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Biochemistry

Regulatory roles of Fnr, Fur, and Arc in expression of manganese-containing superoxide dismutase in *Escherichia coli*

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ABSTRACT **Transcriptional regulation of the *sodA* gene, encoding the manganese superoxide dismutase (superoxide: superoxide oxidoreductase, EC 1.15.1.1) of *Escherichia coli*, was studied by monitoring expression of *sodA–lacZ* in different genetic backgrounds and under different growth conditions. Mutations in the *fnr* gene were found to affect aerobic and anaerobic expression of *sodA–lacZ*. Potential Fnr-binding sites were identified in the promoter region of *sodA*. Strains harboring simultaneous mutations in *arcA/B* and *fur* expressed *sodA–lacZ* under anaerobic growth conditions but were still inducible by iron chelators. However, in the triple mutants (*fnr fur arcA/B*) *sodA–lacZ* was fully expressed under anaerobiosis and was not further induced by the presence of 2,2′-dipyridyl, nitrate, or oxidants. On the other hand, aerobic expression of *sodA–lacZ* from a Fur⁻ strain was ≈3.8-fold higher than that from the wild-type strain but was diminished by introducing mutations in *fnr* or *arcA/B*. In conclusion, Fnr, Arc, and Fur act as anaerobic repressors of *sodA*. Furthermore, the regulation of *sodA* by Fur (ferric uptake regulation protein), Arc (aerobic respiratory control), and Fnr (fumarate nitrate reduction/regulator of anaerobic respiration) is independent of the superoxide response regulon SoxRS.**

Superoxide dismutases (superoxide:superoxide oxidoreductase, EC 1.15.1.1) are ubiquitous among living organisms. They constitute an essential component of the biological defenses against the toxicity of oxygen (1, 2). *Escherichia coli* possesses three isozymic forms of superoxide dismutase (3): a manganese-containing enzyme (MnSOD) encoded by the *sodA* gene (located at 88 min on the *E. coli* chromosome), an iron-containing enzyme (FeSOD) encoded by the *sodB* gene (located at 38 min), and a hybrid enzyme containing one subunit of each of the Fe- and MnSODs.

The expression of *sodA* in *E. coli* is regulated by several environmental stimuli including oxygen (3, 4), compounds capable of increasing the intracellular flux of O_2^- (5, 6), iron chelators (7–9), anaerobic respiration using nitrate as an electron acceptor (10–12), and strong oxidants capable of positively changing the redox potential of the cells (13). These and other results led us (8, 10) to propose that expression of *sodA* in *E. coli* is negatively regulated by an iron-containing trans-acting repressor protein. The proposed model predicts that the repressor protein acts as a sensor for and responds to the redox state of the cells. Studies with ^{59}Fe have identified two iron proteins as being good candidates for playing the role of repressor(s) (10).

In vivo and *in vitro* studies have supported the general concept of a negatively controlled transcriptional model (10, 13–16). Moreover, recent reports (17–19) have pointed out the possibility that the Fur (ferric uptake regulation) protein, which regulates iron uptake in *E. coli* (20), is the same protein proposed for regulation of *sodA* (8). Indeed, Fur has been shown to play a role in *sodA* regulation; however, the absence of Fur did not result in anaerobic expression of *sodA* (17–19).

Recently, H.M.H. and D. Touati (unpublished data) isolated *E. coli* K-12 trans-acting regulatory mutants that are derepressed in anaerobic expression of *sodA*. Further characterization of these mutants revealed that the Fur and the Arc (aerobic respiratory control) proteins are involved in regulation of *sodA* (18, 19). However, it was surprising to note that anaerobic expression of *sodA–lacZ* in these mutants was still inducible by iron chelators (18, 19). These results suggested the possible presence of another repressor protein that is responsive to iron chelation.

