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Novel effect of aromatic compounds on the iron-dependent expression of the *Escherichia coli* K12 manganese superoxide dismutase (*sodA*) gene

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Summary. In *Escherichia coli*, the superoxide dismutase genes (*sodA* and *sodB*) sense the availability of Fe through the action of the *fur* locus [E. C. Niederhoffer, C. M. Naranjo, K. L. Bradley, J. A. Fee (1990) Control of *Escherichia coli* superoxide dismutases (*sodA* and *sodB*) genes by the ferric uptake regulation (*fur*) locus, *J. Bacteriol. 172,* 1930–1938]. Previous work from other laboratories has shown that a variety of metal chelators and of redox-active aromatic compounds can dramatically induce expression of *sodA*. Here we show that non-redox-active, non-metal-chelating aromatic compounds also enhance expression of a chromosomal *sodA* gene fusion and that these effects are strongly modulated by the Fur phenotype ($Fur^{\pm}$) and by the availability of iron in the culture medium. The compounds studied were ethidium bromide, hemin, 2,2′-bipyridine, 1,10-phenantroline, 4,7-phenantroline, rhodamine B1, rhodamine 6G, and, for comparison to previous studies, Paraquat.

Key words: Superoxide dismutases – Aromatic compounds – Ferric uptake regulation

Introduction

Regulation of *sodA* expression in *Escherichia coli* is quite complicated. Recent evidence suggests at least four regulatory loci are involved: *fur* (Niederhoffer et al. 1990; Tardat and Touati 1990), *arcA* and *arcB* (Tardat and Touati 1990), and *soxR* (Tsaneva and Weiss 1990; Greenberg et al. 1990; cf. also Kogoma et al. 1988); the two *arc* genes and *fur* appear to be negative regulators while *soxR* is an activator. How the protein products of these loci function to regulate *sodA* expression remains to be determined, although there is evidence for a direct interaction of holo Fur with an 'iron box' sequence in the *sodA* promoter (Niederhoffer et al. 1990); by this mechanism, iron can be involved. Over the years, evidence has accumulated showing that external stimuli can have dramatic effects on the expression of the *sodA* gene. In general, these are oxidants, such as dioxygen, redox-active compounds, such as Paraquat, and metal chelators, such as 2,2′-bipyridine and 1,10-phenanthroline (cf. Hassan and Fridovich 1979, Hassan 1989 and Touati 1988a for review); unfortunately, only rather focussed interpretations of these observations have been forthcoming. In particular, we have been bothered by the fact, apparently overlooked, that many of the compounds tested are not uni-functional and innocent in all other regards. For example, Paraquat, in addition to undergoing a well-defined and simple set of reactions that can generate superoxide, is a positively charged, aromatic material that is actively pumped into the cell against a very steep gradient (Simons et al. 1976; Kao and Hassan 1985). This is an inherent property of energized lipid vesicles exposed to lipophilic cations. Thus, Chen and his coworkers have shown (1982) that lipophilic cations, such as rhodamine 123, are actively accumulated by energized mitochondria; similarly, Jones and Garland (1977) showed uptake of Paraquat by energized *E. coli*. Further, multiply charged cations are able to interact with many components of the cell (cf. Minton et al. 1990). In a similar vein, 1,10-phenanthroline, in addition to being a powerful chelator of Fe, also binds other metals, forms tris complexes with metal ions having potential for binding to DNA (Sigman and Chen 1990), and is itself a large, planar, hydrophobic, lipid-soluble molecule. For these reasons, we were led to examine briefly the effects of non-redox-active, non-metal-chelating aromatic compounds on *sodA* expression. We avoided the problem of measuring the metal-dependent superoxide dismutase activity through the use of a chromosomal

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Abbreviations. DTPA, diethylenetriaminepentaacetic acid; Paraquat, *N,N′*-dimethyl-1,1-bipyridene; bpy, 2,2′-bipyridine; phen, 1,10-phenanthroline; 4,7-phen, 4,7-phenanthroline.

ΦsodA-lacZ fusion that reveals *sodA* expression by coding for β-galatocidase in an otherwise Lac⁻ genetic background (cf. Touati 1988b). The results indicate that *sodA* expression can be greatly affected by non-redox, non-metal-binding aromatic molecules and that these effects depend on Fe by both Fur-dependent and Fur-independent mechanisms.

