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Lethal Oxidative Damage and Mutagenesis Are Generated by Iron in Δ*fur* Mutants of *Escherichia coli*: Protective Role of Superoxide Dismutase

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The *Escherichia coli* Fur protein, with its iron(II) cofactor, represses iron assimilation and manganese superoxide dismutase (MnSOD) genes, thus coupling iron metabolism to protection against oxygen toxicity. Iron assimilation is triggered by iron starvation in wild-type cells and is constitutive in *fur* mutants. We show that iron metabolism deregulation in *fur* mutants produces an iron overload, leading to oxidative stress and DNA damage including lethal and mutagenic lesions. *fur recA* mutants were not viable under aerobic conditions and died after a shift from anaerobiosis to aerobiosis. Reduction of the intracellular iron concentration by an iron chelator (ferrozine), by inhibition of ferric iron transport (*tonB* mutants), or by overexpression of the iron storage ferritin H-like (FTN) protein eliminated oxygen sensitivity. Hydroxyl radical scavengers dimethyl sulfoxide and thiourea also provided protection. Functional recombinational repair was necessary for protection, but SOS induction was not involved. Oxygen-dependent spontaneous mutagenesis was significantly increased in *fur* mutants. Similarly, SOD deficiency rendered *sodA sodB recA* mutants nonviable under aerobic conditions. Lethality was suppressed by *tonB* mutations but not by iron chelation or overexpression of FTN. Thus, superoxide-mediated iron reduction was responsible for oxygen sensitivity. Furthermore, overexpression of SOD partially protected *fur recA* mutants. We propose that a transient iron overload, which could potentially generate oxidative stress, occurs in wild-type cells on return to normal growth conditions following iron starvation, with the coupling between iron and MnSOD regulation helping the cells cope.

The appearance of oxygen in the earth's atmosphere posed several problems to life-forms: (i) molecular oxygen generates toxic by-products of cell metabolism (21, 33), (ii) oxidation of ferrous iron to insoluble ferric compounds at physiological pH restricts the availability of iron (41), and (iii) free iron potentiates oxygen toxicity by reacting with reduced forms of oxygen, promoting the production of highly deleterious free radicals (24, 26; see also Fig. 8). To live in the presence of oxygen, cells have to (i) detoxify damaging oxygen species (7), (ii) develop strategies to obtain iron from the environment (9, 10), and (iii) tightly regulate the process of iron assimilation to satisfy requirements yet prevent toxic accumulation (5, 11, 37, 46, 57).

Escherichia coli has several pathways for importing iron which are used according to the oxidation state of iron. Iron (III) is transported by iron(III)-chelating compounds called siderophores, whereas iron(II) is directly imported. There are at least five different siderophore-iron(III) transport systems in *E. coli* which deliver iron(III) into the cell by a common process (10). The siderophores carrying iron(III) are taken up by specific high-affinity outer membrane receptors. A common complex, TonB-ExbB-ExbD, provides the energy to cross the outer membrane. The transport is achieved with the aid of specific proteins. The iron(II) uptake system is less well characterized (35). It includes a cytoplasmic membrane protein, FeoB, and possibly a small 75-amino-acid protein, FeoA, of unknown function. The fate of iron released in the cell remains obscure: when and how ferric iron is reduced, whether iron can be directly incorporated into proteins, or whether it is first directed to storage molecules is still unknown. They are at least two iron storage proteins in *E. coli*, bacterioferritin and a ferritin H-like protein, but their function in vivo is unclear (1, 34). The genes for proteins of both iron(III) and iron(II) transport pathways are negatively regulated by the Fur protein (5, 27). When the internal iron(II) concentration reaches a critical level, it associates with Fur, which then binds to a particular sequence (the "iron box") in the promoter of iron-regulated genes, thereby inhibiting transcription (4). A drop in the intracellular iron concentration due to environmental iron limitation leads to Fur inactivation and induction of all genes of the iron transport systems. Fur regulates almost all genes directly involved in iron acquisition and also some toxin genes, including those coding for CFA/I fimbriae (36) or α-hemolysin, which indirectly contribute to the bacterial iron supply by releasing iron from eukaryotic cells (23, 39). An increasing number of genes are being shown to be also Fur regulated, although they have no apparent involvement in the iron supply. They include several regulatory genes involved in metabolic processes; thus, Fur regulation appears to extend to general metabolism, coupling it to the iron supply (54). The genes for superoxide dismutase (SOD), a key enzyme in the defense against oxygen toxicity, are also Fur regulated (47, 55). SOD eliminates superoxide, thereby protecting cells from the damage directly caused by superoxide and inhibiting the superoxide-mediated reduction of ferric iron, which contributes to iron toxicity (20, 25). The *sodA* gene, coding for manganese SOD, is negatively regulated by Fur and five other global transcrip-

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tional regulators in response to environmental variations (15, 47, 55, 56). With sufficient iron in the environment, Fur can completely block *sodA* transcription under anaerobic conditions, although Fur reduces *sodA* transcription only about threefold under aerobic conditions. In contrast, Fur appears to regulate iron SOD positively in a metal-independent manner; the mechanism of this effect is unclear (47).

Several reasons have been suggested to explain the regulation of MnSOD expression in response to iron starvation. Iron starvation could be interpreted as a signal of a more oxidative environment, leading to iron oxidation and consequent iron unavailability. Induction of MnSOD could compensate for FeSOD inactivation due to lack of iron. Gardner and Fridovich (22) showed that aconitase is reversibly inactivated by superoxide and reactivated by iron(II); they suggested that the induction of MnSOD in response to a decrease in the intracellular iron concentration maintains the active aconitase steady-state level by reducing its inactivation by superoxide when iron(II) reactivation is slowed down. We proposed that derepression of iron assimilation in response to intracellular iron depletion may trigger on return to high iron concentrations, an influx of iron into the cell which produces a transient iron overload causing an oxidative burst compensated for by the coordinate MnSOD induction (60).

We report that the permanent derepression of iron assimilation systems in a Δ*fur* mutant produces an oxidative stress leading to various cell damage. Recombinational DNA repair protects against the lethal effect of iron-induced damage but not from lesions leading to mutagenesis. Iron chelation, intracellular iron sequestration, and blockage of an iron transport pathway relieve toxicity, consistent with damage being due to excess iron. The contribution of SOD in reducing iron toxicity is shown. Coordination between iron metabolism and MnSOD expression is discussed in terms of protection against the effects of iron overload.

MATERIALS AND METHODS

Bacterial strains, phages, and plasmids. All strains are *E. coli* K-12 derivatives and are listed in Table 1 or described below. Standard procedures were used for genetic manipulations (45). Mutations in the *fur*, *feo*, *sodB*, *recB*, *recF*, and *sodX* genes were introduced by P1 transduction, selecting for an antibiotic resistance 100% associated with the mutation: resistance to kanamycin for Δ*fur*::*kan*, Δ(*sodB′-′kan*)*2*, and *sodX*::mini-*kan*; resistance to chloramphenicol for *feo*::*cat*, resistance to ampicillin for *recF*::Tn*3*, and tetracycline resistance for *recB268*::Tn*10*. *recA* strains were generated by cotransduction of the *recA* allele with *srl*::Tn*10*, using a P1 lysate grown on GY7096 (Δ*recA*) or on GY5902 (*recA430*) selected for tetracycline resistance. Transduction was confirmed by UV sensitivity. *lexA1* was cotransduced with *malB*::Tn*9*, and chloramphenicol-resistant transductants were screened for UV sensitivity. To construct *sodA sodB* mutants, mutations in the *sodA* gene were introduced into *sodB* strains by transduction, either by direct selection (chloramphenicol resistance) or by cotransduction with *zdh*::mini-*kan*-Ω, selecting for spectinomycin resistance. Transductants were screened for their inability to grow on minimal medium (13).

