

Regulation of iron uptake minimizes iron-mediated oxidative stress

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Sequential accommodation of single electrons by the unpaired orbitals of dioxygen yields oxygen radicals (O_2^-), hydrogen peroxide (H_2O_2), hydroxide radicals (OH^\cdot), and finally water (H_2O). Fe^{2+} catalyses the formation of the most reactive hydroxide radical from hydrogen peroxide and thus contributes substantially to the toxicity of oxygen. Insolubility of Fe^{3+} demands the incorporation of iron into transferrin, lactoferrin, ferritin, iron-sulphur clusters, and heme. Bacteria and fungi synthesize low molecular weight compounds, termed siderophores, which are secreted and used to transport Fe^{3+} into the microbial cells. Iron is economically used and iron toxicity is minimized by the synthesis of siderophores and ferric siderophore transport systems, and by induction of transport gene transcription by certain Fe^{3+} -loaded siderophores. When cells contain sufficient iron, Fe^{2+} -loaded Fur protein and Fe^{2+} -loaded DtxR protein repress gene transcription in Gram-negative bacteria and in most Gram-positive bacteria, respectively. In a recently discovered novel transcription control mechanism, ferric citrate and ferric pseudobactins induce transcription of the iron transport systems by binding to cell surface receptor proteins without entering the cells. Cytoplasmic sigma factors are activated by a signaling device that involves a protein in the outer membrane and a protein in the cytoplasmic membrane. Both proteins extend into the periplasm to transduce the signal through the space between the two membranes. Intracellular iron homeostasis secured by regulation of iron uptake prevents excessive oxidative stress, which could otherwise overcome the cellular defence and repair systems and kill the cells.

1. Reactions of oxygen and oxygen radicals

Aerobic respiration is the most efficient way to produce energy; however, O_2 is not only reduced to O^{2-} , but also to the toxic oxygen radical (O_2^-) and to hydrogen peroxide (H_2O_2). The latter can be converted to the highly reactive and toxic hydroxyl radical (HO^\cdot) by catalysis with Fe^{2+} in the Fenton/Haber-Weiss reaction. Hydrogen peroxide and hydroxyl radicals react with lipids and proteins, and with the most vulnerable molecule, DNA. It has been estimated that 1.5×10^5 oxidative DNA adducts are contained in a human cell (Beckman and Ames 1997; Henle and Linn 1997). Recently, redox-reactive iron was found associated with senile plaques and neurofibrillary tangles of Alzheimer hippocampal tissue, suggesting oxidative damage as a cause of Alzheimer disease (Smith *et al.* 1997). In addition, the superoxide radical converts nitric oxide,

formed by nitric oxide synthase, into the reactive peroxynitrite anion.

Since iron plays an important role in oxygen radical formation, it is important that the cell's iron content is regulated. In mammals, precipitation of the highly insoluble ferric hydroxide at pH 7 is prevented by binding to transferrin. The iron-transferrin complex is taken into cells by endocytosis through the transferrin receptor; iron is released inside the cells, and part of it is incorporated into ferritin. The amount of the transferrin receptor and of ferritin is regulated at the translational level. Under iron limitation, iron binding proteins (IRE-BP) free of iron bind to iron responsive elements (IRE) in the 3'-region of the transferrin receptor mRNA and prevent mRNA degradation. The increased amount of mRNA results in an enhanced synthesis of transferrin receptor. In contrast, binding of IRE-BP to IRE in the 5'-region of ferritin mRNA inhibits translation. This elegant

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mechanism serves to increase the iron supply and avoids synthesis of ferritin when no iron storage is needed. Under iron surplus, IRE-BP is converted to a [4Fe-4S] protein that no longer shows regulatory activities, but displays aconitase activity (Paraskeva and Hentze 1996; Roualt and Klausner 1996).

FNR is another regulatory protein whose activity is controlled by an iron sulphur center. FNR of *Escherichia coli* functions as a transcriptional activator of more than 50 genes that are expressed during anaerobic growth. During aerobic growth, the iron sulphur center, probably [4Fe-4S], is destroyed and thereby inactivates FNR (Lazizzera *et al* 1996; Melville and Gunsalus 1996; Uden *et al* 1995).

