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Control of *Escherichia coli* Superoxide Dismutase (*sodA* and *sodB*) Genes by the Ferric Uptake Regulation (*fur*) Locus

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The ferric uptake regulation (*fur*) gene product participates in regulating expression of the manganese- and iron-containing superoxide dismutase genes of *Escherichia coli*. Examination of β-galactosidase activity coded from a chromosomal ϕ(*sodA'-'lacZ*) fusion suggests that metallated Fur protein acts as a transcriptional repressor of *sodA* (manganese superoxide dismutase [MnSOD]). Gel retardation assays demonstrate high-affinity binding of pure, Mn^{2+}-Fur protein to DNA fragments containing the *sodA* promoter. These data and the presence of an iron box sequence in its promoter strongly suggest that *sodA* is part of the iron uptake regulon. An *sodB'-'lacZ* fusion gene borne on either a low- or high-copy plasmid yielded approximately two- to threefold more β-galactosidase activity in Fur^+ compared with Fur^- cells; the levels of activity depended only weakly on the growth phase and did not change during an extended stationary phase. Measurement of FeSOD activity in logarithmic growth phase and in overnight cultures of *sodA* and *fur sodA* backgrounds revealed that almost no FeSOD activity was expressed in Fur^- strains, whereas wild-type levels were expressed in Fur^+ cells. Fur^+ and Fur^- cells bearing the multicopy plasmid pHS1-4 (*sodB*$^+$) expressed approximately sevenfold less FeSOD activity in the *fur* background, and staining of nondenaturing electrophoretic gels indicates that synthesis of FeSOD protein was greatly reduced in Fur^- cells. Gel retardation assays show that Mn^{2+}-Fur had a significantly higher affinity for the promoter fragment of *sodB* compared with that of random DNA sequences but significantly lower than for the promoter fragment of *sodA*. These observations suggest that the apparent positive regulation of *sodB* does not result exclusively from a direct interaction of holo (metallated) Fur itself with the *sodB* promoter. Nevertheless, the *sodB* gene also appears to be part of the iron uptake regulon but not in the classical manner of Fe-dependent repression.

Bacteria contain two metalloproteins currently classified as superoxide dismutases (SOD; EC 1.15.1.1). These proteins are quite well characterized. Three-dimensional structures have been determined for both Fe and Mn forms (42; see reference 32 and references therein). Detailed mechanistic studies of catalytic superoxide dismutation have been carried out (8; see reference 55 and references therein). The two genes coding for the *Escherichia coli* proteins have been cloned, mapped, and sequenced, and null mutations in each and both genes have been prepared (see reference 56 and references therein). It is widely held that the function of these proteins in bacteria is to provide protection from oxygen poisoning (21, 35). One of us has objected to this belief, suggesting that the functions of SODs are not yet known (18); recent observations (20) that some SOD-less *E. coli* strains can grow in oxygenated glucose minimal medium at rates similar to that of parental cells support this view.

The regulation of SOD activities in *E. coli* is quite complicated (53, 56, 57). Early workers, finding that MnSOD activity is normally not detected in cells grown under anaerobic conditions, suggesting that expression of the *sodA* gene, which codes for MnSOD, was controlled by the presence of O_2^-; in contrast, the *sodB* gene, which codes for FeSOD, appeared to be constitutive (29; see reference 22 and references therein). Compelling evidence that O_2^- was involved seemed to come from observations that culturing cells in the presence of certain redox dyes, notably methyl viologen (paraquat [PQ^{2+}]), caused substantial increases in MnSOD activity (29, 30). More recent studies, however, have suggested that neither O_2 nor O_2^- is involved and that *sodA* expression depends on the nature of the carbon source and the mode of growth, either fermentative or respiratory. Thus, *sodA* expression, as measured by MnSOD activity, occurs during respiratory growth when dioxygen, nitrate, trimethylamine-*N*-oxide, dimethyl sulfoxide, or ferricyanide is used as a terminal electron acceptor (31, 37, 45, 47, 51); it is noteworthy that during either aerobic or anaerobic respiration, MnSOD is induced on the addition of methyl viologen (PQ^{2+}) to the culture (45). MnSOD activity also appears to depend on the nutritional status of the cell in a manner suggesting that involvement of the catabolite activation system (30). Early work also suggested that the concentration of Fe in the culture medium influences levels of SOD activity. In 1976, Simons et al. (50) demonstrated that the level of FeSOD activity was enhanced by increasing the concentration of Fe in an anaerobic culture medium, while in 1984, Moody and Hassan (38) showed that MnSOD activity was enhanced by strongly decreasing the concentration of Fe in the culture medium. In a pioneering study, Touati (57) examined the expression of a ϕ(*sodA'-'lacZ*) chromosomal fusion in which the *lacZ* structural gene is under control of the promoter of *sodA*. In her work, induction of β-galactosidase activity mimicked MnSOD induction: activity was not expressed in anaerobiosis and was induced by oxygen, redox cycling compounds in aerobiosis, and iron chelators in the absence of oxygen. Data were also obtained in agreement with the suggestion of Moody and Hassan (38) of transcriptional repression of an Fe^{2+}-protein complex. The work of Touati (57) further suggested that *sodA* transcription is positively controlled by an activator system and by negative

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autogenous feedback, i.e., direct control by the SodA protein of its own structural gene. The regulation of the *sod* genes thus appears to involve environmental factors in addition to Fe, and there is at present no unifying model.

