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The Flavoenzyme Ferredoxin (Flavodoxin)-NADP(H) Reductase Modulates NADP(H) Homeostasis during the *soxRS* Response of *Escherichia coli*

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***Escherichia coli* cells from strain *fpr*, deficient in the *soxRS*-induced ferredoxin (flavodoxin)-NADP(H) reductase (FPR), display abnormal sensitivity to the bactericidal effects of the superoxide-generating reagent methyl viologen (MV). Neither bacteriostatic effects nor inactivation of oxidant-sensitive hydrolyases could be detected in *fpr* cells exposed to MV. FPR inactivation did not affect the MV-driven *soxRS* response, whereas FPR overexpression led to enhanced stimulation of the regulon, with concomitant oxidation of the NADPH pool. Accumulation of a site-directed FPR mutant that uses NAD(H) instead of NADP(H) had no effect on *soxRS* induction and failed to protect *fpr* cells from MV toxicity, suggesting that FPR contributes to NADP(H) homeostasis in stressed bacteria.**

The *soxRS* regulon positively modulates the expression of at least 17 genes in *Escherichia coli*, mediating an oxidative stress response that protects the bacterial cells against the superoxide anion radical ($O_2^{-}\cdot$), nitric oxide (NO), and redox cycling agents such as the herbicide methyl viologen (MV) (10, 36, 37). The sensor of the regulon is the SoxR protein, a dimeric transcription factor that contains [2Fe-2S] centers and whose only known target is the *soxS* promoter (31, 41). When *E. coli* cells are subjected to a challenge with $O_2^{-}\cdot$, the iron sulfur clusters of SoxR may undergo univalent oxidation to yield the transcriptionally active form of the protein (7, 15). Both oxidized and reduced SoxR are able to interact with the *soxS* promoter, but only binding of the oxidized dimer enhances the synthesis of SoxS, a transcriptional activator of the AraC/XylS family (37). Increased SoxS levels then activate the various regulon genes via σ^{70} RNA polymerase (10, 37). The *soxRS* regulon appears to be specifically tailored to respond to $O_2^{-}\cdot$ (or NO) and is not induced by other sources of oxidative stress such as heat shock or ionizing radiation (10, 37).

E. coli cells exposed to a source of $O_2^{-}\cdot$ may undergo bacteriostatic or bactericidal effects. Bacteriostasis is related to superoxide-mediated inactivation of catalytic [4Fe-4S] clusters in hydrolyases, with the tricarboxylic acid cycle enzyme aconitase being a most sensitive target (9). Inhibition of these enzymes causes a decline in growth rates without affecting cell viability, since oxidized hydrolyases can be reactivated by a reductive system whose components are yet to be identified (12). Bactericidal effects, on the contrary, usually reflect DNA oxidation and cleavage by superoxide derivatives such as the hydroxyl (·OH) and ferryl (FeO^{2+}) free radicals (17, 18). The balance between bacteriostasis and lethality depends on the intensity of the stress imposed, the culture conditions, and the stock of antioxidants present in a given *E. coli* strain, among other factors (16, 19, 30). To cope with the various hazards of $O_2^{-}\cdot$ toxicity, members of the *soxRS* regulon need to operate at different (and complementary) levels of the global cell response to the oxidative challenge. Protective functions include direct $O_2^{-}\cdot$ scavenging by the Mn-containing superoxide dismutase (SOD), replacement of oxidant-sensitive hydrolyases by resistant isoforms, DNA repair activities, diminished uptake, and increased elimination of xenobiotics, etc. (10, 25, 36). As part of the global response, *E. coli* cells also induce the synthesis of several NADP(H)-dependent dehydrogenases and oxidoreductases, including the flavoprotein ferredoxin (flavodoxin)-NADP(H) reductase (FPR) (EC 1.18.1.2) (27). These ubiquitous FAD-containing enzymes catalyze the reversible electron transfer between a single molecule of NADP(H) and two molecules of obligatory one-electron carriers such as ferredoxin or flavodoxin (1). They can also mediate the so-called "diaphorase" reaction, namely, the irreversible oxidation of NADPH by a wide variety of adventitious electron acceptors, such as viologens, quinones, substituted phenols, complexed transition metals and tetrazolium salts, among others (1, 27). The steady-state levels of FPR increased 20-fold on exposure of *E. coli* cells to the $O_2^{-}\cdot$ propagator MV (27, 38), and FPR-deficient strains (*fpr*) proved to be abnormally sensitive to oxidants (3, 22). Expression of this reductase from a multicopy plasmid provided increased MV tolerance to *E. coli* cells displaying wild-type levels of FPR synthesis and induction, indicating that the antioxidant effect was dose dependent even beyond physiological levels of the flavoenzyme (3, 21). The nature and mechanism of this defensive action,