Specific strain constructions. (i) Δ*fur* deletion. An approximately 1,800-bp *Hin*dIII-*Bam*HI fragment containing the *fur*$^+$ gene derived from pF3 was cloned into the M13mp18 polylinker. A 173-bp deletion within the structural gene, starting 8 bp from the ATG start codon, was generated by site-directed mutagenesis, creating a *Pst*I restriction site and introducing a stop codon just downstream of the deletion. The deletion was verified by DNA sequencing (52), and the absence of Fur protein synthesis was verified by expression in maxicells. The *Hin*dIII-*Bam*HI fragment carrying the deletion was cloned between the corresponding sites of pBR322, giving pBT2-1, and the kanamycin resistance gene block (Pharmacia) was inserted as a *Pst*I fragment into the *Pst*I site created by the deletion (pBT2-2). The Δ*fur*::*kan* allele was then transferred to the chromosome by transformation of a *recD* strain (49) with plasmid DNA linearized by *Pvu*II. Loss of the *fur*$^+$ allele in Kan^r Ap^s recombinants was verified by their ability to confer a Fur$^-$ phenotype on a Δ*arcA sodA-lacZ* strain by transduction or on a *fhuF*::λ*plac* Mu strain by cotransduction with *zbf*::Tn*10* (60% cotransducible with *fur*$^+$).

(ii) Cloning of the *sodX* (*feo*) gene. The *sodX* mutant was previously identified as exhibiting a Fur$^-$-like phenotype and mapped in the *ompB* region (55). To localize the gene more precisely, mini-*kan* transposons were randomly inserted into the chromosome of a *sodX* strain, and several insertions linked to *sodX* were selected. *sodX* was thus mapped between *ompR* and *malQ*, a region covered by the lambda phages 2F6 and E3C10 from the library constructed by Kohara et al. (38). *sodX* was further mapped to the *Eco*RI-*Bam*HI DNA fragment from lambda 2F6 (Fig. 1) by subcloning partially deleted DNA fragments and complementation assays of the SodX$^-$ phenotype (derepression of *fhuF*::λ*plac* Mu and of Δ*arcA sodA-lacZ*). The *feo* gene (previously erroneously mapped at 38 min [28]) sequence was published during this work (35). Comparison of the restriction maps and phenotypes indicate that *sodX* and *feo* are the same gene, and this conclusion was confirmed by exchanges of mutants with K. Hantke. *sodX* was therefore renamed *feo.*

(iii) Δ*feo* deletion. The *cat* gene was obtained as a 2-kb *Hin*dIII-*Bam*HI fragment of pPR3 and was inserted into the *Eco*RV site of pSD1-2 after treatment with Klenow enzyme to generate blunt ends (pLB1). The Δ*feoB*::*cat* allele was then transferred to the chromosome by transformation of a *recD* strain with plasmid DNA linearized with *Bam*HI. Loss of the *feo*$^+$ allele in Cm^r Ap^s transductants was verified by the ability to confer a Fur$^-$-like phenotype to an *arcA sodA-lacZ* or *fhuF*:λ*plac*Mu strain by transduction. Transduction of the Δ*feo*::*cat* allele into strain H5124 (*feoB*::Tn*5*) resulted in 100% loss of kanamycin resistance.

Media, growth conditions, and general methods. Cells in liquid culture were grown in LB broth (45) or, as indicated, in M63 (45) containing 0.2% glucose, at 37°C with shaking at 200 rpm. Medium was supplemented with tetracycline (10 or 6 μg/ml for mutagenesis experiments), chloramphenicol (20 μg/ml), kanamycin (40 μg/ml), spectinomycin (35 μg/ml), rifampin (100 μg/ml), and/or ampicillin (50 μg/ml in solid medium, 500 μg/ml in liquid medium, 20 μg/ml for strains carrying mini-F and 25 μg/ml for *recF*::Tn*3*) as needed; ferrozine was added to 5×10^{-4} M. Anaerobic cultures were grown in a Forma Scientific anaerobic chamber in LB medium containing 1% glucose. Media and materials were equilibrated in the anaerobic chamber before use.

β-Galactosidase assays were performed by method of Miller (45). Growth was monitored by measuring optical density at 600 nm.

Mini-F curing (61) was done as follows: a colony was used to inoculate LB at 37°C without shaking; the culture, grown overnight, was diluted to a final concentration of approximately 100 cells per ml and grown with shaking. After 2 or 3 h, novobiocine was added to 50 μg/ml to samples, which were further incubated at 37°C for 18 to 24 h and plated on LB. Colonies were screened by replica plating for loss of ampicillin resistance. Curing efficiency was usually between 85 and 95%.

H_2O_2 challenge. Cells grown in LB medium to an optical density of 0.5 were distributed into 50-ml Erlenmeyer flasks (5 ml each), and H_2O_2 was added to various concentrations from 0 to 35 mM. After 20 min of incubation with shaking at 37°C, treatment was stopped by addition of catalase (Boehringer-Mannheim) at 400 U/ml and chilling, followed by plating on LB plates. Samples were diluted in cold 10^{-2} M $MgSO_4$ containing 400 U of catalase per ml. Colonies were counted after 18 h of incubation at 37°C.

Mutation frequency measurements. Three forward mutation tests were used.

(i) Tet^s to Tet^r. Plasmid pPY98 is a derivative of pBR322 in which the *tet* gene is under the control of the Mnt-regulated *ant* promoter of P22 (43) such that it confers a Ap^r Tet^s phenotype. Mutations in the *mnt* gene or in its operator confer tetracycline resistance (8). Fresh cultures grown overnight in LB medium containing 25 μg of ampicillin per ml were plated on LB plates containing 6 μg of tetracycline plus 25 μg ampicillin per ml. Tet^r colonies were scored after 24 h of incubation at 37°C.

(ii) Val^s to Val^r. Valine-resistant mutants were scored by plating cultures grown overnight (one colony inoculated into 10 ml of LB medium), washed twice in cold buffer, on M63 plates containing 0.2% glucose and 40 μg of valine per ml. Val^r colonies were scored after 48 h of incubation at 37°C.

(iii) Rif^s to Rif^r. Rif^r mutants were scored by plating a culture grown overnight on LB plates containing 100 μg of rifampin per ml. Rif^r colonies were scored after 24 h of incubation at 37°C.

For measurement under anaerobic conditions, 10-ml cultures were inoculated with 10 to 100 bacteria grown under anaerobic conditions to saturation and plated on selective medium under anaerobic conditions. Glucose (1%) was added to all media under anaerobic conditions. Control cultures with similar inocula were grown and plated under aerobic conditions.

