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TITLE: Relationship of the Superoxide Dismutase Genes, *sodA* and *sodB*, to the Iron Uptake (*fur*) Regulon in *Escherichia coli* K-12

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RELATIONSHIP OF THE SUPEROXIDE DISMUTASE GENES, *sodA* AND *sodB*, TO THE IRON UPTAKE (*fur*) REGULON IN *ESCHERICHIA COLI* K-12[1]

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ABSTRACT Expression of *sodA*, as indicated by MnSod activity is normal in *fur* mutants. This suggests that *sodA* is not a member of the *fur* regulon and that the putative Fe-binding, regulatory protein of *sodA*, suggested by Moody and Hassan (1984 J Biol Chem 259:12821), is not the Fur protein. By contrast, expression of *sodB*, as indicated by FeSod activity, is completely blocked in *fur* mutants and the effect is restored by transformation with a plasmid having a normal *fur* locus. The observations suggest that Fur, either directly or indirectly, controls SodB biosynthesis. Additional observations are described which indicate that SodB and Fur act together in a complicated fashion to control the biosynthesis of enterobactin.

INTRODUCTION

Metalloproteins that catalyze the disproportionation of superoxide anion are denoted superoxide dismutases (EC 1.15.1.1) (1-3), which function by the following catalytic cycle.

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$$E_{ox} + O_2^- \rightarrow E_{red} + O_2$$
$$E_{red} + O_2^- + 2H^+ \rightarrow E_{ox} + H_2O_2$$

There are three forms: an iron-requiring protein (FeSod) found in bacteria and plants, a manganese-containing protein (MnSod) present in bacteria and mitochondria, and a copper-zinc protein (CuZnSod) found in the cytosol of eukaryotic cells. In *Escherichia coli*, the 38.7-kDa FeSod and 45.8-kDa MnSod are dimeric structures with one iron or manganese atom bound to each subunit; the bacterial enzymes have remarkably similar structures (4). Their common physical structures and catalytic properties have suggested a common biological function, namely protection against oxygen toxicity through superoxide dismutation. This idea has been criticized by Fee (5).

The regulation of Sod activities in *E. coli* is remarkably complex. Expression of the *sodA* gene, which codes for the Mn-protein, is apparently coupled to respiration. Thus, *sodA* is expressed during respiratory growth with dioxygen, nitrate, trimethylamine oxide, dimethyl sulfoxide, and ferricyanide acting as electron acceptors (6-10); during either aerobic or anaerobic respiration, MnSod is induced by added methyl-viologen (11). Contrary to previous claims (11), *sodA* expression is not dependent on O_2^- (12). The work of Moody and Hassan (13) suggested that *sodA* expression was enhanced by removing Fe^{2+} from the culture medium. By contrast, expression of sodB appears to be enhanced with increased levels of Fe^{2+} in the medium (14, and references in 15). Thus, both genes may be responsive to Fe in the environment.

In this work we present evidence that *sodB* is closely related to the iron uptake systems of *E. coli*. Iron is required for growth of the cell and is available through several genetically unlinked uptake systems (16-18). These include the siderophores; enterobactin, aerobactin, and ferrichrome; the ferric citrate transport system; and a low-affinity, iron transport apparatus. The ferric ion uptake regulatory locus (*fur*) negatively

controls expression of these systems through the Fur protein (19). *In vivo*, the Fur protein binds, as a Fe^{2+}-containing dimer, to a specific sequence of DNA (20), denoted the "iron box" (GATAATGATAATCATTACT), which is in the promoter regions of genes coding for iron uptake. An apparent "iron box" can be found in the *sodA* promoter region (21) suggesting the possibility that *sodA* may interact with Fur. By contrast, the *sodB* promoter region does not contain this iron-regulatory element (15).

Recognition of the "iron box" in *sodA* prompted us to examine *sodA* and *sodB* regulation in *fur* mutants. We predicted that *sodA* would be constitutively expressed in *fur* mutants while *sodB* would be unchanged (wild-type phenotype) in *fur* mutants. Our predictions were completely wrong.