Materials and methods

Bacterial strains and sources of materials. Bacterial strains used in this work were described previously (Niederhoffer et al. 1990) and are the isogenic pair: ENF19 [BN402 but *Φ(sodA'-'lacZ)* Cam^r] and ENF13 [BN402 but *fur*::Tn5 Kan^r *Φ(sodA'-'lacZ)* Cam^r]. The following companies served as sources of the listed reagents: Aldrich Chemical Co. Inc. (Milwaukee, WI), diethylenetriaminepentaacetic acid (DTPA) and 4,7-phenanthroline (4,7-phen); Bethesda Research Laboratories Inc. (Gaithersburg, MD), 5-bromo-4-chloro-3-indolyl-β-D-galactoside; Difco Laboratories (Detroit, MI), Bactotryptone and yeast extract; Eastman Chemical Products Inc. (Kingsport, TN, a divison of Eastman Kodak Co.), rhodamine 6G; E. I. du Pont de Nemours & Co. Inc. (Wilmington, DE), rhodamine B1 base; J. T. Baker Chemical Co. (Phillipsburg, NJ), 2,2'-bipyridine (bpy) and 1,10-phenanthroline (phen); Sigma Chemical Co. (St. Louis, MO), cytochrome *c*, ethidium bromide, hemin, and methyl viologen. All other chemicals were of the highest commercial quality.

Spectrophotometric determinations. The purity of 4,7-phen was determined by measuring the concentration of contaminating $Fe(phen)_3^{2+}$, which has a molar absorption coefficient at 510 nm of 11100 M^{-1} cm^{-1}. A 10 mM potassium phosphate pH 7 solution containing 100 μM, 4,7-phen in a total volume of 1 mL was brought to 50 μM $FeSO_4$ from a concentrated stock. The absorption spectrum was recorded before and after the Fe addition to determine the contribution to the spectrum of $Fe(phen)_3^{2+}$. No contamination was detected in our stocks of 4,7-phen. In a separate study, the possibility of Fe binding 4,7-phen was explored by preparing a 1 mL solution of 10 mM potassium phosphate pH 7 containing 12.5 mM 4,7-phen and 12.5 mM $FeSO_4$. The absorption spectrum was recorded before and after Fe addition. The spectra contained no evidence for the formation of an Fe complex with either of these ligands.

Cell growth. For aerobic induction experiments, overnight cultures of L broth plus glucose (0.2%) were grown under appropriate antibiotic pressure (chloramphenicol, 15 μg/ml; kanamycin, 50 μg/ml; and/or tetracycline, 12.5 μg/ml) in a rotary shaker and used to inoculate prewarmed (37° C) L broth/glucose medium to $A_{600} \approx 0.05$. The cultures were grown under normal aeration for 30 min before being divided into separate flasks containing prewarmed (37° C) L broth/glucose supplemented to either 100 μM DTPA, 500 μM bpy, 100 μM EB, 100 μM hemin, 100 μM phen, 100 μM 4,7-phen, 20 μM Paraquat, 100 μM rhodamine, or 100 μM B1 rhodamine 6G. Aliquots were removed at the indicated times, immediately cooled with ice water, and kept on ice until assayed for β-galactosidase activity. For anaerobic induction studies, aerobically grown overnight cultures were used to inoculate prewarmed (37° C) medium containing the above supplements, and the culture tubes were sealed. When the anaerobic cultures reached late exponential phase (as followed with a Klett-Summerson colorimeter), they were cooled with ice water and assayed for enzyme activity.

Assays. Cells extracts were prepared as described previously and assayed for total protein content by the bicinchoninic acid method using bovine serum albumin as the calibration standard. Permeabilized cells were used to assay for β-galactosidase. Duplicate or triplicate determinations were performed with an average deviation of 10%.

Results

We surveyed a number of aromatic compounds for their possible effect on *sodA* expression. Aerobically grown cells were collected at $A_{600} \approx 0.3$–0.7 and β-galactosidase activity measured. The results are shown in Table 1. All showed significant effects on *sodA* expression in Fur$^+$ cells while in Fur$^-$ cells these were generally decreased, if not absent. The final concentrations of chelators used in these experiments exceeds the ambient concentration of Fe in L broth which ranges over about 10–20 μM; adding additional Fe, up to 50 μM, was without effect. A few of these compounds were chosen for more detailed study.

