Characterization of regulatory mutations causing anaerobic derepression of the *sodA* gene in *Escherichia coli* K12: cooperation between *cis*- and *trans*-acting regulatory loci

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The genetic loci leading to anaerobic derepression of a *sodA::lacZ* protein fusion in a UV-generated mutant strain (UV14) of *Escherichia coli* were identified. The mutant (UV14) was found to harbour two altered loci: one is in the *trans*-regulatory gene *fnr* (fumarate nitrate reduction) where leucine-129 was changed to glutamine (*fnr14*), and the second (*sodA14*) is in the promoter region (*cis*) of the *sodA* gene apparently affecting the binding of the Fur (ferric uptake regulation) protein. Introduction of an *fnr*⁺ gene into UV14 restored anaerobic repression of *sodA::lacZ* and restored the ability of the cells to reduce nitrate. However, when either the *fnr14* or the *sodA14* mutation was introduced into an otherwise wild-type background, only slight anaerobic derepression of *sodA* was observed. When both the *cis*- and *trans*-acting mutations (i.e. *sodA14* and *fnr14*) were combined simultaneously in an otherwise wild-type background, the specific activity of *sodA::lacZ* expression was comparable to that of the original mutant strain (UV14). Furthermore, a genetically confirmed *fur fnr* double mutant was also similarly derepressed in anaerobic *sodA::lacZ* expression. The data presented suggest that the *cis*-mutation in UV14 (*sodA14*) affects the Fur-binding site in the *sodA* promoter, while having no effect on Fnr or Arc mediated repression. Also, a second putative Fnr-binding site that straddles the ribosomal binding-site was identified in the *sodA* gene.

Introduction

Catalytic removal of the superoxide radical (O₂⁻), the first product in the univalent pathway for oxygen reduction, is accomplished by superoxide dismutases (EC 1.15.1.1; SODs) (McCord & Fridovich, 1969). These metallo-enzymes dismutate superoxide to H₂O₂ and O₂, therefore providing the first line of protection against oxygen toxicity (McCord & Fridovich, 1969; Hassan, 1989). In the facultatively aerobic micro-organism *Escherichia coli*, three forms of SOD are found (Hassan, 1989): a constitutive iron-containing isozyme (FeSOD), an inducible manganese-containing enzyme (MnSOD), and a hybrid form composed of subunits from both the Mn- and FeSODs. MnSOD is encoded by the *sodA* gene which is mapped at 88 minutes on the *E. coli* chromosome (Touati, 1983; Bachmann, 1990).

Conditions known to induce *sodA* include: presence of

oxygen (Gregory & Fridovich, 1973), changes in cellular redox potential (Schiavone & Hassan, 1988), the addition of iron chelators (Hassan & Moody, 1984; Moody & Hassan, 1984; Pugh & Fridovich, 1985), increased intracellular flux of O₂ by the presence of redox cycling compounds (Hassan & Fridovich, 1977, 1979) and depletion of cellular reduced glutathione (Gardner & Fridovich, 1987). Studies with a *sodA::lacZ* protein fusion (Touati, 1988) have confirmed previous conclusions that *sodA* is transcriptionally regulated (Moody & Hassan, 1984).

Moody & Hassan (1984) proposed that transcriptional repression of *sodA* is mediated by an iron-binding redox-sensitive protein, where the putative repressor is presumed to be active when reduced iron is bound, but is inactivated under oxidizing conditions or by chelation of iron. Recent investigations have refined this model and implicated the products of several genes in the regulation of *sodA*, including: the superoxide responsive regulon (*soxRS*; Greenberg *et al.*, 1990; Tsaneva & Weiss, 1990), the ferric uptake regulation gene (*fur*; Neiderhoffer *et al.*, 1989, 1990; Tardat & Touati, 1991; Beaumont & Hassan,

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Abbreviation: SOD, superoxide dismutase.

