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Interaction of Six Global Transcription Regulators in Expression of Manganese Superoxide Dismutase in *Escherichia coli* K-12

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Transcription of the *sodA* gene of *Escherichia coli*, which encodes manganese superoxide dismutase, is governed by six global regulators: the product of the *soxRS* locus (superoxide response) and mutated alleles of the *soxQ* locus (such as *cfxB*) act as activators; the products of the *fur* (ferric uptake regulation), *arcA* (aerobic regulation control), and *fnr* (fumarate nitrate reductase) genes and the integration host factor (IHF) negatively regulate *sodA*. The action of these effectors on the *sodA* promoter was investigated by using chromosomal *sodA-lacZ* operon fusions with intact or deleted promoters, different environmental conditions, and strains carrying different combinations of null mutations in the effector genes. The data allow us to assign target regions in the *sodA* promoter for activation by SoxRS and CfxB and for repression by Fur and ArcA. In aerobiosis, activation of *sodA* transcription by SoxRS was compatible with CfxB activation or Fur repression, whereas *cfxB* and *fur* controls were mutually exclusive. Repression by Fnr appeared, at least in part, to be ArcA dependent. IHF enhanced aerobic Fur repression, and in the absence of Fur, it enhanced anaerobic repression by ArcA. The DNA targets for Fur (encompassing the −35 region) and ArcA (from and downstream of the −35 region) appear to overlap, suggesting that Fur and ArcA repressions are mutually exclusive. Fur (in response to the iron pool) or ArcA, acting with Fnr and IHF (in response to the redox state of the cells), can block anaerobic *sodA-lacZ* expression with about equivalent efficiencies. The possible biological significance of this result is discussed.

Superoxide dismutases (SODs) protect cells from oxidative damage by removing superoxide radicals. Cell damage can be due directly to superoxide or indirectly to even more reactive oxygen species such as hydroxyl radicals, whose formation via a Fenton reaction is favored by excess superoxide (6).

There are two SODs in *Escherichia coli*. Whereas the activity of iron-containing SOD is similar under almost all growth conditions, the activity of manganese-containing SOD (MnSOD) is greatly modulated by environmental oxidative threats (21, 30, 34). MnSOD is encoded by the *sodA* gene, which is regulated transcriptionally (47) and posttranslationally in a metal-dependent fashion (5, 35). Six global effectors of transcription are presently known to affect MnSOD gene expression. They are the products of the *soxRS* (18, 48) and *soxQ* (17) loci; of the *fur* (20), *arcA* (25), and *fnr* (43) genes; and of the integration host factor (IHF) (15). The *sox* products activate *sodA* expression, and the others repress it.

The Fur (ferric uptake regulation) protein, in the presence of a divalent metal, likely iron in vivo (2), represses the transcription of genes involved in iron assimilation (1, 20) and negatively regulates *sodA* expression (31, 45). Under conditions of iron deficiency (which can result from iron oxidation), *sodA* is induced together with the genes of iron assimilation. The ArcA (aerobic regulation control) protein is the regulatory protein of a two-component system, *arcA-arcB*, which controls the expression of numerous genes of aerobic metabolism (26). ArcA represses *sodA* in anerobiosis, coupling its expression to aerobic respiration (45). IHF is a DNA-binding protein which induces a bend in the DNA at its target binding site (11). Mutations in one IHF subunit gene, *himA*, lead to increased expression of *sodA* (39a). The Fnr (fumarate nitrate reductase) protein is involved in the anaerobic activation or repression of numerous genes and binds iron (40). Fnr has recently been shown to influence *sodA* expression (22). The *soxRS* (superoxide response) locus mediates part of the global response to superoxide (13, 48). It includes two genes, *soxR* and *soxS*, which code for a two-stage regulation system. SoxR is a sensor-regulatory protein which upon activation by superoxide (or some product thereof) stimulates the transcription of *soxS*, the product of which in turn activates transcription of the various *soxRS* regulon genes, including *sodA* (33, 52). Certain mutated alleles of the *soxQ* locus (known as *soxQ1* and *cfxB*) activate the transcription of several proteins, including oxidative stress proteins controlled by the *soxRS* locus, such as MnSOD (17). Since the environmental signal which triggers SoxQ activation is unknown, it is still uncertain whether the activation by mutated *soxQ* alleles reflects an activation normally mediated by the *soxQ* locus or whether it is allele-specific cross-talk. SoxRS-mediated activation and activation by *soxQ* alleles act independently (17).

Except for repression by Fur, very little is known about the way in which *sodA* transcription is controlled by these various effectors. Furthermore, the mode of DNA binding of the regulatory proteins ArcA and SoxS and their DNA targets in the promoter regions of the genes that they regulate are not known. The Fur protein has been shown to bind to *sodA* DNA by gel retardation assays (31). Examination of the *sodA* promoter sequence (44) reveals two potential promoters, P and P′; two overlapping putative Fur binding sites, "iron boxes" (12), which overlap with the −35 P and −35 P′; and potential IHF (15) and Fnr (43) binding sites. Only the promoter P has been found to be functional under the usual aerobic growth conditions (44), but this does not rule out P′ functioning in other growth conditions.

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To gain insight into the organization of these multiple effectors controlling *sodA* transcription, we have examined the effect of promoter deletions on *sodA* expression in various environmental conditions, using as reporters chromosomal *sodA-lac* operon fusions. The capacity of each effector to control transcription from the wild-type and deleted promoters was assessed by using combinations of null mutants in different regulators.

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. All strains were *E. coli* K-12 derivatives. Strains, phages, and plasmids are listed in Table 1; see below for specific constructions. Basic genetic manipulations were carried out by standard procedures (29). Mutations in the *soxRS* locus and the *himA*, *arcA*, and *fur* genes were introduced by P1 transduction, selecting for antibiotic resistance 100% associated with the mutation: resistance to chloramphenicol for Δ*sox-8*::*cat* (Δ*soxRS*), Δ*soxR4*::*cat* [*soxR*(Con)], and *himA*::*cat* mutations; resistance to tetracycline for *sox-3*::Tn*10* (*soxS*), *himA*Δ*82*-Tn*10* (Δ*himA*), and Δ*arcA*::*tet* (Δ*arcA*) mutations; and resistance to kanamycin for Δ*fur*::*kan* (Δ*fur*). The *cfxB* mutation was introduced by cotransduction with *zdd-2207*::Tn*10*Km (≈40% cotransduction), and *cfxB* cotransductants were screened for resistance to 6 μg of chloramphenicol per ml. *fnr* mutations were introduced by cotransduction with *zjc*::Tn*10* (≈50% cotransducible with *nirR22*) or *zdc-235*::Tn*9* (cotransduction with Δ*fnr* ≈ 5%). *fnr* mutants were screened for their ability to confer the Fnr^- phenotype to LCB79. The Δ*fur*::*kan* mutation was introduced into kanamycin-resistant strains by cotransduction with *zjc*::Tn*10* (62% cotransduction). *arcA*Δ*194* was introduced by cotransduction with *zjj*::mini-*tet* (60% cotransducible with *arcA*), and Δ*arcA* in the case of a Tet^r recipient strain was introduced by cotransduction with *zjj*::mini-*kan* (75% cotransduction); Tet^r or Kan^r transductants were screened for the $ArcA^-$ phenotype as previously described (17). Δ*fur*::*kan* is a 181-bp internal deletion of the *fur* gene generated by site-directed mutagenesis in which a *kan* gene block was inserted (47a).