Aerobic conditions

We previously showed that growth of cells in 100 μM DTPA caused an approximately threefold induction of *sodA* in Fur$^+$ cells, to roughly the same level observed with Fur$^-$ cells, while causing no increased expression of *sodA* in Fur$^-$ cells. A time-dependent expression of *sodA* was noted previously (Niederhoffer et al. 1990), and these results are taken to represent the effect of Fe depletion from the culture medium and to indicate that the increase in *sodA* expression is mediated by the Fur system. Preliminary results also indicated an influence of Fe availability on Paraquat-mediated induction of *sodA* (Niederhoffer et al. 1990); we explore that further here. The results illustrated in Fig. 1 show the classical effect (Hassan and Fridovich 1977) of Paraquat on expression of *ϕ(sodA'-'lacZ)* in Fur$^+$ and Fur$^-$ strains

Table 1. Effect of various aromatic organic compounds on the specific β-galactosidase activity from a chromosomal fusion *ϕ(sodA'-'lacZ)* in Fur$^+$ and Fur$^-$ strains

Compound	β-Galactosidase activity (U/A_{600})			
	Fur$^+$	+C/−C	Fur$^-$	+C/−C
—	340	1	1200	1
DTPA	1000	2.9	900	0.8
EB	910	2.7	1800	1.5
R6G	810	2.4	1800	1.5
RB	900	2.6	1500	1.0
Heme	670	1.9	970	0.8
4,7-phen	910	2.7	1200	1.0
phen	630	1.8	650	0.5
PQ	1500	4.4	2900	2.4

Cells were grown in the presence of 100 μM of each compound (EB = ethidium bromide, R6G = rhodamine 6G, RB = rhodamine B, PQ = Paraquat) and harvested during logarithmic growth, $A_{600} \approx 0.3$–0.7. +C/−C indicates ratio of results with/without the designated compound. Results similar to those obtained with 4,7-phen were also obtained with 1,7-phen

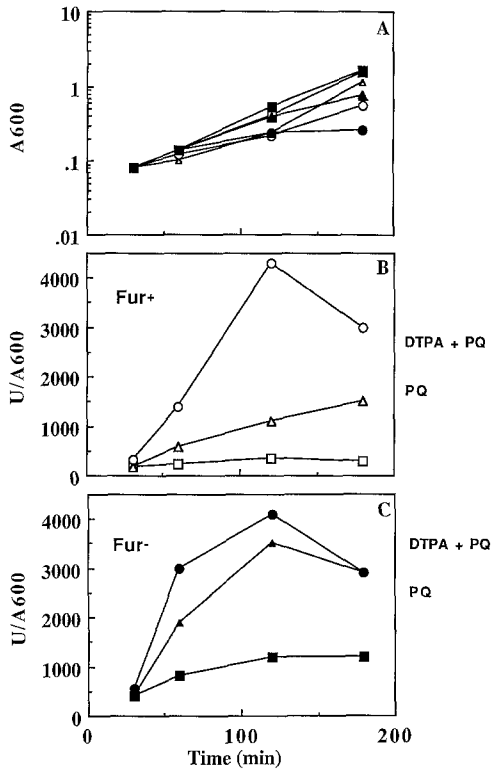

Fig. 1. Effect of Fe, Paraquat (PQ), and Paraquat plus DTPA on the growth (**A**) and the β-galactosidase activity from chromosomal fusion ϕ(*sodA'-'lacZ*) in Fur$^+$ (**B**) and Fur$^-$ (**C**) strains. Fur$^+$ (□, △, ○) and Fur$^-$ (■, ▲, ●) cells were grown in L broth plus glucose (□, ■), 20 μM PQ (△, ▲), or 20 μM PQ and 100 μM DTPA (○, ●). Growth is expressed as absorbance at 600 nm (A_{600}) and β-galactosidase activity as units (U)