Table 1. *Strains and plasmids*

Strain/ plasmid	Relevant genotype	Derivation	Source (reference)
GC4468	F ⁻ Δ <i>lacU169 rpsL</i>		Touati (1988)
QC774	As GC4468 but <i>φ(sodA::lacZ)</i> 49 <i>φ(sodB-Kan)</i> 1-Δ ₂ Cm ^R Km ^R		Touati (1988)
NC3	As GC4468 but (<i>sodA-lacZ</i>)49 Cm ^R Lac ⁺		This laboratory
NC53	As NC3 but (<i>sodB-Kan</i>) 1-Δ ₂ Cm ^R Km ^R		This laboratory
UV14	As NC53 but Lac ⁺ anaerobically		H. M. Hassan
UV16	As NC53 but Lac ⁺ anaerobically		H. M. Hassan
NC147	As UV14, but <i>sodA</i> ⁺ -Tn10 nearby		D. Touati
NC210	CAG5053 KL208, <i>zbc3105::Tn10</i>		Singer <i>et al.</i> (1989)
NC211	CAG5054 KL96, <i>trpB83::Tn10</i>		Singer <i>et al.</i> (1989)
NC212	CAG5055 KL16, <i>zed3069::Tn10</i>		Singer <i>et al.</i> (1989)
NC400	ECL512, <i>fmr-1</i> , <i>zci::Tn10</i>		Iuchi <i>et al.</i> (1989)
NC402	ECL594, <i>arcB1 zgi::Tn10</i>		Iuchi <i>et al.</i> (1989)
NC411	As NC3, but <i>fmr-1</i>	P1(NC400) × NC3	Hassan & Sun (1992)
NC439	As NC3, but <i>fur::Tn5</i>	P1(728) × NC3	Hassan & Sun (1992)
NC504	As GC4468, but Δ <i>fmr</i>	P1(NC400) × GC4468, then Tn10 cured	H. M. Hassan & H. C. H. Sun
NC506	As GC4468, but Δ <i>arcB</i>	P1(NC402) × GC4468, cured for Tn10	H. M. Hassan & H. C. H. Sun
NC511	Km ^R <i>fur::Tn5</i>	P1(NC728) × GC4468	H. M. Hassan & H. C. H. Sun
NC600	W3110 Δ <i>lacU169 tna2</i>		Bagg & Neilands (1987)
NC658	Cm ^R (<i>sodA14::lacZ</i>)	P1(UV14) × GC4468	This study
NC667	<i>fmr</i> ⁺ <i>zci::Tn10</i>	P1(NC400) × GC4468 select for Tet ^R /NR ⁺⁺	This study
NC672	UV14 <i>sodA</i> ⁺ Cm ^S	P1(NC147) × UV14 select Cm ^S lac ⁻	This study
NC675	UV14 <i>zci::Tn10 fmr</i> ⁺	P1(NC667) × UV14 select Tet ^R and NR ⁺⁺	This study
NC676	NC675 but nitrate reductase ⁻ (<i>fmr14</i>)	P1(NC667) × UV14 select Tet ^R and NR ⁻	This study
NC677	UV14 (<i>sodA::lacZ</i>)-wt	P1(NC3) × NC672	This study
NC685	Δ <i>fmr sodA14</i>	P1(UV14) × NC504	This study
NC690	<i>fur sodA14</i>	P1(UV14) × NC511	This study
NC691	Δ <i>arcB sodA14</i>	P1(UV14) × NC506	This study
NC696	GC4468 <i>sodA14/fmr14 zci::Tn10</i>	P1(NC676) × NC658	This study
NC701	as NC696, independent isolate	P1(NC676) × NC658	This study
NC704	as NC729, but <i>fmr-1</i>	P1(NC411) × NC729	This study
NC707	as NC727 but <i>fmr-1</i>	P1(NC411) × NC727	This study
NC727	as NC600 but <i>sodA::lacZ</i>	P1(NC3) × NC600	This study
NC728	as NC600 but <i>fur::Tn5</i>		Bagg & Neilands (1987)
NC729	<i>fur::Tn5 sodA::lacZ</i>	P1(NC3) × NC728	This study
pDT1.5	<i>sodA</i> ⁺ plasmid		Touati (1983)
p ⁺ <i>Fmr</i> +	pBR322 <i>fmr</i> ⁺		Jones & Gunsalus (1983)

*NR⁺, nitrate reductase positive anaerobically; NR⁻, nitrate reductase negative anaerobically.