During the course of investigating the role of DNA topology in the expression of *sodA–lacZ* (21), we noted that strains harboring mutations in the *fnr* (fumarate nitrate reduction) gene consistently express *sodA–lacZ* at a slightly higher level than the isogenic *fnr*⁺ strains (L. Schrum and H.M.H., unpublished data). In this report, we demonstrate the regulatory role of Fnr in expression of *sodA* and show that it acts as a repressor or as an activator in the absence or presence of oxygen, respectively. A preliminary report of these results has been presented (22).

MATERIALS AND METHODS

Materials. Chlortetracycline hydrochloride, sulfanilamide, *N*-(1-naphthyl)ethylenediamine dihydrochloride, methyl viologen (paraquat), 2,2′-dipyridyl, tetracycline (Tet), kanamycin (Km), chloramphenicol (Cm), and *o*-nitrophenyl β-D-galactoside were purchased from Sigma. Quinaldic acid was from Aldrich.

Media and Growth Conditions. *E. coli* cultures were grown at 37°C in LB broth (pH 7.3) containing, per liter, 10 g of Bacto-tryptone (Difco), 5 g of yeast extract (BBL), and 10 g of NaCl supplemented with sterile glucose to a final concentration of 1% (LBG). Solid media were prepared by adding 1.5% Bacto-agar before autoclaving. Where indicated, the following antibiotics were added: Cm at 20 μg/ml, ampicillin (Ap) at 100 μg/ml; Km at 50 μg/ml, and Tet at 15 μg/ml. Media used in anaerobic experiments were preequilibrated in a Coy anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) for at least 2 days, as described (8).

Overnight cultures grown in LBG, supplemented with the appropriate antibiotics, were used to inoculate fresh prewarmed media of the same composition to an initial OD_{600} equal to 0.02–0.04. The cultures were allowed to grow to an OD_{600} of 0.08–0.1 before any treatment began. To ensure sufficient aeration, liquid cultures were shaken at 200 rpm and the culture/flask ratio was 1:5 (8, 10). Anaerobic cultures were grown in the anaerobic chamber in test tubes without shaking. Growth was monitored by measuring OD_{600} using an HP8452A diode-array spectrophotometer.

Bacterial Strains and Plasmids. The bacterial strains and plasmids used are listed in Table 1. Preparation of phage (P1 *vir*) stocks and transduction procedures were performed by

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Abbreviations: Cm, chloramphenicol; Ap, ampicillin; Km, kanamycin; Tet, tetracycline.

Table 1. *E. coli* K-12 strains and plasmids

Strain/ plasmid	Relevant genotype (derivation)	Source or ref.
NC400	ECL512, *fnr-1 zci*::Tn*10*	E. C. C. Lin
NC401	ECL585, *arcA1 zjj*::Tn*10*	Lin (23)
NC402	ECL594, *arcB1 zgi*::Tn*10*	Lin (24)
NC369	W3110, Δ*lacU169, tna2, fur*::Tn*5*	Neilands (20)
NC3	QC772-F$^-$, Δ*lacU169 rpsL*, (*sodA*::*lacZ*)49 Cm^r	Touati (15)
NC410	Same as NC3 but *fnr-1 zci*::Tn*10* [NC400(P1)XNC3]	This study
NC411	Same as NC3 but Δ*fnr* (deletion of Tn*10* from NC410)	This study
NC412	Same as NC3 but Δ*fnr, fur*::Tn*5* [NC369(P1)XNC411]	This study
NC414	Same as NC3 but Δ*arcB* [NC402(P1)XNC3, then deletion of Tn*10*]	This study
NC416	Same as NC3 but Δ*arcB, fnr-1 zci*::Tn*10* [NC402(P1)XNC414]	This study
NC435	Same as NC3 but *arcA1 zjj*::Tn*10*	M. Beaumont (this laboratory)
NC437	Same as NC3 but *arcA1 zjj*::Tn*10, fur*::Tn*5*	M. Beaumont (this laboratory)
NC438	Same as NC3 but *arcB1 zgi*::Tn*10, fur*::Tn*5*	M. Beaumont (this laboratory)
NC439	Same as NC3 but *fur*::Tn*5*	M. Beaumont (this laboratory)
NC417	Same as NC3 but Δ*arcA, fur*::Tn*5* (deletion of Tn*10* from NC437)	This study
NC418	Same as NC3 but Δ*arcB, fur*::Tn*5* (deletion of Tn*10* from NC438)	This study
NC419	Same as NC3 but Δ*arcA, fur*::Tn*5, fnr-1 zci*::Tn*10* [NC400(P1)XNC417]	This study
NC420	Same as NC3 but Δ*arcB, fur*::Tn*5, fnr-1 zci*::Tn*10* [NC400(P1)-XNC418]	This study
NC413	NC417/pfnr2	This study
NC415	NC419/pfnr2	This study
pfnr2	pBR322 *fnr*$^+$ Ap^r	Gunsalus (25)