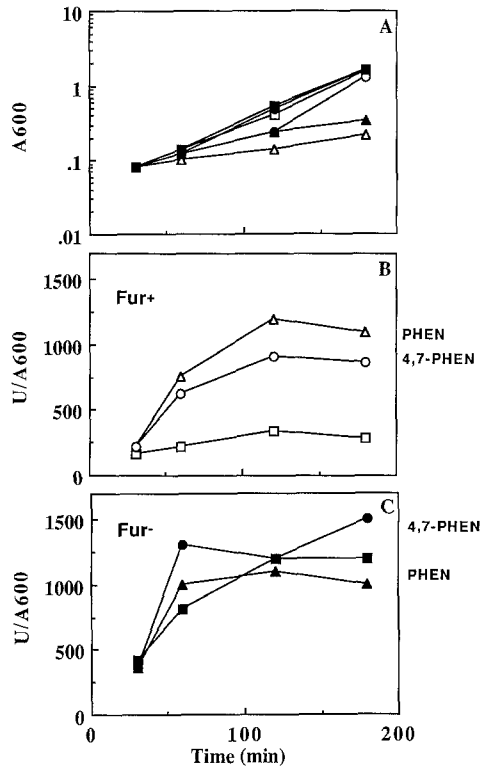

Fig. 2. Effect of Fe, ethidium bromide (EB), and ethidium bromide plus DTPA on the growth (**A**) and the β-galactosidase activity from chromosomal fusion ϕ(*sodA'-'lacZ*) in Fur$^+$ (**B**) and Fur$^-$ (**C**) strains. Fur$^+$ (□, △, ○) and Fur$^-$ (■, ▲, ●) were grown in L broth plus glucose (□, ■), 100 μM EB (△, ▲), or 100 μM EB plus 100 μM DTPA (○, ●)

and how that is influenced by iron depletion. In Fig. 1A it is shown that 20 μM Paraquat has little affect on growth of Fur$^+$ or Fur$^-$ cells in the presence of ambient Fe; however, it greatly reduces growth in the presence of DTPA, which by itself has little effect on growth rate (Niederhoffer et al. 1990). As can be seen from Fig. 1B, Paraquat induces *sodA* ≈6-fold in the presence of ambient Fe and chelation of residual Fe with DTPA permits ≈14-fold induction. A similar pattern is seen with Fur$^-$ cells with the obvious differences being that *sodA* is expressed about 4-fold more in Fur$^-$ than in Fur$^+$ cells and that ambient Fe shows less interference in the Paraquat-dependent induction of *sodA* in Fur$^-$ cells. The maximum level of *sodA* expression appears to be independent of *fur*.

Ethidium bromide is a positively charged aromatic compound known to be taken up by cells and bind to DNA. Data in Fig. 2 show the effect of 100 μM ethidium bromide on expression of *sodA*. As shown in Fig. 2A, ethidium bromide had no effect on the growth of either Fur$^+$ or Fur$^-$ cells except when DTPA was present. In the presence of Fe, ethidium bromide has only a modest induction effect on *sodA* expression (≈3-fold in Fur$^+$ and ≈1.5-fold in Fur$^-$) while in the presence of DTPA, it increases *sodA* expression ≈8-fold in Fur$^+$ and ≈2.5-fold in Fur$^-$ cells. The qualitative pattern is very similar to that seen with Paraquat although the maximum specific activities obtained with the latter are ≈2-fold greater than with ethidium bromide.

o-Phenanthroline has been widely used to depauperate cultures of Fe (cf. Bagg and Neilands 1987). We noted that bpy and phen strongly affected growth of Fur$^+$ and Fur$^-$ cells. Indeed, in minimal medium bpy and phen will prevent growth of these strains (unpublished observations). The two compounds have similar effects, but they occur at 500 μM for bpy and only 100 μM for phen; we present only the results with phen. In Fig. 3 the effects of these compounds on cell growth are shown; Fig. 3B, C show that phen also increases *sodA* expression, as originally found by others (Moody and Hassan 1984), ≈5-fold in Fur$^+$ cells with no change in Fur$^-$ cells. It is noteworthy that 4,7-phen, a non-chelating analog of phen, increases *sodA* expression ≈3-fold in Fur$^+$ cells but has little or no effect in Fur$^-$ cells. A mixture of 4,7-phen and DTPA had no addition effect on *sodA* expression in aerobic Fur$^-$ cells over 4,7-phen alone (data not shown). It appears that enhancement of *sodA* expression by DTPA in Fur$^-$ cells grown under aerobic conditions occurs only with the positively charged compounds: Paraquat (Fig. 1), ethidium bromide (Fig. 2), and rhodamine 6G (not shown).