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TABLE 1. *E. coli* strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics[a]	Source and/or reference
Strains		
GC4468	F^- Δ*lac U169 rpsL*	4
QC772	GC4468 *sodA49* Cam^r	4
C-6007	Cla *fpr* Kan^r Rif^r	3
RR6A	GC4468 *fpr* Kan^r	This study
RR64	QC772 *fpr* Kan^r Cam^r	This study
B247	MC4100 (λJW2) (lysogen) λJW2:ϕ (*soxS*′::*lacZ*′) Amp^r Kan^r	29, 41
Plasmids		
pEC105	pSU18 carrying the *E. coli fpr* gene, Cam^r	This study
pDR105	pSU19 carrying the pea *fpr* gene, Cam^r	D. Rial and E. A. Ceccarelli, unpublished data
pY308S	pSU19 carrying the Y308S mutant version of the pea *fpr* gene, Cam^r	32; This study
pTN1530	pNK1415 Δ*soxR soxS*′::*lacZ* Amp^r	31

[a] Amp^r, ampicillin resistance; Rif^r, rifampin resistance; Cam^r, chloramphenicol resistance.

however, remain elusive, although a number of hypotheses have been advanced.

In their seminal work, Liochev et al. (27) proposed that *E. coli* FPR might be involved in the reduction of SoxR once the oxidative condition has subsided, so that the function of the reductase would be to provide for self-regulation of the entire *soxRS* system. Alternatively, FPR could participate in the reductive healing of $O_2^{-}\cdot$ -damaged hydrolyases (10, 24). A function of this type would be in agreement with recent observations showing that aconitase activities but not aconitase protein levels were severely depressed in a conditional yeast mutant lacking the adrenodoxin reductase homologue, a mitochondrial flavoenzyme with FPR activity (23). Still other proposals posed a role for FPR in the modulation of the $NADP(H)^+$ homeostasis or in the reduction of an abundant cellular scavenger (22). The various invoked mechanisms are based on the promiscuity exhibited by FPR at its acceptor side, but empirical evidence for any of these contentions is scant. To gain further insight into the protective role of this reductase, we probed the effects of FPR inactivation, FPR overexpression, and FPR mutation on growth, survival, *soxRS* induction, and NADP(H) levels in MV-treated *E. coli* cells.

All strains used in this work were derivatives of *E. coli* K-12, and their relevant features are summarized in Table 1. The *fpr* mutation of strain C-6007 (3) was transferred into strains QC772 (*sodA*) and GC4468 (parental) by P1 transduction (28). Transductants were screened for kanamycin resistance (Kan^r), and the properties of the presumptive *fpr*-inactivated clones were assessed by PCR analysis (3) and FPR inmunoreaction (38). Recombinant plasmids employed in the present study are also described in Table 1. Briefly, pEC105 contains the entire sequence of the *E. coli fpr* gene (3), with the initial ATG fused in-frame to codon 13 of the *lacZ* gene in pSU18. Plasmid pDR105 harbors a full-length cDNA encoding the mature, processed region of pea FPR (5), linked in-frame to the first 16 triplets of the β-galactosidase gene in pSU19. A site-directed mutant version of this fused gene, containing a Tyr-to-Ser replacement at position 308 (35), was also prepared in pSU19, yielding recombinant vector pY308S. Chimeric genes were placed under the control of the *lacZ* promoter in the three plasmids. Finally, pTN1530 contains a *soxS*′::*lacZ* operon fusion expressing β-galactosidase activity under the control of the *soxS* promoter (31). Transformation of competent *E. coli* cells with the various plasmids, isolation of genomic and plasmidic DNA, and miscellaneous recombinant DNA techniques were carried out according to established procedures (2). Unless otherwise stated, *E. coli* cells were grown at 37°C in Luria-Bertani (LB) broth (2). Antibiotics (100 μg of ampicillin, 100 μg of kanamycin, and/or 20 μg of chloramphenicol ml^{-1}) were added when required (Table 1).