Specific strain constructions. (i) Δ*sodA* deletion. The *sodA* gene is carried on a 1,050-bp *Ava*I-*Ava*I fragment starting 135 bp upstream of the ATG. This fragment was deleted from pDT1-5, and the deleted allele was exchanged with the wild-type allele as previously described (8), using a *polA*(Ts) strain carrying a resistance marker (*rha*::Tn*5*) linked to *sodA*. A P1 lysate was made on the strains potentially containing the Δ*sodA* deletion and used to transduce a *sodB*::MudPR3(Cm^r) strain. The Kan^r transductants were further screened for their inability to grow aerobically on minimal medium (8). The $sodB^+$ and rha^+ alleles were then reintroduced in two successive transductions; *zdh*::mini-*tet* or the *zdh*::mini-*kan*-Ω was used as a marker to cotransduce $sodB^+$ (selection for Tet^r Cm^s or Kan^r Cm^s, respectively). Deletion of the *sodA* allele (Δ*sodA3*) was further verified by Southern blot, using fragments of the *Ava*I-*Ava*I 1,050-bp *sodA* region as a probe, and by loss of MnSOD activity and immunoreactivity with MnSOD antibodies (Western blot [immunoblot]).

(ii) Δ*arcA* deletion. A 2,523-bp *Hin*dIII-*Pvu*II fragment containing the $arcA^+$ gene derived from pRB38 was cloned from pBT1-1 into the M13mp18 multisite. A 167-bp deletion of the structural gene starting 1 bp after the ATG codon was generated by site-directed mutagenesis. The *Eco*RI-*Hin*dIII fragment carrying the Δ*arcA* deletion was cloned into pTZ19R, giving pIC6-3, and the *Eco*RI-*Ava*I fragment from pBR322, encoding tetracycline resistance, was inserted into the *Sma*I site created by the deletion (pIC6-4). The Δ*arcA*::*tet* allele was then transferred to the chromosome by transformation of a *recD* strain (37) with plasmid DNA linearized by *Sac*I digestion. Tet^r recombinants were selected on 6 μg of tetracycline per ml, and loss of the $arcA^+$ allele was further verified by the ability to confer an $ArcA^-$ phenotype to a strain carrying an *sdh-lacZ* or *sodA-lacZ* fusion by transduction.

Media, growth conditions, and β-galactosidase assays. Cells in liquid culture were grown in LB broth (29) at 37°C with shaking at 200 rpm. Medium was supplemented with tetracycline (10 or 6 μg/ml for Δ*arcA*::*tet* selection), chloramphenicol (20 μg/ml), and/or kanamycin (40 μg/ml) as needed. Minimal medium was M63. Anaerobic cultures were grown in a Forma scientific anaerobic chamber, in LB medium containing 1% glucose, buffered with 50 mM MOPS (morpholinepropanesulfonic acid) (pH 7) or phosphate buffer when indicated. All media and materials were equilibrated in the anaerobic chamber for 3 days before use. Paraquat (methyl viologen) was purchased from Sigma.

For aerobic β-galactosidase assays, precultures grown in LB broth were stopped by chilling on ice at an optical density at 600 nm of 1 and were diluted (1/20) the following day in 15 ml of prewarmed medium in a 100-ml Erlenmeyer flask. After 1 h, samples were taken at 20-min intervals for 100 min and assayed for β-galactosidase activity according to the method of Miller (29). Growth was monitored by measuring the optical density at 600 nm at 40-min intervals. No more than 3 ml was removed during the experiment to avoid drastic changes in culture volume and consequent culture aeration. In anaerobiosis, 10-ml flasks were inoculated with an anaerobic overnight culture diluted 1/10. Samples for measuring optical density and β-galactosidase were taken after 60, 120, and 180 min or, for *fnr* mutants, after 60, 120, 220, and 300 min.

Operon fusion constructions. A DNA fragment containing the wild-type or deleted promoter region of *sodA* was generated by creating a *Bam*HI restriction site in the ribosome binding site sequence (GGAGAT→GGATCC) and an *Eco*RI restriction site 854 bp upstream (GCGTTC→GAATTC) by site-directed mutagenesis. The *Eco*RI-*Bam*HI fragments from the wild-type or deleted promoters (Fig. 1) were inserted into the multisite of pRS415 in front of the *lac* operon and transferred to the chromosome as described previously (41). The fusions were verified by sequencing the two strands on the plasmid; they were then transferred by homologous recombination onto λLRS45, which was used to lysogenize a $sodA^+$ or Δ*sodA* strain.

Construction of upstream deletions. Unidirectional upstream deletions were generated in the *sodA* promoter by following the method described by Barcak and Wolf (3) with a few modifications. Briefly, the *Eco*RI-*Bam*HI fragment (851 bp) containing *sodA* promoter generated for the operon-fusion constructions was introduced into the multisite of M13mp18. An *Xho*I restriction site was created by site-directed mutagenesis 24 bp downstream of the *Eco*RI site. The plasmid was linearized with *Eco*RI, the 3′ ends were then filled in with deoxynucleoside triphosphate (dNTP) cocktail containing [α-S]dATP and PolIk, and the DNA was then digested with *Xho*I to generate an unprotected 3′ end. Deletions were created from the *Xho*I restriction site by digestion for various times with the 3′ exonuclease activity from T4 DNA polymerase, followed by digestion with S1 nuclease. After treatment with Klenow fragment poly-