For measurements with ferrozine, cultures were inoculated as above (10 to 100 bacteria in 10 ml), distributed into Erlenmeyer flasks with or without ferrozine, and grown to saturation under aerobic conditions.

Two dilutions (10^7 to 10^9 bacteria) were spread, each on five plates, in each experiment to score resistant mutants. Culture concentrations were measured by plating on LB or M63 plus glucose; colonies were counted after overnight or 24-h incubation at 37°C. Titrations were done in duplicate.

For each of the three tests, it was verified that the resistant mutants had no growth advantage over the sensitive strain by measuring the ratio of sensitive to resistant cells in mixed cultures.

Site-directed mutagenesis and general molecular biology methods. (i) Mutagenesis. The *fur*$^+$ region carried in M13mp18 was mutagenized with the oligonucleotide-directed in vitro mutagenesis kit RPN1523, version 2 (Amersham, Buckinghamshire, United Kingdom). Sequence of candidate mutant was verified

TABLE 1. Bacterial strains, phages, and plasmids

Bacterium, phage, or plasmid	Genotype or relevant characteristic(s)	Source or reference
E. coli strains[a]		
GC4468	F$^-$ Δ(*argF-lac*)*U169 rpsL*	R. D'Ari
K-12	Prototroph	C. Di Russo
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 deoC1 ptsF25 flbB5301*	G. Weinstock
AB2847	*aroB malT tsx*	K. Hantke
H2300	Same as AB2847 but with Δ(*tonB-trp*)	K. Hantke
BR158	Same as AB2847 but with *tonB*	K. Hantke
BN4020	*fur*::Tn*5*	J. B. Neilands
QC1732	Same as GC4468 but with Δ*fur*::*kan*	This work
QC1464	Same as GC4468 but with *sodX*::mini-*kan*	This work
QC1949	Same as GC4468 but with Φ(*sodA-lacZ*)*1 arcA*::*tet*	15
H1717	Same as MC4100 but with *aroB fhuF*::λp*lac* Mu	K. Hantke (29)
KSL68	*metB thi pyrE rpsL lacMS286* ϕ80d11*lac*BK1 Δ*recA306 srl*::Tn*10*	A. Bailone
GY7096	Same as KSL68/pGY5353 (*bla*$^+$ *recA*$^+$), Ap^r (20 μg/ml)	A. Bailone (17)
QC1720	Same as GY7096 but with *fur*::Tn*5*	This work
QC1721	Same as GY7096 cured of pGY5353 but with Δ*recA306*	This work
QC1722	Same as QC1720 cured of pGY5353 but with *fur*::Tn*5* Δ*recA306*	This work
GC7365	Same as C600 but with *recD1009*	A. Jaffé (49)
QC2130	Same as C600 but with *recD1009* Δ*feoB*::*cat*	This work
H5124	Same as MC4100 but with *aroB feoB*::Tn*5*	K. Hantke (35)
GC2281	*lexA1*(Ind$^-$) *malB*::Tn*9*	R. D'Ari
N2101	*recB268*::Tn*10*	42
GY5902	*recA430 srl*::Tn*10*	A. Bailone (17)
JCD1420	*recF*::Tn*3*	14
AB1157	*thr-1 leu-6 proA2 thi-1 argE3 his-4 lacY1 galK2 ara-14 xyl-5 mtl-1 ton-33 str-31 supE44*	3
JC5519	Same as AB1157 but with *recB21 recC22*	42
JC7623	Same as AB1157 but with *recB21 recC22 sbcB15 sbcC201*	42
QC772	Same as GC4468 but with Φ(*sodA'-'lacZ*)*49* (Cm^r)	13
QC773	Same as GC4468 but with Φ(*sodB-kan*)Δ*2* (Kan^r)	13
QC789	Same as GC4468 but with *sodB*::MudPR3 (Cm^r)	13
QC1436	Same as GC4468 but with Φ(*sodA'-'lacZ*)*49 zij*::mini-*kan*-Ω2(80% cotransducible with *sodA*)	This work
QC1725	Same as GC4468 but with Δ*sodA3*	15
QC1726	Same as GC4468 but with Δ*sodA3 sodB*::MudPR3	This work
QC1736	Same as GC4468 but with Δ*sodA3 sodB*::MudPR3 *fur*::*kan*	This work
QC1799	Same as GC4468 but with Δ*sodA3* Φ(*sodB-kan*)Δ*2*	This work
Phages and plasmids		
λ 2F6	Lambda phage carrying DNA fragment bearing the *sodX* (*feo*) gene	38
λ E3C10	Lambda phage carrying DNA fragment bearing the *sodX* (*feo*) gene	38
pGY5353	Mini-F *bla*$^+$ *recA*$^+$ Ap^r	17
pPR3	Source of *cat* cassette	48
pSD1-1	pBR322 derivative carrying *feo* (*sodX*) region, Ap^r	This work
pSD1-2	pSD1-1 with the 1.5-kb *Eco*RV-*Eco*RV DNA fragment deleted, Ap^r	This work
pLB1	pSD1-2 with a *cat* cassette at *Eco*RV site, Ap^r Cm^r	This work
pF3	pBR322 derivative carrying a 2,700-bp fragment bearing the *fur*$^+$ region	5
pBT2-1	pBR322 with the 1,800-bp *Hin*dIII-*Bam*HI DNA fragment from pF3 with 173 bp deleted within the *fur* gene	This work
pBT2-2	pBT2-1 with a cassette conferring kanamycin resistance in *fur*	This work
pBFR.GEN	pBC (high-copy-number vector) carrying the *bfr* gene	54
pBFR.5	pBC derivative carrying the complete *bfr* region	54
pSU.FTN2	pCYC184 derivative carrying the *rsgA* gene	54
pDT1-19	pBR322 carrying the *sodA*$^+$ gene under *tac* promoter control and the *lacI* gene	58
pPY98	pBR322 derivative in which the *tetA* gene is under the *mnt*-regulated *ant* promoter	43

[a] Strains used for the construction of the set of isogenic strains in Table 2 and 3.

by nucleotide sequencing by using Sequenase, version 2.0 (U.S. Biochemical Corp.), and the method of Sanger et al. (51).

The oligonucleotide used to generate the deletion was a 30-mer with a 15-bp left arm and a 15-bp right arm, 5′ GATTCCGCATGA**CTGCAG**TTTGACGA CGCT3′, hybridizing on either side of the region to be deleted. The left-right junction creates a *Pst*I site (indicated in boldface characters).

(ii) General methods. DNA preparations and gel electrophoresis were carried out by using Qiagen kits and standard procedures (50). Restriction enzymes, T4 DNA ligase, and DNA polymerase Klenow fragment were purchased from Biolabs; [α-^{35}S]dATP for DNA sequencing was purchased from Amersham.