E. coli requires Fe for growth and elaborates a remarkably complex machinery to extract it from the environment (59). Some 30 genes of the *E. coli* chromosome are involved in iron uptake, and all are in some way regulated by the ferric uptake regulation (*fur*) locus (9, 25, 26, 39, 41, 44, 46, 59). The mechanism of this regulation is now understood in broad outline (2, 3, 9, 14–16, 25, 26, 39, 41, 44, 46, 58, 59): the product of the *fur* gene, Fur, is a 17-kilodalton polypeptide that binds Fe^{2+}, forming a dimeric protein (metallated or holo Fur) that associates with a specific DNA sequence in the operator-promoter regions of *fur*-controlled genes; there Fur in conjunction with Fe acts as a repressor of transcription (16, 58). When the Fe concentration within the cell is lowered beyond a certain level, this repression is relieved (2, 16). In all of the genes examined so far, metallated Fur recognizes an operator sequence called the iron box (3, 9, 25, 26, 39, 41, 44, 46), having a consensus sequence GATAAT GAT(A/T)AT(T/C)ATT(T/A)(T/G)C (17). In a preliminary communication, we noted (40) that *sodA* contains an iron box (GATAATCATTTTCAATATC) that is ~85% similar to the consensus sequence. The only significant deviation occurs at position 15 (shown in boldface), where *sodA* has an A while all other sequences contain a T. This sequence overlaps the RNA polymerase-binding (−35) region of the promoter of *sodA*. A corresponding sequence is not found in the *sodB* gene (10). By analogy to other genes bearing this sequence, the presence of an iron box suggested to us that the *sodA* gene would be derepressed in strains carrying a null mutation in *fur*, while the *sodB* gene, which lacks the iron box, would be unaffected. Here we show that the *fur* locus plays an important, though not dominant, role in the expression of *sodA* and a dominant role in the expression of *sodB*.

(Various aspects of this work were presented at the 1988 UCLA Symposium on Metal Ion Homeostasis: Molecular Biology and Chemistry, Frisco, Colorado, 10 through 16 April 1988 [40].)

MATERIALS AND METHODS

Bacterial strains, plasmids, and sources of materials. The *E. coli* strains and the plasmids used in this work appear in Table 1. All chemicals were of the highest commercial quality. Restriction endonucleases and exonucleases were obtained from New England BioLabs, Inc., Beverly, Mass.; Bethesda Research Laboratories, Inc., Gaithersburg, Md.; and Boehringer Mannheim Biochemicals, Indianapolis, Ind. Radioisotopes were obtained from New England Nuclear Corp., Boston, Mass., and nitrocellulose was provided by Schleicher & Schuell, Inc., Keene, N.H.

General recombinant DNA methodology. The procedures described by Maniatis et al. (33), Miller (36), Berger and Kimmel (5), Ausubel et al. (1), and Silhavy et al. (49) were followed.

Sequence analysis. Analyses of gene sequences were performed with the University of Wisconsin Genetics Computer Group sequence analysis software package and the GenBank and National Biomedical Research Foundation data bases (17).

Construction of protein fusions. A *sodB'-'lacZ* protein fusion was constructed from the *sodB* promoter and the *lacZ'* structural gene. Plasmid pHS1-8 was digested with *Bst*XI and *Nde*I restriction endonucleases; the ~200-base-pair (bp) fragment containing the *sodB* promoter and the first eight amino acids of the structural gene was isolated, and the 5′ overhangs were removed with mung bean nuclease. The Casadaban β-galactosidase fusion vector pMC1871 (12) was digested with *Sma*I restriction endonuclease, dephosphorylated with calf intestine alkaline phosphatase, mixed with the *sodB* promoter fragment and T4 DNA ligase, and incubated at 12°C for ~14 h. The DNA was transformed into Lac^- strains in order to select for complementation of β-galactosidase activity on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) indicator plates. Plasmid DNA was isolated from blue colonies and used to transform Fur^+ and Fur^- strains. Two potential colonies were identified by the slot lysis method (48); their plasmids (later shown to be identical) were isolated and transformed into the *fur* isogenic strains for replica plating. When grown on X-Gal plates, Fur^- cells bearing this plasmid, pMN3, were light blue whereas Fur^+ cells bearing the plasmid formed dark blue colonies. Restriction analysis and hybridization to an oligodeoxynucleotide probe directed to the *sodB* promoter were used to demonstrate the presence of the promoter insert upstream of *lacZ'* and to show that pMN3 and pMN9 were identical. The resulting protein fusion was then transferred to the low-copy vector pHSG575 (54) by in vitro methods to yield pDM28.

TABLE 1. Strains and plasmids

Strain or plasmid	Genotype	Reference
Strains		
GC4468	F^- Δ*lacU169 rpsL*	11
QC774	GC4468 Φ(*sodA'-'lacZ*)*49* Φ(*sodB'-'kan*)*1*-Δ_2 Cam^r Kan^r	11
AB1157	*leuB6*(Am SuIII) *thr-1* Δ(*gpt-pro*)*62* *hisG4*(Oc)[a] *argE3*(Oc) *lacY1 galK2* *ara-14 mtl-1 xyl-5 thi-1 tsx-33* *rpsL31 supE44*(Am SuII) Rec^-	2
BN402	AB1157 Δ*lacU169 galK*	2
BN4020	AB1157 Δ*lacU169 galK fur*::Tn*5* Kan^r	2
ENF13	BN4020 Φ(*sodA'-'lacZ*)*49* Cam^r	This work
ENF17	BN402 Φ(*sodB'-'kan*)*1*-Δ_2 Kan^r	This work
ENF19	BN402 Φ(*sodA'-'lacZ*)*49* Cam^r	This work
Plasmids		
pHS1-4	*sodB*$^+$ Tc^r	11
pHS1-8	*sodB*$^+$ Tc^r	11
pDT1-5	*sodA*$^+$ Ap^r	11
pMN3	*sodB'-'lacZ* Tc^r	This work
pDM28	*sodB'-'lacZ* Cm^r	This work
pABN203	*fur*$^+$ Tc^r	15
pCON6	*iucA'-'lacZ* Ap^r	14

[a] Oc, Ochre.