Synthesis of SodA, one of the three superoxide dismutases of *E. coli*, is induced by the superoxide radical. Transcription of the *sodA* gene is activated by SoxS, and *soxS* gene transcription is activated by SoxR. The superoxide radical activates SoxR by oxidation of its [2Fe-2S] cluster, which is not destroyed, but functions reversibly. The iron sulphur center responds to oxidative stress and mediates the expression of defense genes (Gaudu *et al* 1997; Hidalgo *et al* 1997).

2. Protection against oxidative stress

Bacteria have to cope not only with metabolic oxygen radicals, but also with hydrogen peroxide formed by neutrophils to combat bacterial infections. *E. coli* reacts to oxidative stress by using two major regulatory devices: the peroxide stress response via *oxyR*, and the superoxide stress response via *soxR* and *soxS*. The *oxyR* response is stimulated by hydrogen peroxide and organic peroxides, and the *soxRS* response is induced by the superoxide radical. A set of proteins is synthesized—approximately 40 in each regulon—some of which are enzymes that destroy reactive oxygen species (Lynch and Lin 1996). Three superoxide dismutases convert the superoxide radical into hydrogen peroxide, and two catalases convert hydrogen peroxide into oxygen and water. Damaged DNA is repaired by several mechanisms, e.g., by a methylation-directed mismatch repair system encoded by the *mutH*, *mutL*, and *mutS* genes, by a mismatch repair DNA glycosylase encoded by *mutY*, and by exonuclease III, which displays a 3' to 5' exonuclease activity on double-stranded DNA and removes 3'-terminal phospho-monoester groups. The Dps protein protects DNA against the toxic and mutagenic effects of oxygen species by binding nonspecifically to DNA (Martinez and Kolter 1997). Large amounts of Dps are formed during the stationary growth phase in which cells display a higher resistance to hydrogen peroxide than in the exponential growth phase. Synthesis of Dps and of exonuclease III depends on a specific sigma factor, σ^S , which directs the RNA polymerase core enzyme to stress-related and

other promoters. Dps synthesis is also induced via *oxyR* during exponential growth by low doses of hydrogen peroxide. Metabolites such as NADH, NADPH, glutathione, ascorbate, and thioredoxin add to the overall defense by reacting stoichiometrically with harmful oxygen species.

3. Iron overload may override the oxygen protection devices

The iron content of *E. coli* is regulated by the activity of the iron transport systems of which seven exist for Fe^{3+} and at least two exist for Fe^{2+} in the *E. coli* K-12 laboratory strain (Braun and Hantke 1997; Earhart 1996; Crosa 1997). The iron transport rates are determined by the amounts of the iron transport proteins, which are controlled by the transcription rates of the transport genes. In Gram-negative bacteria such as *E. coli*, the transcription rates of iron transport genes are regulated by the Fur protein, and in most Gram-positive bacteria, by the DtxR protein. Fur and DtxR bind Fe^{2+} and are thereby converted into transcription repressors. Fe^{2+} -Fur and Fe^{2+} -DtxR bind to the promoters upstream of the transport gene operons and inhibit transcription. The number of iron-loaded Fur and DtxR proteins depend on the iron content of the cells. Under iron-limiting growth conditions, transcription of the transport genes can be more than 20-fold higher than under iron-replete growth conditions. In *E. coli*, approximately 40 genes are regulated by Fe^{2+} -Fur (Stojiljkovic *et al* 1995). In *fur* mutants, transcription of iron-regulated genes is derepressed, and cells become iron overloaded if sufficient iron is provided in the growth medium. *fur* mutants display an increased oxygen-dependent mutation rate and are not viable under toxic conditions if the cells are also mutated in the *recA* gene, which is involved in DNA repair. Cells are no longer killed if ferric iron uptake is impaired by a mutation in the *tonB* transport gene, if intracellular iron is deposited in the overproduced iron storage protein FTNF or chelated by ferrozine, or if hydroxyl radicals are scavenged by dimethylsulphoxide and thiourea (Touati *et al* 1995). Another means of iron overload was recently found when transport of Fe^{2+} by divalent cation transport systems was studied. These transport systems are usually not specific for a single cation, but accommodate several metal ions such as Mg^{2+} , Mn^{2+} , Co^{2+} , Ni^{2+} , and also Fe^{2+} , although with different efficiencies. Most artificial growth media for *E. coli* contain 1 mM Mg^{2+} , but 30 μM is sufficient to support growth. At this low Mg^{2+} concentration, Fe^{2+} is rapidly taken up provided reducing conditions are maintained in the transport assay by 1 mM sodium ascorbate (Hantke 1997). Under these conditions, the level of internalized iron is rather high. Plating of the cells after the transport assay revealed a death rate of more than