1990, 1991, 1992), the aerobic respiratory control (*arc*; Tardat & Touati, 1991; Beaumont & Hassan, 1991, 1992) and fumarate nitrate reduction (*fmr*; Hassan & Sun, 1991, 1992). Additionally, post-translational activation (Privalle *et al.*, 1989), integration host factor (IHF; Presutti, 1992) and DNA supercoiling (Schrum & Hassan, 1990, 1992) have been implicated in the regulation of MnSOD biosynthesis. Recently, we characterized two mutants of *E. coli* partially derepressed in the anaerobic expression of a *sodA::lacZ* protein fusion (Beaumont & Hassan, 1991, 1992). One of the mutants (UV16) was shown to be defective in *fur* and *arcA* and to be similar to another previously reported mutant (Tardat

& Touati, 1991). The second mutant (UV14) was characterized as *fur*⁺ and *arcA*⁺, yet remained partially derepressed in anaerobic expression of *sodA::lacZ* (Beaumont & Hassan, 1992).

The present study further examines UV14, and shows that it harbours two mutations: one affecting the *trans*-acting element, *Fmr*, and the other affecting the *cis*-acting regulatory region of *sodA* apparently required for the binding of the *trans*-acting regulatory element, *Fur*. Either of the *cis*- or the *trans*-mutation alone confer only moderate anaerobic derepression of *sodA::lacZ*, while together they allow synergistic expression of *sodA* anaerobically.

Methods

Materials. *o*-Nitrophenyl β -D-galactoside was from Sigma. The iron chelator 2,2'-dipyridyl was obtained from Aldrich. All other chemicals were of the highest quality available.

Strains and plasmids. Strains used are all derivatives of *E. coli* K12, and are listed in Table 1. Ultraviolet (UV)-generated regulatory mutants were isolated (H. M. Hassan, unpublished) based on anaerobic expression of β -galactosidase from a chromosomal *sodA::lacZ* protein fusion.

Media and growth conditions. LB medium was prepared as described by Miller (1972). Where indicated the medium was supplemented with 1% glucose (LBG), and with 1 mM-MnCl₂ when MnSOD was to be assayed. MacConkey agar base from Difco was supplemented with 1% lactose. Growth of the cultures was measured as optical density at 600 nm (OD₆₀₀). Anaerobic growth was carried out in a Coy anaerobic chamber as previously described (Moody & Hassan, 1984). All experiments were carried out at 37 °C.

Genetic techniques and strain construction. P1 transduction was as described by Miller (1972). Transformation and plasmid preparation followed protocols described by Sambrook *et al.* (1989). Mapping of the *sodA* trans-acting regulatory mutations was carried out by Hfr conjugational crosses as described by Singer *et al.* (1989). Marker exchange in transconjugants was observed by anaerobic Lac⁻ phenotype (expressed from *sodA::lacZ*) in colonies which harboured both donor (Tet^R) and recipient (Cm^R) markers.

Enzyme assays. β -Galactosidase was assayed as described by Miller (1972), and the data were plotted in the form of differential plots (i.e. changes in U ml⁻¹ vs. changes in OD₆₀₀). Superoxide dismutase was assayed by the xanthine oxidase/cytochrome *c* method of McCord & Fridovich (1969) using cell-free extracts prepared as previously described (Moody & Hassan, 1984). Nitrite was determined by the method of Hanson & Phillips (1981). Enzyme assays were carried out at 25 °C using a Kontron spectrophotometer. Protein was estimated by the Lowry method with a bovine serum albumin standard. Activity gels for resolution of active forms of SODs were carried out as described in Beauchamp & Fridovich (1971), and quantified by an LKB Laser densitometer.