Cell growth. Isogenic *E. coli* strains (BN402 and BN4020 and ENF19 and ENF13) were grown at 37°C in a rotary shaker in L broth plus glucose (0.2%) medium and under appropriate antibiotic pressure (chloramphenicol, 15 μg/ml; kanamycin, 50 μg/ml; and/or tetracycline, 12.5 μg/ml). Cultures were grown under anaerobic, aerobic (0.8 atm [ca. 80 kPa]), or aerobic plus PQ^{2+} conditions according to need. The anaerobic cultures were grown in sealed culture flasks and tubes. Iron concentrations were measured by the method of Blair and Diehl (6). For induction experiments, overnight L broth-glucose (0.2 %) cultures were grown and used to inoculate prewarmed (37°C) L broth-glucose medium to an A_{600} of ~0.05. The cultures were grown under normal aeration for 30 min before being divided into separate flasks containing prewarmed L broth-glucose supplemented with

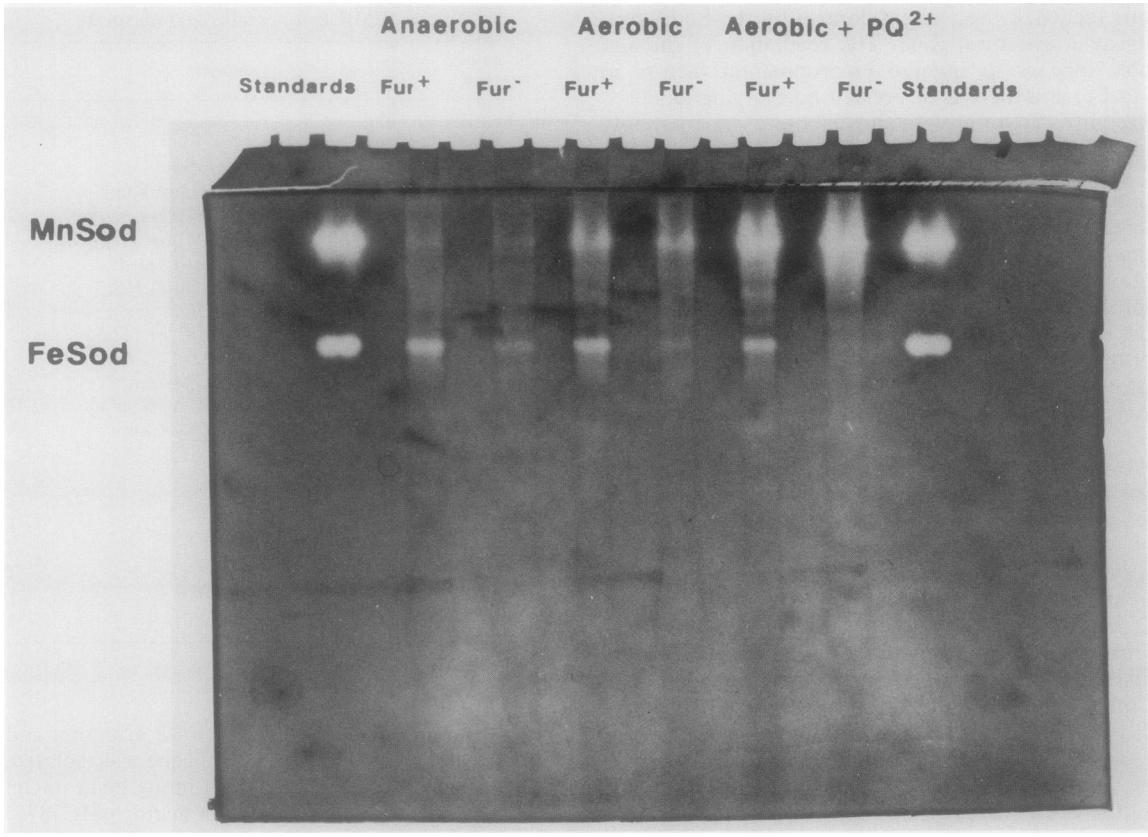

FIG. 1. Visualization of SOD activity from extracts of *E. coli* cells having Fur^+ and Fur^- phenotypes. Purified proteins were placed in the leftmost and rightmost lanes (*E. coli* MnSOD [3.5 μg per lane] and FeSOD [6 μg per lane]). Extracts were prepared from cells grown to stationary phase as described in the text, and 120 μg of protein was applied in each lane. Electrophoresis was carried out in a nondenaturing, discontinuous, 10% polyacrylamide gel, and SOD activity was detected by the method of Beauchamp and Fridovich (4).

20 μM PQ^{2+}, 50 μM $FeSO_4$, or 100 μM diethylenetriaminepentaacetic acid (DTPA). Samples were removed at the indicated times, immediately cooled with ice water, and kept on ice until assayed for β-galactosidase activity.

Preparation of cell extracts. Cells were collected at the indicated times by centrifugation at 6,000 × *g*, washed and suspended in a pH 8 solution containing 50 mM potassium phosphate and 1 mM EDTA, mixed with lysozyme, ruptured by sonication at 4°C for two 30-s intervals with a Sonifier (Branson Sonic Power Co., Danbury, Conn.) equipped with a standard microtip, and centrifuged at 10,000 × *g* to remove cell debris. Protein concentrations were determined by the bicinchoninic acid method (52); bovine gamma globulin or bovine serum albumin served as the calibration standard.

Electrophoresis. Cell extracts and purified protein were electrophoresed under nondenaturing discontinuous conditions (27) on 10% polyacrylamide gels. The gels were stained for protein with 26% isopropanol–11% acetic acid–0.1% Coomassie blue R and destained with 10% isopropanol–10% acetic acid, or stained for SOD activity by the method of Beauchamp and Fridovich (4).

Enzyme activities. The xanthine-xanthine oxidase-coupled reduction of cytochrome *c* assay as described by McCord and Fridovich (34) was used to quantify soluble SOD activity. β-Galactosidase activity was measured by the sodium dodecyl sulfate-$CHCl_3$ method of Miller (36).

Protein-DNA gel retardation. DNA fragments were prepared as follows by working from the known sequences. *iucA* plasmid pCON6 (14), harboring the aerobactin operator, was digested with *Eco*RI and *Bam*HI endonucleases to generate a 160-bp fragment containing the promoter-operator sequences to which holo Fur has previously been shown to bind (14). *sodA* treatment of pDT1-5 (11) with *Ava*I and *Taq*I endonucleases yielded 195- and 252-bp fragments containing the promoter and part of the *sodA* structural gene, respectively. *sodB* digestion of pHS1-8 (11) with *Bst*XI, *Eco*RI, and *Nde*I endonucleases resulted in a 200-bp promoter fragment and a 530-bp *sodB* structural gene fragment. The desired DNA fragments were isolated from low-melting-point agarose gels and purified by phenol-chloroform extraction and ethanol precipitation. These fragments were end labeled with [γ-^{32}P]ATP by using the exchange reaction catalyzed by T4 polynucleotide kinase and purified by centrifugation through a Sephadex G-50 spin column as described by Maniatis et al. (33) or by centrifugation through a 30-kilodalton cutoff Centricon filter (Amicon Corp., Lexington, Mass.). Purified Fur protein was generously provided by the J. B. Neilands laboratory in the demetallated form as a lyophilized powder. Apo Fur at ~20 μM was reconstituted in incubation buffer for ~30 min at room temperature; small samples of solution were stored in sealed tubes at −20°C until needed. The labeled DNA fragments were incubated at 37°C for a minimum of 30 min with increasing amounts of freshly thawed solutions of holo Fur protein. The incubation buffer contained 10 mM BisTris-borate [bis(2-hydroxyethyl) imino tris(hydroxymethyl)-methane] (pH 7.5), 100 μM $MnCl_2$, 5 μg of sonicated salmon sperm DNA per ml, 100 μg of bovine serum albumin per ml, 40 mM KCl, 1 mM $MgCl_2$, and