To study the induction of the *soxRS* regulon by MV, saturated cultures of *E. coli* were diluted (100-fold) into fresh LB broth supplemented with the corresponding antibiotics and grown for 2 to 4 h at 37°C to reach an optical density at 600 nm (OD_{600}) of 0.3 to 0.4. Aliquots (50 ml each) of these cultures were placed in 250-ml flasks, MV was added to a final concentration of 100 μM, and the samples were shaken vigorously (300 rpm) at 37°C for the times indicated in Fig. 2 and Table 2. For experiments on the rate of *soxRS* deactivation, cells induced for 1 h were collected by centrifugation, washed twice with LB broth to remove the viologen, resuspended in 50 ml of the same medium, and incubated at 37°C with vigorous shaking. Aliquots were withdrawn at the times indicated and assayed for β-galactosidase activity by the procedures of Miller (28). The OD_{600} was used as a measure of cell density. To evaluate MV toxicity, appropriate dilutions of cells grown to stationary phase were spread onto LB agar plates containing various concentrations of the herbicide. Colonies were enumerated after 16 and 40 h of incubation at 37°C. When required, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the media to induce expression of genes placed under control of the *lacZ* promoter (Table 1).

The presence of bacterial or plant-derived FPR in cleared supernatants from *E. coli* cell lysates was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting (22, 38). Ferredoxin-dependent cytochrome *c* reductase and ferricyanide-dependent diaphorase activities of FPR were determined as described before (32). Aconitase activities were assayed by the method of Flint et al. (9). The NADP(H) levels were estimated in the various strains by a redox cycling assay (40), after alkaline extraction of the pyridine nucleotides. Portions (200 μl) of cells grown to an OD_{600} of ~0.5, treated or not with 100 μM MV (1 h at 37°C), were mixed with an equal volume of 0.5 M KOH. For the

TABLE 2. Effect of FPR expression on NADP(H) levels and *soxRS* induction in various *E. coli* strains[a]

Strain	Plasmid	Plasmid-borne activity	Mean FPR-dependent activity (U/mg) ± SE with:		Mean NADPH/$NADP^+$ ratio[b] ± SE with:		*soxRS* induction in 0.1 mM MV[c]
			No MV	0.1 mM MV	No MV	0.1 mM MV	
B247	pSU18	None	4.8 ± 0.4	6.9 ± 0.5	4.4 ± 0.6	1.6 ± 0.4	1
B247	pEC105	*E. coli* FPR	20.2 ± 1.4	23.8 ± 1.0	1.3 ± 0.3	0.7 ± 0.1	2.7
B247	pDR105	Pea FPR	27.1 ± 0.4	30.5 ± 3.1	2.1 ± 0.3	1.1 ± 0.1	2.8
B247	pY308S	NADH-dependent pea FPR	5.0 ± 0.8, 19.0 ± 1.6[d]	6.3 ± 0.7, 20.3 ± 2.1[d]	6.0 ± 0.6	2.0 ± 0.2	0.8
GC4468	pTN1530	SoxS-driven β-galactosidase	6.5 ± 0.2	8.0 ± 0.7	4.6 ± 1.4	1.9 ± 0.3	1
RR6A (*fpr*)	pTN1530	SoxS-driven β-galactosidase	5.2 ± 0.8	4.2 ± 1.0	6.1 ± 1.5	3.2 ± 0.3	1.1

[a] *E. coli* cells were grown in LB broth to an OD_{600} of ≈0.4, incubated for an extra hour in the same medium with or without MV and assayed for FPR- dependent cytochrome *c* reductase activity, β-galactosidase activity, and NADP(H) contents by the procedures described in the text. Values are the means of four experiments ± the SE.

[b] Total NADP(H) levels ranged from 6.0 to 12.9 nmol per 10^9 cells, with <15% variation between isogenic strains.

[c] The β-galactosidase activities of the derivative strains B247/pEC105, B247/pDR105, B247/pY308S, and RR6A were referred to those of the corresponding controls, B247/pSU18 and GC4468, respectively, which were taken as unity.

[d] FPR activity was estimated by using 5 mM NADH as an electron donor.

estimation of total NADP(H) amounts, the mixture was immediately neutralized by the addition of 100 μl of a solution containing 0.5 M triethanolamine, 0.4 M KH_2PO_4, and 0.1 M K_2HPO_4. To determine the NADPH contents, alkaline mixtures were incubated for 60 min at 60°C prior to neutralization in order to selectively destroy the $NADP^+$. Proteins were then extracted twice with 500 μl of chloroform, and pyridine nucleotides were assayed in the upper aqueous phase. Each determination was made in triplicate.