TABLE 1. Bacterial strains, phages, and plasmids

Bacterium, phage, or plasmid	Genotype or relevant characteristics	Derivation	Source or reference
E. coli strains[a]			
K1299	N99 *himA*Δ*82*-Tn*10*		28
HN1491	N99 *himA*::*cat*		H. Nash
LCB79	Δ*lacU169*(*chlI-lacZ*)		9
JRG1728	MC1000 Δ*fnr zdc-235*::Tn*9*		42
GC4468	F$^-$ Δ*lacU169 rpsL*		8
GC7365	C600 *recD1009*		A. Brachet
BW829	GC4468 Δ*sox-8*::*cat*[b]		48
BW831	GC4468 *sox-3*::Tn*10*[b]		51
BW847	GC4468 Δ*soxR4*::*cat*[b]		48
JHC1068	GC4468 *cfxB zdd-2207*::Tn*10*Km		17
JHC1096	GC4468 Δ*soxQ*		17
QC1191	GC4468 *zdh*::mini-*tet* (42% cotransducible with *sodB*$^+$)		D. Touati
QC1251	GC4468 Φ(*sodA'-'lacZ*)*49 zbf*::Tn*10* (60% cotransducible with *fur*$^+$)		D. Touati
QC1262	GC4468 Φ(*sodA-lacZ*)*49 arcA*Δ*194* (*sodZ*) *zjj*::mini-*tet* (60% cotransducible with *arcA*)		45
QC1402	GC4468 *zdh*::mini-*kan*-Ω (60% cotransducible with *sodB*$^+$) Spc^r		D. Touati
QC1424	GC4468 *fur zjj*::mini-*kan* (75% cotransducible with *arcA*$^+$)		45
QC1682	GC4468 Δ*sodA3 sodB*::MudPR3 (Cm^r) *rha*::Tn*5*		This study
QC1712	GC4468 Δ*sodA3 zdh*::mini-*tet rha*::Tn*5*	P1 (QC1191) × QC1682	This study
QC1725	GC4468 Δ*sodA3 zdh*::mini-*tet*	P1 (GC4468) × QC1712	This study
QC1732	GC4468 Δ*fur*::*kan*		B. Tardat
QC1745	GC4468 Δ*sodA3 zdh*::mini-*kan*-Ω (Spc^r)	P1 (QC1402) × QC1725	This study
QC1749	QC1709 Δ*soxR4*::*cat*	P1 (BW847) × QC1709	This study
QC1754	QC1751 Δ*sox-8*::*cat*	P1 (BW829) × QC1751	This study
QC1755	QC1751 Δ*soxR4*::*cat*	P1 (BW847) × QC1751	This study
QC1758	QC1751 Δ*fur*::*kan*	P1 (QC1732) × QC1751	This study
QC1759	QC1751 Δ*fur*::*kan* Δ*soxR4*::*cat*	P1 (BW847) × QC1758	This study
QC1772	QC1769 Δ*sox-8*::*cat*	P1 (BW829) × QC1769	This study
QC1773	QC1769 Δ*soxR4*::*cat*	P1 (BW847) × QC1769	This study
QC1774	QC1769 Δ*fur*::*kan*	P1 (QC1732) × QC1769	This study
QC1776	QC1770 Δ*sox-8*::*cat*	P1 (BW829) × QC1770	This study
QC1777	QC1770 Δ*soxR4*::*cat*	P1 (BW847) × QC1770	This study
QC1782	GC4468 Δ*fur*::*kan zbf*::Tn*10*	P1 (QC1251) × QC1732	This study
QC1794	QC1793 Δ*fur*::*kan*	P1 (QC1732) × QC1793	This study
QC1801	QC1793 Δ*fur*::*kan arcA*Δ*194 zjj*::mini-*tet*	P1 (QC1262) × QC1794	This study
QC1804	QC1793 Δ*fur*::*kan* Δ*sox-8*::*cat*	P1 (BW829) × QC1794	This study
QC1808	QC1793 Δ*sox-8*::*cat*	P1 (BW831) × QC1793	This study
QC1835	QC1793 *zdc-235*::Tn*9*	P1 (JRG1728) × QC1793	This study
QC1842	QC1793 *himA*Δ*82*-Tn*10*	P1 (K1299) × QC1793	This study
QC1860	QC1832 Δ*fur*::*kan*	P1 (QC1732) × QC1832	This study
QC1879	QC1739 Δ*soxR4*::*cat*	P1 (BW847) × QC1739	This study
QC1880	QC1739 *sox-3*::Tn*10*	P1 (BW831) × QC1739	This study
QC1881	QC1739 Δ*sox-8*::*cat*	P1 (BW829) × QC1739	This study
QC1882	QC1709 Δ*sox-3*::Tn*10*	P1 (BW831) × QC1709	This study
QC1888	QC1793 *himA*::*cat*	P1 (HN1491) × QC1793	This study
QC1889	QC1769 Δ*sox-8*::*cat* Δ*fur*::*kan*	P1 (QC1732) × QC1772	This study
QC1890	QC1793 Δ*sox-8*::*cat himA*Δ*82*-Tn*10*	P1 (K1299) × QC1808	This study
QC1896	QC1751 Δ*sox-8*::*cat* Δ*fur*::*kan*	P1 (QC1732) × QC1754	This study
QC1908	QC1793 Δ*fur*::*kan himA*::*cat*	P1 (QC1732) × QC1888	This study
QC1942	*recD* Δ*arcA*::*tet*		This study
QC1943	QC1793 Δ*fur*::*kan* Δ*arcA*::*tet*	P1 (QC1942) × QC1994	This study
QC1949	QC1793 Δ*arcA*::*tet*	P1 (QC1942) × QC1793	This study
QC1950	QC1885 Δ*arcA*::*tet*	P1 (QC1942) × QC1885	This study
QC1951	QC1793 *himA*::*cat* Δ*arcA*::*tet*	P1 (QC1942) × QC1888	This study
QC1953	QC1793 Δ*fur*::*kan himA*::*cat* Δ*arcA*::*tet*	P1 (QC1942) × QC1908	This study
QC1963	QC1793 Δ*fnr zdc-235*::Tn*9*	P1 (JRG1728) × QC1793	This study
QC1964	QC1793 Δ*fnr zdc-235*::Tn*9* Δ*fur*::*kan*	P1 (QC1732) × QC1963	This study
QC1965	QC1793 Δ*fnr zdc-235*::Tn*9* Δ*arcA*::*tet*	P1 (QC1942) × QC1963	This study
QC1966	QC1793 Δ*fnr zdc-235*::Tn*9* Δ*fur*::*kan* Δ*arcA*::*tet*	P1 (QC1942) × QC1964	This study
QC1968	QC1793 Δ*fnr zdc-235*::Tn*9* Δ*fur*::*kan himA*Δ*82*-Tn*10*	P1 (K1299) × QC1964	This study
QC1974	QC1793 Δ*fnr zdc-235*::Tn*9 himA*Δ*82*-Tn*10*	P1 (K1299) × QC1963	This study
QC1978	QC1793 Δ*fur*::*kan* Δ*sox-8*::*cat himA*Δ*82*-Tn*10*	P1 (K1299) × QC1804	This study
QC1980	QC1885 Δ*fur*::*kan* Δ*arcA*::*tet*	P1 (QC1732) × QC1950	This study
QC1993	QC1793 Δ*arcA*::*tet zjj*::mini-*kan*	P1 (QC1424) × QC1949	This study