40% glycerol (final volume, 10 μl) and was mixed with 1 μl of freshly prepared 20 mM dithiothreitol immediately before use to ensure stable formation of holo Fur. Samples (5 μl) of DNA-protein solution or of tracking dyes were loaded into separate lanes of a 5% polyacrylamide gel that was prepared with 40 mM BisTris-borate (pH 7.5) and 100 μM $MnCl_2$, polymerized with riboflavin and *N,N,N',N'*-tetramethylethylenediamine, and run at constant 200 V for 2 to 3 h to minimize heat generation. The electrophoresis was discontinued when the xylene cyanole dye had migrated to the leading edge of the gel. The gels were transferred to filter paper and placed on film at −80°C with intensifying screens.

RESULTS

Qualitative indications of MnSOD and FeSOD levels in cell extracts of isogenic strains BN402 (Fur^+) and BN4020 (Fur^-) grown to stationary phase under anaerobic, aerobic, and aerobic plus PQ^{2+} conditions are shown in Fig. 1. FeSOD levels were atypical in Fur^- cells (virtually absent) and normal in Fur^+ cells, whereas MnSOD levels in both Fur^+ and Fur^- strains appeared to be roughly equal and typical of cells harvested in the stationary phase of growth. Because MnSOD activity was greatly enhanced by PQ^{2+} in Fur^- cells and because MnSOD was not present at significant levels in Fur^- cells grown under anaerobic conditions (Fig. 1), it is likely that the *fur* locus is not the major controlling factor of the cell of *sodA* expression; by contrast, *fur* must be regulating *sodB* expression in a very robust manner.

Expression of ϕ(*sodA'-'lacZ*). On the basis of qualitative indications of MnSOD activity, as shown in Fig. 1, we previously suggested that *sodA* may not be regulated by *fur* (40). To examine the question further, we quantified the influence of *fur* on *sodA* transcription by transferring the chromosomal fusion, ϕ(*sodA'-'lacZ*), of Carlioz and Touati (11) by P1 transduction (33) to BN402 and BN4020. This transduction nulls the *sodA* gene and replaces it with *'lacZ* under the control of the *sodA* promoter (11, 57). Figure 2 shows the levels of β-galactosidase activity as a function of cell growth in L broth-glucose supplemented to 50 μM Fe. A number of observations can be made from these curves. First, Fur^+ and Fur^- cells grew at essentially identical rates in this medium. Second, *sodA* was substantially derepressed in Fur^- cells in comparison with Fur^+ cells; during the period of balanced growth there was approximately threefold more *sodA'-'lacZ* gene product in the Fur^- strain than in the Fur^+ strain. Third, as the cells entered stationary phase the β-galactosidase activity decreased, indicating that *sodA* is turned off in some manner or that the fusion protein is degraded at a greater rate; specific β-galactosidase activity in both strains decreased to the zero-time levels in overnight cultures (data not shown). Although we have no explanation for this behavior, some classes of *E. coli* proteins behave in this manner (43). This behavior might account for the apparent lack of regulation when MnSOD activity is observed on activity gels. We have also examined expression of the *sodA'-'lacZ* fusion gene under anaerobic conditions. As expected, the absolute level of activity was much lower in anaerobically grown cells (compare aerobic Fur^+ cells grown to an A_{600} of 1.7 having 320 U/A_{600} with anaerobic Fur^+ cells grown to a A_{600} of 1.5 having 7 U/A_{600}), consistent with the idea that another regulatory system must be involved. Nevertheless, in anaerobic conditions, Fur^- cells have approximately fourfold more activity than Fur^+ cells (compare Fur^- cells grown to an A_{600} of 1.3 having 34 U/A_{600} with Fur^+ cells grown to an A_{600} of 1.5 having 7 U/A_{600}). Because the *fur* and *sodA* genes are widely distributed on the chromosome (3, 56), these observations indicate that *sodA* is *trans* regulated, at least in part, by *fur*.

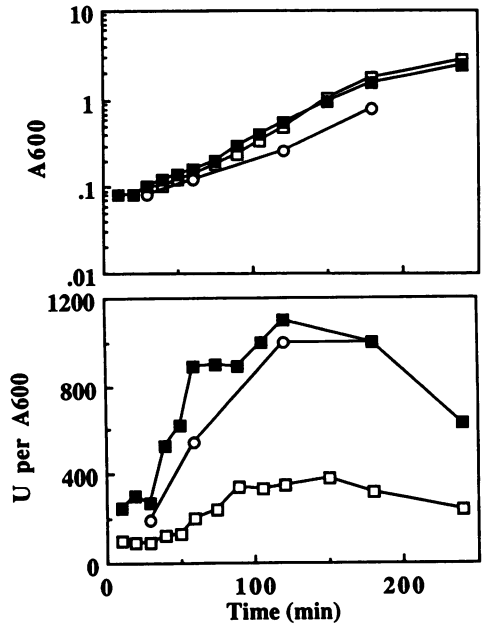

FIG. 2. Expression of the chromosomal fusion gene ϕ(*sodA'-'lacZ*) as a function of growth in isogenic Fur^+ and Fur^- strains of *E. coli*. Symbols: □, strain ENF19 Fur^+; ■, strain ENF13 Fur^-; ○, strain ENF19 Fur^+ cells grown in the presence of 100 μM DTPA. DTPA had very little effect on the β-galactosidase activity of Fur^- cells. No antibiotics were included in the final growth medium.