Elimination of FPR increases the susceptibility of *E. coli* cells to the bactericidal effect of MV. If FPR were involved in the reductive activation of oxidized hydrolyases, bacteriostatic effects should become apparent when FPR-deficient bacteria are challenged with low doses of MV. Figure 1A shows the behavior of *E. coli* cells from *fpr* and *sodA* strains, and from a *sodA fpr* double mutant, when exposed to MV in solid media. Bacteria carrying the *sodA* mutation lack the Mn-containing SOD (4, 16). They showed a clear growth inhibition when exposed to MV, reflecting the abnormal accumulation of $O_2^{-}\cdot$ in the cytosol. At 16 h after challenge, only a few colonies were visible in the MV plates, but at 40 h the numbers of developing colonies were similar to those of the untreated controls (Fig. 1A), although of a smaller size (data not shown). These results indicate that the effect of MV was reversible in this strain and of a static nature.

Survival of the *fpr* mutants also declined as the MV concentration was raised, but the numbers of colonies counted at 16 or 40 h postchallenge were essentially the same (Fig. 1A), indicating that MV caused irreversible damage to these bacteria. Moreover, the average sizes of colonies surviving a 40-h treatment with 100 μM MV were not significantly different from those obtained in the absence of the herbicide (data not shown), suggesting that the growth rates of the spared cells were not affected by the stress imposed. Both the bactericidal and the bacteriostatic effects of MV were apparent in *fpr sodA* double mutants (Fig. 1A).

MV-mediated damage to aconitase, a typical [4Fe-4S]-containing hydrolyase belonging to the tricarboxylic acid cycle, could be demonstrated in *sodA* mutants, whereas introduction of FPR deficiency had no further effect on aconitase inactivation (Fig. 1B). Similar results were obtained when we assayed other sensitive hydrolyases, such as the 6-phosphogluconate dehydratase of the Entner-Doudoroff pathway or the dihydroxy acid dehydratase of branched amino acid synthesis (data not shown). Accordingly, *fpr* mutants failed to show added auxotrophies related to respiratory substrates (i.e., glycerol), gluconate, or amino acids (data not shown). The collected results indicate that *E. coli* FPR is either not involved in reactivation of oxidized hydrolyases or, if it does play any role, is not limiting under physiological or stressed conditions.

Effect of ferredoxin-NADP(H) reductase levels on *soxR* activation. We then evaluated whether FPR levels could affect the activation state of the *soxRS* regulon. Bacterial or plant-type FPR versions were overexpressed in wild-type *E. coli* cells containing a single chromosomal copy of a *soxS′*::*lacZ* operon fusion (41). Transformed bacteria could accumulate large amounts of recombinant FPR from either origin when supplemented with the gratuitous *lacZ* inducer IPTG. Under the conditions employed here, plasmid-borne FPR expression levels were 5- to 10-fold greater than those observed upon maximal induction of the indigenous *fpr* gene in MV-treated cells (Fig. 2A) and were accompanied by a concomitant increase in FPR-dependent activities (Table 2).

The extent of SoxR activation in the presence of 100 μM MV (as monitored by the β-galactosidase activity assay) was significantly higher in cells expressing a recombinant FPR relative to those transformed with the supporting vector (Fig. 2B). Even in the absence of the herbicide, FPR expression led to a major increase of SoxS-dependent activities, from 165 ± 20 to 321 ± 48 β-galactosidase units. Similar results were obtained irrespective of whether the pea (Fig. 2B) or the *E. coli* (Table 2) reductase isoforms were used. As expected, MV removal resulted in a rapid decline of the activated state of SoxR (Fig. 2B). Although cells overexpressing FPR displayed consistently higher β-galactosidase activities compared to those transformed with pSU supporting plasmids, the kinetics of *soxRS* deactivation were identical in the two types of bacteria (Fig. 2B, inset).

The effect of decreasing FPR concentrations below physiological levels was also investigated by using the *fpr* mutant strain RR6A (Table 1) and a *soxS′*::*lacZ* operon fusion encoded by a multicopy plasmid (31). Neither the extent nor the