the method of Miller (26). Plasmid isolation and transformation procedures were essentially as described by Sambrook *et al.* (27).

Construction of Isogenic Strains Harboring Mutations in *arcA/B, fur, fnr*, and Combinations Thereof. P1 lysates were prepared on strain NC369 (*fur*::Tn*5*) and used to transduce NC3; Km-resistant (Km^r) transductants were selected and confirmed for the Fur$^-$ phenotype (28, 29). P1 lysates prepared on NC400 (*fnr*), NC401 (*arcA*), and NC402 (*arcB*) were used to transduce NC3, and transductants were selected on LB plates containing Tet. To combine mutations in *fnr* and *arcA/B* within the same strain, it was necessary to delete the Tet^r (Tn*10*) marker before introducing another Tn*10*-linked mutation. Selection for loss of Tet resistance was achieved by selecting for quinaldic acid (100 μg/ml) resistance (30). The mutant phenotype of the Tet-sensitive (Tet^s) clones was confirmed by the dye (toluidine blue) sensitivity method (23, 31) for *arcA/B* strains and by the lack of nitrate reductase activity (32) for the *fnr* mutants.

β-Galactosidase Assay. The enzyme was assayed in permeabilized whole cells as described by Miller (26). For measuring β-galactosidase in anaerobically growing cells, 0.05–0.10 ml of culture was added anaerobically to the cold Z buffer before removal from the anaerobic chamber and was immediately treated with chloroform and SDS (26). Enzyme activity was monitored during the entire logarithmic growth phase of the cells. Data were plotted in the form of differential plots (i.e., changes in units/ml vs. changes in OD_{600}). The slope of the differential plot represents the activity of β-galactosidase per unit of cell density (units/OD_{600}) determined over the entire logarithmic growth phase of the culture. Variations within the same experiment were <5% in duplicate samples.

RESULTS

Effect of *fnr* on Anaerobic Expression of *sodA*. Expression of the *sodA* gene was examined in *E. coli* strains harboring a *sodA–lacZ* protein fusion, which has been shown (15) to faithfully report the activity of the *sodA* promoter. Fig. 1 shows that, under anaerobic conditions, expression of *sodA–lacZ* in the *fnr* mutant strain (NC411) was significantly higher than that seen in the isogenic *fnr*$^+$ (NC3). Addition of the iron chelator 2,2′-dipyridyl (0.25 mM) increased the level of *sodA–lacZ* expression in both strains (*fnr*$^-$/*fnr*$^+$); however,