RESULTS

Oxygen-dependent lethality of a Δ*fur* Δ*recA* mutant. We were able to construct a Δ*fur* Δ*recA* mutant only under anaerobic conditions, not under aerobic conditions. A *fur*::Tn*5* Δ*recA*/F′ *bla recA*$^+$ strain was efficiently cured of the episome under anaerobic conditions (90% curing), while all survivors after curing treatment under aerobic conditions retained the episome (data not shown). A *fur*::Tn*5* Δ*recA* strain, con-

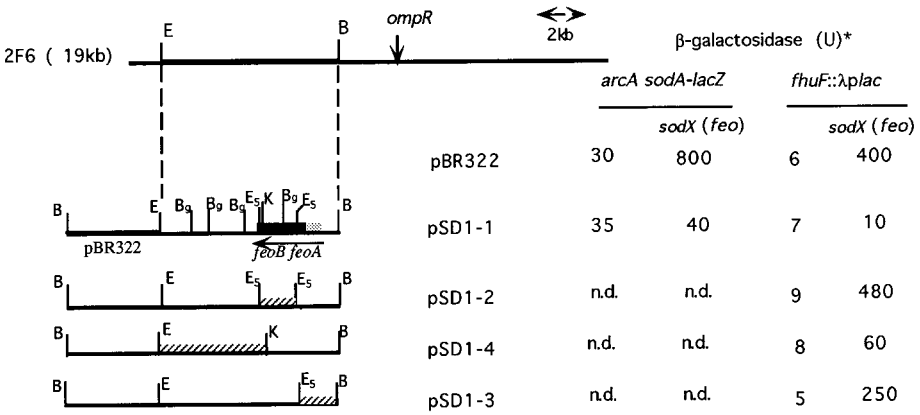

FIG. 1. Cloning of the *feo* (*sodX*) gene. Hatched lines indicate deleted DNA fragments. *Bam*HI (B), *Eco*RI (E), *Eco*RV (E_5), *Bgl*I (B_g), and *Kpn*I (K) sites are shown. *feoA feoB* localization is according to Kammler et al. (35). The results of complementation assays of *sodX* with various plasmids under anaerobic conditions are shown on the right. Cultures grown overnight anaerobically in medium containing 500 μg of ampicillin per ml were diluted 10-fold. Samples were taken after 1, 2, and 3 h for measurements of optical density and β-galactosidase activity. β-Galactosidase activity is expressed in Miller units (41). n.d., not determined.

structed anaerobically, died after the shift to aerobic conditions (Fig. 2). The growth of *fur*::Tn*5* single mutant was not affected by the shift, and Δ*recA* mutant showed only a growth lag, indicating that the lethality results from the combination of the *fur* and *recA* mutations.

Role of iron in oxygen sensitivity of the Δ*fur* Δ*recA* mutant. Physiologically, Fur is inactivated by iron starvation. A possible explanation for the oxygen-dependent lethality of the *fur recA* mutant is that the increased influx of iron in the absence of Fur repression leads, with sufficient iron in the environment, to an intracellular iron overload. In the presence of oxygen, this excess iron could generate oxidative stress and consequent DNA damage, lethal in the absence of RecA. We therefore investigated the effect of diminishing the intracellular iron pool by reduction of the exogenous iron concentration by iron chelators, blockage of iron transport pathways, or intracellular iron sequestration. To avoid possible side effects of other known or cryptic mutations, experiments were done in several different genetic backgrounds.

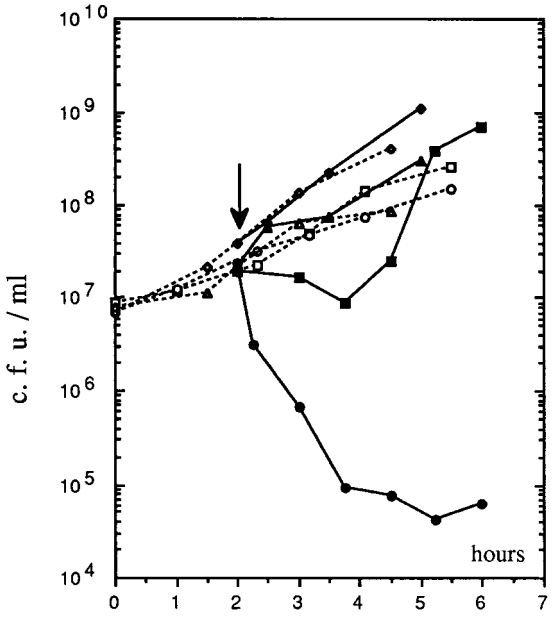

FIG. 2. Survival after a shift from anaerobiosis to aerobiosis. Cultures grown overnight in an anaerobic chamber were diluted 50-fold in fresh medium. After 2 h (arrow), samples were divided into two parts and one part was rapidly removed from the anaerobic chamber (5 ml in a 50-ml Erlenmeyer flask) and transferred to a rotary shaking water bath in air at 37°C. Samples were taken at intervals, and CFU under anaerobic conditions were determined. Values are the means of three experiments. The arrow indicates the time of shift to aerobiosis. Open symbols and dotted lines, anaerobiosis; solid symbols, aerobiosis. Symbols: ◇ and ◆, GY7096 (parent); △ and ▲, QC1720 (*fur*::Tn*5*); □ and ■, QC1721 (Δ*recA*); and ○ and ●, QC1722 (*fur*::Tn*5* Δ*recA*).

(i) Effect of iron chelators on aerobic survival of the Δ*fur* Δ*recA* mutant. Cells grown in the presence of the ferrous iron chelator ferrozine recovered normal growth shortly after a shift from anaerobiosis to aerobiosis (Fig. 3a). Interestingly, there was a lag in the growth of the *recA* strain after a shift from anaerobiosis to aerobiosis which disappeared when the cells were grown with ferrozine (not shown), suggesting that even in *fur*$^+$ strains, the shift to aerobiosis is accompanied by an iron-mediated oxidative stress and that RecA protects from that stress. The growth of *fur*::Tn*5* and wild type strains after a shift to aerobiosis was unchanged by the presence of ferrozine (not shown).

(ii) Effect of inhibition of ferric iron uptake on aerobic survival of the Δ*fur* Δ*recA* mutant. Ferric iron transport can be inhibited by suppressing the activity of the TonB protein, involved in the transport of iron(III) by siderophores. A *tonB* mutation totally suppressed the oxygen sensitivity of the Δ*fur* Δ*recA* strain; colonies appeared after overnight aerobic growth (Table 2), although a growth lag of about 1 h was observed in cultures after the shift from anaerobiosis to aerobiosis (not shown).

Under usual growth conditions, most ferric iron transport is mediated by enterobactin, an endogenously synthesized siderophore. A mutation in *aroB*, which inhibits enterobactin biosynthesis, greatly reduced the oxygen sensitivity, although it did not completely restore normal aerobic growth. There were few colonies (10^{-5} to 10^{-4}) after overnight aerobic incubation, but 1 to 20% of the cells grew, forming small colonies after 2 days. In the *aroB*$^+$ strain, survival remained at 3×10^{-5}. When survivors from several *aroB* cultures were inoculated under anaerobic conditions and plated again for aerobic survival after overnight aerobic growth, they grew better (20 to 75% large colonies after 48 h), suggesting the acquisition of suppressor mutations (data not shown).