Continued on following page

TABLE 1—*Continued*

Bacterium, phage, or plasmid	Genotype or relevant characteristics	Derivation	Source or reference
QC1994	QC1793 Δ*fnr zdc-235*::Tn*9 himA*Δ*82*-Tn*10* Δ*arcA*::*tet zjj*::mini-*tet*	P1 (QC1993) × QC1974	This study
QC1998	QC1885 Δ*fnr zdc-235*::Tn*9*	P1 (JRG1728) × QC1885	This study
QC1999	QC1885 Δ*fnr zdc-235*::Tn*9* Δ*arcA*::*tet*	P1 (JRG1728) × QC1950	This study
QC2007	QC1793 *zdd-2207*::Tn*10*Km	P1 (JHC1068) × QC1793	This study
QC2008	QC1793 *cfxB zdd-2207*::Tn*10*Km	P1 (JHC1068) × QC1793	This study
QC2009	QC1793 Δ*fur*::*kan zdd-2207*::Tn*10*Km	P1 (QC1732) × QC2007	This study
QC2010	QC1793 Δ*fur*::*kan cfxB zdd-2207*::Tn*10*Km *zbf*::Tn*10*	P1 (QC1782) × QC2009	This study
Phages and plasmids[c]			
λLRS45			41
λIC0	λLRS45 recombinant carrying Φ(*sodA-lacZ*)*1*[d]		This study
λIC143	λLRS45 recombinant carrying Φ(*sodA-lacZ*)Δ*143*		This study
λIC209	λLRS45 recombinant carrying Φ(*sodA-lacZ*)Δ*209*		This study
λIC1	λLRS45 recombinant carrying Φ(*sodA-lacZ*)Δ*1*		This study
pTZ19R			Pharmacia
pDT1-5	pBR322 derivative carrying *sodA*$^+$ region		46
pRB38	Carries *arcA*$^+$ region		14
pBT1-1	pBR322 carrying the 2,523-bp *Hin*dIII-*Pvu*II fragment from pRB38 containing *arcA*$^+$		45
pIC6-1	M13mp18 with the *Hin*dIII-*Pvu*II fragment from pBT1-1 into the multisite		This study
pIC6-2	As pIC6-1, but deleted for 167 bp starting 1 bp after ATG codon of *arcA*		This study
pIC6-3	pTZ19R with the *Eco*RI-*Hin*dIII fragment from pIC6-2		This study
pIC6-4	As pIC6-3, but with the *Eco*RI-*Ava*I fragment from pBR322 conferring tetracycline resistance		This study
pIC3-2	pTZ19R with the *Eco*RI-*Bam*HI fragment from pDT1-5 deleted for the 1,050-bp *Ava*I-*Ava*I fragment containing *sodA*		This study
pRS415			41
pIC4	pRS415 with the 851-bp *Eco*RI-*Bam*HI fragment containing the wild-type *sodA* promoter		This study
pIC4-143	As pIC4, but with the Δ143 deletion		This study
pIC4-209	As pIC4, but with the Δ209 deletion		This study
pIC4-1	As pIC4, but with the Δ1 deletion		This study
pIC5	M13mp18 containing the *Eco*RI-*Bam*HI fragment from pIC4		This study
pIC7	As pIC5 with an *Xho*I restriction site		This study
pIC7-143	As pIC7, but deleted from *Xho*I site to the end point of Δ143		This study
pIC7-209	As pIC7, but deleted from *Xho*I site to the end point of Δ209		This study
pIC8	As pIC5, but with the Δ1 deletion		This study

[a] Strains K1222 to QC1682 were used for construction; strains QC1712 to QC2010 are fusion strains used in this study.

[b] *sox-3*::Tn*10* is a mutation in *soxS*; Δ*soxR4*::*cat* is a *soxR* constitutive mutant; Δ*sox-8*::*cat* is a deletion of *soxR* and *soxS*. The mutations are referred as *soxS*, *soxR4*, and Δ*soxRS* in the text.

[c] Strains GC4468, JHC1068, JHC1096, QC1725, and QC1745 were lysogenized with λIC0 (QC1709, QC1739, QC1740, QC1751, and QC1793, respectively), with λIC143 (QC1886, QC1846, QC1847, QC1769, and QC1885, respectively), and with λIC209 (QC1887, QC1848, QC1849, QC1770, and QC1884, respectively); QC1745 was lysogenized with λIC1 (QC1832).

[d] Φ(*sodA-lacZ*)*1* refers to the operon fusion with the wild-type promoter.

merase, *Eco*RI linkers were introduced at the extremities to permit plasmid circularization. Plasmids were further analyzed by restriction endonuclease digestion and sequencing of the junction region; selected deleted promoters were fused to *lacZ* by cloning the *Eco*RI-*Bam*HI fragment into the multisite of pRS415, and the fusion was transferred onto the chromosome as described above.

Site-directed mutagenesis and general molecular biology methods. (i) Mutagenesis. The target for mutagenesis was M13mp18 containing either the *sodA* region or the *arcA* region, and mutagenesis was carried out using the oligonucleotide-directed in vitro mutagenesis system Kit RPN1523, version 2, from Amersham, Buckinghamshire, United Kingdom. Potential mutants were analyzed by nucleotide sequencing using Sequenase, version 2.0 (U.S. Biochemical Corp.), and the method of Sanger et al. (38). The oligonucleotide sequences used to generate restriction sites (in boldface characters) were as follows: a 28-mer sequence, 5′TCGGTTTTGCG**GAATTC**ATCGCAAAGGA3′, was used to generate the *Eco*RI site at position −810 from the transcription starting point of *sodA* (bases underlined are those that differ from the original sequence); a 25-mer, 5′GCG GCATTTT**CTCGAG**CGGGATGTC3′, was used to generate an *Xho*I site at position −786; a 20-mer, 5′CATATTCG **GATCC**AGTATTG3′, was used to generate a *Bam*HI site at position +42. The Δ1 deletion was generated with a 32-mer with a 17-bp left arm and a 15-bp right arm, 5′GAAAAT GATTATCAATGTTTTGCAGGAAAATG3′ hybridizing on either side of the region to be deleted. For the Δ*arcA* deletion we used a 30-mer, 5′GGTAGCAAACATGC **CCGGG**TAAGAACGGTC3′, containing a 14-bp left arm and a 16-bp right arm on either side of the sequence to be deleted. The two-arm junction creates a *Sma*I site (indicated in boldface characters).