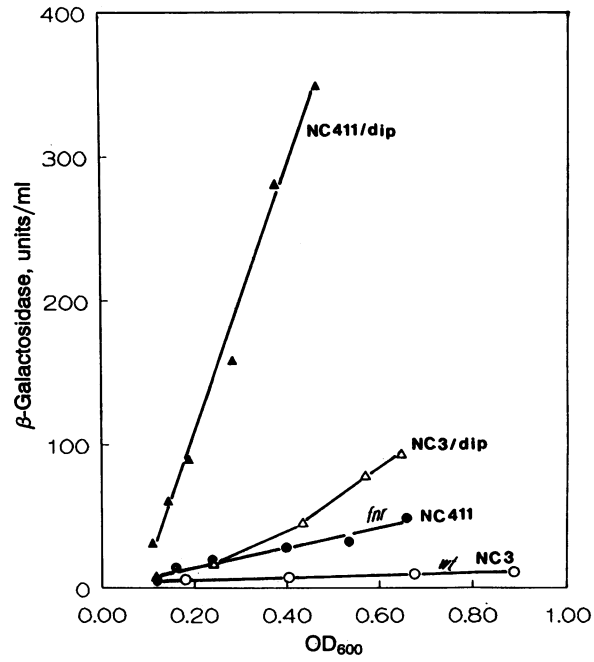

FIG. 1. Effect of *fnr* on anaerobic expression of *sodA–lacZ*. *E. coli* K-12 strains NC3 (*fnr*$^+$) and NC411 (*fnr*$^-$) were grown anaerobically in LBG in the absence (circles) and presence (triangles) of 0.25 mM 2,2′-dipyridyl (dip). Cells were removed at intervals and assayed for growth (OD_{600}) and for β-galactosidase (units/ml). Data are presented in the form of differential plots.

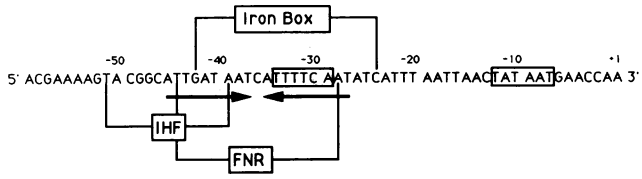

FIG. 2. Nucleotide sequence of the 5' regulatory region of *sodA* (33). Only nucleotides −59 to +1 are listed. The +1 nucleotide designates the start point of transcription. The −35 and −10 regions are boxed. Arrows indicate the 19-base-pair palindrome. The potential integration host factor (IHF)-, Fur-, and Fnr-binding sites are bracketed.

the inductive effect of iron chelation was more pronounced in the *fnr* mutant strain (NC411). These results suggested that the *fnr* gene product (Fnr) may play a role in regulation of *sodA*. Indeed, examining the DNA sequence of the promoter region of the *sodA* gene (33) for the presence of an Fnr-binding site(s) using the symmetrical consensus sequence proposed by Spiro and Guest (34, 35) revealed the presence of potential Fnr-binding site(s) (Fig. 2; See also *Discussion*). These findings in conjunction with the fact that anaerobic expression of *sodA–lacZ* in the trans-acting regulatory mutants is still inducible by iron chelators (18, 19) and that Fnr-regulated operons are induced by iron chelation (36, 37) led us to examine the role of Fnr in expression of *sodA–lacZ* in strains lacking the *sodA* putative repressors (i.e., Fur and/or ArcA/B).

Effects of *fnr* on Anaerobic Expression of *sodA–lacZ* in Strains Harboring Mutations in *fur* and/or *arc*. Data in Fig. 3 show that single mutations in either *arcA*, *arcB* (data not shown), or *fur* had no affect on anaerobic expression of *sodA–lacZ*. Mutations in *fnr* (NC411) or *fnr fur* (NC412) showed a significant increase (8- to 9-fold) in β-galactosidase activity compared to the isogenic wild-type strain (NC3). The double mutant *arcA fur* (NC417) expressed *sodA–lacZ* at a much higher level than any of the single or double mutations studied, in agreement with previous findings (18). Similar results were obtained with the double mutant *arcB fur* (NC418) (data not shown). However, the simultaneous deficiency in ArcA, Fur, and Fnr (strain NC419) resulted in the highest level of anaerobic expression of *sodA–lacZ* (Fig. 3) [i.e., ≈4-fold higher than that seen in the double mutant *arcA fur* (NC417)]. Furthermore, transforming NC419 with a multicopy plasmid bearing the wild-type *fnr*$^+$ gene (pfnr2) in trans (strain NC415) reduced the level of β-galactosidase from

