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MINIREVIEW

Iron and Oxidative Stress in Bacteria[1]

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The appearance of oxygen on earth led to two major problems: the production of potentially deleterious reactive oxygen species and a drastic decrease in iron availability. In addition, iron, in its reduced form, potentiates oxygen toxicity by converting, via the Fenton reaction, the less reactive hydrogen peroxide to the more reactive oxygen species, hydroxyl radical and ferryl iron. Conversely superoxide, by releasing iron from iron-containing molecules, favors the Fenton reaction. It has been assumed that the strict regulation of iron assimilation prevents an excess of free intracellular iron that could lead to oxidative stress. Studies in bacteria supporting that view are reviewed. While genetic studies correlate oxidative stress with increase of intracellular free iron, there are only few and sometimes contradictory studies on direct measurements of free intracellular metal. Despite this weakness, the strict regulation of iron metabolism, and its coupling with regulation of defenses against oxidative stress, as well as the role played by iron in regulatory protein in sensing redox change, appear as essential factors for life in the presence of oxygen.
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Reactive oxygen species (ROS)[3] are deleterious in excess. They are naturally produced by aerobic metabolism and are a permanent threat to living organisms (1). All organisms have developed ways of protecting themselves against ROS, including specific defenses and global responses that enable cells to survive periods of oxidative stress. Both types of protection are regulated and respond to the environment-associated oxidative threat (2–4). Iron, as a partner of the Fenton reaction, potentiates oxygen toxicity (5). It is thought that the strict regulation of iron metabolism, and its coupling with regulation of defenses against oxidative stress, is an essential factor for life in the presence of oxygen. *In vivo* evidence in bacteria, supporting this idea, are reviewed: effect of deregulation of iron homeostasis leading to oxidative stress, coupling between response to and protection against oxidative stress, and regulation of iron metabolism. The difficulties to assay intracellular free iron content lead to contradictory results, not presently permitting us to clearly support or counter genetic evidence. Although most of the studies have been done in *Escherichia coli*, it is likely that similar effects and coordination exist in numerous bacteria. Fur-like proteins coordinating regulation of iron assimilation with defense against oxidative and acid stresses and with general metabolism are found in a constantly increasing number of evolutionarily unrelated bacteria, thereby enabling cells to adapt their metabolism to the iron supply and protect against iron toxicity.

EXCESS OF SUPEROXIDE FAVORS THE FENTON REACTION

Superoxide is not extremely reactive by itself; for instance, it cannot attack DNA. However, it has been shown *in vitro* that it favors the Fenton reaction by reducing free ferric iron, leading to production of hydroxyl radicals which can damage any biological macromolecules:

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[3] Abbreviations used: ROS, reactive oxygen species; SOD, superoxide dismutase.

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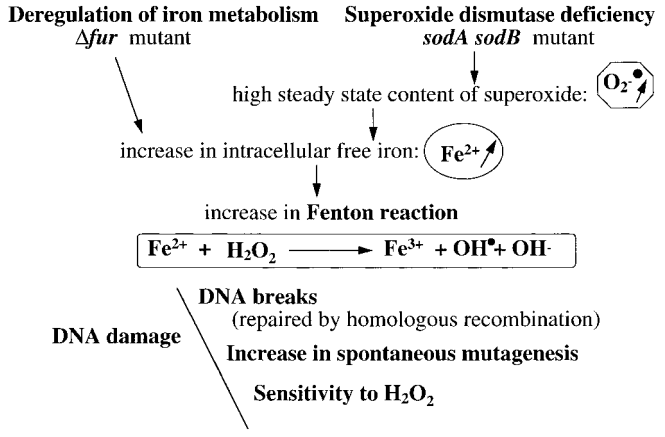

FIG. 1. Similar effects of iron homeostasis deregulation and superoxide-mediated oxidative stress in *E. coli.*

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-} \quad \text{Fenton reaction.}$$

A definitive *in vivo* demonstration of iron-mediated superoxide toxicity was provided by analysis of the phenotype of *sodA sodB E. coli* mutants (6). These mutants are completely devoid of cytoplasmic superoxide dismutase (SOD) and consequently achieved a much higher steady-state content of superoxide (7). This excess of superoxide causes DNA damage that cannot be due to direct effects of superoxide itself (6, 8, 9) (Fig. 1).