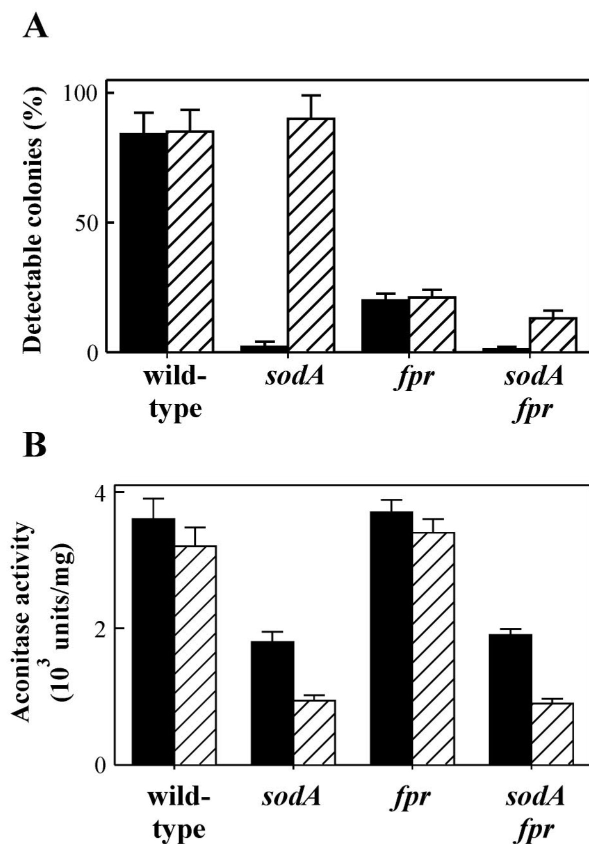

FIG. 1. Cell survival and aconitase levels in *fpr* and *sodA* single and double *E. coli* mutant bacteria exposed to MV. (A) Growth of *E. coli* mutants in the presence of MV. Cells from strains GC4468 (wild type), RR6A (*fpr*), QC772 (*sodA*), and RR64 (*sodA fpr*) were grown (16 h at 37°C) in LB broth with antibiotics (Table 1), and appropriate dilutions were then spread onto LB agar plates containing 100 μM MV. Colonies were counted after 16 h (closed bars) and 40 h (hatched bars) of incubation at 37°C, and percentages refer to the colony numbers obtained in plates lacking the herbicide. Results from four different experiments were averaged, and the standard error (SE) values are indicated in the middle of the bar. (B) Effect of MV on aconitase activities of *E. coli* mutants. Aerobic cultures of the various *E. coli* strains, initiated with 5% (vol/vol) inocula from overnight-grown cells, were incubated for an additional 4 to 6 h at 37°C in glucose minimal medium (12) either in the absence (solid bars) or in the presence (hatched bars) of 3 μM MV. Extracts were prepared from the collected bacteria essentially as described by Gardner and Fridovich (11). Aconitase activities were measured in the soluble fractions and refer to total protein contents, as determined by a dye-binding assay (39). One activity unit is defined as the amount of enzyme capable of catalyzing the formation of 1 nmol of aconitate per min under the conditions of the reaction. Bars represent the averages of three experiments ± the SE.