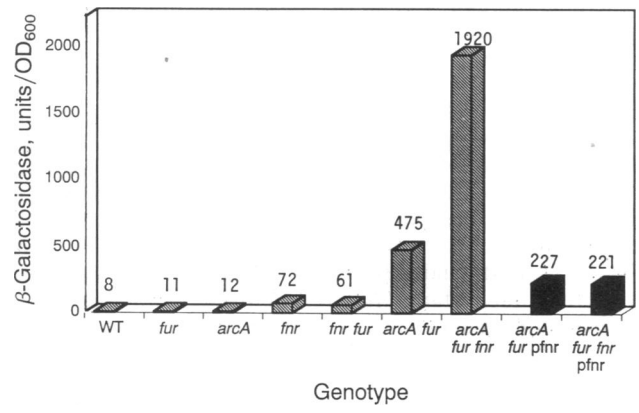

FIG. 3. Effects of mutations in *fur*, *fnr*, and/or *arcA* on anaerobic expression of *sodA–lacZ*. Strains harboring mutations in *fur*, *fnr*, *arcA*, or combinations thereof were grown anaerobically in LBG and assayed for growth (OD_{600}) and for β-galactosidase (units/ml). Data were plotted as in Fig. 1 and the slopes of the lines (units/OD_{600}) are presented in the form of a bar graph. WT, wild type.

Table 2. Effect of 2,2'-dipyridyl and nitrate on anaerobic expression of *sodA–lacZ* in different *fur/fnr/arc* mutants

		β-Galactosidase, units/OD_{600}		
Strain	Genotype	Control	+Dip	+NO_3^-
NC3	wt	8	190	60
NC417	*arcA fur*	500	1390	1110
NC418	*arcB fur*	670	1480	1130
NC419	*arcA fur fnr*	1940	1900	1900
NC420	*arcB fur fnr*	1600	1580	1600
NC415	*arcA fur fnr*/pfnr2	220	1670	400

Cultures were grown anaerobically in LBG as described in *Materials and Methods* and in Fig. 3 in the presence of 0.25 mM 2,2'-dipyridyl (Dip) or 10 mM KNO_3. At 15- to 30-min intervals, samples were removed and assayed for OD_{600} and β-galactosidase (units/ml). Data were plotted in the form of differential plots and the slopes (units/OD_{600}) were calculated.

≈1920 to ≈220 units/OD_{600}. Similarly, transforming NC417 (*arcA fur*) with pfnr2 (strain NC413) reduced the level of β-galactosidase from ≈480 to ≈230 units/OD_{600} (52%). These results suggested that *sodA* repression is partially mediated by Fnr and that increased levels of Fnr (due to the presence of a multicopy plasmid bearing the *fnr*$^+$ gene) can partially (≈50%) substitute for the absence of Fur, ArcA, or both.

Effects of 2,2'-Dipyridyl, Nitrate, and Oxidants on Anaerobic Expression of *sodA–lacZ*. Data in Table 2 show that the anaerobic expression of *sodA–lacZ* in the triple mutants *arcA/B fur fnr* (NC419 and NC420) was uninducible by 2,2'-dipyridyl or by nitrate, while the double mutants *arcA/B fur* (NC417 and NC418) and the triple mutant harboring pfnr2 (NC415) were significantly induced by either iron chelation or nitrate. Similar results were obtained when 0.125 mM $CuSO_4$/0.25 mM KCN was used as an oxidant/anaerobic inducer (13) of *sodA–lacZ* (data not shown). These results show that the triple mutants (NC419 and NC420) were fully derepressed with respect to anaerobic expression of *sodA–lacZ*.