Cloning and sequencing. Total chromosomal DNA from both the wild-type (GC4468) and mutant (UV14) strains was isolated as described in Sambrook *et al.* (1989) and used to amplify the *fnr* gene by the *Taq*-polymerase chain reaction in a thermocycler (Precision Scientific) using 18 bp primers annealing to both the 5' end (bases 180 to 197) and the 3' end (bases 1380 to 1363) based on the published *fnr* sequence (Shaw & Guest, 1982). Typical reaction conditions were: 10 ng template, 1 μ M primers, with 94 °C denaturation (1 min), 54 °C annealing (1 min) and 74 °C extension (1.5 min). The amplified fragments were cloned and sequenced by single stranded, M13 sequencing using described protocols and reagents (USB). Dideoxy sequencing reactions (Sanger *et al.*, 1977) were resolved on a 5% Long Ranger (AT Biochem) acrylamide gel and autoradiographed using Kodak X-omat AR film. The mutation was confirmed in two independent genomic DNA isolations and two independent PCR reactions.

Results

Mapping of trans-acting regulatory loci in UV14

The Hfr strain kit provided by C. Gross (Singer *et al.*, 1989, and Table 1) was used to map the mutant phenotype in UV14. Conjugation between UV14 and

Hfr strains NC210, NC211 and NC212 led to conversion of UV14 to a wild-type phenotype (i.e. conversion of a Lac⁺ to Lac⁻ phenotype on anaerobic MacConkey agar plates) at a frequency of 62%, 80% and 10%, respectively (representative values from two crosses). NC210 transfers a region of DNA (12' $\rightarrow$ 35') that overlaps with that transferred by NC211 (25' $\rightarrow$ 45') in the region between (25' $\rightarrow$ 35'). This overlapping region is known to include the locus for *fnr* located at 29.5' (Bachmann, 1990), which has been recently implicated in *sodA* regulation (Hassan & Sun, 1991, 1992). Therefore,

Table 2. Effect of mutation(s) in UV14 on nitrate reduction

Cells were grown anaerobically at 37 °C in LB medium with 1% glucose (LBG) and 0.1% KNO₃. Overnight cultures were used to inoculate LBG and samples were withdrawn for determination of both OD₆₀₀ and nitrite. Values are taken from the mid-exponential phase of growth.

Strain	Phenotype	Nitrite [mmol (OD ₆₀₀) ⁻¹]
NC53	Wild-type Fnr ⁺	5.98
UV14	<i>sodA</i> derepressed mutant	0.84
UV14/pFnr ⁺	<i>sodA</i> derepressed mutant/Fnr ⁺	3.30
NC675	UV14 Fnr ⁺	5.54
NC411	Fnr ⁻	1.05
NC411/pFnr ⁺	Fnr ⁺	3.97

Fig. 1. Effects of the *fnr*⁺ gene on anaerobic expression of *sodA::lacZ* in UV14. UV14 was transformed with plasmid-borne *fnr*⁺ (pFnr⁺), or transduced with a P1 lysate from a strain carrying a *zci::Tn10* marker closely linked to *fnr*⁺, to give strain NC675. Culture conditions and assay procedure are as described in Table 3 and in Methods.

Fig. 2. (a) Autoradiogram of a sequencing gel showing the mutated region of the UV14 *fnr* gene and the corresponding region of the cloned and sequenced wild-type gene from GC4468. The portion of the sequence shown corresponds to bases 887 through 926 of the published sequence (Shaw & Guest, 1982). The change of CTG to CAG (reading from top to bottom) is the mutated codon at amino acid 129, with the T to A change at base 905. (b) Upstream regulatory region of the *sodA* gene (Takeda & Avila, 1986) showing the proposed Fur-binding 'iron box'; the -10 and -35 regions (boxed); the ribosomal binding-site (underlined); the two potential Fnr binding-sites (overlined; sites I and II); and the base changes (A to T and a A deletion) found in *sodA14* (arrows; from S. Bowen & H. N. Hassan, in preparation).