How an excess of superoxide *in vivo* favors the Fenton reaction is still an object of debate. It is generally accepted that there is an increase in intracellular toxic iron with excess superoxide, but the long-standing hypothesis that superoxide mediates the reduction of ferric iron, which has been shown to occur *in vitro,* has been called into question *in vivo* (10). It is indeed highly doubtful that "free ferric iron" (iron available for catalysis of Fenton-type reactions) would be maintained in the intracellular environment, in the presence of the many potential reductants, several of which are more efficient reductants than superoxide, for example, NADPH and glutathione. It has been suggested that iron regulation ensures that there is no free intracellular iron and that, in stress conditions, only an excess of superoxide releases free iron from iron-containing molecules. The release of iron by superoxide has been demonstrated for [4Fe–4S] cluster-containing enzymes of the dehydratrase-lyase family, which on oxidation by superoxide, release a "free iron" that could readily enter the Fenton reaction (11). Consistent with this notion, SOD deficient mutants showed defects in activity of enzymes containing [4Fe–4S] clusters, and defect-associated phenotypes. Such enzymes include aconitase, α,β-dihydroxyacid dehydratase, 6-phosphogluconate dehydratase, and fumarase A and B [reviewed in (12)].

Fur IRON REGULATION

Fur has been identified as a regulatory protein controlling the genes involved in iron acquisition (13). Fur is active as a dimer, with one Fe^{2+} per monomer as a cofactor (14) and a structural zinc site essential for activity (15). It acts as a transcriptional repressor (14). It binds to DNA at a specific sequence, the iron box (16). In some cases, Fur has been shown to have a positive effect on transcription (17–19; S. Dubrac and D. Touati, unpublished data). Genes positively regulated by Fur do not have iron boxes in their 5′ regions. It is therefore unclear whether these positive regulations involve a different molecular mechanism or whether they result from regulatory cascades.

Other regulators of iron acquisition, such as DtxR, have been found in a few bacteria (20, 21), but Fur appears to be the most widespread regulator of iron homeostasis in bacteria. Fur-like proteins and/or *fur*-like regulations have been found in a large, constantly increasing, number of bacteria (Table I).

Fur regulatory functions, which were initially thought to be limited to the control of iron acquisition, seem to be more extensive, suggesting a role for Fur as a general regulator. In iron scarcity, which results in Fur inactivation, all the genes involved in iron acquisition are derepressed (14). All genes encoding proteins involved in the import of elemental Fe^{3+} by various siderophores or in the direct import of Fe^{2+} are negatively regulated by Fur (22, 23). In *Helicobacter pylori*, Fur regulates the expression of the *ribBA* gene involved in iron acquisition via ferric iron reduction (24). Several pathogens, such as *Shigella dysenteriae, E. coli* O157/H7 (enterohemorrhagic), *Yersinia enterolytica, Neisseria gonorrheae, Haemophilus influenzae,* and others, are able to take iron from their host (25). They use hemin, hemoglobin, transferrin, and lactoferrin as alternative iron sources and the corresponding acquisition systems are Fur regulated (26–29). Conversely and consistently Fur positively (directly or indirectly) regulates iron storage in *E. coli* (30). The production of several toxins and pathogenicity factors, such as colicin, α-hemolysin, and pectate lyases, is under Fur control and is derepressed in conditions of iron deficiency (31–35). Their release, as harming cells, gain access to host intracellular iron.

Fur also regulates several functions that enable bacteria to adapt to or escape the poor environmental conditions caused by iron scarcity. These include general metabolism functions such as the regulation of purine biosynthesis (36), the replacement of ferredoxin by flavodoxin (37), and functions controlling bacterial motility (36). In *Pseudomonas* sp., Fur acts at the first

TABLE I

Bacteria in Which Fur-Like Proteins and/or *fur*-Like Regulations Have Been Found

Escherichia coli (54, 55), *E. coli* O157:H7 (28) (enterohemorrhagic)	*Salmonella typhimurium* (18, 56)
Bordetella pertussis (57), *B. bronchiseptica* (57)	*Legionella pneumophila* (58)
Vibrio cholerae (59), *V. parahaemoliticus* (60), *V. vulnificus* (61), *V. anguillarum* (63), *V. fisheri* (64)	*Shigella flexineri, S. dysenteriae* (62), *Yersinia pestis* (65), *Y. enterolytica* (66), *Brucella abortus* (unpublished)
Pseudomonas aeruginosa (67), *P. fluorescens* (68), *P. putida* (69)	
Helicobacter pylori (69)	*Campylobacter jejuni* (70)
Haemophilus ducreyi (71), *H. influenzae* (72)	*Klebsiella pneumoniae* (73)
Neisseria gonorrhoeae (74), *N. meningitidis* (75)	*Morganella morganii* (76)
Bacillus subtilis (43)	*Staphylococcus epidermidis* (77)
Streptococcus pyrogenes (43)	*Edwarsella tarda* (34)
Erwinia chrysanthemi (35)	*Rhizobium leguminosarum* (40)
Actinobacillus actinomycetemcomitans (78)	*Aeromonas species* (79)
Desufovibrio vulgaris (44), *Desulfoarculus baarsii* (unpublished)	*Synechococcus* sp. strain PCC 7942 (37)

step of regulatory cascades involving several global regulators, including sigma factors (38).