METHODS

Some of the *E. coli* K-12 strains and the multicopy plasmids used in this work are listed in Table 1. Cells were grown in LB medium supplemented with glucose (0.2%) and the appropriate antibiotics. Cultures were induced with 100 μM methylviologen. Anaerobic cultures were grown in sealed flasks. Minimal medium consisted of Chelex-treated M9, glucose, thiamine, amino acids, $MgCl_2$, $CaCl_2$, $FeCl_2$, and the appropriate antibiotics. Catechol production was assayed by the Arnow method (22).

Saturated cultures were harvested, washed twice with 50 mM KPi, 0.5 mM EDTA, 1 mM $MnCl_2$, pH 8.0 buffer, lysed with lysozyme, treated with p-toluenesulfonyl fluoride, sonicated at 4°C for 90 s, and centrifuged at 10 000 x *g* for 10 min to remove cell debris. Protein concentrations were determined by the BCA method (23); bovine gamma globulin served as the calibration standard. Proteins were analyzed on 10% polyacrylamide gels (24). The gels were stained for protein with 26% isopropanol, 11% glacial acetic acid, and 0.1% Coomassie blue R, and destained with 10%

isopropanol and 10% glacial acetic acid, or stained for Sod activity with nitro blue tetrazolium (25).

TABLE 1
Strains And Plasmids

Strains	Genotype	Source or Reference
GC4468	*F⁻ Δlac4169 rps L*	D. Touati
QC774	GC4468Φ(*sodA-lacZ*)49 Φ(*sodB*-Km)1-Δ_2 Cm^R Km^R	D. Touati
AB1157	*his arg thr*	G. Walker
BN402	P1 *lac*ΔU169xAB1157 *galK⁻*	J. Neilands
BN407	P1 *lac*ΔU169xAB1157 *galK⁻* pColVA3:Tn10 Tc^R	J. Neilands
BN4020	P1 *lac*ΔU169xAB1157 *galK⁻* *fur*:Tn5 Km^R	J. Neilands
BN4023	P1 *lac*ΔU169xAB1157 *galK⁻* *fur*:Tn5 Km^R pColVA3:Tn10 Tc^R	J. Neilands
EN3	P1 QC774xBN407 Km^R	J. A. Fee
EN5	P1 QC774xBN402 Km^R	J. A. Fee
EN9	P1 QC774xBN402 Cm^R	J. A. Fee
EN13	P1 QC774xBN4020 Cm^R	J. A. Fee
Plasmids		
pHS1-4	*sodB⁺* Tc^R	D. Touati
pJM19	*sodB⁺* Am^R	J. A. Fee
pABN203	*fur⁺* Tc^R	J. Neilands

The xanthine-xanthine oxidase coupled reduction of cytochrome *c* was used to quantify soluble Sod activity (26).

RESULTS

As determined by PAGE using the assay of Beauchamp and Fridovich (24) (not shown), strains BN402 (wild-type) and BN 4020 (*fur*⁻) showed the normal phenotype for MnSod activity. No MnSod was produced under anaerobic growth, but O_2 and methylviologen induced MnSod activity. The FeSod activity was present in BN402, but FeSod activity was not detected in BN4020. Transformation of BN4020 with pABN203 (*fur*⁺) restores the wild-type levels of FeSod activity. The plasmid pHS1-4 (*sodB*⁺) produced large amounts of FeSod activity in BN402 but barely detectable levels of FeSod activity in BN4020.

Both MnSod and FeSod contribute to the total activity in cell extracts. In *sodA* mutants, the cytosolic activity is due only to FeSod; specific activities for *sodA* mutants appear in Table 2. The values directly parallel the observation made in the PAGE analysis and provide a quantitative base for our conclusions (see below).