90%. It is not clear whether the high concentration of imported iron or its subcellular localization is toxic because most of Fe^{2+} taken up by the cells can be chased with 3 mM Mg^{2+} , in contrast to Fe^{3+} transported by ferrichrome, which cannot be released from the cells and is not toxic. Although a mutation in one of the Mg^{2+} transport genes, *corA*, reduces Fe^{2+} transport by only 30%, it prevents cell killing. The residual Fe^{2+} transport is not catalyzed by the Feo transport system, which displays a rather high specificity for Fe^{2+} (Kammler *et al* 1993).

4. Fe^{3+} transport systems of *E. coli*

Ferric siderophore transport systems of *E. coli* are the best-characterized bacterial iron transport systems. Ferric siderophores of the hydroxamate type used by *E. coli* are ferrichrome and the related compounds aerobactin, coprogen, and ferrioxamine B. Only aerobactin is synthesized by certain *E. coli* strains; ferrichrome and coprogen are formed by fungi and ferrioxamine B by Actinomycetes. The ferric hydroxamates are all taken up across the cytoplasmic membrane by a single transport system that consists of the soluble periplasmic binding protein FhuD, the integral cytoplasmic membrane protein FhuB, and the FhuC ATPase (figure 1) located at the inner side of the cytoplasmic membrane (Schultz-Hauser *et al* 1992). Although the structures of the ferric hydroxamates differ considerably (Drechsel and Winkelmann 1997), they are all recognized by the FhuD protein and donated to the FhuB protein, which presumably also

recognizes the substrates it transports. Upon binding of the substrates, FhuD alters conformation (Köster and Braun 1990; Rohrbach *et al* 1995). Interaction between FhuD and FhuB occurs in the periplasm and also with a transmembrane region and a cytoplasmic region of FhuB (Mademidis *et al* 1997). This unexpected finding is based on the determination of the binding of synthetic 10-residue and 20-residue peptides, identical in sequence to FhuB segments, to isolated FhuD. FhuB peptides that bind to FhuD also inhibit transport when transferred into the periplasm through the channels of a FhuA outer membrane protein derivative with a deletion of the gating loop (see below). These permanently open channels are three times larger than the porin channels (Killmann *et al* 1993) and are therefore suitable for bringing compounds into the periplasm for which the outer membrane is not permeable. The cytoplasmic segment of FhuB that interacts with FhuD is only a few amino acids apart from the site that is assumed to interact with the FhuC ATPase (Köster and Böhm 1992). As has been shown by reconstituted transport systems of histidine (Bishop *et al* 1989) and maltose (Davidson *et al* 1992), binding of substrate-loaded binding protein to the integral membrane proteins triggers ATP hydrolysis by the transport ATPase. A signal from the periplasmic to the cytoplasmic side of the cytoplasmic membrane is assumed to activate the ATPase. In contrast, FhuD comes so close to FhuC that FhuD can physically interact with FhuC to initiate ATP hydrolysis. Since these are the first data on the interacting regions of a periplasmic protein and a transmembrane protein of the common ATP transporters, the conclusions drawn for ferrichrome transport may well apply to the other ABC transporters. By this transport mechanism, bacteria take up ferric siderophores, vitamin B_{12} , certain amino acids, peptides, phosphate, and sulphate, and export certain proteins, polysaccharides, and toxic compounds. Eukaryotic members of the superfamily include the multi-drug resistance P-glycoprotein (MDR), the cystic fibrosis transmembrane conductance regulator (CFTR), and the α -factor secretory yeast protein (Fath and Kolter 1993).

