A)XCAAAX$_3$(A/T)(A/T)-3′ (46). +1, predicted transcriptional start site for *fldB*.

equivalents while forming superoxide. To test this hypothesis, the *fldB*::*tet* insertion/deletion mutation was transduced into a *soxS′*-*′lacZ* fusion strain. The specific activities of β-galactosidase in the *fldB*$^+$ and *fldB* mutant strains (BW1157 and BW1534) were the same (86 U). Inducibility by paraquat was tested at concentrations of from 0.01 to 1.0 mg/ml. Similar levels of induction were observed in both strains. For example, at 0.01 mg/ml, which produced about 40% of maximum induction, values for the wild type and mutant were 2,626 and 2,418 U, respectively.

The gene dose of *fldB* does not affect paraquat sensitivity. The *soxRS* regulon protects the cell against univalent redox cycling compounds. Thus, *soxS* mutants display an increased sensitivity to killing by paraquat (47). This is also a property of an *fpr* mutant (7) which lacks a *soxS*-regulatable NADPH: ferredoxin (flavodoxin) reductase. It was therefore reasonable to suspect that the copy number of *fldB* might affect paraquat sensitivity. The gradient plate technique (15) was used to test strain W3110 (*fldB*$^+$) together with three of its derivatives: BW1531 (*fldB*::*tet*), W3110(pPG2[*fldB*$^+$]), and W3110(pPG5 [*fldB*::*tet*]). The LB agar contained a gradient of from 0 to 120 μg of paraquat/ml and, for the plasmid-bearing strains, a uniform concentration of ampicillin. All four strains showed the same degree of sensitivity: 60 to 70 mm of growth along the gradient.

DISCUSSION

fldA is 348 nt upstream of *fur*, which encodes an iron-responsive regulatory protein. The two genes form an operon belonging to the *soxRS* regulon, and *fur* is also transcribed independently from an OxyR-regulated promoter (49). In our experiments, the *fldA*::*cat* mutation was complemented by an *att*λ element that contained *fldA*. Therefore, the lethality of *fldA*::*cat* must be directly due to a loss of *fldA* function. However, we have not excluded the formal possibility that the lethal effect is due to the loss of *fldA* combined with the noninducibility of *fur*.

In in vitro reactions, the anaerobic ribonucleoside triphosphate reductase (encoded by *nrdD* and *nrdG*) requires FldA; ferredoxin cannot be substituted for it (9). It should be an essential enzyme for anaerobic growth. However, *nrdD* and *nrdG* mutants fail to grow only under the most stringent anaerobic conditions, in a low-redox-potential broth medium containing Na_2S (20). These were conditions that we could not apply to our plating experiments (Tables 2 and 3). The *nrd* mutants grew well on solid media in oxygen-depleted chambers. Therefore, the lethality of an *fldA*::*cat* mutation under our plating conditions cannot be attributed to its effect on anaerobic nucleotide reduction alone; there must be at least one other essential pathway requiring FldA. Similarly, inviability could not be explained by the requirement of pyruvate formate-lyase for flavodoxin (36). A mutant lacking the lyase gene grows anaerobically, displaying only a mild requirement for acetate (35). The results of anaerobic transductions, similar to those in Table 2, were not significantly altered by the addition of 5 mM acetate to the medium (results not shown).

Although there is as yet no known phenotype associated with an *fldB* mutation, there is strong evidence that the wild-type allele is a functional gene in *E. coli*. Our *fldB′*-*′lacZ* fusion depended for its expression on both the *fldB* promoter and *fldB* ribosome binding site, and the hybrid protein was expressed constitutively at a high level. In addition, its expression was regulated by SoxS (Fig. 3). Even if the gene product itself is not functional in *E. coli*, it is likely to be closely derived from one that is functional in another organism. *Salmonella enterica* serovar Typhimurium, a close relative of *E. coli*, also has an *fldB* gene next to *xerD* (BLAST program, version 2.0).

Apart from *S. enterica* serovar Typhimurium FldB, there are no known or predicted proteins that have a degree of identity with *E. coli* FldB that exceeds that of FldA (43%). There are, however, known or predicted proteins from other organisms that are about 39 to 49% homologous to both FldA and FldB of *E. coli*. They include putative flavodoxins from *Anabaena* sp., *Synechoccus* sp., *Klebsiella aerogenes*, *Azotobactor* sp., and *Haemophilus influenzae*. In the paralogs, the regions of sequence similarities appear to be distributed throughout the length of the polypeptides. In summary, the available evidence based on current sequencing data does not indicate that FldB is a member of a distinct subclass of flavodoxins found among distantly related bacteria.

The *xerD*-*fldB* intergenic region (Fig. 4) contains sequences that are similar to those of known regulatory regions. Two sources using different algorithms predicted an *fldB* promoter with the same transcriptional start site (Fig. 4) (M. G. Reese, Neural network promoter prediction tool, http://www.fruitfly.org/seq_tools/promoter.html [revision date, 18 December 1999; last date accessed, 29 December 1999]; GenBank accession no. AE000373) predict an *fldB* promoter with the same transcriptional start site (Fig. 4). The putative −10 hexamer 5′-TACACT-3′ is preceded by a 5′-TGN-3′ sequence characteristic of "extended −10" regions that are found in promoters lacking recognizable −35 hexamers (5). Near the −35 region, on the antisense strand, are two overlapping sequences that resemble a SoxS-binding site, or "soxbox" (26, 46). Although they might regulate the *xerD* gene, they appear to be too close to it, and there is no apparent reason why *xerD* should belong to an oxidative stress regulon. Physical and genetic studies are needed to confirm that these are indeed regulatory sequences.

The inclusion of *fldB* together with *fldA* and *fpr* in the *soxRS*

regulon underscores the importance of flavodoxins in this global response to oxidative stress. The *soxRS* regulon is induced by the depletion of reducing equivalents through univalent redox reactions. The induced flavodoxins together with their reductase may facilitate the restoration of the redox equilibrium that must accompany recovery from oxidative stress.

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