we explored the possibility that UV14 contains a mutation affecting the expression of Fnr.

fnr mutation found in UV14

The *fnr* locus has been shown to be an indispensable up-regulator for the function of anaerobic respiratory systems, including nitrate reductase, fumarate reductase and DMSO/TMAO reductases (Spiro & Guest, 1990). Data in Table 2 show that the ability of UV14 to reduce nitrate was significantly lower than that of the wild-type strain but similar to a well-defined *fnr* mutant (NC411) (compare lines 2 and 5, Table 2). Furthermore, the *fnr*⁺ gene provided either by P1 transduction or on a multicopy plasmid led to wild-type levels of *sodA::lacZ* anaerobically (Fig. 1) and restoration of nitrite production (Table 2, lines 3 and 4).

Cloning and sequencing of the *fnr* gene from UV14 and its wild-type parent identified a single base change in the coding region that differed from the published sequence (Shaw & Guest, 1982) and from the cloned wild-type gene (Fig. 2a). The single base change (T at position 905 to A) led to a leucine-129 to glutamine conversion. This mutant allele is termed *fnr14*. Additionally, we found that base 1316, in the termination loop of *fnr*, was changed from a T to a G (data not

shown). We also noted an error of TCG to TGC at amino acid 29 in the published *fnr* sequence. This error has also been noted by Trageser *et al.* (1990) and Kiley & Reznikoff (1991).

A second mutation discovered in UV14

The fact that mutations in the *fnr* gene do not cause anaerobic derepression of the *sodA* gene (Hassan & Sun, 1991, 1992), suggested that UV14 must also possess other mutation(s) causing the observed phenotype. Concurrently, it was discovered that UV14 harboured a *cis*-acting mutation which was subsequently cloned and sequenced (S. Bowen & H. M. Hassan, unpublished; Fig. 2b). The effect of this *cis*-mutation (termed *sodA14*) on *sodA::lacZ* expression was examined in a wild-type background (Fig. 3a) (strain NC658 was constructed by transduction of GC4468 with a P1 lysate prepared on UV14 and selecting for Cm^R/Lac⁺ aerobically). Very low levels of anaerobic β -galactosidase activity were observed in the constructed *sodA14* mutant (NC658), and the addition of iron chelator did not increase the expression of *sodA::lacZ*; instead it decreased the level of expression for some unknown reason (Fig. 3a).

Conversely, a strain was constructed (NC672) in which the defective *sodA14* locus was replaced by the

Fig. 3. Anaerobic derepression of *sodA::lacZ* due to *cis*-acting (*sodA14*; NC658; panel a) and *trans*-acting (*fnr14*; NC677; panel b) mutant elements of UV14. Culture conditions, addition of the iron chelator 2,2'-dipyridyl (dip), and determination of β-galactosidase were as described in Fig. 1 and in Methods.

corresponding wild-type locus to observe the effects of the *trans*-acting regulatory mutation from UV14 (i.e. *fnr14*) on *sodA::lacZ* expression. Again, no significant derepression of *sodA::lacZ* activity was observed, but induction by 2,2'-dipyridyl was restored (Fig. 3b; repeated three times, representative values shown).

Synergistic effects of *cis*- and *trans*-mutations of UV14 on anaerobic expression of *sodA::lacZ*

The lack of significant derepression of *sodA* as a result of either the *sodA14* or *fnr14* mutation (Fig. 3) led us to investigate the combined effects of these two mutations in a genetically clean wild-type background. Strains were constructed (NC696 and NC701) which harboured the *sodA14* along with the *fnr14* mutations. We also combined the *sodA14* mutation with a confirmed *fnr* mutation (NC685). In all three constructed strains, the anaerobic levels of *sodA::lacZ* expression were elevated about 10- to 15-fold over the additive effects of either *sodA14* or *fnr14* mutation alone (Fig. 3), and the level of expression of NC696 and NC701 was similar to that of UV14 (Table 3; representative values for three determinations shown).