Transport of ferric siderophores across the cytoplasmic membrane appears to be the same for all ferric siderophores—in Gram-negative and—in Gram-positive bacteria. The binding protein, which occurs in free form in the periplasm of Gram-negative bacteria, in Gram-positive bacteria which are devoid of an outer membrane, is fixed by a lipid anchor to the outer surface of the cytoplasmic membrane (Schneider and Hantke 1993).

Bacteria also use iron sources provided by their hosts, such as heme, haemoglobin, haemopexin, transferrin, and lactoferrin. Heme and iron released from the proteins are transported by ABC transporters, as was for the first time shown for heme uptake of *Yersinia enterocolitica*

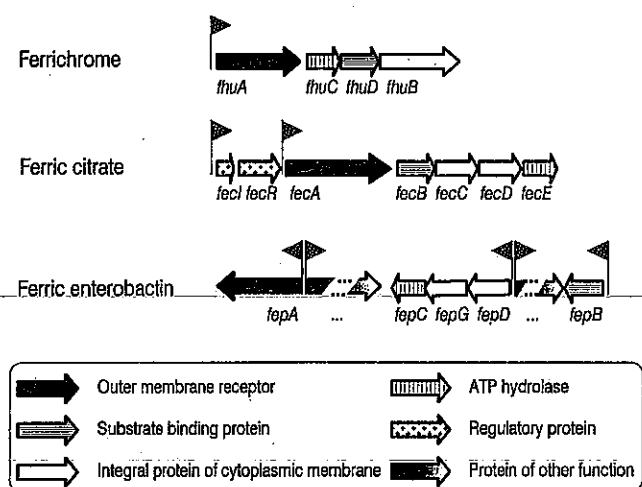


Figure 1. Arrangement of ferric siderophore transport genes of *E. coli*. The arrows indicate the transcription polarities; the flags indicate promoter regions to which Fe^{2+} -Fur binds. Enterobactin synthesis genes (not shown) are located between the ferric enterobactin transport genes (devised by W Köster).

(Stojiljkovic and Hantke 1994) and iron uptake of *Serratia marcescens* (Angerer *et al* 1990).

The outer membrane of Gram-negative bacteria forms a permeability barrier for substrates larger than 600 Da, which is determined by the size of the water-filled channels formed by the porin proteins. Most of the ferric siderophores are excluded from diffusion through the porins. For this reason, but also because of their low abundance in the vicinity of cells, ferric siderophores bind to highly specific outer membrane receptor proteins. For example, *E. coli* K-12 contains three receptor proteins designated FhuA, IutA, and FhuE for the ferric hydroxamates ferrichrome, ferric aerobactin, and ferric coprogen, which are all taken up via the same FhuD/FhuB/FhuC

transport system across the cytoplasmic membrane. After binding to the receptors, the ferric siderophores have to be released and translocated vectorially from the cell surface into the periplasm. This step consumes energy, which is provided by the electrochemical potential of the cytoplasmic membrane. Energy transfer from the cytoplasmic membrane to the receptors in the outer membrane is probably mediated by a complex consisting of the proteins TonB, ExbB, and ExbD (Ton system). It has been proposed that TonB assumes an energized conformation that interacts with the outer membrane receptors and opens their channels. Interaction of FhuA with TonB (Schöffler and Braun 1989; Günter and Braun 1990) is enhanced when ferrichrome binds to FhuA