TABLE 2
Specific Iron Superoxide Dismutase Activities[a] For *sodA* Mutants

	fur⁺			*fur*⁻		
	BN402	pABN203	pHS1-4[b]	BN4020[c]	pABN203	pHS1-4
No O_2	15	40	1700	<2	38	36
O_2	29	170	1800	<2	29	36
O_2 + PQ^{2+}	56	41	1700	<7	12	49

[a] Sod activity measured by the xanthine-xanthine oxidase coupled cytochrome *c* method (26); units/mg protein; 5-28 µg protein/mL in assay
[b] 0.2 µg protein/mL in assay
[c] 50-250 µg protein/mL in assay

Enterobactin production assayed by the method of Arnow (22) indicates total catechol content of the sample. Selective strains were tested and the results appear in Table 3. As expected, *fur*$^+$ strains did not produce catechol in iron sufficient conditions while *fur* mutant strains excreted catechol independently of iron in the medium. Unexpectedly, interuption of the *sodB* gene led to an apparent *fur* phenotype, and this was not restored by transformation of *sodB* strains with pJM19 *sodB*$^+$. Indeed, multiple copies of either *fur*$^+$ (pABN203) or *sodB*$^+$ (pJM19) led to the apparently complete suppression of enterobactin biosynthesis in all strains tested.

TABLE 3
Production of Catechols[a] by Selected Strains

Strain Genotype	-Fe	+Fe	Phenotype
fur$^+$	+	-	wt
fur$^-$	+	++	*fur*
fur$^-$ pABN203 *fur*$^+$	-	-	
fur$^+$*sodB*$^-$	+	++	*fur*
fur$^+$*sodB*$^-$ pJM19 *sodB*$^+$	-	-	
fur$^+$ pJM19 *sodB*$^+$	-	-	

[a] Ref. (22); + indicates a wild-type level of catechol production, ++ indicates an obvious increase in catechol production, - indicates that no catechol was present.

DISCUSSION

Three general conclusions can be drawn from our observations: First, expression of *sodA* is not affected by mutation in the *fur* locus. Second, *sodB* expression is directly or indirectly controlled by the *fur* locus. Finally, *sodB* (or downstream cistrons) is somehow controlling

expression of enzymes involved with enterobactin biosynthesis.

Based on the observation that *E. coli* expresses MnSod under anaerobic conditions in iron-depleted media, Moody and Hassan (13) postulated the existence of an Fe-binding protein that acts as a repressor of *sodA*. To account for the apparent "oxidative" induction of MnSod activity, it was further speculated that oxidation of Fe^{2+} to Fe^{3+} relieved repression of *sodA* (8). The recognition of a nearly perfect "iron box" in the -35 region of *sodA* prompted us to test the idea that *sodA* was part of the *fur* regulon. The data indicate that *sodA* is not regulated by *fur;* it is possible that another iron-binding protein is involved.

In contrast to *sodA*, *sodB* expression is dramatically influenced by the *fur* locus. Thus, the *sodB* gene product, FeSod, is absent in *fur* mutants and is restored when supplemented with pABN203 *fur*$^+$. The return of FeSod activity parallels the appearance of protein (not shown) indicating that *sodB* regulation by fur involves synthesis of new enzyme rather than activation of incompetent enzyme. Assuming regulation of transcription, our observations indicate that *fur* through its product Fur, is acting in trans to control *sodB* expression. The question of whether this is a direct activation of *sodB* by Fur or an indirect activation through some other loci, is presently under experimental consideration.

The *ent* operon, responsible for the biosynthesis of enterobactin, is central to *E. coli*'s strategy for obtaining Fe from its environment (18). It has been known for some time that expression of *ent* is controlled by the *fur* locus whose gene product, in combination with Fe^{2+}, acts as a repressor of *ent*. As shown in Table 3, our observations confirm this basic hypothesis and extend it to include a role for *sodB* (or downstream cistrons). Excess *fur* in the cell, as obtains in the presence of the multicopy plasmid, pABN203 *fur*$^+$, suppresses enterobactin biosynthesis even in iron poor medium. Similarly, the multicopy

plasmid, pJM19, bearing *sodB*$^+$ also suppresses enterobactin biosynthesis. Broadly speaking, cells lacking either Fur or SodB appear to sense iron starvation and the *ent* operon is turned on, while cells having excess Fur or SodB sense iron sufficiency and expression of the *ent* operon is shut down. We are currently exploring the genetic and molecular basis of *sodB* in these regulatory processes.