Fur is also involved in protecting against stresses, by regulating defenses against oxidative stress (discussed below) and the acid stress response (39).

The wide spectrum of genes regulated by Fur suggested that defects in this regulation would have serious consequences. Attempts to obtain *fur* mutants by allelic exchange have been unsucessful in *P. aeruginosa, N. gonorrhoeae, N. meningitidis,* and *V. anguillarum*. The failure to eliminate wild-type copies of the *fur* gene in *R. leguminosarum* and *Synechococcus* 7942 *fur* mutants strongly suggests that Fur plays an essential role in these bacteria (37, 40).

It is noteworthy that a Fur-like protein has been found in cyanobacteria which diverged from purple bacteria before the development of an oxidizing atmosphere, suggesting that need for iron regulation even existed in the absence of oxygen (37).

LOSS OF IRON REGULATION CAUSES OXIDATIVE STRESS

Evidence that loss of iron regulation led to oxidative stress and consequent deleterious effects was provided by a study of *E. coli fur* mutant (9). The mutant was viable but had impaired growth, and there was evidence for oxidative DNA damage (Fig. 1). As in SOD-deficient mutants, there was an oxygen-dependent increase in spontaneous mutagenesis in the *fur* mutant. The mutant was sensitive to hydrogen peroxide and could not survive in the presence of oxygen if the ability to repair DNA breaks by homologous recombination was impaired, in Δ*fur recA* or Δ*fur recB* strains.

As summarized in Fig. 2 excess free iron was responsible for those deleterious effects, which can be suppressed by scavenging iron with ferrozine, a ferrous iron chelator, by blocking ferric iron import into a *tonB* mutant, or by increasing the iron storage capicity of the cell by overproducing the ferritin H-like protein. Thus, the deregulation of the control of iron acquisition in conditions of iron sufficiency leads to oxidative stress in an aerobic environment.

Attempts to correlate excess of intracellular free iron with oxidative stress by direct intracellular iron measurements in various mutants led to contradictory results because of the difficulty in properly assaying free iron concentration. Keyer and Imlay observed an increase in intracellular loosely bound iron detected by EPR in *fur* and *sod* mutants (41). In contrast Abdul-Tehrani and collaborators reported an iron deficiency in *fur* mutant measured by chemical and physicochemical methods (Mössbauer spectroscopy) (42). The cause of the discrepancy might be that only reactive iron was supposed to be measured in the former experiment while total iron was likely measured in the later ones.

RECIPROCAL COUPLING BETWEEN THE CONTROL OF IRON HOMEOSTASIS AND DEFENSE AGAINST OXIDATIVE STRESS

Fur regulation of SODs has been observed in several bacteria. The expression of MnSOD is induced on Fur inactivation (*fur* mutant or iron deficiency) in *E. coli, S. typhimurium, B. pertussis, P. aeruginosa, P. putida, C. jejuni,* and *S. epidermidis* and direct Fur repression has been demonstrated in several cases [reviewed in (12)]. In contrast, FeSOD expression in *E. coli* is positively controlled by active Fur, via the presumably indirect stabilization of RNA (S. Dubrac and D. Touati, unpublished data). These opposing patterns of regulation keep global SOD activity high enough to cope with any iron environment and the related threat of oxidative stress.

In *B. subtilis,* genome sequencing has identified multiple Fur homologs, dissociating Fur-like functions (43). One Fur-like protein regulates iron uptake; another (PerR) negatively regulates the peroxide regulon in response to divalent cation availability (manganese and iron), in a Fur-like manner. A sequence encoding a