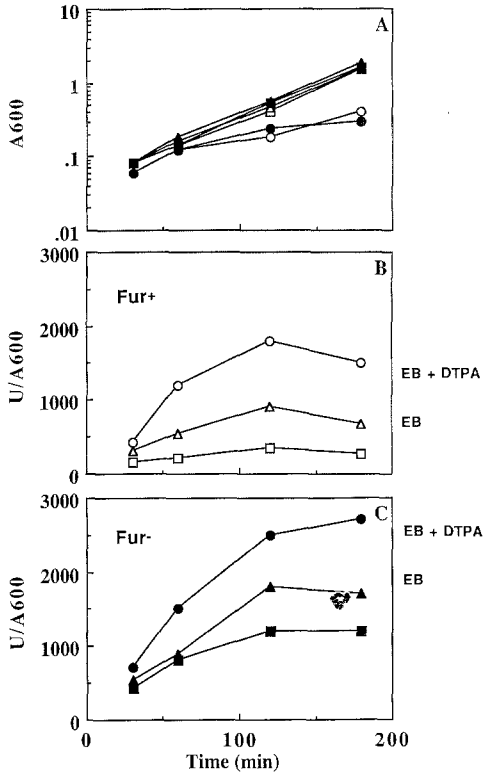

Fig. 3. Effect of Fe, 1,10-phenanthroline (phen) and 4,7-phenanthroline (4,7-phen) on the growth (**A**) and the β-galactosidase activity from chromosomal fusion ϕ(*sodA'-'lacZ*) in Fur$^+$ (**B**) and Fur$^-$ (**C**) strains. Fur$^+$ (□, △, ○) and Fur$^-$ (■, ▲, ●) were grown in L broth plus glucose (□, ■), 100 μM phen (△, ▲), or 100 μM 4,7-phen (○, ●)

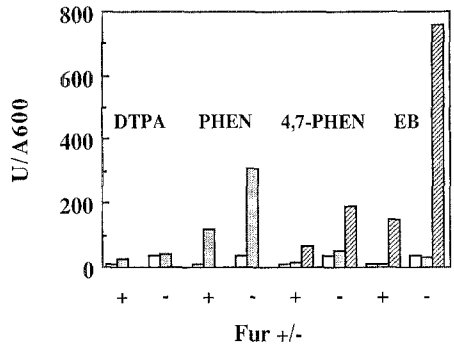

Fig. 4. Effect of aromatic compounds and metal chelators on anaerobic expression of β-galactosidase activity from the chromosomal fusion ϕ(*sodA'-'lacZ*) carried in Fur$^+$ (+) and Fur$^-$ (−) strains. Cells were grown in L broth plus glucose (open boxes); 100 μM DTPA, 100 μM phen, 100 μM 4,7-phen, or 100 μM ethidium bromide EB (dotted boxes); or 100 μM DTPA plus 4,7-phen or EB (hatched boxes)

Anaerobic conditions

Under anaerobic, fermentative growth *sodA* is expressed at very low levels (Gregory and Fridovich 1973). Moody and Hassan (1984) found that chelating agents added to cells grown under such conditions caused induction of MnSOD activity. We extend their observations here to include the effects of the non-chelating 4,7-phen analog and ethidium bromide. Under anaerobic conditions these chemicals have virtually no effect on cell growth (not shown). Cells were harvested at $A_{600} \approx 0.5$–1.0; their specific β-galactosidase activities are presented as histograms in Fig. 4. The effect of added DTPA is shown in the leftmost clusters of Fig. 4. The enhancement of *sodA* expression in Fur$^+$ cells is ≈3-fold, increasing to that seen with iron-sufficient Fur$^-$ cells. As reported earlier (Niederhoffer et al. 1990), DTPA has no effect on *sodA* expression in Fur$^-$ cells. Because DTPA is a very strong metal chelator and, at this concentration, is not seen as otherwise interfering in cell behavior, we view these data as representing the effect of iron deficiency on *sodA* expression in anaerobiosis. Second from the left in Fig. 4 are data indicating the effect of added phen on the response of *sodA* in Fur$^+$ and Fur$^-$ cells. The open bars, in accord with previous observations, show that *sodA* is only weakly expressed in Fur$^+$ cells (≈10 U/A_{600}) and is derepressed somewhat in Fur$^-$ cells (≈38 U/A_{600}). Addition of phen to the medium causes ≈12-fold increase of *sodA* expression in Fur$^+$ cells and ≈8-fold in Fur$^-$ cells. While these ratios are large, *sodA* is still substantially repressed compared to the maximum possible value (≈4000 U/A_{600}). Nevertheless, phen definitely interferes with the normal repression mechanism operative under anaerobic conditions.