(ii) General methods. DNA preparations, Southern blots, and gel electrophoresis were as described elsewhere (27);

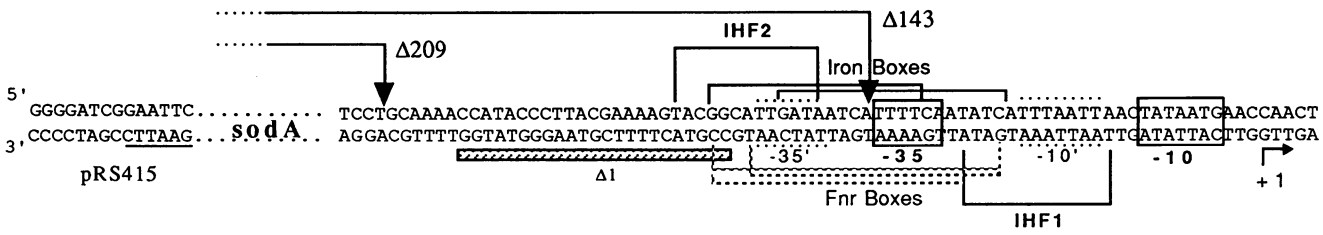

FIG. 1. Nucleotide sequence of the *sodA* promoter region. +1 designates the start point of transcription. The −35 and −10 regions are boxed; −35′ and −10′ (dotted boxes) indicate the putative P′ promoter. Putative binding sites are bracketed: iron boxes in thick lines, Fnr sites in dotted lines, and IHF sites in thick lines labeled IHF1 and IHF2. Arrows indicate the end points of Δ209 and Δ143 upstream deletions. The hatched bar represent the Δ1 deletion. The junction (*Eco*RI site) between pRS415 and the *sodA* promoters is underlined.

cell extracts (47), Western blots (8), and nondenaturing gel activity (4) have been described previously. Restriction enzymes, T4 DNA ligase, nuclease S1, T4 DNA polymerase, Klenow fragment DNA polymerase (PolIk), and dNTPs were purchased from Boehringer Mannheim; *Eco*RI linker d(GGAATTCC) was from Biolabs; and [α-S]dATP was from Pharmacia-LKB. DNA for hybridization (Southern blot) was labelled by random-primed, no-radioactive-DNA labelling with dioxigenin-UTP (Boehringer Mannheim). The Sequenase kit, version 2.0 (U.S. Biochemical Corp.), was used for sequencing; [α-^{35}S]dATP for DNA sequencing was purchased from Amersham.

Nucleotide sequence accession number. The sequence of *sodA* including the upstream region not previously published has been registered in the GenBank library under the number M94879.

RESULTS

Target region of the *sodA* promoter for activation by SoxS. SoxS is the regulatory protein of the two-stage system *soxR-soxS* (33, 52). In order to delimit the region of *sodA* containing the target sequence for activation by SoxS, we attempted to generate a series of upstream deletions into the *sodA* promoter region by unidirectional nuclease digestion (see Materials and Methods). Surprisingly, although various times of digestion were used and numerous deletions were analyzed, we could not obtain a deletion with an end point between −74 and −33 bp, whereas many deletions had end points upstream of −74 or downstream of −33. Similar difficulty in obtaining upstream deletions ending between −74 and −33 was encountered in a previous attempt to generate deletions by digestion with the nuclease *Bal* 31, suggesting that the DNA structure in this region impairs the normal progression of nucleases. The upstream deletions used in this study, Δ143 and Δ209, are shown in Fig. 1; the corresponding deleted promoters are denoted p143 and p209. Genes of the *soxRS* regulon are inducible by redox cycling agents such as paraquat. We examined whether the ability to be induced by paraquat was retained by the deleted *sodA* promoters, p209 and p143 (Fig. 2). Expression of *sodA* was monitored by β-galactosidase assays in *sodA-lacZ* operon fusions carrying the various *sodA* promoters. The Δ209 deletion did not affect inducibility by paraquat. Furthermore, *sodA* expression in constitutive (*soxR4*) or defective (Δ*soxRS*) mutants was the same from p209 as from the wild-type promoter. In contrast, the p143-deleted promoter was no longer inducible by paraquat, and the expression of *sodA* from p143 was the same in a *soxRS*-defective mutant and in a constitutive mutant. This localizes the DNA target for SoxS activation on the wild-type *sodA* promoter to between positions −74 and −33.

Comparison of the sequence between −74 and −33 with the promoter sequence of *zwf*, another gene of the *soxRS* regulon (36), revealed an 18-bp sequence presenting homology with the *sodA* sequence from −64 to −46. In a further attempt to locate the SoxS recognition site more precisely on *sodA* DNA, an internal deletion, Δ1 (Fig. 1), covering this region was made. The deletion completely prevented the paraquat inducibility of the *sodA-lacZ* operon fusion (data not shown), suggesting that Δ1 overlaps the region necessary for SoxS binding.

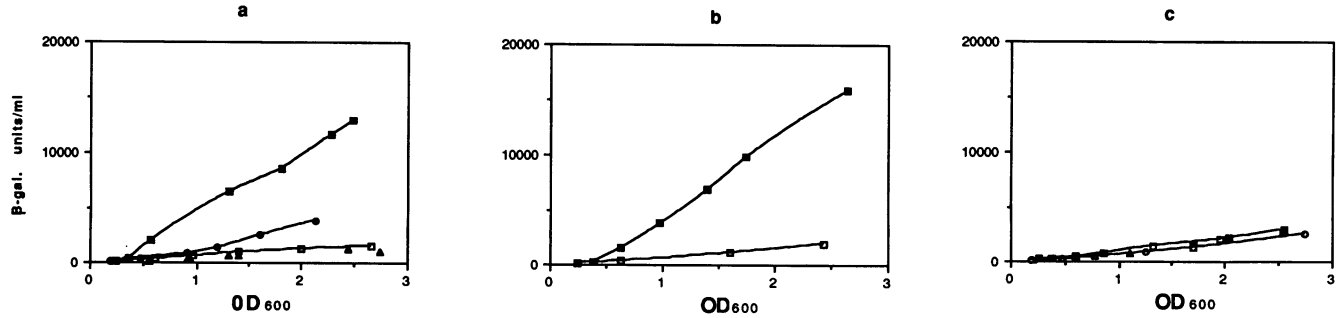

FIG. 2. Effects of Δ209 and Δ143 deletions on *sodA* promoter sensitivity to SoxRS regulation. Cells carrying various operon fusions and mutations at the *soxRS* locus were grown and assayed for β-galactosidase (β-gal) as described in Materials and Methods. β-gal U/ml = β-gal expressed in Miller units × optical density at 600 nm (OD_{600}). Promoters in the fusion: wild-type promoter (a), p209 promoter (b), and p143 promoter (c). Symbols: □, wild type (strains 1751, 1770, and 1769); ■, wild type plus 50 μM paraquat, added when the optical density at 600 nm was ≈0.2, as previously described (6); △, Δ*soxRS* strains (strains 1754, 1772, and 1776); ▲, Δ*soxRS* strains plus paraquat; ● and ○, *soxR4* strains (strains 1755, 1773, and 1777).