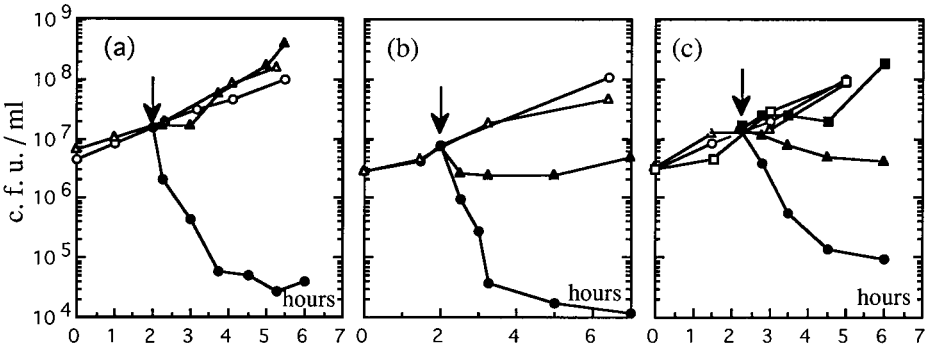

FIG. 3. Effect of iron chelator and hydroxyl radical scavengers on aerobic survival of the Δ*fur* Δ*recA* strain. A culture of QC1722 grown overnight was diluted in medium containing various additions and shifted to aerobiosis as described in the legend to Fig. 2. The arrow indicates the time of shift to aerobiosis. Open symbols, anaerobiosis; solid symbols, aerobiosis. Symbols: (a) ○ and ●, no addition; △ and ▲, ferrozine (5×10^{-4} M) added; (b) ○ and ●, no addition; △ and ▲, 5% dimethyl sulfate added; (c) ○ and ●, no addition; △ and ▲, 10 mM thiourea added; □ and ■, 100 mM thiourea added.

(iii) Effect of inhibition of ferrous iron transport on aerobic survival of the Δ*fur* Δ*recA* mutant. Mutations in *feoB* inhibit ferrous iron transport. The aerobic survival of a Δ*feo* Δ*fur* Δ*recA* strain was as low as that of Δ*fur* Δ*recA* mutant, indicating that direct import of ferrous iron is not a major factor in the oxygen sensitivity of the Δ*fur* Δ*recA* mutant (Table 2). The Δ*feo* Δ*fur* Δ*recA* strain was obtained by cotransduction of Δ*recA* with *srl*::Tn*10* into the Δ*feo* Δ*fur* strain under anaerobic conditions. *recA* mutants were obtained at the expected frequency among Tet^r transductants, and all (20 of 20) were oxygen sensitive. When transduction was done under aerobic conditions, a few fast-growing Tet^r *recA*$^+$ transductants appeared after overnight incubation; then, after 3 days, small Tet^r colonies appeared, with a RecA$^-$ phenotype. The ratio of *recA* to *recA*$^+$ among Tet^r transductants was similar to the *recA*/*recA*$^+$ ratio when the experiment was performed under anaerobic conditions (about 9/1). The *recA* colonies obtained under aerobic conditions and grown under anaerobic conditions exhibited nearly 100% aerobic survival (normal-size colonies after overnight growth, not shown), indicating that the strains had acquired suppressor mutation(s). This phenomenon explains a previous report of apparent suppression of Δ*fur* Δ*recA* oxygen sensitivity by the Δ*feo* mutation (59). The efficient selection of suppressor mutation(s) under aerobic conditions was not observed in a *feo*$^+$ background; attempts to introduce the *recA* allele by aerobic transduction of a Δ*fur* strain were unsuccessful, whereas plating the same transduction under anaerobic conditions gave the expected frequency of *recA* transductants. Furthermore, all survivors of a *fur* Δ*recA*/F′*recA*$^+$ strain retained the *recA*$^+$ episome after aerobic curing treatment. Thus, the Δ*feo* mutation presumably reduces the intracellular ferrous iron concentration sufficiently to allow the expression of suppressor mutations under aerobic selective pressure. The nature of the suppressor mutations has not yet been identified.

The Δ*feo* mutation also enhanced the protective effect of the *aroB* mutation: 44% large colonies appeared after aerobic overnight plating of Δ*feo* Δ*fur* Δ*recA* *aroB* mutants, whereas small heterogenous colonies appeared only after 2 days at a lower frequency in the *aroB* Δ*fur* Δ*recA* strain, against suggesting a lower intracellular toxic iron overload in the Δ*feo* strain (Table 2).

(iv) Effect of overexpression of iron storage proteins on aerobic survival of the Δ*fur* Δ*recA* mutant. Plasmids carrying bacterioferritin and ferritin H-like genes were introduced by anaerobic transformation into Δ*fur* Δ*recA* strains. Neither the plasmids carrying the *bfr* gene or the complete *bfr* region (including *gen-64*) reduced the oxygen sensitivity (Table 2). Conversely, the plasmid carrying the gene encoding a ferritin H-like protein (*rgsA* or *gen-165*) almost completely suppressed oxygen sensitivity in three different genetic backgrounds. Thus,

TABLE 2. Effect of iron transport blocks or iron sequestration on the oxygen sensitivity of the Δ*fur* Δ*recA* mutant[a]

Allele[b]			Plasmid	Survival[c]			
tonB[d]	*aroB*	Δ*feo*::*cat*		GC4468	K-12	MC4100	AB2847
+	+	+	None	6×10^{-5} ($\pm 4.5 \times 10^{-5}$)	8×10^{-6} ($\pm 3.0 \times 10^{-6}$)	3×10^{-6} ($\pm 2.0 \times 10^{-6}$)	2×10^{-5} ($\pm 2.5 \times 10^{-5}$)
+	+	+	pBFR.GEN	9×10^{-5} ($\pm 1.0 \times 10^{-5}$)	9×10^{-6} ($\pm 2.0 \times 10^{-6}$)	2×10^{-6} ($\pm 3.0 \times 10^{-6}$)	ND[e]
+	+	+	pBFR.5	9×10^{-5} ($\pm 1.5 \times 10^{-5}$)	2×10^{-5} ($\pm 1.0 \times 10^{-5}$)	2×10^{-6} ($\pm 2.0 \times 10^{-6}$)	ND
+	+	+	pSU.FTN2	0.62 ($\pm$ 0.06)	0.53 ($\pm$ 0.02)	0.14 ($\pm$ 0.06)	ND
+	+	−	None	7×10^{-5} ($\pm 3.0 \times 10^{-5}$)	1×10^{-5} ($\pm 0.5 \times 10^{-5}$)	4×10^{-6} ($\pm 1.0 \times 10^{-6}$)	3×10^{-5} ($\pm 2.0 \times 10^{-5}$)
+	−	+	None	ND	ND	ND	0.01 to 0.20[f]
+	−	−	None	ND	ND	ND	0.44 ($\pm$ 0.14)
−	−	+	None	ND	ND	ND	1.0 ($\pm$ 0.05)

[a] Anaerobic cultures were plated under anaerobic and aerobic conditions, and CFUs were counted.
[b] Strains are Δ*recA* Δ*fur* derivatives of GC4468, K-12, MC4100, and AB2387 with wild type (+) or mutated (−) alleles of *tonB*, *aroB*, and *feo*.
[c] Survival is the ratio of the number of colonies under aerobic conditions to those under anaerobic conditions. Colonies were counted after overnight incubation; there was no change in CFU after 48 h except as specified. Values are means of three to six experiments, and standard errors are in parentheses. Strain names of derivatives are, from top to bottom, as follows: GC4468 derivatives, QC2124, QC2334, QC2397, QC2311, and QC2392; K-12 derivatives, QC2194, QC2355, QC2296, QC2354, and QC2357; MC4100 derivatives, QC2367, QC2394, QC2395, QC2393, and QC2370; and AB2387 derivatives, QC2427, QC2424, QC2184, QC2420, and QC2183/QC2185.
[d] *tonB* or Δ(*tonB-try*).
[e] ND, not determined.
[f] Small colonies, counted after 48 h.