sodA14 Mutation prevents induction of *sodA::lacZ* by iron chelation

In order to explore the reasons for lack of induction of *sodA::lacZ* by 2,2'-dipyridyl in UV14, we examined the effect of *sodA14* on the expression of a *sodA*⁺ gene from a multicopy plasmid. Thus, the wild-type strain (NC53) and the mutant strain (UV14) were transformed with plasmid pDT1.5 (Touati, 1983; harbouring *sodA*⁺), and the effects of 2,2'-dipyridyl on the simultaneous anaerobic expression of β-galactosidase from the chromosomal *sodA::lacZ* and MnSOD from the plasmid, pDT1.5, were observed. Data in Table 4 show that

Table 3. Anaerobic expression of *sodA::lacZ* in strains harbouring both *sodA14* and *fnr14* mutations from UV14

Strains were grown anaerobically at 37 °C in LBG. Overnight cultures were used to inoculate 10 ml cultures, from which samples were drawn at time intervals for determination of both OD₆₀₀ and β-galactosidase as described in Methods.

Strain	Genotype	β-Galactosidase [U (OD ₆₀₀) ⁻¹]
NC53	Wild-type	5
UV14	<i>sodA</i> derepressed mutant	1603
NC685	Δ <i>fnr</i> / <i>sodA14</i>	2688
NC696*	<i>fnr14</i> / <i>sodA14</i>	1609
NC701*	<i>fnr14</i> / <i>sodA14</i>	1606

* Independent isolates from two separate constructions.

Table 4. Effect of 2,2'-dipyridyl on the anaerobic expression of *sodA*⁺/*sodA::lacZ* from wild-type and mutant (UV14) strains harbouring pDT1.5

Cultures were grown anaerobically in LBG at 37 °C with 1 mM-MnCl₂. Overnight cultures were used to inoculate 50 ml of the same medium. At time intervals, samples were withdrawn for determination of both OD₆₀₀ and β-galactosidase. When cells reached mid-exponential phase, they were harvested by centrifugation and cell-free lysates prepared and assayed for MnSOD as described in Methods.

Strain	MnSOD (U mg ⁻¹)		β-Galactosidase [U (OD ₆₀₀) ⁻¹]	
	-DIP	+DIP*	-DIP	+DIP*
NC53/pDT1.5	0.4	33.7	26	352
UV14/pDT1.5	25.8	392.2	1321	1348

* Where indicated, 2,2'-dipyridyl (0.5 mM) was added when cells reached an OD₆₀₀ of about 0.1.

UV14/pDT1.5 responded to chelator addition with an 11-fold increase in MnSOD, while β-galactosidase, driven from the mutated *cis*-acting regulatory region

Fig. 4. Expression of *sodA::lacZ* from a strain harbouring *sodA14* mimics that from a known *fur* mutation. (a) Combined effects of *sodA14* with *fur* (NC690) (O), *arcB* (NC691) (+) or wild-type (NC658) (+) background on anaerobic expression of *sodA::lacZ*. (b) Effects of *fur* and *sodA14* on aerobic expression of *sodA::lacZ*: O, *fur⁻* (NC439); Δ, *sodA14* (NC658); +, *fur⁺* (NC3). Culture conditions and assay of β -galactosidase were as described in Fig. 1 and in Methods.