Previous results of others showed that depletion of Fe from the medium leads to derepression of *sodA* (38, 57). Indeed, we also find that treating cells with metal-chelating agents that do not inhibit growth, such as DTPA, significantly derepressed *sodA'-'lacZ* in Fur^+ but not in Fur^- strains (Fig. 2). A similar effect was seen in Fur^+ cells grown under anaerobic conditions (compare Fur^+ cells grown in the absence of DTPA to an A_{600} of 1.5 having 7 U/A_{600} with Fur^+ cells growing in the presence of 100 μM DTPA to an A_{600} of 0.9 having 26 U/A_{600}). As noted in the legend to Fig. 2, DTPA has little effect on expression of the fusion gene in Fur^- cells (data not shown). As to the well-established inductive effect of PQ^{2+}, Fur^+ and Fur^- cells, containing *sodA'-'lacZ* and growing in the same conditions and at the same rates (Fig. 2), were similarly induced for β-galactosidase in the presence of 20 μM PQ^{2+}. Thus, at an A_{600} of ~1, cells growing without and with PQ^{2+} had increased β-galactosidase activity (Fur^+, 300 to 1,600 U/A_{600}; Fur^-, 1,200 to 2,500 U/A_{600}, respectively). Under these conditions, holo Fur diminishes approximately fivefold the extent to which PQ^{2+} can induce *sodA* expression. Potential relationships between PQ^{2+}, Fe, and Fur will be difficult to sort out in a chemically defined medium, such as M9, as Fe deficiency and PQ^{2+} (19) have profound effects on cell growth.

Expression of *sodB*. Figure 3 shows the level of β-galactosidase expressed from the *sodB'-'lacZ* fusion gene carried by the low-copy plasmid pDM28. Note that there was approximately two- to threefold more β-galactosidase activity in Fur^+ cells compared with Fur^- cells. There appears to be a small decrease in β-galactosidase activity during expo-

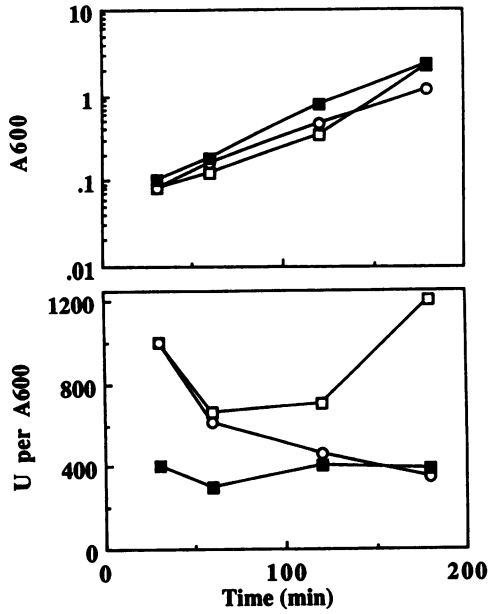

FIG. 3. Expression of the plasmid-borne fusion gene *sodB'-'lacZ* in various strains of *E. coli*. Symbols: □, strain BN402 Fur$^+$; ■, strain BN4020 Fur$^-$; ○, strain BN402 Fur$^+$ cells grown in the presence of 100 μM DTPA. DTPA had very little effect on the β-galactosidase activity of Fur$^-$ cells. No antibiotics were included in the final growth medium.

nential growth, with a return of activity as the cells approach stationary phase; overnight cultures of the Fur$^+$ and Fur$^-$ strains had specific activities of 830 ± 80 (Fur$^+$) and 340 ± 30 (Fur$^-$) U/A_{600}. Similar results were obtained with the multicopy plasmid pMN3, which yielded ~2,000 ± 200 U/A_{600} in Fur$^+$ cells and ~500 ± 50 U/A_{600} in Fur$^-$ cells when harvested at an A_{600} of ~1. Null mutation of the *sodB* gene decreased expression of the *sodB'-'lacZ* fusion gene in pDM28 approximately 20% at each point shown in Fig. 3 (data not shown). Increasing the concentration of Fe in L broth-glucose from ~10 to 50 μM Fe had no effect on the expression of the *sodB* fusion gene in any of these strains (data not shown), although treatment of Fur$^+$ cells with DTPA diminished the levels of β-galactosidase to Fur$^-$ levels (Fig. 3); the latter result suggests that very different plasmid copy numbers are not responsible for the observed differences between Fur$^+$ and Fur$^-$ cells. The expression of the *sodB* fusion gene appears not to be self-regulated and is only modestly dependent on cell growth phase, consistent with previous conclusions that FeSOD is constitutive (29). The data also suggest that control of *sodB* by *fur* is positive in nature, occurs in *trans*, and is not exclusively posttranslational. In earlier work, Simons et al. (50) found approximately fourfold more FeSOD activity in cells grown anaerobically on an Fe-enriched medium compared with those grown anaerobically on an unsupplemented but not Fe-depleted medium. Our data suggest that their observation is best explained by an increased loading of apo FeSOD with Fe.

The availability of *sodA* mutants in Fur$^+$ and Fur$^-$ cells allowed us to quantify accumulation of FeSOD in stationary-phase cells without interference from MnSOD activity. Qualitative indications of SOD activity in extracts from such cells are shown in Fig. 4A; Fig. 4B shows protein staining of