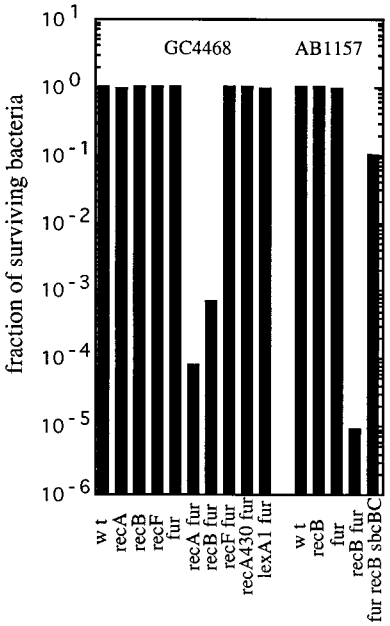

FIG. 4. Effect of mutations in recombination pathways and SOS induction on the aerobic survival of Δ*fur* mutants. Cultures grown overnight anaerobically were plated under anaerobic and aerobic conditions. CFU in aerobic to anaerobic conditions are reported (means of at least three experiments each in duplicate). Strains are derivatives of GC4468 or AB1157. Parental strains are indicated at the top of the figure. Additional mutations are indicated at the bottom of the figure. wt, no additional mutation.

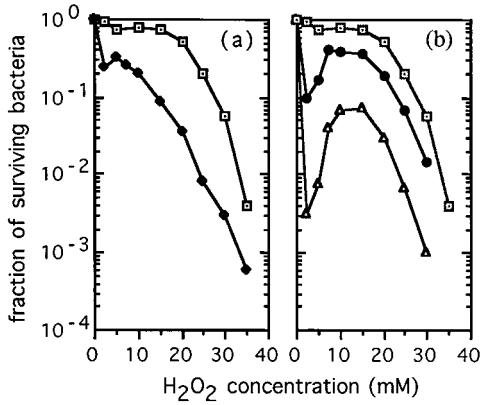

FIG. 5. Sensitivity of Δ*fur* and *sodA sodB* mutants to H_2O_2. Surviving fraction is the ratio of number of bacteria in the sample challenged with H_2O_2 to the untreated sample after 20 min of challenge. Symbols: (a) ⊡, GC4468 (wild type); ◆, QC1732 (Δ*fur*); (b) ⊡, GC4468 (wild type); ●, QC1726 (Δ*sodA sodB*); △, QC1736 (Δ*fur* Δ*sodA* Δ*sodB*).

overexpression of this iron storage protein from a multicopy plasmid prevents toxic iron accumulation.

Effect of hydroxyl radical scavengers on aerobic survival of Δ*fur* Δ*recA* mutants. Oxygen-dependent toxicity of iron is mediated by the products of the Fenton reaction, including hydroxyl radicals. Dimethyl sulfoxide, a hydroxyl radical scavenger, protected the Δ*recA* Δ*fur* strain from death (Fig. 3b). Thiourea exhibits both hydroxyl radical-scavenging and metal-chelating activities and also had a protective effect (Fig. 3c). In contrast, cells grown in LB plus 100 mM mannitol were killed as rapidly as those grown in LB plus glucose (data not shown). This absence of protection by mannitol (an efficient hydroxyl radical scavenger in vitro) may be due to its low intracellular concentration.

Role of recombinational DNA repair in protection from iron-induced oxygen-dependent death of Δ*fur* mutants. The RecA protein induces the SOS system in response to blockage of DNA replication fork progression and is required for homologous recombination, which contributes to the repair of single- and double-strand breaks in DNA. We investigated whether the sensitivity of Δ*fur* Δ*recA* strains to aerobic conditions was due to the inability to induce the SOS response or to carry out DNA recombination or both by testing the effect of mutations affecting one or other function.

In the SOS response, RecA is activated to a coprotease state and mediates the cleavage of LexA, the repressor of SOS genes. A *lexA1* Δ*fur* mutant, defective in induction of the SOS response, was oxygen insensitive (Fig. 4). The *recA430* mutation renders RecA deficient for SOS induction but not recombination. A *recA430* Δ*fur* strain was not killed by the shift from anaerobiosis to aerobiosis (Fig. 4).

In *E. coli* there are two pathways for homologous recombination, both of which use the RecA protein. The major pathway utilizes the *recBC* functions. The other, minor pathway involves *recF* function. When *sbcBC* suppressor mutations are introduced into a *recBC* strain, the recombinational system is switched to the activated *recF* pathway (42). Δ*fur* mutants lacking RecBC died upon shift to aerobiosis, and the survival curve closely resembled that of the Δ*fur* Δ*recA* mutant (not shown and Fig. 4), whereas a *recF* Δ*fur* mutant was viable (Fig. 4). The Δ*fur recBC sbcBC* strain was weakly sensitive to aerobic conditions (Fig. 4), indicating that much of the damage could be repaired by the *recF* pathway, when active.

Thus, RecA protection of Δ*fur* mutants against oxygen is due to recombinational repair, mediated through the *recA-recBC* pathway, and does not require induction of SOS functions.

Further evidence for oxidative stress in Δ*fur* mutants: sensitivity to hydrogen peroxide. Challenge with low doses of hydrogen peroxide produces DNA damage, via the Fenton reaction, which are almost totally repaired in wild-type cells. However, *recA* mutants are exquisitely sensitive to hydrogen peroxide, with two modes killing (30–32). The Δ*fur* mutant similarly showed increased sensitivity to H_2O_2, suggesting that damage produced in this strain exceeds its repair capacity (Fig. 5a).

Increased spontaneous mutagenesis in a Δ*fur* mutant. Iron has been demonstrated to induce DNA base modifications and mutagenesis via the Fenton reaction (2, 12, 44). We examined mutagenesis in Δ*fur* mutant by three different tests (Fig. 6). The frequency of Val^s to Val^r, forward mutations, which could arise by deletion, insertion, or base substitutions, increased by more than threefold. There was no significant difference ($P > 0.7$ by t test) in the mutation frequency in cultures in late exponential phase (optical density at 600 nm of 0.8) or stationary phase (overnight). The increased mutagenesis was oxygen dependent. The frequency of Rif^s to Rif^r mutations, produced by base substitutions, was also increased about threefold, slightly less than the frequency of oxidative mutations induced by lack of SOD (18). Similarly, there was a significant increase of Tet^s to Tet^r forward mutations in the pPY98 test; however, this system is suitable only for qualitative estimates of mutagenesis efficiency. Surprisingly, adding ferrozine to the cultures did not significantly reduce the frequency of mutagenesis, indicating that DNA lesions leading to lethal events in the *recA* strain and mutagenic lesions are different. The iron responsible for mutagenic lesions was presumably not accessible to the chelator.