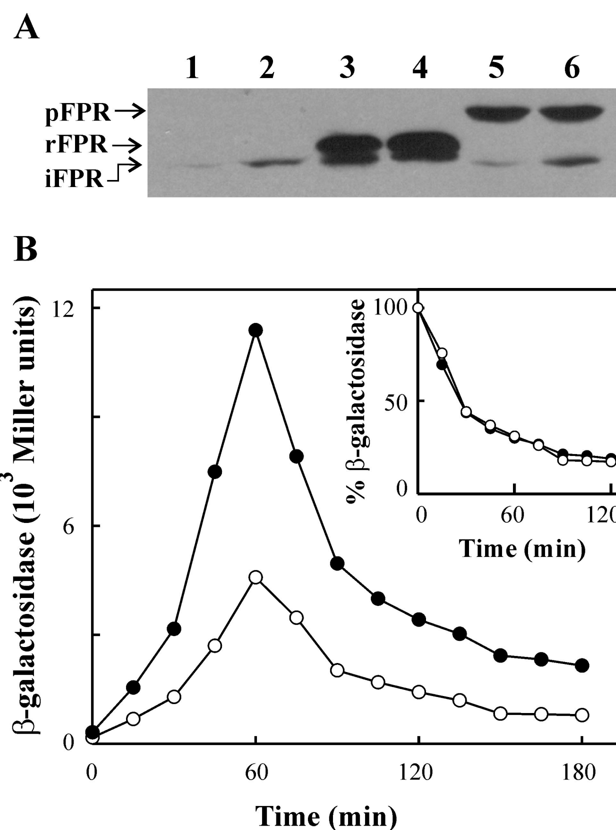

FIG. 2. Expression of FPR stimulates the *soxRS* response in *E. coli*. (A) Accumulation of indigenous and recombinant FPR in *E. coli*. Cells from strain B247, transformed with either pSU19 (lanes 1 and 2), pEC105 (lanes 3 and 4), or pDR105 (lanes 5 and 6), were cultured in LB broth containing 0.5 mM IPTG. The samples corresponding to lanes 2, 4, and 6 were further induced by exposure to 100 μM MV for 1 h. Cleared supernatants corresponding to 15 μg of protein were resolved by SDS-PAGE and blotted onto nitrocellulose membranes. Filters were probed with a succession of antisera raised against both *E. coli* and pea FPR. The electrophoretic mobilities of the various FPR variants are indicated on the left. The slower migration of the recombinant *E. coli* FPR (rFPR), relative to the indigenous form (iFPR), is caused by the extra residues present at the amino terminus of the plasmid-borne reductase. pFPR, recombinant pea FPR. (B) Effect of FPR overproduction on MV-induced *soxRS* activation in wild-type *E. coli* cells. Strain B247, containing a chromosome copy of a *soxS′::lacZ* operon fusion (41), was transformed with the FPR-producing plasmid pDR105 (●), or with the supporting plasmid pSU19 (○). Cells were cultured in LB medium supplemented with 0.5 mM IPTG, 100 μM MV, and the corresponding antibiotics (Table 1). After 1 h of treatment with MV and IPTG, cells were collected by centrifugation, washed, and assayed for β-galactosidase as described in the text. (Inset) Relative decrease of *soxS′::lacZ* expression in each strain was calculated as the fraction of the β-galactosidase activity induced by MV treatment for 1 h.

time course of the *soxRS* response elicited by MV was significantly affected by FPR inactivation in the mutant cells compared to FPR-proficient bacteria (data not shown). Similar results had been obtained by P. Gaudu and D. Touati (unpublished data), who studied the MV-dependent induction of a *sodA′::lacZ* operon fusion in FPR-deficient cells. Moreover, Demple and coworkers also failed to observe changes in *soxRS* activation in both *fpr* and *fnr E. coli* strains compared to the corresponding parentals (E. Hidalgo, B. Gonzalez Flecha, and B. Demple, unpublished data).

FPR modulates NADP(H) levels during the *soxRS* response of *E. coli*. The results summarized in the previous section indicate that FPR overproduction stimulates the induction of the *soxRS* system. It is unlikely, however, that this effect could involve direct participation of the reductase in SoxR oxidation. An alternative possibility is that FPR could modulate the *soxRS* response through indirect mechanisms, namely, by regulating the accumulation of electron donors in the bacterial

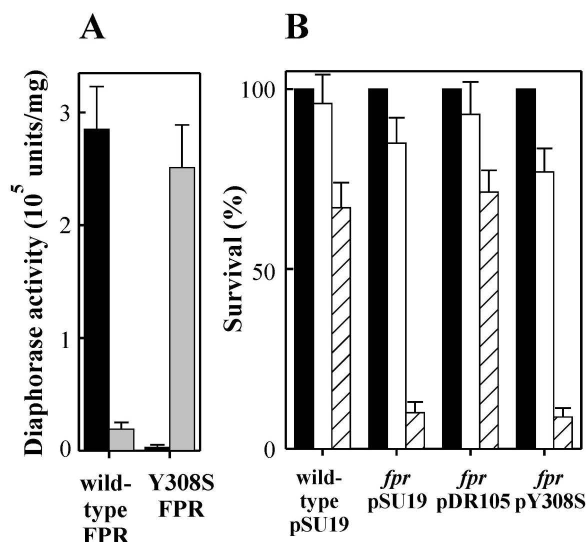

FIG. 3. A site-directed NAD(H)-dependent ferredoxin-NADP(H) reductase mutant does not complement the *fpr* deficiency in *E. coli*. (A) NADP(H)-dependent (solid bars) and NAD(H)-dependent (shaded bars) diaphorase activities of purified wild-type and Y308S FPR, assayed with potassium ferricyanide as an electron acceptor (32). The Y308S FPR mutant, in which the carboxyl-terminal tyrosine residue was replaced by a serine, was designed and purified as reported elsewhere (32, 35). (B) Survival of *fpr* mutant bacteria expressing NADP(H)- or NAD(H)-dependent FPR versions to MV toxicity. Parental cells from strain GC4468 were transformed with pSU19, whereas bacteria from the *fpr* mutant genotype RR6A were transformed with pSU19 or with the recombinant plasmids pDR105 or pY308S harboring the wild-type or Y308S mutant FPR genes, respectively. Plating experiments were carried out as described in the legend to Fig. 1A, with the addition of 0.5 mM IPTG and either no (solid bars), 50 μM (open bars), or 100 μM (hatched bars) MV added to the agar broth. The heights of the bars represent the averages of three experiments ± the SE.

cytosol. The inhibitory effect of NADPH (but not of NADH) on SoxR activation has been recognized (13). To evaluate this possibility, we probed the effect of a site-directed mutant version of pea FPR that displays a shift in substrate preference from NADP(H) to NAD(H) (Fig. 3A [see also reference 35]). The expression of this mutant FPR in wild-type *E. coli* cells did not affect the magnitude (Table 2) or the time course (data not shown) of *soxRS* induction by the herbicide. Moreover, the NAD(H)-dependent flavoenzyme provided no additional protection to *fpr* mutant bacteria exposed to MV toxicity (Fig. 3B).