Effects of Fnr, Fur, and Arc on Aerobic Expression of *sodA–lacZ*. Data in Table 3 (column 3) show that aerobic expression of *sodA–lacZ* in response to mutations in *fnr*, *arcA/B*, *fur*, and combinations thereof is more complex than that seen under anaerobiosis. Thus, the level of expression of *sodA–lacZ* in strains harboring a single mutation in either *fnr* (NC411), *arcA* (NC435), or *arcB* (NC414) was similar to that of the wild-type strain, while a mutation in *fur* alone (NC439) increased the level of expression by ≈3.8-fold (Table 3). These results suggest that aerobic expression of *sodA–lacZ* is

Table 3. Effect of paraquat on aerobic expression of *sodA–lacZ* in the different *fur/fnr/arc* mutants

		β-Galactosidase, units/OD_{600}		
Strain	Genotype	$-PQ^{2+}$ (*a*)	$+PQ^{2+}$ (*b*)	*b* − *a*
NC3	wt	1180 (1)	7,100	5920
NC411	*fnr*	1000 (1)	7,000	6000
NC435	*arcA*	1010 (1)	6,150	5140
NC414	*arcB*	1160 (1)	7,750	6590
NC439	*fur*	4440 (3.8)	10,520	6080
NC412	*fur fnr*	1250 (1)	6,000	4750
NC417	*fur arcA*	2430 (2)	8,360	5930
NC418	*fur arcB*	1750 (1.5)	7,440	5690
NC419	*fur arcA fnr*	2200 (1.9)	8,360	6160
NC420	*fur arcB fnr*	1370 (1.2)	6,780	5410

Cultures were grown aerobically at 200 rpm in LBG in the presence and absence of 0.1 mM paraquat (PQ^{2+}) and were assayed at 15- to 30-min intervals for OD_{600} and β-galactosidase (units/ml). Data were calculated as described in Table 2. Numbers in parentheses indicate -fold increase above the level of wild type.

partially repressed by Fur. However, the expression of *sodA–lacZ* in a strain lacking Fur (NC439) was diminished by introduction of mutations in *fnr* (NC412), *arcA* (NC417), *arcB* (NC418), or both *fnr* and *arcA/B* (NC419 and NC420). The lack of both Fur/Fnr reduced the level of β-galactosidase to that of the wild type, while the lack of Fur/Arc or Fur/Arc/Fnr reduced the level of expression from 4440 to 1400–2400 units/OD_{600} (i.e., 1.2- to 2.0-fold higher than wild type). Thus, for maximum aerobic expression of the *sodA* gene both ArcA and Fnr must be present, while Fur must be absent.

Effect of Paraquat on Aerobic Expression of *sodA–lacZ*. The aerobic induction of the *sodA* gene in response to an increased rate of intracellular superoxide generation, caused by the presence of paraquat or other redox cycling compounds (5, 6, 15), has been shown to be positively regulated by the *soxRS* gene products (SoxRS) (38–41). Therefore, it was of interest to study the effect of paraquat (0.1 mM) on aerobic expression of *sodA–lacZ* in the different mutant strains (Table 3, columns 4 and 5) in order to determine whether the positive regulation of *sodA* by SoxRS is dependent on the regulatory elements Fur, Arc, and Fnr. The data show that all constructs were inducible by paraquat and that the difference in β-galactosidase levels in paraquat-treated minus untreated cells (Table 3, *b* − *a*) was relatively constant (range, 4750–6590 units/OD_{600}; average, ≈5770 units/OD_{600}).

DISCUSSION

The data presented show that expression of *sodA* is regulated by three complex global control systems (Fur, Arc, and Fnr), which coordinate the synthesis of MnSOD with iron uptake and the cell's ability to switch between aerobic and anaerobic respiration. This complex, yet delicate, regulatory circuit would ensure optimal growth and cell yield under both aerobic and anaerobic growth conditions, and it would provide maximum protection against the toxicity of molecular oxygen.

Role of Fur. Fur protein, the product of the *fur* gene, negatively controls transcription of many genes required for synthesis of iron siderophores in *E. coli* (20, 28). Recently, a potential Fur binding site was identified in the untranslated 5′ region of *sodA* (Fig. 2; refs. 17 and 42), and a pure Fur protein was shown to bind to the *sodA* promoter region (17). The present findings (Fig. 3 and Table 3) show that Fur acts as a negative regulator (i.e., repressor) of *sodA* under both aerobic and anaerobic conditions. The regulation of *sodA* by Fur represents a unique example of a Fur-regulated gene that is not involved in the supply of iron to the cell.