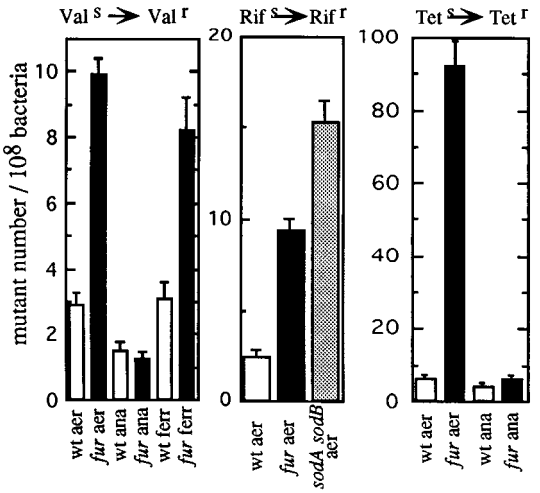

FIG. 6. Mutagenesis in a Δ*fur* mutant. GC4468 (wild type [wt]), QC1732 (Δ*fur*), and QC1726 (*sodA sodB*) were tested. For measurements of Tet^s to Tet^r, GC4468 and QC1732 were transformed with pPY98. The bacteria were tested under aerobic (aer) and anaerobic (ana) conditions and under aerobic conditions in the presence of 5×10^{-4} M ferrozine (ferr). Values are the means of n (3 to 9) experiments, depending on the assay. Bars represent standard errors. Differences are significant (t test) for Val^s to Val^r under aerobic conditions ($n = 8$, $P < 0.001$) with ferrozine ($n = 5$, $P = 0.05$); Rif^s to Rif^r, *fur* versus wt ($n = 4$, $P < 0.001$) and *sodA sodB* versus wt ($n = 5$, $P < 0.001$); and Tet^s to Tet^r under aerobic conditions ($n = 9$, $P < 0.001$). They are not significant for the experiments done under anaerobic conditions for Val^s to Val^r ($n = 5$, $P = 0.75$) and Tet^s to Tet^r ($n = 3$, $P = 0.15$).

Role of SOD in protection against iron-induced oxidative DNA damage. The Fenton reaction can be enhanced in vivo by an increase in either intracellular H_2O_2 or iron(II). SOD-deficient mutants are sensitive to H_2O_2 (Fig. 5b) because of the ability of superoxide to drive the Fenton reaction by reducing ferric complex (13, 31). The sensitivity of the Δ*fur* Δ*sodA sodB* mutant to H_2O_2 was greater than what could be expected from additive effects of *fur* and *sodA sodB* mutations (Fig. 5b).

A plasmid overexpressing MnSOD from p*tac* promoter partially protected the Δ*fur* Δ*recA* strain from death in medium containing 0.1 mM manganese (Fig. 7a), suggesting that SOD contributes to lowering the iron(II) pool in a Δ*fur* mutant.

sodA sodB Δ*recA* mutants cannot grow under aerobic conditions (Table 3); however, in contrast to Δ*fur* Δ*recA* mutants, they do not die after a shift from anaerobiosis to aerobiosis (Fig. 7b). A block (*tonB* mutants) or reduction (*aroB* mutants) of iron(III) transport suppressed oxygen sensitivity, but neither overexpression of storage proteins (bacterioferritin or ferritin H-like), iron chelation (Fig. 7b), or blockage of ferrous iron transport had any protective effect (Table 3). The toxicity is therefore presumably due to increased (superoxide-mediated) iron reduction and not to an iron overload.

Figure 8 depicts a scheme of metabolism of iron under aerobic conditions in *E. coli.*

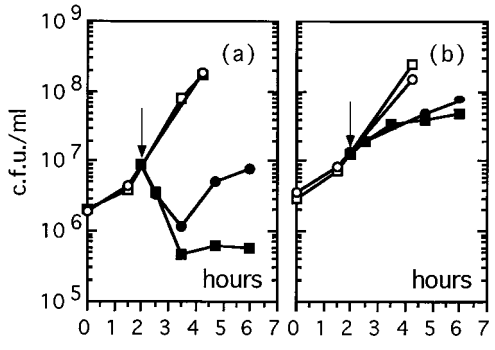

FIG. 7. Effect of SOD expression on the oxygen sensitivity of Δ*recA* strains. (a) As in Fig. 2. Symbols: □ and ■, QC2124 (Δ*fur* Δ*recA*) plus 10^{-4} M $MnSO_4$ and 2×10^{-3} M isopropyl-β-D-thiogalactopyranoside (IPTG); ○ and ●, QC2124/pDT1-19 plus 10^{-4} M $MnSO_4$, 2×10^{-3} M IPTG, and 500 μg ampicillin per ml; (b) □ and ■, QC2125 (Δ*recA sodA sodB*); ○ and ●, QC2125 plus ferrozine (5×10^{-4} M).

DISCUSSION

Iron stimulates the generation of highly reactive oxygen species in vitro such as hydroxyl radicals, causing DNA damage (2, 16, 40). The strict regulation of assimilation and storage of iron in both prokaryotic and eukaryotic cells is believed to prevent free intracellular iron accumulation, protecting cells from iron toxicity (37, 46). We show that the deregulation of iron assimilation in *E. coli fur* mutants produces an oxidative stress due to iron overload.

fur mutants were not viable in oxygen in the absence of efficient recombinational repair and showed an increased frequency of spontaneous mutagenesis. These results suggested DNA damage. The oxygen-dependent lethality of Δ*recA* mutants in a *fur* genetic background was due to the recombination defect and did not depend on impaired SOS function. Furthermore, damage in *recA*$^+$ *fur* mutants did not induce the SOS response, as shown by measuring expression of an *sfi-lacZ* reporter fusion (29a); cultures of *fur* mutants exhibit *lexA*-independent filamentation (unpublished observation), similar to the filamentation observed after exposure to H_2O_2 (31). A functional *recA-recBC* pathway was sufficient for aerobic viability of the *fur* mutant, whereas the *recA-recF* pathway was insufficient. Stimulation of *recA-recF* pathway in the absence of RecB almost completely restored aerobic growth, showing that both RecBC and RecF are able to repair the *fur*-induced lesions. The lethal lesions were presumably DNA strand breaks, since functional recombination repair restored viability. We failed to detect double-strand breaks in *fur recA* mutants after a shift from anaerobiosis to aerobiosis by analyzing chromosomal DNA by pulsed-field electrophoresis or plasmid DNA by agarose gel electrophoresis. This result suggests that there are few lesions.

The effects of a *fur* mutation are reminiscent of mode one killing by H_2O_2 described by Imlay and Linn (30–32). This result is consistent with the deleterious species being produced by the Fenton reaction in both cases.

The *fur* mutation leads to a permanent influx of iron which overwhelms the iron storage capacity of the cells, leading to an intracellular overload of iron. The addition of ferrozine to the medium reduced the free iron(II) pool and thereby restored viability to the *fur recA* mutant. Similarly, increasing the iron storage capacity of the cell by overproducing the ferritin H-like storage protein also restored viability. Surprisingly, bacterioferritin overproduction had no effect.