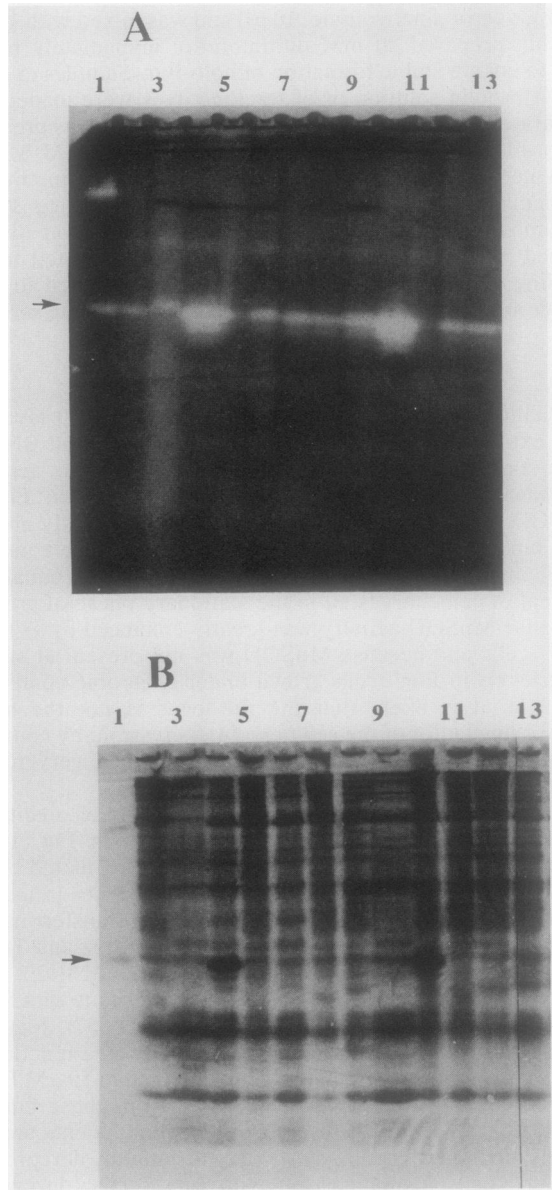

FIG. 4. Activity and protein-stained gels of cell extracts of *E. coli* bearing various plasmids. The corresponding lanes in both gels contain the same quantities of protein. (A) Gel stained for SOD activity as described in the legend to Fig. 2; (B) gel stained with Coomassie blue as described in Materials and Methods. Each lane was loaded with following amounts (in micrograms) of protein (cell extract): 1, 5 μg each of pure *E. coli* MnSOD and FeSOD; 2, 180; 3, 200; 4, 190; 5, 270; 6, 190; 7, 160; 8, 230; 9, 180; 10, 250; 11, 270; 12, 210; 13, 160. Extracts in lanes 2 through 7 were from cells grown anaerobically; extracts in lanes 8 through 13 were from cells grown aerobically. The strain and plasmids used are as follows: lanes 2 and 8, ENF19; lanes 3 and 9, ENF19(pABN203); lanes 4 and 10, ENF19(pHS1-4); lanes 5 and 11, ENF13; lanes 6 and 12, ENF13 (pABN203); lanes 7 and 13, ENF13(pHS1-4). The arrow on the left of each gel represents the position of FeSOD activity.

nondenaturing electrophoretograms of the corresponding extracts. Examination of Fig. 4A shows that we were indeed working with *sodA* mutant cells and that the expression of FeSOD activity appears to be largely repressed in SodA$^-$ Fur$^-$ strains (Fig. 4A, lanes 5 and 11), consistent with the

TABLE 2. FeSOD activity in Fur$^+$ and Fur$^-$ strains of *E. coli* devoid of MnSOD (*sodA*)[a]

E. coli strain	FeSOD activity (U/mg)	
	Fur$^+$	Fur$^-$
sodA	38 (9)[b]	<2 (1)[c]
sodA(pHS1-4)	320 (100)	45 (10)
sodA(pABN203)	7 (1)	7 (1)
sodA(pCON6)	15 (1)	<2 (1)
sodA(pMN3)	7 (1)	<2 (1)

[a] Strains Fur$^+$ and Fur$^-$ were prepared and grown to stationary phase (18 h, A_{600} of ~5); cell extracts were assayed for FeSOD by the method of McCord and Fridovich (34). The amount of protein used in each assay ranged from 5 to 28 μg for Fur$^+$ cells and from 50 to 250 μg for Fur$^-$ cells; the assays were carried out in triplicate, and the mean errors are indicated in parentheses.

[b] Activities varied slightly during growth. Cultures having an A_{600} of 0.7 had 17 U/mg while cultures having an A_{600} of 2.1 and 23 U/mg.

[c] Apparent activity was independent of growth.

results shown in Fig. 1. The quantitative data of Table 2 indicate that the appearance of FeSOD activity requires an intact *fur* locus. Thus, the level of expression in Fur$^+$ cells is ≥20-fold higher than in the Fur$^-$ strain. Moreover, when expression of FeSOD from the multicopy plasmid pHS1-4 (*sodB*$^+$) is measured in these two strains, the ratio is ~7. When one examines the level of FeSOD protein expressed in cells bearing pHS1-4, one can clearly observe less protein in Fur$^-$ cells (Fig. 4B, compare lanes 4 and 7 and lanes 10 and 10). As expected, the presence of pABN203 (Fur$^+$) increased the activity of FeSOD in Fur$^-$ cells. However, the presence of this plasmid decreased FeSOD activity in Fur$^+$ cells, indicating that the plasmid does not restore the parental condition. There is also a discrepancy between the data of Table 2, according to which FeSOD appears to be strongly repressed (≥20-fold) in Fur$^-$ compared with Fur$^+$ cells, and the fusion gene data shown in Fig. 3, in which the ratio is ~2- to 3-fold. Additional data in Table 2 suggest that plasmid-borne, Fur-binding DNA sequences may affect *sodB* expression. Thus, if the Fur protein is itself an activator of *sodB* expression, lowering intracellular Fur levels, by transformation with a multicopy plasmid known to bind holo Fur, should lead to a decrease in FeSOD activity in Fur$^+$ cells. Accordingly, transformation with pCON6 (*iucA'-'lacZ*) (14) lowered FeSOD activity approximately twofold, consistent with a *trans* action of *fur* on *sodB*. To test whether the promoter of *sodB* might have a similar effect, Fur$^+$ and Fur$^-$ cells were transformed with the multicopy plasmid pMN3 (*sodB'-'lacZ*) and examined for FeSOD activity in the stationary phase. The approximately fivefold-lower FeSOD activity in cells bearing pMN3 is consistent with the idea that Fur protein may bind to the *sodB* promoter, although indirect control is not excluded.