TABLE 2. Effect of the *cfxB* mutation on aerobic expression of *sodA-lacZ* from wild-type and p143 promoters

Strain(s)[a]	Relevant genotype	β-Galactosidase (U)[b] in strains with:	
		Wild-type promoter	p143 promoter
QC1709, QC1886	Wild type	500	820
QC1740, QC1847	Δ*soxQ*	470	830
QC1739, QC1846	*cfxB*	2,200	820
QC1739, QC1846 (with paraquat)	*cfxB*	4,240	840
QC1749	*soxR4*	1,850	
QC1879	*cfxB soxR4*	2,850	
QC1882	*soxS*	400	
QC1880	*cfxB soxS*	1,600	
QC1881	*cfxB* Δ*soxRS*	1,500	

[a] Strains are derivatives of GC4468.

[b] β-Galactosidase assays were done as described in the legend to Fig. 2. Values (in Miller units) are deduced from the slope of the curves and represent the average from at least two experiments with variations of no more than 15% from the mean.

TABLE 3. Effect of the Δ*fur* mutation on aerobic expression of *sodA-lacZ* from wild-type and p143 promoters

Strain(s)	Paraquat	Relevant genotype	β-Galactosidase (U)[a] in strains with:	
			Wild-type promoter	p143 promoter
QC1725 derivatives				
QC1751, QC1769		Wild type	510	800
QC1751, QC1769	+	Wild type	3,370	810
QC1758, QC1774		Δ*fur*	1,650	810
QC1758	+	Δ*fur*	6,250	
QC1755, QC1773		*soxR4*	1,900	830
QC1759		Δ*fur soxR4*	3,000	
QC1754, QC1772		Δ*soxRS*	380	810
QC1896, QC1889		Δ*fur* Δ*soxRS*	900	840
QC1745 derivatives				
QC2007		Wild type	500	
QC2009		*cfxB*	2,250	
QC2008		Δ*fur*	1,700	
QC2010		*cfxB* Δ*fur*	2,400	

[a] β-Galactosidase assays were done as described in the legend to Fig. 2; values were calculated as described in Table 2, footnote *b*.

SoxRS- and CfxB-mediated activations are cumulative. *cfxB* mutation causes transcriptional activation of genes of the *soxRS* regulon and increases the synthesis of other proteins not influenced by *soxRS* (17). The effect of a *cfxB* mutation was assessed with wild-type, p209, and p143 *sodA* promoters (Table 2). The Δ209 deletion did not impair the CfxB-mediated activation (data not shown), whereas the p143 promoter was not activated via CfxB; aerobic expression was identical in wild-type (*soxQ*$^+$), Δ*soxQ*, and *cfxB* strains. This places the target for *cfxB* activation in the same region as the target for SoxS.

Greenberg et al. (17) showed previously that SoxRS and CfxB activations act independently; activation via *soxRS* did not depend on *soxQ*, while activation by *cfxB* did not depend on *soxRS*. We further questioned whether the two activations could occur simultaneously. The results (Table 2) indicate (i) that inactivation of SoxS reduces expression of *sodA* in a *cfxB* mutant as in the wild type, (ii) that *cfxB* mutants are still inducible by paraquat, and (iii) that expression in a double mutant, a *cfxB soxR4* strain, is significantly higher than in either single mutant. We thus conclude that *cfxB* and *soxRS* activations are cumulative.

Interference of aerobic *fur* repression with *soxRS* and *cfxB* activation. A putative Fur binding site or iron box, GATAA CATTTTCAATATC (mismatches with the consensus sequence are underlined), was previously reported in the *sodA* promoter (31). We have indicated in Fig. 1 a second iron box, GGCATTGATAATCATTTTC, which overlaps the first one, a configuration observed in several promoters of Fur-regulated genes. The deletion in p143 destroyed a large part of those two putative Fur binding sites. Fur was therefore expected to be inactive on the p143 promoter. Indeed, aerobic and anaerobic expression from the p143 promoter was the same in Δ*fur* mutants as in otherwise isogenic *fur*$^+$ strains (Tables 3 and 4). Similarly, the Δ1 deletion, which slightly overlaps with the upstream iron box, rendered the *sodA* promoter insensitive to aerobic or anaerobic repression by Fur (data not shown), showing that this sequence is necessary for Fur binding to DNA.

The effects of *soxRS* activation and *fur* derepression are cumulative: (i) the level of Φ(*sodA-lacZ*) induction by paraquat was 2-fold higher in a Δ*fur* strain than in the wild type (Table 3), (ii) expression in the double mutant, the Δ*fur soxR4* strain, was twice that in a *soxR4* constitutive mutant, and (iii) expression in a Δ*soxRS* Δ*fur* strain was about 2.5-fold higher than in a Δ*soxRS* strain. Conversely, expression in a Δ*fur* strain was higher than expression in a Δ*fur* Δ*soxRS* strain. This suggests that the two regulations act independently and can be simultaneous.

In contrast, *sodA* expression was nearly the same in *cfxB* and *cfxB* Δ*fur* strains, suggesting that CfxB activation interferes with aerobic *fur* repression (Table 3). Expression in the *cfxB* mutant is significantly higher than in the *fur* mutant, showing that apparent *sodA* activation in *cfxB* is not due to an indirect effect involving Fur inactivation.

Interaction of Fur, ArcA, and Fnr in anaerobic repression of *sodA*. In a previous study we showed that *fur* and *arcA* mutations act synergistically in derepressing *sodA* in anaerobiosis (45). Single mutants were almost completely repressed, showing that either Fur or ArcA alone can preclude

TABLE 4. Effect of mutations in *arcA*, *fur*, and *fnr* on anaerobic expression of *sodA-lacZ* from wild-type and p143 promoters

Strain(s)[a]	Relevant genotype	β-Galactosidase (U)[b] in strains with:	
		Wild-type promoter	p143 promoter
QC1793, QC1885	Wild type	16	145
QC1949, QC1950	Δ*arcA*	47	870
QC1834, QC1945	*TcarcAΔ194*	35	870
QC1794, QC1946	Δ*fur*	50	150
QC1943, QC1980	Δ*fur* Δ*arcA*	820	860
QC1801	Δ*fur arcAΔ194*	560	
QC1963, QC1998	Δ*fnr*	48	500
QC1964	Δ*fur* Δ*fnr*	250	
QC1965, QC1999	Δ*arcA* Δ*fnr*	140	900
AC1966	Δ*fur* Δ*arcA* Δ*fnr*	900	

[a] Strains are derivatives of QC1745. Δ*fnr* strains carry the *zdc*::Tn*9* transposon.