Role of Arc. ArcA protein, the product of the *arcA* gene, is known to repress the expression of many genes of *E. coli* that are not required for anaerobic growth (23). ArcA and ArcB are members of the two-component class of regulatory proteins, where ArcB is the sensor partner for ArcA (43, 44). Mutations in either *arcA* or *arcB* alone had no effect on expression of *sodA–lacZ* (Fig. 3 and Table 3). Similar results have been reported (24). In agreement with the results reported by others (18), our data showed that the effects of mutations in *arcA* or *arcB* are manifested only in strains harboring a *fur*$^-$ background (Fig. 3 and Table 3). Thus, under anaerobic conditions, the simultaneous inactivation of *fur* and *arcA/B* genes resulted in expression of *sodA–lacZ*, which is in agreement with the conclusion that ArcA/B and Fur are negative regulators (i.e., repressors) of the *sodA* gene (18, 19). Under aerobic conditions, ArcA$^+$ in a *fur*$^-$ *fnr*$^+$ background allowed for higher expression of *sodA–lacZ* (compare NC439 vs. NC417; Table 3, column 3), while this was not the case in a *fur*$^-$ *fnr*$^-$ background (compare NC412 and NC419; Table 3, column 3). The aerobic role of ArcA, if any, seems to be dependent on the absence of Fur, and on whether Fnr is present or absent. Recently, ArcA was shown to activate the *cyd* operon encoding cytochrome *d* (43). Thus, it is conceivable that ArcA can act as an aerobic activator in addition to its classical role as anaerobic repressor.

Role of Fnr. Fnr protein, the product of the *fnr* gene, is generally recognized as a positive activator of anaerobic respiration (25, 45). The *fnr* gene is expressed under both aerobic and anaerobic growth conditions (35, 46). A Fnr consensus sequence has been identified in the promoters of genes regulated by Fnr (34, 35). The proposed sequence is an inverted repeat of 22 base pairs with 4 bases spacing the half-sites, 5′-ANANTTGATNNANATCAATNNN-3′ (where boldface letters indicate well-conserved bases and N indicates any base). The promoter region of the *sodA* gene contains the two highly conserved half-sites (Fig. 2) with a spacing of 7 bases instead of the conserved half-site spacing of 4 bases, 5′-GGCA<u>TTGAT</u>N_7<u>TTCAAT</u>ATC-3′ (where underlined bases indicate identity). However, if the spacing between the half-sites is kept at 4 bases, two potential Fnr-binding sites can be located: (*i*) 5′-<u>A</u>TTGA<u>TAAT</u>-CATTT<u>TCAAT</u>ATC-3′ (located between −45 and −24) and (*ii*) 5′-GGCA<u>TTGAT</u>AATC<u>ATTTT</u>CAAT-3′ (located between −48 and −27). Overall, site *i* has a better homology (9/14) than site *ii* (7/14); however, each of the two sites has a homology in 7/10 of the most conserved bases. The two potential Fnr-binding sites found in the *sodA* promoter sequence do not show the high degree of homology found in the promoters of Fnr-regulated operons such as *narG, cyd, ndh,* or *fnr*; however, their degree of homology is equal to or better than those found in other Fnr-regulated operons such as *aspA, fumB, frdA,* or *glpA* (34). At the present time, the significance of these potential Fnr-binding sites is not clear due to lack of knowledge about specific interactions between the *sodA* promoter and Fnr. In general, attempts to demonstrate specific Fnr–DNA interactions have been unsuccessful (34, 47), which may be due to the lack of appropriate effector(s) and/or incubation conditions. The optimum conditions for binding of Fnr to *sodA* promoter remain to be defined.