(*sodA14*), remained unchanged. In contrast, NC53/pDT1.5 demonstrated inducibility of both the plasmid-borne *sodA* (MnSOD) and the chromosomal *sodA::lacZ* (Table 4; repeated twice, representative values shown). These data show that the *sodA14* mutation acts only in *cis* in abolishing the induction of *sodA* by iron chelation.

cis Mutation in UV14 mimics *fur* mutation

Because the base changes in the *sodA14* mutation fell within the Fur-binding site 'iron box' of the *sodA* regulatory region (S. Bowen & H. M. Hassan, unpublished; Fig. 2b), strains were constructed to assess the phenotype conferred by this mutation. If the *sodA14* mutation does prevent binding of and repression by Fur protein, *sodA::lacZ* expression in such a mutant would be similar to that previously observed in a *fur* mutant. Indeed, the introduction of *sodA14* into an *Arc⁻* background (NC691) resulted in partial anaerobic derepression of *sodA::lacZ* (Fig. 4a), as would be expected from a strain lacking repression by Fur and Arc (Tardat & Touati, 1991; Beaumont & Hassan, 1992). Also, cells harbouring the *sodA14* region demonstrated higher aerobic expression of *sodA::lacZ* (Fig. 4b), as was reported for *fur* mutants (Neiderhoffer *et al.*, 1990; Tardat & Touati, 1991; Beaumont & Hassan, 1992; Hassan & Sun, 1992).

Strains harbouring mutations in both *fur* and *fnr* show elevated anaerobic expression of *sodA::lacZ*

UV14 contains two mutations; a promoter mutation which appears to effect binding of Fur to the *sodA* promoter, and a *trans*-mutation which disables Fnr function. It remains to be determined if the *sodA* phenotype of UV14 is a result of the combined effects of only the *sodA14* and the *fnr14* mutations. If so, a double *trans*-acting mutant (*fur fnr*) should phenotypically

Table 5. Anaerobic expression of *sodA⁺* and *sodA::lacZ* in *fur*, *fnr*, and *fur fnr* mutant strains and effect of 2,2'-dipyridyl

Isogenic strain pairs harbouring *sodA::lacZ* (NC727, 707, 729, 704) were grown overnight in LBG (with 1 mM-MnCl₂ when MnSOD assayed) at 37 °C. Culture conditions and assay procedures were as described in Table 4 and in Methods.

Strain	Genotype	β -Galactosidase [U (OD ₆₀₀) ⁻¹]	
		- DIP	+ DIP*
NC727	Wild-type	6	139
NC707	<i>fnr</i>	37	366
NC729	<i>fur</i>	65	517
NC704	<i>fur fnr</i>	857	378

* The iron chelator 2,2'-dipyridyl was added at an OD₆₀₀ of approximately 0.1.

behave like UV14, with respect to the anaerobic expression of *sodA*. The data (Table 5; representative values of two experiments) clearly show that the absence of both functional Fur and Fnr proteins resulted in anaerobic expression of *sodA::lacZ*, as well as loss of induction by 2,2'-dipyridyl as observed in UV14.

Discussion

The mutant strain UV14 was found to harbour two mutations: one is in the *fnr* gene and the second is in the promoter region of the *sodA* gene apparently affecting the binding of the Fur protein. A *fur fnr* double mutant behaved similarly to UV14, and also lacked responsiveness to iron chelation. These results are in contrast to earlier findings (Hassan & Sun, 1992) which showed lack of anaerobic expression of *sodA::lacZ* in a *fur fnr* double mutant. It was subsequently found that the *fur fnr* strain used by Hassan & Sun (1992) was a 'leaky' mutant with respect to the *fur* locus (H. M. Hassan & H. C. H. Sun,

unpublished); however, this was not the case for the double mutant used in the present study.

The *fnr* mutation in UV14 was found to be a leucine to glutamine conversion at amino acid 129, and resulted in loss of a Fnr-mediated function (i.e. anaerobic nitrate reduction). The mutation does not fall within the proposed secondary structures of Fnr postulated to bind iron or DNA on Fnr-regulated genes, but falls near the nucleotide binding domain in the homologous Crp protein (Spiro & Guest, 1990). It is interesting to note that a region of Fnr adjacent to this has been implicated in subunit association (Kiley & Reznikoff, 1991). If the *fnr14* mutation alters the association constant of the Fnr subunit and subsequent active Fnr formation, repression of *sodA* may be affected. The ability to directly assess the *in vitro* binding of the Fnr protein to the *sodA* promoter is hampered by the difficulty in isolating a biologically active Fnr protein (Kiley & Reznikoff, 1991).