Inhibition of siderophore-mediated ferric iron transport by a *tonB* mutation suppressed the lethality of *fur recA*. Inhibition of ferrous iron transport in a *feo* mutant did not. Thus, imported ferric iron was responsible for toxicity. Furthermore, a mutation in the *aroB* gene impairing the synthesis of enterobactin, the major siderophore in normal growth conditions, sufficiently reduced iron influx to permit partial growth of *fur recA* cells in air.

Ferrous iron is mainly transported under anaerobic conditions. However, there is some aerobic transport of ferrous iron

TABLE 3. Effect of iron transport blocks or iron sequestration on the oxygen sensitivity of the *sodA sodB* Δ*recA* mutant

Allele[b]			Plasmid	Survival[c]		
tonB	*aroB*	Δ*feo*::*cat*		GC4668	K-12	AB2387
+	+	+	None	2×10^{-4} ($\pm 0.5 \times 10^{-4}$)	7×10^{-5} ($\pm 5.0 \times 10^{-5}$)	ND
+	+	+	pBFR.GEN	9×10^{-5} ($\pm 3.5 \times 10^{-5}$)	ND	ND
+	+	+	pSU.FTN2	8×10^{-5} ($\pm 3.0 \times 10^{-5}$)	ND	ND
+	+	−	None	3×10^{-4} ($\pm 1.0 \times 10^{-4}$)	5×10^{-5} ($\pm 4.0 \times 10^{-5}$)	ND
+	−	+	None	ND[d]	ND	0.82 ($\pm$ 0.10)
−[e]	−	+	None	ND	ND	0.72 ($\pm$ 0.10)
−[f]	−	+	None	ND	ND	1.0 ($\pm$ 0.25)

[a] The experiment was carried out as described in footnote *a* of Table 2.
[b] Strains are *sodA sodB* Δ*recA* derivatives of GC4468, K-12, and AB2387 with wild type (+) or mutated (−) alleles of *tonB*, *aroB*, and *feo*. GC4468 derivatives carry the Δ*sodA3* and Φ(*sodB'-'kan*)Δ*2* alleles (derived from QC1799); K-12 and AB2847 carry Φ(*sodA-lacZ*)*49* and the Φ(*sodB'-'kan*)Δ*2* alleles.
[c] Survival was measured as in footnote *c* of Table 3. Strain names are, from top to bottom as follows: GC4468 derivatives, QC2326, QC2335, QC2336, and QC2337; K-12 derivatives, QC2375 and QC2387; and AB2387 derivatives, QC2346 and QC2345/QC2347.
[d] ND, not determined.
[e] *tonB*.
[f] Δ(*tonB-try*).

via the *feo* pathway and the intracellular iron(II) concentration is reduced in *feo* mutants under aerobic conditions. Thus, *feo* mutants show a *fur*-like phenotype on expression of *fur*-regulated genes (55 and unpublished observations), and a *feo* mutation had a cumulative effect with the *aroB* mutation, restoring viability to a *fur recA* strain. The *feo* mutant facilitated the appearance of suppressor mutations in a *feo fur recA* strain under aerobic conditions. This result again suggests a reduction of toxic iron in the mutant, allowing residual growth during which mutations can be selected.

Surprisingly, spontaneous mutagenesis in *fur* mutants was only slightly reduced by the presence of ferrozine. Possibly the amount of iron(II) required to induce mutations is very low, and chelation by ferrozine did not reduce the intracellular concentration under this threshold. Alternatively, mutagenic iron might be bound to DNA complexes such that it cannot be chelated by ferrozine.

Deficiency in SOD expression causes DNA damage in the presence of oxygen (increased spontaneous mutagenesis and H_2O_2 sensitivity) (13, 18), which has been attributed to the ability of superoxide to enhance the Fenton reaction (20, 25). *sodA sodB recA* mutants are not viable under aerobic conditions. The discrepancy between this result and a previous report (18) is presumably due to the use of a *recA* point mutation in the first study, allowing either *recA* revertants or the appearance of phenotypic suppressors. Aerobic growth was restored by mutations (*tonB* or *aroB*) which reduce ferric iron import,

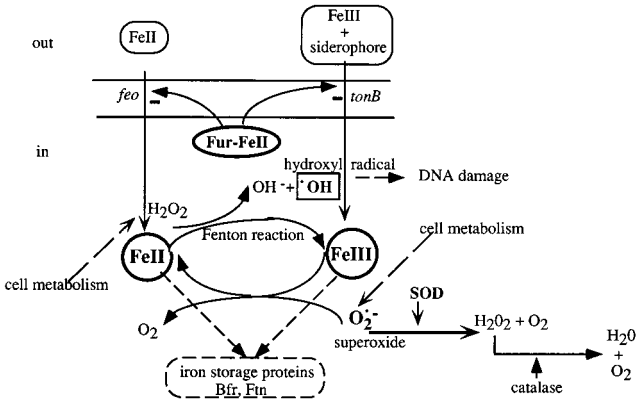

FIG. 8. Scheme of aerobic iron metabolism in *E. coli*.

showing that the lethal events were iron dependent. However, neither iron chelation nor iron storage suppressed lethality, consistent with the elevated steady-state level of superoxide in *sodA sodB* mutants not increasing the overall intracellular iron concentration but rather enhancing superoxide-mediated iron reduction. This consequently may increase the relative concentration of toxic iron(II).

It has been reported that there is a reduced activity of FeSOD in the *fur* strain (47). Moreover, Schrum and Hassan (53) reported that MnSOD activity was not correlated with *sodA* transcription in *fur* mutants because of partial loading of MnSOD apoprotein with iron, resulting in enzymatically inactive or partially active forms (6). This altogether may lead to reduced SOD global activity in *fur* mutants. However, oxidative stress in *fur recA* mutants is stronger than in *sodA sodB recA* mutants and thus cannot be entirely mediated by reduced SOD activity.

In wild-type strains, Fur is inactivated in response to iron starvation and the derepression of iron assimilation systems allows the cell to maintain its intracellular iron concentration. When iron availability returns to normal, ferrous iron activates Fur and switches off the system. However, depletion of transport proteins is not likely to be immediate. The situation observed in *fur* mutants, namely, stimulation of iron assimilation with sufficient iron in the environment, is presumably established for a period before siderophores and transport proteins return to their basal level. This phenomenon might create a transient iron overload and generate oxidative stress. The induction of SOD may provide protection against this iron stress.

The effects of MnSOD induction upon iron starvation may thus include (i) compensating for a reduction in FeSOD activity (47) and limiting aconitase inactivation during iron starvation (22), and (ii) upon return to high iron concentrations in the environment, preventing effects of iron overload, including MnSOD inactivation.

To avoid intracellular iron overload, the return to normal iron availability must be sensed extremely rapidly. The mechanism of Fur activation by its Fe(II) cofactor is poorly understood, and the origin of the ferrous iron pool, the source of Fur cofactor, is unknown. The Fur$^-$-like phenotype of *feo* mutants suggests that this pool is at least partly filled by import of external ferrous iron, even under aerobic conditions. This would provide a direct and rapid way for the Fur protein to sense iron repletion.

We show that fulfilling the requirement for iron can be damaging for the cell by producing an oxidative stress and thus increasing mutagenesis. The coupling of superoxide dismutase expression to iron metabolism regulation appears to deal with this "iron stress."

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