The central histograms of Fig. 4 show the effects of the non-metal binding analog, 4,7-phen and a mixture of this and DTPA. Again, the open boxes show the effect of *fur* mutation on *sodA* expression, that 4,7-phen effects a modest increase in *sodA* expression (≈1.7-fold in Fur$^+$ and ≈1.4-fold in Fur$^-$cells). Noting above that a DTPA/4,7-phen mixture had rather little additional effect on *sodA* expression in Fur$^-$ cells grown aerobically, such a mixture produced a large increase in *sodA* expression (compared to the unperturbed anaerobic condition): ≈7-fold in Fur$^+$ and ≈5-fold in Fur$^-$ cells. These values are comparable for those observed with phen alone, suggesting that a combination of the aromatic and iron-binding properties of is responsible for its inductive effect, that a non-chelating aromatic compound can itself disrupt cellular measures to repress *sodA* in anaerobiosis, and that this effect is greatly enhanced by iron depletion.

The rightmost cluster of histograms in Fig. 4 shows the effects of ethidium bromide on anaerobic *sodA* expression. By itself, it has no effect under these conditions, but a mixture of ethidium bromide and DTPA has a dramatic effect. In Fur$^+$ cells, we saw a ≈15-fold increase and a ≈20-fold increase in Fur$^-$ cells. These ratios correspond to ≈5% and ≈20% of full *sodA* expression (using 4000 U/A_{600} as the maximum).

Discussion

There are several features of the data that deserve comment. Organic compounds having significant aromatic character, that do not undergo redox chemistry at cellu-

lar potentials, and have no obvious capacity for metal binding can substantially enhance expression of *sodA*. The presence of intact Fur protein influences this apparent induction. During aerobic growth, the absence of Fur appears to suppress the ability of the various compounds to enhance *sodA* expression. This effect extends to all compounds tested and is most obvious in the data of Table 1. Iron depletion has a modest effect on the enhancement of *sodA* expression in Fur^- cells by charged aromatics but offers no enhancement with uncharged aromatics. By comparison, during anaerobic growth, an intact Fur protein seems to interfere with the ability of aromatic compounds to induce *sodA* expression while Fe depletion by DTPA greatly stimulates expression of *sodA*; the latter is most obvious with 4,7-phen and ethidium bromide, as shown in Fig. 4.

Assuming that the observed changes in *sodA* expression are not due to pleitropic disturbances, it is reasonable to offer some general, hopefully heuristic, molecular interpretation of the data. We suggest that simple chelators, such as DTPA, which do not penetrate the cell (cf. Nikaido and Vaara 1985), exert their effect on *sodA* primarily through the Fur system; this occurs in both aerobic and in anaerobic growth and with DTPA corresponds to $\approx$3–4-fold effect. Aromatic chelators like phen and bpy will also scavenge Fe (and other metals) with a basal response through Fur, and their additional effects might involve direct interaction with other regulatory protein(s); for example, binding to a repressor protein, such as ArcA, or an activator protein, such as SoxR, could account for the observations. The Fe dependence of the effects of these aromatic substances suggests that holo Fur may be interacting with the other regulatory proteins. That would be consistent with the observations of Tardat and Touati (1990) who found an apparent synergism between the *fur* and *arc* genes in the regulation of *sodA* expression. In the case of ethidium bromide, intercalation into the DNA with a direct influence on its supercoiling cannot be dismissed; however, this seems unlikely with 4,7-phen and other neutral aromatics. [We note recent evidence that ethidium bromide interacts with the *E. coli* RecA protein, thereby facilitating its binding to DNA (Thresher and Griffith 1990)].

In capsule, our data support the notion that aromatic compounds may, in cooperation with iron, interact with one or more regulatory proteins of *sodA*, and other genes regulated by these proteins can be expected to behave similarly. Such non-redox processes should be considered when studying the effects on cell behavior of redox active aromatics, such as Paraquat.

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