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REFERENCES

1. Rotilio G (1986). "Superoxide and Superoxide Dismutases in Chemistry, Biology and Medicine." Amsterdam: Elsevier/North Holland Biomedical Press.
2. Fridovich I (1986). Superoxide dismutases. Adv Enzymol 58:61.
3. Fee JA (1981). The copper/zinc superoxide dismutase. In Sigel H (ed): "Metal Ions in Biological Systems," New York: Dekker, p 259.
4. Stallings WC, Pattridge KA, Strong RK, Ludwig ML (1984). Manganese and iron superoxide dismutases are structural homologs. J Biol Chem 259:10695.
5. Fee JA (1982). Is superoxide important in oxygen poisoning? TIBS 7:84.
6. Smith MW, Neidhardt F (1983). Proteins induced by aerobiosis in *Escherichia coli*. J Bacteriol 154:344.
7. Miyake K (1986). Effect of nitrate on the level of superoxide dismutase in anaerobically grown *Escherichia coli*. J Gen Appl Microbiol 32:527.

8. Hassan HM, Moody CS (1987). Regulation of manganese-containing superoxide dismutase in *Escherichia coli*: anaerobic induction by nitrate. J Biol Chem 262:17173.
9. Schiavone JR, Hassan HM (1988). The role of redox in the regulation of manganese-containing superoxide dismutase biosynthesis in *Escherichia coli*. J Biol Chem 263:4269.
10. Privalle CT, Fridovich I (1988). Inductions of superoxide dismutases in *Escherichia coli* under anaerobic conditions: accumulation of an inactive form of the manganese enzyme. J Biol Chem 263:4274.
11. Hassan HM, Fridovich I (1977). Regulation of the synthesis of superoxide dismutase in *Escherichia coli*: induction by methyl viologen. J Biol Chem 252:7667.
12. Nettleton CJ, Bull C, Baldwin TO, Fee JA (1984). Isolation of the *Escherichia coli* iron superoxide dismutase gene: evidence that intracellular superoxide concentration does not regulate oxygen-dependent synthesis of the manganese superoxide dismutase. Proc Natl Acad Sci USA 81:4970.
13. Moody CS, Hassan HM (1984). Anaerobic biosynthesis of the manganese-containing superoxide dismutase in *Escherichia coli*. J Biol Chem 259:12821.
14. Simons RS, Jackett PS, Carroll MEW, Lowrie DB (1976). Superoxide independence of paraquat toxicity in *Escherichia coli*. Toxicol Appl Pharmacol 37:271.
15. Carlioz A, Ludwig ML, Stallings WC, Fee JA, Steinman HM, Touati D (1988). Iron superoxide dismutase: nucleotide sequence of the gene from *Escherichia coli* K12 and correlations with crystal structures. J Biol Chem 263:1555.
16. Neilands JB (1982). Microbial envelope proteins related to iron. Ann Rev Microbiol 36:285.
17. Crichton RR, Charloteaux-Wauters M (1987). Iron transport and storage. Eur J Biochem 164:485.
18. Winkelmann G, vander Helm D, Neilands JB (eds) (1987): "Iron Transport in Microbes, Plants, and Animals," Weinheim, Federal Republic of Germany: VCH Publishers.

19. Bagg A, Neilands JB (1987). Molecular mechanism of regulation of siderophore-mediated iron assimilation. Microbiol Rev 51:509.
20. Bagg A, Neilands JB (1987). Ferric uptake regulation protein acts as a repressor, employing iron(II) as a cofactor to bind the operator of an iron transport operon in *Escherichia coli*. Biochemistry 26:5471.
21. Takeda Y, Avila H (1986). Structure and gene expression of the *E. coli* Mn-superoxide dismutase gene. Nucl Acids Res 14:4577.
22. Arnow LE (1937). Colorimetric determination of the compounds of 3,4-dihydroxyphenylalanine-tyrosine mixtures. J Biol Chem 118:531.
23. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985). Measurement of protein using bicinchoninic acid. Anal Biochem 150:76.
24. Hames BD (1987). An introduction to polyacrylamide gel electrophoresis. In Hames BD, Rickwood D (eds): "Gel Electrophoresis of Proteins: A Practical Approach," Oxford: IRL Press, p 1.
25. Beauchamp C, Fridovich I (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 44:276.
26. McCord JM, Fridovich I (1969). Superoxide dismutase: an enzymatic function for erythrocuprein (hemocuprein). J Biol Chem 244:6049.