[b] Cells were grown anaerobically and assayed for β-galactosidase as described in Materials and Methods. Values were calculated as described in Table 2, footnote *b*. Values for strain QC1793 *zdc*::Tn*9* (QC1835) were similar to those for QC1793 (not shown).

TABLE 5. Effect of a *himA* mutation on aerobic and anaerobic expression of *sodA-lacZ*

Strains[a]	Genotype	β-Galactosidase (U) during:			
		Aerobiosis of:		Anaerobiosis of:	
		himA$^+$ strains	*himA*::*cat* or *himA*Δ*82*::Tn*10* strains	*himA*$^+$ stains	*himA*::*cat* or *himA*Δ*82*::Tn*10* stains
QC1793, QC1842	Wild type	510	720	16	16
QC1794, QC1908	Δ*fur*	1,650	1,460	50	110
QC1808, QC1890	Δ*soxRS*	380	800		
QC1804, QC 1978	Δ*fur*Δ*soxRS*	900	900		
QC1949, QC1951	Δ*arc*	500		47	53
QC1963, QC1974	Δ*fnr*	470		48	60
QC1943, QC1953	Δ*fur* Δ*arc*	1,700		820	1,150
QC1964, QC1968	Δ*fur* Δ*fnr*			250	850
QC1965, QC1994	Δ*arc* Δ*fnr*			140	160

[a] Strains are derivatives of QC1745.

sodA transcription. The results in Table 4 show that although the deleted promoter p143 is completely insensitive to Fur repression, it is still subject to ArcA repression in anaerobiosis. Thus, *sodA* expression is much higher in a Δ*arcA* background than in an *arcA*$^+$ background and is identical to expression from the wild-type *sodA* promoter in a Δ*fur* Δ*arcA* background. It seems, however, that deletion p143 slightly affected ArcA binding to the *sodA* promoter. Indeed, in an *arcA*$^+$ background, expression from p143 was higher than expression from the wild-type *sodA* promoter in the absence of Fur (Δ*fur* strain), showing that the p143 deletion impairs repression by an effector other than Fur, presumably ArcA. Furthermore, an *arcA* mutant isolated previously as "*sodZ*" (45) and renamed *arcAΔ194* after characterization (45a) had a leaky phenotype as judged by *sodA* expression from the wild-type promoter (derepression of *sodA* in *arcAΔ194* is about half of derepression in the Δ*arcA* null mutant; Table 4) but had the same phenotype as the null mutant with respect to expression of *sodA* from the p143 promoter. This indicates that the altered ArcA protein in the *arcAΔ194* mutant still had some repressive effect on the wild-type promoter but none on p143, suggesting again that the ArcA target is slightly impaired at the p143 promoter.

Hassan and Sun have shown that anerobic expression of *sodA* is increased in *fnr* mutants (22). Indeed, a Δ*fnr* deletion caused derepression of the operon fusion with the wild-type *sodA* promoter, but the effects of *fnr* are reduced or disappear in a Δ*arcA* background (Table 4). Surprisingly, the Δ*fnr* mutation still caused a threefold derepression from the p143 promoter, in which the putative Fnr binding sites (22) have been largely destroyed. All together, this suggests that part of the Fnr repression of *sodA* is indirect and is mediated by ArcA, suggesting that Fnr might act as an activator of *arcA* transcription or that the Fnr and ArcA proteins interact. It should be pointed out that in our experimental conditions of fermentative growth in strict and prolonged anaerobiosis, *fnr* mutants always grow more poorly than their *fnr*$^+$ counterparts. This growth inhibition becomes drastic for strains carrying multiple mutations, and such extensive growth perturbation could bias the results. Since pH has been reported to have a drastic effect on the expression of certain genes in *fnr* mutants and affected growth (10), we buffered the growth medium at pH 7; that did not change the results described above (data not shown).

Aerobic and anaerobic effects of IHF on *sodA* expression. Schrum and Hassan (39a) reported that IHF was a negative effector of *sodA* expression. The results in Table 5 show that the effect of IHF on the wild-type promoter differs in aerobiosis and anaerobiosis. In aerobiosis, a deletion of the *himA* gene, which codes for a subunit of IHF, produced a 1.5- to 2-fold increase of *sodA* expression in a wild-type or Δ*soxRS* background, but this effect disappeared in Δ*fur* strains. Thus, in aerobiosis IHF seems to enhance Fur repression. In anaerobiosis IHF had an effect only in Δ*fur* mutants. In the *arcA*$^+$ Δ*fur* strain, a null mutation in the *himA* gene (Δ*himA* or *himA*::*cat*) produced a 3- to 3.4-fold increase in the expression of *sodA*. In Δ*arcA* Δ*fur* strains there was only a small effect (*sodA* expression was 1.4-fold higher in Δ*fur* Δ*arcA* Δ*himA* strains than in Δ*fur* Δ*arcA* strains), suggesting that the major effect of IHF in anaerobiosis is to enhance ArcA repression.

DISCUSSION

The goal of this study was to shed light on the organization, within the promoter region, of the multiple effectors which regulate the expression of the *E. coli sodA* gene, coding for MnSOD. We therefore undertook a systematic analysis of *sodA* expression in strains which were strictly isogenic except for the regulators under study.

The data suggest that the DNA targets of several of the regulatory proteins overlap, indicating competition between regulators responding to different signals. In all of this work we did not find any situation in which expression of the *sodA-lacZ* fusion from the promoter carrying the Δ209 deletion was different from expression from the wild-type promoter. Thus, all transcriptional controls on *sodA* are exerted on a DNA fragment of less than 120 bp, not extending beyond position −76.

SoxRS activation is triggered in response to superoxide generation. We located the target for SoxS activation between positions −74 and −33 in the *sodA* promoter, and more precisely we observed that deletion of the sequence from −69 to −46 (Δ1) was enough to suppress activation. The deletion encompasses the ACCCTTACGAAAAGT ACG sequence, which presents 61% identity (underlined bases) with a sequence found at positions −171 to −184 in the *zwf* promoter region (36), pinpointing it as a possible candidate for the binding recognition site by the SoxS regulatory protein. However, no homologous sequence could be found in the other sequenced promoters of genes of the *soxRS* regulon, and further work is needed to determine whether this homology is significant. SoxRS activation shows no competition with other aerobic regulations. As