In the absence of oxidants, 80 to 85% of the NADP(H) pool of the wild-type *E. coli* cells was in the reduced state (Table 2). Disruption of the *fpr* gene resulted in a moderate increase of the NADPH/$NADP^+$ ratio (Table 2), reflecting a minor contribution of FPR to NADP(H) homeostasis in unstressed *E. coli* cells. The observed effect, albeit small, supports the notion that this flavoenzyme behaves as an NADPH consumer in the *E. coli* cytosol (22). Accordingly, overexpression of FPR in an otherwise wild-type background resulted in a significant decline of the cytosolic NADPH pool (Table 2). Exposure of the cells to MV resulted in a further decrease of NADPH relative to $NADP^+$ in all strains assayed, with the FPR-producing bacteria displaying the lowest levels (Table 2). As could be anticipated, expression of the NADH-consuming Y308S FPR version had little or no effect on the redox state of the NADP(H) pool (Table 2).

Concluding remarks. Based on the ability of FPR to mediate rapid electron transfer from NADPH to different types of acceptors, several authors have suggested that the FPR antioxidant role could be related to its participation in a putative redox pathway leading to reductive reconstitution of $O_2^{-}\cdot$-damaged hydrolyases (10, 24) or to reductive deactivation of the sensor protein SoxR (27, 42, 43). Although either of the two mechanisms could explain the need for FPR recruitment during the *soxRS* adaptive response, our results argue against both possibilities. Indeed, growth arrest and hydrolyase inactivation did not contribute significantly to the MV sensitivity of *fpr* mutant bacteria (Fig. 1). This behavior contrasts with that exhibited by a conditional yeast mutant with reduced FPR activity, which developed a complex syndrome including hydrolyase inactivation and abnormal iron trafficking (23). On the other hand, FPR did interfere with the SoxR reduction pathway but not in the way predicted originally. Accumulation of recombinant reductase above MV-induced levels led to stimulation of the *soxRS* response (Fig. 2) rather than to the depression that could have been expected from FPR involvement in SoxR reduction and/or inactivation (27, 42).

In general, FPR's protective effects were linked to its NADP(H)-dependent activities. Predictable changes in the redox state of the NAD(H) pool resulting from overproduction of an NADH-oxidizing FPR mutant had no effect on either *soxRS* induction or MV tolerance (Table 2, Fig. 3). The ability of the flavoenzyme to reduce its physiological substrates ferredoxin or flavodoxin also seems to be dispensable for its protective action (22). The question remains open as to how the FPR-mediated oxidation of NADPH, by using whatever electron acceptor is available, relates to its well-documented antioxidant role. One possible mechanism is proposed in Fig. 4. FPR induction at the onset of the *soxRS* response might be instrumental to keep NADPH at tolerable levels during the progress of the stress condition. The potential toxicity of NADPH (and NADH) derives from its ability to rapidly reduce iron and other transition metals required for the synthesis of highly toxic hydroxyl radicals through Fenton-type chemistry (Fig. 4). As early as 1988, Imlay and Linn (17) proposed that cells might deliberately deplete the pyridine nucleotide pool to impair production of Fenton-class radicals. The identity of the electron donor(s) driving iron reduction in vivo remains unclear but noteworthy NADPH concentrations may rise to 150 μM in the *E. coli* cytosol, compared to $<$20 μM NADH (33). It should be kept in mind, however, that other possible protective mechanisms cannot be ruled out by these experiments and that further research will be required to evaluate the general validity of this proposal.