DNA- and protein-binding assays. The question whether Fur might act by directly binding to the promoter sequences of the two *sod* genes was addressed by gel retardation assays (23, 24). The results presented in Fig. 5 show high-affinity binding of metallated Fur to the promoter regions of *iucA* and *sodA* and a somewhat lower affinity for the promoter region of *sodB*. The Fur protein has only recently been purified and has not been well characterized (58). It appears to be a complicated protein, sensitive to a variety of environmental perturbations, including the ability to associate with several different bivalent metal ions and a sensitivity to air oxidation. J. B. Neilands kindly supplied us with a sample of highly purified material. We handled this according to his published methods to demonstrate specific associ-

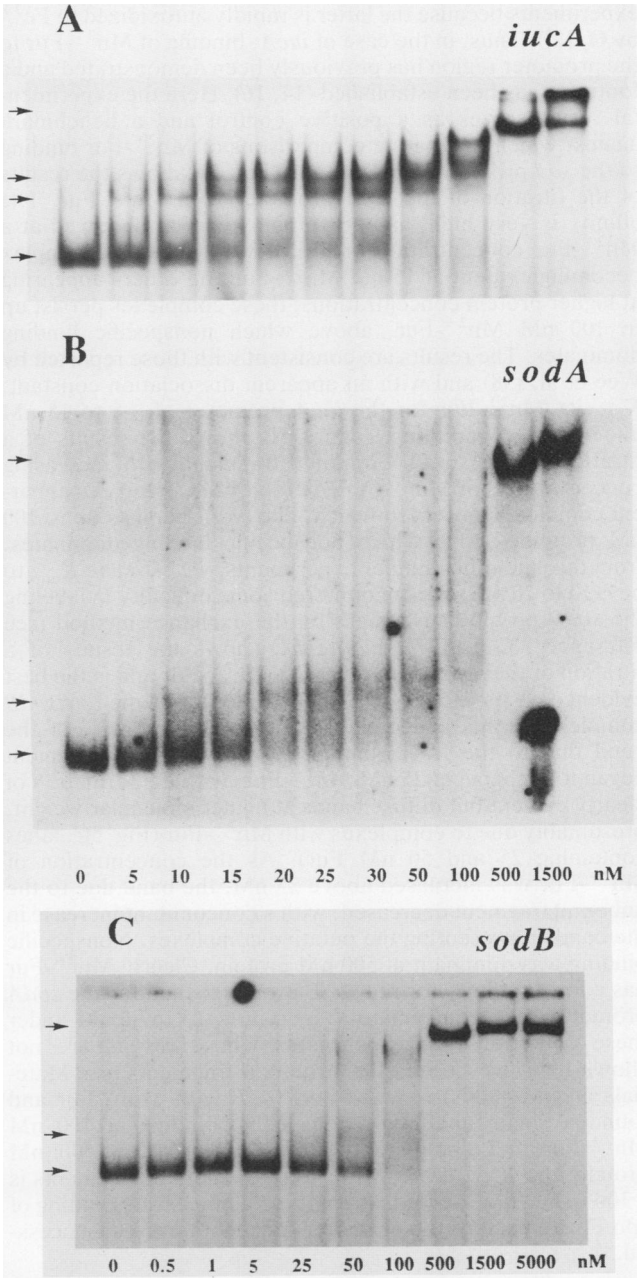

FIG. 5. Evidence for physical binding of Mn^{2+}-Fur protein to the promoter-operator regions in *iucA*, *sodA*, and *sodB* genes of *E. coli*. (A) Each lane was loaded with ~0.01 pmol of end-labeled DNA and increasing amounts of Mn^{2+}-Fur up to 1,500 nM, as indicated at the bottom of the figure. (B) Each lane contains equal amounts (~0.01 pmol) of the 195-bp promoter fragment of the *sodA* gene. The concentration of Mn^{2+}-Fur was increased as indicated at the bottom of the figure. (C) Each lane contains ~0.01 pmol of the 200-bp promoter fragment of the *sodB* gene. The molar ratio of Fur to promoter fragment varied from 0 to ~750. All other conditions were as described above or in the text. Note the different concentration range of Mn^{2+}-Fur in panel C. The bottom arrow on the left of each panel represents free promoter, the top arrow represents nonspecific promoter-Fur complexes, and the middle arrow(s) indicates specific promoter-Fur complex(es).

ation of Mn^{2+}-Fur with DNA fragments containing iron box sequences. (Mn^{2+} rather than Fe^{2+} was used in these experiments because the latter is rapidly autoxidized to Fe^{3+} by O_2 [2]). Thus, in the case of *iucA*, binding of Mn^{2+}-Fur to the promoter region has previously been demonstrated and a footprint has been established (14, 16). Here the experimental result serves as a positive control and a benchmark against which qualitative comparisons of Mn^{2+}-Fur binding to the *sod* promoter can be made. Fig. 5A shows the results of the titration of the *iucA* promoter with Mn^{2+}-Fur. The affinity is very high, as one complex can be observed at a Mn^{2+}-Fur concentration of 5 nM, with a second complex becoming evident at 15 nM Mn^{2+}-Fur and others appearing at higher protein concentrations; these complexes persist up to 100 nM Mn^{2+}-Fur, above which nonspecific binding dominates. The results are consistent with those reported by Wee et al. (58) and with an apparent dissociation constant, K_{app}, of Mn^{2+}-Fur for the *iucA* promoter of ~5 to 15 nM under these conditions. Figure 5B shows the results of a titration of the *sodA* promoter fragment with increasing concentrations of Mn^{2+}-Fur. At 15 nM Mn^{2+}-Fur, an apparent complex is observed which, like *iucA*, persists up to 100 nM protein, above which nonspecific binding dominates; from this and other similar experiments, we estimate K_{app} to be ~10 to 20 nM. We encountered some difficulty in labeling the *sodB* promoter fragment by the exchange method (see reference 33, p. 127). Figure 5C shows the results of a titration of the *sodB* promoter with Mn^{2+}-Fur and is the best evidence we have been able to obtain for a specific Fur:*sodB* complex. In this and other titrations, the intensity of the band due to the free-promoter-containing DNA fragment began to diminish at 25 nM Mn^{2+}-Fur, with the formation of clearly evident but diffuse bands at higher molecular weight, presumably due to complexes with Mn^{2+}-Fur (Fig. 5C, lanes containing 25 and 50 nM Fur). As the concentration of Mn^{2+}-Fur was increased above 25 nM, the band due to the unbound fragment decreased, with a concomitant increase in the bands representing the putative complexes. Nonspecific binding was dominant at 500 nM protein. Clearly Mn^{2+}-Fur has a lower affinity for the *sodB* promoter than for the *sodA* promoter, and we estimate K_{app} to be $\approx$ 25 to 50 nM under these conditions. In experiments whose results are not shown here, we titrated the structural fragments (see Materials and Methods) of *sodA* and *sodB* with Mn^{2+}-Fur and found a small amount of nonspecific binding at 100 nM Mn^{2+}-Fur and complete nonspecific binding at 500 nM protein; the K_{app} of Mn^{2+}-Fur to the structural fragments is ~100 to 500 nM. Several attempts to demonstrate binding of apo Fur to each of the promoter fragments were unsuccessful.