The data (Fig. 3) showed that Fnr acts as an anaerobic repressor of *sodA*. Thus, under anaerobic conditions, the expression of *sodA–lacZ* in the double mutant NC417 (*fur arcA*) was further increased by inactivation of the *fnr* gene as in NC419 (*fur arcA fnr*). Furthermore, transforming the triple mutant (NC419) with a multicopy plasmid bearing the *fnr*$^+$ gene (pfnr2) resulted in lowering *sodA–lacZ* expression to a level ≈50% lower than that seen in the double mutant (NC417). These results suggested that excess Fnr protein may partially substitute for Fur and/or ArcA in their roles as anaerobic repressors of *sodA*. Support for this notion came from the finding that excess Fnr also reduced the anaerobic expression of *sodA–lacZ* in the double mutant (*fur arcA*) by ≈50% (Fig. 3). It is interesting to note that the potential Fnr binding sites overlap the iron box (Fig. 2), thus suggesting some kind of competition between Fnr and Fur during their regulation of *sodA* expression. The finding that Fnr may contain iron (34, 36, 37) is further supported by our finding that the anaerobic expression of *sodA–lacZ* in the double mutants (*fur arcA/B*) or the triple mutant harboring pfnr2 (NC415) was still inducible by the addition of 2,2′-dipyridyl, while the triple mutants (*fur arcA/B fnr*) were not responsive to such a treatment (Table 2). Nitrate had the same effect as 2,2′-dipyridyl on anaerobic expression of *sodA–lacZ* (Table 2). The anaerobic induction of MnSOD by nitrate (10–12) is dependent on a functional nitrate reductase (12), which is controlled by Fnr (25, 45). Thus, Fnr plays both direct (repressor) and indirect roles in regulation of *sodA*.

Role of SoxRS. Recent studies have shown that induction of *sodA*, in response to an increased flux of intracellular superoxide radicals (5, 6), is positively regulated by *soxRS* gene products (SoxRS) (38–41). The data presented in Table

3 (columns 4 and 5) showed that induction of *sodA–lacZ* by paraquat is independent of mutations in *fur, fnr, arcA/B*, or combinations thereof. Therefore, regulation of the *sodA* gene by Fur, ArcA/B, and Fnr is independent of SoxRS.

Conclusion. The concentration of iron and the redox state in cells play an important role in regulation of *sodA* (8–10, 13–19). The iron in Fnr and Fur seems to act as an effector/sensor molecule that can sense changes in the redox state of the cells. Moreover, the valence of the iron (Fe^{2+}/Fe^{3+}) or its limitation can modulate the regulatory function of both Fur and Fnr. Thus, in the absence of oxygen both Fur and Fnr will contain Fe^{2+} and will act as repressors of *sodA* with Fur being dominant over Fnr. The presence of iron chelators will inactivate both iron-containing repressors (Fur and Fnr). Also, in anaerobiosis, reduced intermediates of the electron-transport chain will trigger phosphorylation of ArcB, which in turn activates ArcA to become a repressor of both aerobic respiration (43, 44) and the *sodA* gene (refs. 18 and 19; Fig. 3). The data presented here showed that, in air, ArcA and Fnr have a moderate effect on the rate of expression of *sodA–lacZ*; however, no definite pattern was discernible.

The coordinate regulation of the synthesis of MnSOD and the cell's ability to acquire iron and to switch between aerobic and anaerobic metabolism is indeed complex, but intriguing. It is interesting to note that Fur, Fnr, and ArcA/B all affect the status of the redox components of the electron-transport chain. Thus, Fur controls the concentration of iron in the cells, while Arc and Fnr control aerobic/anaerobic respiration, which require iron for the synthesis of heme/nonheme proteins. Indeed, Iuchi *et al.* (43) have recently proposed that a component of the electron-transport chain provides the signal required for activation of the Arc system. Moreover, expression of the cytochrome *d* complex (encoded by the *cyd* operon) in *E. coli* is under the control of both ArcA and Fnr (48). Therefore, it is possible that communication between Fur, Fnr, and ArcA/B, which is required for the delicate coordination between synthesis of the enzymes needed for respiration and for detoxification of oxygen toxicity, takes place at some common site involving iron and the electron-transport chain.

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