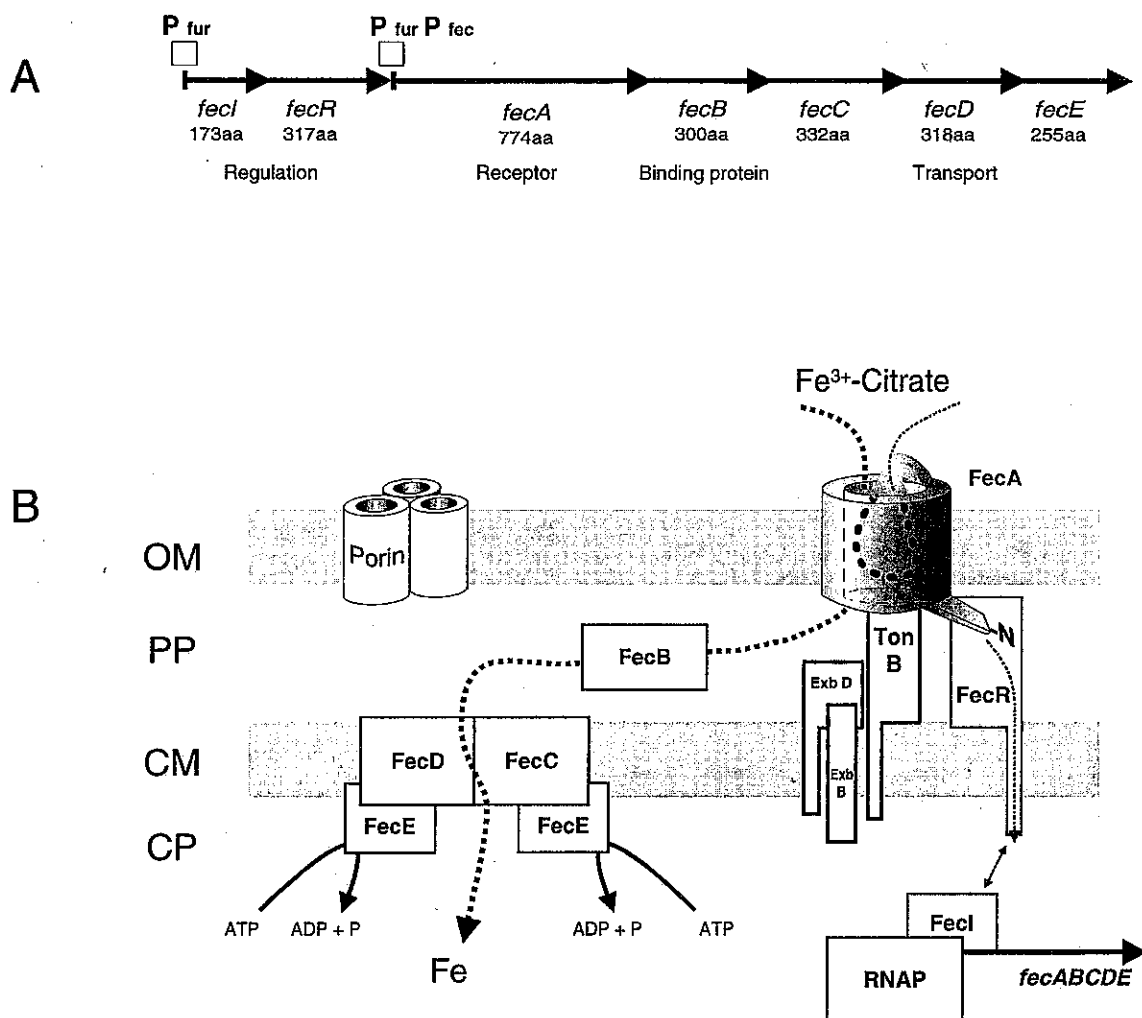


Figure 2. (A) Arrangement of the *fecIR* regulatory genes and the *fecABCDE* transport genes on the chromosome of *E. coli* K-12. P_{fur} and P_{fec} indicate promoters regulated by Fe^{2+} Fur and ferric citrate, respectively. (B) Location of the Fec regulatory proteins and the Fec transport proteins in the outer membrane (OM), the periplasm (PP), the cytoplasmic membrane (CM), and the cytoplasm (CP). The left panel illustrates transport of iron and the proteins involved, the right panel illustrates transcription initiation of *fec* transport gene transcription by ferric citrate bound to FecA. N, N-terminal end of FecA; RNAP, RNA polymerase core enzyme. The arrows on the gene maps indicate the transcription polarities (devised by S Plantör).