DISCUSSION

The results indicate an interesting and relatively complicated relation between the *fur* gene and the two *sod* genes of *E. coli*. The simplest interpretation of our observations suggests that *sodA* is negatively regulated by *fur* through the now-classical mechanism of Fe^{2+}-dependent repression, although there are clearly other cellular factors involved in its total regulation, and that *sodB* is positively regulated by *fur*, though perhaps by an indirect mechanism. In her comprehensive study of *sodA* regulation, Touati (57) showed that *tac-'sodA* operon fusions yield active MnSOD, indicating that cellular regulation of *sodA* is primarily (though not exclusively) at transcription. The physical evidence presented here for specific binding of Mn^{2+}-Fur to the promoter fragment of *sodA* indicates that the in vivo control of *sodA* by *fur* is most likely due to a direct interaction of metallated Fur with *sodA*. Indeed, the Fe dependence (metal chelate treatment) of ϕ(*sodA'-'lacZ*) regulation in Fur^+ cells and the presence of an apparent iron box in this gene support the notion that repression of *sodA* by *fur* occurs through a mechanism similar to that of other *fur*-regulated genes (2, 3, 9, 14–16, 25, 26, 39, 41, 44, 46, 58). Moody and Hassan (38) found that treatment of anaerobically grown cells with powerful iron chelators resulted in a large induction of MnSOD activity. Our observations suggest that their data can be only partially understood in terms of the classical mechanism by which Fe depletion derepresses *fur*-controlled genes. The role of PQ^{2+} remains an enigma, as it can activate *sodA* expression both aerobically and anaerobically (45) and therefore does not appear to involve dioxygen. While holo Fur does interfere with the activating effect of PQ^{2+} on *sodA* expression, it seems likely that PQ^{2+} interacts with another *sodA* regulation system and/or that internalized PQ (50) affects local DNA structure (13).

The relation between *fur* and *sodB* is evidently more complicated than that between *fur* and *sodA*. While our data do not provide a clear picture of this gene-gene interaction, certain aspects deserve comment. Because the level of expression of the *sodB'-'lacZ* gene on pDM28 was only slightly depressed in $SodB^-$ strains, *sodB* is probably not significantly autoregulated; moreover, this fusion gene was only modestly regulated by *fur* (Fur^+/Fur^- ratio, ~3 to 5) (Fig. 3). By contrast, the results of Fig. 1 and 4 and Table 2 show that null mutation of the *fur* locus dramatically reduced the amount of FeSOD activity produced by the cell, and Fig. 4B shows that the level of FeSOD protein was also greatly reduced. The discrepancy between the induction of *sodB'-'lacZ* in Fur^+ and Fur^- strains and the amounts of FeSOD in Fur^+ and Fur^- strains is bothersome. Because readthrough (7) on the plasmids bearing *sodB'-'lacZ* is not likely to be a factor, there must be some other explanation; it is possible that posttranscriptional events are important in the regulation of *sodB* by *fur*.

Because *fur* plays a dominant role in cellular regulation (3, 9, 15, 25, 28, 59) and null mutations may be highly pleiotropic, the question arises as to whether the effect of *fur* on *sodB* expression results from a direct interaction of Fur protein with the DNA of *sodB*, its mRNA, or its apo protein. Our data do not provide overwhelming evidence for direct activation of *sodB* by Fur, but there are three lines of evidence which support direct control. The first deals with the data of Table 2. If holo Fur is constrained to act as a repressor, Fur^- cells will contain an excess of a regulatory protein responsible for repressing either transcription or translation of *sodB*. It follows that Fur^- cells containing multiple copies of a transcribable DNA molecule capable of lowering the concentration of this putative repressor protein, such as the *sodB* promoter, should express FeSOD activity; this did not occur. If Fur is positively regulating *sodB* at transcription, a plasmid bearing fur^+ should restore FeSOD activity in Fur^- cells; this was observed. Moreover, if Fur is a direct activator, plasmids able to bind Fur should lower FeSOD activity in Fur^+ cells but have no effect in Fur^- cells; this was observed. Although other workers (26) have been unable to observe titration effects with classical *fur*-regulated genes, *sodB* does not fall into this class. Second, by lowering the Fe in the culture medium, through treatment with DTPA, expression of *sodB'-'lacZ* in Fur^+ cells was lowered to that in Fur^- cells (Fig. 3). This result implies that holo Fur is involved in the expression of *sodB*, through a

direct interaction either with the *sodB* gene or with its mRNA.

Third, we consider the results of the band shift assays. The conditions we used in these experiments were optimized for binding Mn^{2+}-Fur to the *iucA* operator (14), which contains a typical iron box sequence. Assuming that the iron box of *sodA* is the binding site of holo Fur, the affinity of Mn^{2+}-Fur for the *sodA* promoter is significantly less than for the *iucA* promoter; we believe this results from the different sequence of the *sodA* iron box and reflects nonoptimal solution conditions for Mn^{2+}-Fur binding to *sodA*. The promoter of *sodB* does not contain an iron box. Therefore, one would expect that rather different conditions must be found to optimize the binding of Fur to this promoter. In the conditions of the experiment whose results are shown in Fig. 5, we see a much lower apparent affinity of Mn^{2+}-Fur for the promoter fragment of *sodB*, though clearly distinguishable from nonspecific binding. This weaker binding is also apparent in an increased diffuseness of the bands we believe to be Fur:*sodB* complexes. We are currently seeking conditions that will stabilize the putative Fur:*sodB* complex, and we are examining the effect of Fur on in vitro transcription.

In summary, our observations show that the *sod* genes of *E. coli* are part of the ferric uptake regulon, thereby suggesting a possible functional role for the Sod proteins in iron metabolism.

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