The second mutation (Fig. 2*b*; S. Bowen & H. M. Hassan, unpublished) falls within the proposed binding site of the Fur protein to the *sodA* promoter region (Neiderhoffer *et al.*, 1989; Naik & Hassan, 1990). This mutation resulted in moderate anaerobic derepression of *sodA*, and is refractory to chelator induction. When the *sodA14* and *fnr14* mutations were present together, synergistic expression of *sodA* was observed anaerobically (Table 3). The data also suggest that the *sodA14* mutation does not affect the putative binding sites for Fnr and/or Arc (Table 3 and Fig. 4*a*). The lack of chelator enhanced expression when the *sodA14* mutation is present (Fig. 3*a*; Table 4) could also be interpreted such that the mutated site might be important for the binding of an activator molecule. The ability of a strain harbouring *sodA14* (NC658) to be induced by paraquat in air (data not shown) suggests that this mutation does not fall within the site required for SoxS mediated activation of *sodA* (Greenberg *et al.*, 1990; Tsaneva & Weiss, 1990).

The *fnr* locus encodes a redox-sensitive, iron-binding protein which regulates cellular electron flow based on the level of available electron acceptors (Spiro & Guest, 1990; Uden & Trageser, 1991). It is postulated that the metallated protein binds to a 22 bp consensus sequence upstream of regulated genes, located near the -35 region in up-regulated genes, and close to the +1 site in repressed genes (Spiro & Guest, 1990). It is interesting to note that, based on sequence homology to the Fnr consensus sequence, two putative Fnr-binding sites (Fig. 2*b*; sites I and II) are found in the *sodA* gene; one previously identified in the -35 region (Hassan & Sun, 1992), and one identified in this study that straddles the ribosomal binding-site. Previous studies (Hassan & Sun, 1992) suggested that *fnr* might function as both a repressor and activator in *sodA* regulation. It is inter-

esting to speculate on the function of these two Fnr-binding sites in the regulatory region of the *sodA* gene; thus 'site I' may serve for binding of a repressor form of Fnr (i.e. metallated Fnr) while 'site II' may allow the binding of an activator form of Fnr (i.e. demetallated Fnr). This conclusion is supported by the fact that the *cis*-mutation in UV14 (*sodA14*), which overlaps the iron-box and 'site II' (Fig. 2), was still responsive to *fnr* mutations with respect to expression of *sodA::lacZ*. Also, recent studies have shown that *sodA* mutants deleted in the iron-box and 'site II' were still induced for *sodA::lacZ* in response to a deletion in the *fnr* gene (Compan & Touati, 1993). Therefore, it is more likely that 'site I' is the site essential for negative regulation of *sodA* by Fnr. The presence of 'site I' in the untranslated region of *sodA* mRNA may suggest post-transcriptional regulation of *sodA* by Fnr. Work is in progress to further examine this possibility.

The synthesis of these data into a comprehensive model will require further studies into the mode of action of the multiple elements implicated in *sodA* regulation. Perhaps most important will be sorting true repressor/activator elements from pleiotropic effectors which only modify cellular physiology, and hence activity of the true effector(s). However, present and previous findings clearly indicate that mutational loss of Fur function (i.e. as in *fur* or *sodA14*) in combination with a second mutation in either *fnr* or *arc* is necessary for partial anaerobic derepression of *sodA*, while full derepression requires the simultaneous inactivation of all three repressors (Fur, Arc and Fnr) (Hassan & Sun, 1992; Compan & Touati, 1993). Recent studies have suggested a functional or physical linkage between Arc and Fnr proteins in regulatory processes (Sawers & Suppmann, 1992). However, in the absence of direct evidence for Fnr or Arc binding to *sodA*, one cannot rule out the possibility of Arc or Fnr modulating an independent effector molecule in response to either signals of respiratory or oxidative environment.

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