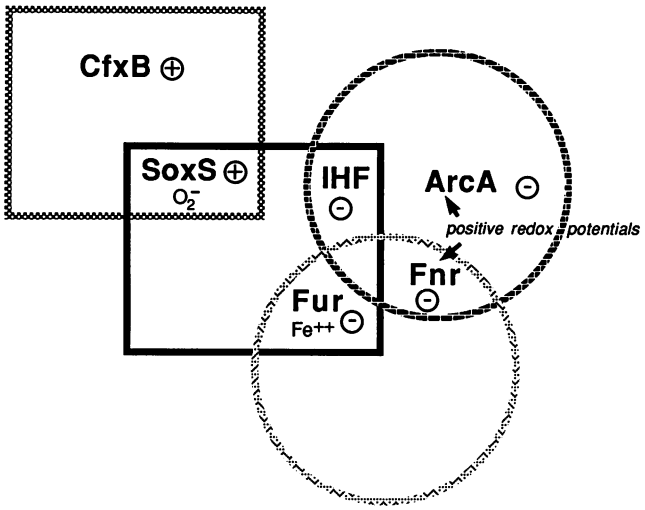

FIG. 3. Schematic representation of possible combinations of regulators governing *sodA* expression. Each diagram (square or circle) encloses the regulatory elements which can act simultaneously on *sodA* transcription (direct or indirect effect). Squares represent combinations occurring in aerobiosis, and circles indicate combinations occurring in anaerobiosis; regulators which are exclusive are enclosed in different boxes. The symbols ⊕ and ⊖ indicate positive and negative effects observed in the presence of the regulator on *sodA* expression. Signals triggering activation of regulators are in italic characters.

schematized in Fig. 3, SoxRS activation occurs simultaneously with Fur repression or with CfxB-mediated activation and permits IHF to act, suggesting either that its DNA target is different from the targets of these effectors or that the way in which the regulatory proteins are bound to the DNA allows simultaneous binding to the same target.

In contrast, activation mediated by CfxB occurs together with SoxS activation but interferes with Fur repression (schematized in Fig. 3 by putting CfxB and SoxS in the same box and Fur and CfxB in different ones). This could reflect competition for the same DNA target between Fur and the regulatory protein triggered in the *cfxB* mutant, or it could indicate that *cfxB* in addition to its ability to activate *sodA* has a negative effect on Fur activity.

In anaerobiosis, *fur* and *arcA* mutations have a synergistic effect on *sodA* expression, suggesting that either Fur or ArcA alone can repress *sodA* with the same efficiency and that their targets on *sodA* DNA may overlap. The deletion Δ143, which destroys both overlapping iron boxes, completely abolished Fur repression, as expected. Overlapping iron boxes are found in several promoters of Fur-regulated genes, but the correlation between the presence of two putative binding sites and the strength of Fur binding to DNA is still unclear (7, 12, 19). The Δ1 deletion, which touches only the upstream iron box, also abolishes Fur repression, suggesting that at least this sequence is necessary for in vivo binding of Fur to *sodA* DNA. Our data locate the ArcA binding site downstream of −33. The less efficient repression of the altered protein encoded by *arcAΔ194* represses less efficiently with the p143 promoter than with the wild-type promoter, indicating that the ArcA binding site on *sodA* DNA is slightly impaired by the deletion Δ143. Thus, the Fur and ArcA binding sites on *sodA* appear to overlap.

IHF has been found to alter DNA structure and influence the function of other DNA-binding proteins (15); in other cases a direct regulatory effect was hypothesized (49). Although some direct effect of IHF on the *sodA* promoter cannot be excluded, its major effect appears to be to enhance Fur repression in aerobiosis and ArcA repression in anaerobiosis. We identified two potential IHF binding sites in the *sodA* promoter, with opposite orientations. The site denoted IHF1 in Fig. 1 overlaps only the downstream iron box and, presumably, the ArcA binding site. IHF2 overlaps both iron boxes but not the ArcA binding site. Our data do not permit a conclusion as to the in vivo utilization of IHF1, IHF2, or both. Since only the IHF2 site was impaired by the Δ143 deletion, we could hope to use Δ143 to determine whether this sequence was involved in the enhancement of *arcA* repression. Unfortunately, careful examination of the sequence around and upstream of the −35 region generated by the deletion revealed new potential IHF binding sites, albeit with lower homology with the consensus sequence, preventing any conclusions.

The effects of *fnr* mutations on *sodA* expression do not allow clear-cut conclusions and might be partially biased by the extremely poor growth of some *fnr* mutants under drastic anaerobic growth conditions. A conjugated action of Fnr and ArcA has now been described for numerous genes (16, 24, 39). However, the ArcA-dependent derepression caused by Δ*fnr* on the Δ143 promoter, in which putative Fnr binding sites have been largely deleted, suggests an indirect action of Fnr via ArcA, possibly in addition to a direct action on the wild-type promoter. Sawers and Suppmann (39) proposed that a functional interaction between Fnr and ArcA proteins might explain the effect of *fnr* and *arcA* mutations on the transcription of the pyruvate formate-lyase gene. An alternate hypothesis is that Fnr positively regulates ArcA transcription; experiments in progress (9a) support such a hypothesis in apparent contradiction to the report that a Δ*fnr* deletion did not influence the expression of an *arcA-lacZ* fusion (33a).

SOD, which maintains a low steady-state concentration of superoxide in the cell (23), plays an essential role in the defense against oxygen toxicity (8). Induction of MnSOD synthesis in response to superoxide (*soxRS* control) is logical. The Fur control is more subtle. In the presence of oxygen, iron becomes a threat, since it catalyzes the production of reactive hydroxyl radicals via the Fenton reaction. Fur regulation permits iron to be maintained at the minimum necessary level, thus minimizing this threat. We have shown (47a) that permanent derepression of iron assimilation (in Δ*fur* mutants) produces oxidative stress, suggesting that a transient oxidative stress occurs whenever iron limitation, via Fur inactivation, triggers an influx of iron into the cell. The coordinated derepression of MnSOD synthesis would reduce the steady-state concentration of superoxide, permitting the cell to deal with this oxidative stress that is not primarily produced by an excess of superoxide. The apparent overlapping of Fur and ArcA targets on *sodA* DNA and the synergy between the effects of Δ*fur* and Δ*arcA* mutations strongly suggest competition between the two repressors and support the view that Fur, or ArcA together with Fnr and IHF, can repress *sodA* in anaerobiosis with about the same efficiency. Both the *fur* and the *arc-fnr* systems respond to the redox state and to iron, but the hierarchy of responses is inverted. For Fur activation, iron content appears to be the primary signal, amplified in anaerobic conditions presumably through an increase in the intracellular Fe^{2+}/Fe^{3+} ratio. The primary signal for Arc-Fnr is the redox state of the cells (24, 32, 50). Although iron could be an oxygen sensor, Fur inactivation under aerobiosis

is governed not by more oxidizing conditions but by iron depletion (45b). When both Fur and ArcA are present in active forms in vivo in anaerobiosis, it is not known which effectively represses *sodA*. The scheme that we favor postulates that *sodA* expression is first coupled to aerobic respiration, which generates oxidative species, and it is only when ArcA repression is relieved, opening the way to formation of toxic oxidative species, that Fur, triggered by iron, starts to interact. Experiments are currently in progress to test this hypothesis.

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