The response of the *soxRS* regulon to abnormal accumulation of NADPH-consuming FPR illustrates further the complex nature of this adaptive system, whose general features are also described schematically in Fig. 4. A common property of many *soxRS* inducers, including nitric oxide, redox cycling compounds, and even superoxide itself, is that they can deplete the cellular pool of reduced metabolites. This led Liochev and Fridovich (26) to postulate that SoxR might be actually sensing changes in the redox status of the cell. In vitro, both NADPH (13) and GSH (6) are able to inhibit SoxR activity. Thus, even

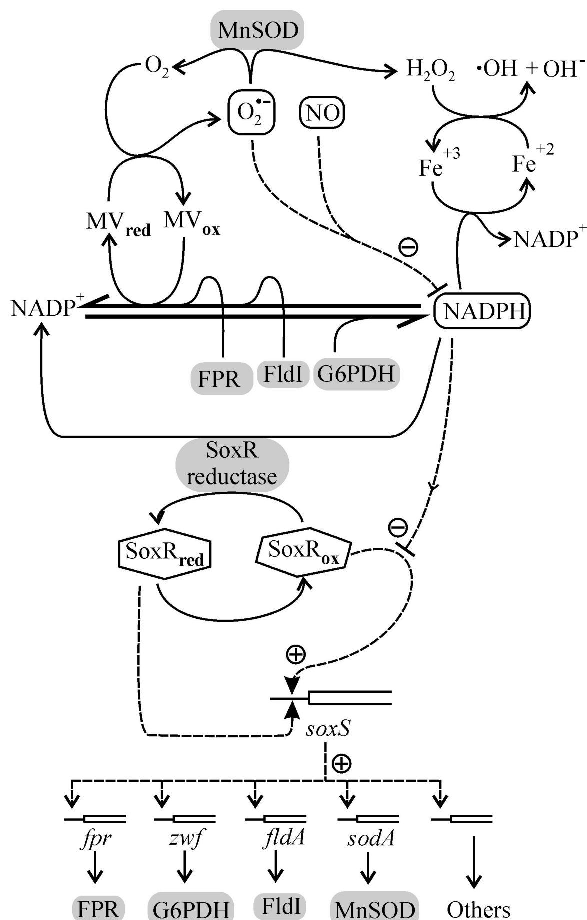

FIG. 4. Integrated model for FPR participation in the *soxRS* response of *E. coli*. At least part of the FPR protective action is proposed to be related to its ability to oxidize the NADPH pool, since NADPH accumulation during the oxidative stress condition might favor propagation of active oxygen species such as ·OH through the reduction of Fe^{3+} and other transition metals (17). FPR is not required for regulation of the *soxRS* response, although depletion of the electron source might contribute to SoxR activation (see below). Under conditions of normal aerobic growth, a reductive pathway presumably involving a SoxR reductase (20) maintains the SoxR dimer in a reduced inactive state ($SoxR_{red}$). During oxidative stress, the dimer is converted into the transcriptionally active form with oxidized iron-sulfur centers ($SoxR_{ox}$). Both forms of SoxR bind to the *soxS* promoter, but only the interaction of $SoxR_{ox}$ stimulates transcription (arrow with plus symbol). SoxS then activates expression of the other members of the regulon, including FPR, glucose-6-phosphate dehydrogenase (G6PDH), flavodoxin I (FldI), and Mn-dependent SOD. The balance between $SoxR_{ox}$ and $SoxR_{red}$ will be biased toward SoxR oxidation whenever electrons are drained from the cellular pool of reduced metabolites. Factors affecting this balance comprise inactivation of key enzymes involved in NADPH synthesis (G6PDH) and the accumulation of chemicals or proteins causing the oxidation or destruction of reduced metabolites. The latter class includes oxygen-centered radicals such as superoxide or nitric oxide, redox cycling compounds such as MV, and electron acceptor proteins and enzymes such as FldI, desulfoferrodoxin, or FPR.

though specific mechanisms such as SoxR nitrosylation may be operating under certain circumstances (8), many oxidants could trigger induction of the regulon simply by draining the cellular electron source. The accumulation of flavodoxin I, one of the FPR physiological substrate acceptors and a recently added member of the *soxRS* system, is indeed reported to cause enhanced induction of the regulon upon MV treatment of the bacterial host (43). Similar results have been obtained when *E. coli* cells were transformed with a plasmid expressing desulfoferrodoxin from sulfate-reducing bacteria, whose accumulation triggers the *soxRS* response presumably by impairing SoxR reduction (14, 34). A comparable stimulation of the regulon was also observed in *zwf* cells, deficient in the SoxS-dependent NADPH producer glucose-6-phosphate dehydrogenase (14, 26). The molecular events involved in that type of SoxR sensing are most probably related to faulty reduction of the sensor protein rather than to SoxR oxidation itself. The sensitivity of the *soxRS* response to multiple signals most certainly confers evolutionary advantages to cells thriving in changing media by allowing the system to adapt to a number of environmental and physiological challenges other than oxidative stress.

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