(Moeck *et al* 1997). Conformational changes in FepA, the receptor of ferric enterobactin, have been observed during transport by electron spin resonance spectroscopy by monitoring the mobility of covalently bound nitroxide spin labels (Jiang *et al* 1997).

Heme, haemoglobin, transferrin, and lactoferrin receptors have been identified in the outer membrane of a number of Gram-negative bacterial families and genera such as Neisseriaceae (e.g., *Neisseria gonorrhoeae* and *Neisseria meningitidis*), Pasteurellaceae (e.g., *Haemophilus influenzae*) (Gray-Owen and Schryvers 1996; Genco and Desai 1996), *Yersinia* (Stojiljkovic and Hantke 1994), and *Serratia marcescens* (Ghigo *et al* 1997).

5. Regulation of iron uptake by ferric siderophores

Under iron limitation, synthesis of ferric siderophores is derepressed. In addition, some ferric siderophores released into the growth medium induce transcription of the transport genes. This double check guarantees that the very costly synthesis of siderophores occurs only when they are needed, and that the transport systems are turned on only when the iron-loaded substrates are available. The underlying control device has been studied most extensively in the ferric citrate transport system of *E. coli* (Braun 1997). Ferric citrate added to the growth medium induces transcription of the five *fec* transport genes (figure 2). Ferric citrate binds to the FecA outer membrane receptor protein and this binding, without further transport, induces transcription (Härle *et al* 1995). The question arises how the *fec* transport genes in the cytoplasm are informed of the presence of ferric citrate at the cell surface. Two proteins, FecA in the outer membrane and FecR in the cytoplasmic membrane, convey the information into the cytoplasm. Cells lacking *fecR* do not respond to ferric citrate and do not transcribe the *fec* transport genes. A cytoplasmic N-terminal FecR fragment of 56 amino acids induces *fec* transport gene transcription independent of ferric citrate (Ochs *et al* 1996). Signal transduction across the outer membrane has the same energy requirement as ferric citrate transport across the outer membrane (Kim *et al* 1997). Transcription is mediated by the cytoplasmic FecI protein, which acts as a sigma factor and directs the RNA polymerase core enzyme to the promoter upstream of the *fec* transport gene cluster (Angerer *et al* 1995; Enz *et al* 1995). FecI belongs to the σ^{70} factors of the ECF type, which respond to extracytoplasmic functions (Lönetto *et al* 1994). The molecular events underlying signal transduction across the outer membrane, the periplasm, and the cytoplasmic membrane, and conversion of FecI to an active sigma factor are not known. A series of conformational changes initiated by binding of ferric citrate to FecA, which in turn changes the conformation of FecR, and then the

activation of FecI by activated FecR is conceivable, but chemical modification of the proteins has not been excluded. Transcription of the regulatory genes *fecI* and *fecR* (figure 2) is repressed by Fe^{2+} -Fur and is not induced by ferric citrate. Fe^{2+} -Fur represses *fec* transport gene transcription via *fecI* and *fecR* transcription and also represses *fec* transport gene transcription directly by binding to the promoter upstream of *fecA* (Angerer A and Braun V, unpublished results).

Various mechanisms of ferric siderophore induction of ferric siderophore transport systems have been found in *Pseudomonas* strains. Synthesis of the PupB receptor of *P. putida* WCS358 is induced by its cognate pseudobactins BN7 and BN8. Two regulatory proteins, PupI and PupR, with sequence similarities to FecI and FecR, positively regulate PupB synthesis. PupB is directly involved in induction; this indicates that the control of *pupB* transcription is similar to the regulation of *fecABCDE* transcription (Koster *et al* 1994). Ferric enterobactin induces synthesis of the Pfe receptor in *P. aeruginosa* through a phosphorylation/dephosphorylation relay (Dean and Poole 1993), and pyochelin activates synthesis of the receptor by an AraC-type mechanism (Heinrichs and Poole 1993).

Iron is provided to bacteria in many forms which require as many transport systems. All the transport systems have to be adapted to the iron supply to guarantee sufficient iron for growth and to avoid iron toxicity by iron overload.

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