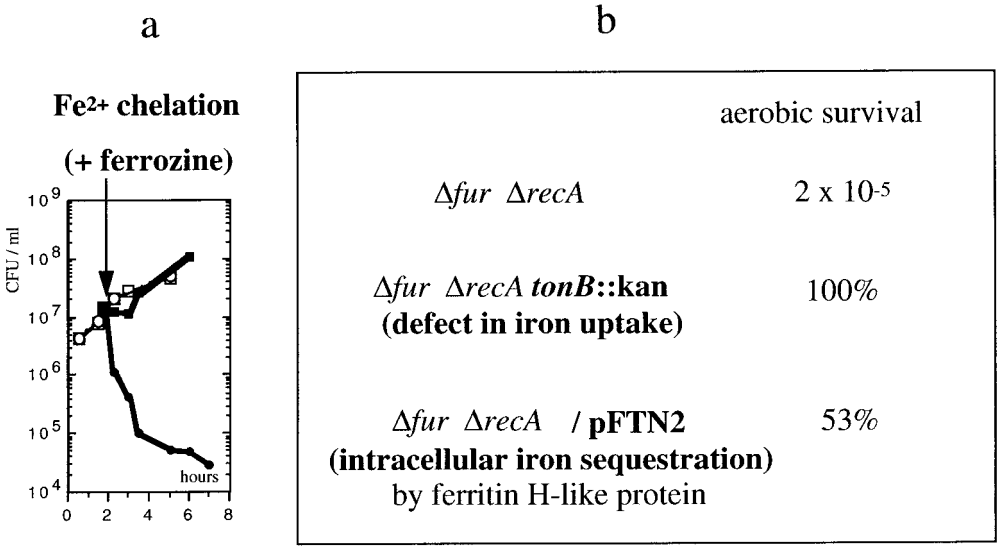

FIG. 2. Reversion of aerobic lethality of a *recA* Δ*fur E. coli* strain by limiting excess intracellular iron. Three different means (indicated in bold characters) to limit excess intracellular iron are shown: (a) Cultures of *recA* Δ*fur* strain with no ferrozine (squares) and 10^{-4} M ferrozine (circles) were grown in anaerobiosis (open symbols) and shifted to aerobiosis (thick lines and closed symbols). The surviving bacteria were counted by plating in anaerobiosis and are expressed in CFU/ml. The arrow indicates the time of transfer to aerobiosis. (b) Overnight cultures grown in anaerobiosis were plated for cell counts in aerobiosis and in anaerobiosis. Aerobic survival is the ratio of the number of colonies under aerobic conditions to that under anaerobic conditions. Data taken from Touati *et al.* (9).

Fur-like (or Per-like) protein has been found in several *Desulfovibrio* species (44; M.J. Pianzzolla and D. Touati, unpublished) upstream from rubrerytrin, which was recently shown to have an NADPH peroxidase activity *in vitro* (45). In *Mycobacterium tuberculosis,* a *fur*-like gene is cotranscribed with the *katG* gene, which encodes a catalase peroxidase involved in virulence (46). Putative per- or fur boxes are found upstream from the catalase operon of *Listeria seeligeri* and the alkylhydroperoxidase operons of *Clostridium pasteurianum* and *Enterococcus faecalis.*

Conversely, *fur* expression is increased in *E. coli* in response to oxidative stress as *fur* gene being a member of soxRS and oxyR regulons (47). Consistently, *fur* mutants defective in either of these protective responses against superoxide or hydrogen peroxide stress, although viable, have impaired growth and are subject to higher levels of oxidative damage (M. Jacques and D. Touati, unpublished data).

IRON–SULFUR CLUSTERS AS SENSORS OF CELL REDOX STATE: REGULATORY EFFECTS

Iron–sulfur centers are involved in the sensing of redox state and/or oxidative stress in several regulatory proteins (48, 49). Their activity is dependent on both iron and environmental oxidant. In *E. coli* the [4 Fe–4S] cluster of the Fnr protein, which activates the expression of numerous anaerobic metabolism genes, is an oxygen sensor. In the presence of oxygen, the cluster disintegrates and the protein ceases to bind DNA (50). The SoxR protein, the sensor of the global protective response against superoxide, contains two [2Fe–2S] clusters. It has been shown that the mechanism of SoxR activation is based on the oxidation–reduction of its iron–sulfur cluster (51, 52). The sensitivity of the [4Fe–4S] cluster of aconitase to superoxide has been proposed to act as a circuit breaker. The partial inactivation of aconitase by reducing the flow of electrons under oxidative stress largely halting the production of superoxide (53). Similar coupling between iron and oxidant sensing also exists in eukaryotic cells. In mammals the bifunctional iron-responsive element-binding protein 1(IRP1) functions as a sensor of both oxygen and iron levels, with the holoprotein (aconitase) functioning as a sensor of oxidants and the apoprotein (IRE-binding protein) as a sensor of iron (49).

CONCLUDING REMARKS

The lack of iron availability in the presence of oxygen is a major problem, but it is also a necessity. Soluble reduced iron in the presence of oxygen, due to the Fenton reaction, would have prevented aerobic life. Consistent with this, well waters from anoxic regions under the earth and containing large amounts of ferrous iron (up to 500 μM) rapidly kill immersed bacteria such as *E. coli* and *S. typhimurium,* by taking up too much ferrous iron in the presence of oxygen, resulting in such well waters being highly pure.

Strict and elaborate iron regulation enables cells to acquire the iron necessary for survival and maintain low levels of "free" iron, which could cause oxidative stress and damage. Iron is sensed as a signal of potential oxidative stress and defenses against oxidative stress are adapted to the iron environment. The interrelationship between the control of iron homeostasis and protection against oxidative stress is not unique to bacteria. Several recent studies have reported parallel effects and coupling in eukaryotic cells [see, for instance, (80)].

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