Bacterial approaches for assembling iron-sulfur proteins.

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Abstract

Building iron-sulfur (Fe-S) clusters and assembling Fe-S proteins are essential actions for life on Earth. The three processes that sustain life, photosynthesis, nitrogen fixation, and respiration, require Fe-S proteins. Genes coding for Fe-S proteins can be found in nearly every sequenced genome. Fe-S proteins have a wide variety of functions, and therefore, defective assembly of Fe-S proteins results in cell death or global metabolic defects. When compared to alternative essential cellular processes, there is less known about Fe-S cluster synthesis and Fe-S protein maturation. Moreover, new factors involved in Fe-S protein assembly continue to be discovered. These facts highlight the growing need to develop a deeper biological understanding of Fe-S cluster synthesis, holo-protein maturation, and Fe-S cluster repair. Herein we outline bacterial strategies used to assemble Fe-S proteins and the genetic regulation of these processes. We focus on recent and relevant findings and discuss future directions including the proposal of using Fe-S protein assembly as an anti-pathogen target.

1. Iron-Sulfur proteins

Iron (Fe) is an essential nutrient for nearly all organisms. The importance of Fe for the survival of microbes is highlighted by the fact that many organisms encode multiple Fe acquisition systems. These acquisition systems aid in competition and allow cells to acquire Fe under a variety of conditions to meet demand.

A large proportion of internalized Fe is housed within inorganic prosthetic groups called iron-sulfur (Fe-S) clusters, which are utilized by organisms in the three primary branches of life. Protein Fe-S clusters are typically ligated using cysteine thiolates and are commonly found as rhombic [2Fe-2S] or cubic [4Fe-4S] clusters; however, more complex Fe-S cofactors are utilized for specialized processes such as nitrogen fixation and hydrogen metabolism. When *Escherichia coli* is cultured in a defined medium with glucose or acetate as a carbon source approximately 30% of the intracellular Fe is located in Fe-S clusters and low-spin ferrous heme centers (1).

As a result of their structural and electronic plasticity, Fe-S clusters are utilized for a variety of cellular functions. The genome of *E. coli* is predicted to encode ~140 Fe-S proteins (out of the ~4,300 total protein coding ORFs (2)) that have wide-ranging functions including carbon transformations, environmental sensing, DNA repair, and respiration (3). Likewise, the metabolisms of most organisms are highly reliant on the functionalities of the Fe-S proteins. Failure to properly maturate Fe-S proteins results in widespread metabolic disorders and in some cases can lead to cell death (4-6). *Bacillus subtilis* strains (lacking Suf) and *E. coli* strains (lacking Suf and Isc) cannot build Fe-S clusters and are non-viable because they cannot properly maturate the essential Fe-S proteins IspG and IspH, which are required isoprenoid synthesis (7, 8). Bypassing the need for IspG and IspH, by engineering the organisms to utilize the eukaryotic Fe-S protein-independent mevalonate pathway for isoprenoid synthesis, circumvents the necessity for Fe-S biosynthesis for survival (9).

2. Building iron-sulfur clusters

Because of the toxic nature of free Fe^{2+} and sulfide (S^{2-}), tightly controlled mechanisms have evolved to synthesize Fe-S clusters from its monoatomic precursors, thereby minimizing the cytosolic concentrations of these elements not ligated to macromolecules (10). Three multiprotein assembly systems (nitrogen fixation (NIF), sulfur mobilization (SUF), and iron-sulfur cluster (ISC)) have been described in bacteria and archaea for the synthesis of Fe-S clusters for assembly of [2Fe-2S] and [4Fe-4S] proteins (11-13). These systems function similarly, but they are biochemically discrete. Additional systems have been described for building more complex Fe-S clusters such as those found in dinitrogenase reductases and hydrogenases (reviewed here (14, 15)).

The NIF system was the first described Fe-S synthesis system. NIF was discovered because it is essential for nitrogen fixation (13). NIF functions to provide basic Fe-S clusters for nitrogenase maturation and it is often found in diazotrophs (16). SUF and ISC are responsible for building the Fe-S clusters for the maturation of the majority of non-nitrogenase Fe-S proteins. Bioinformatic analyses have identified the SUF system as the most prevalent machinery in prokaryotic genomes (17). Bacterial genomes can encode one (*Staphylococcus aureus*), two (*Escherichia coli*), or all three (*Erwinia chrysanthemi* and some nitrogen fixing Cyanobacteria) of the synthesis systems ((17, 18) and regulation section).

The SUF, NIF, and ISC macromolecular machines all use a common strategy to synthesize Fe-S clusters (Figure 1). Iron, sulfur, and electrons are combined upon a cytosolic molecular scaffolding protein(s) to form an Fe-S cluster. SufBCD, IscU, and NifU are the scaffold proteins for the SUF, ISC, and NIF systems, respectively (Figure 2) (16, 19, 20). Although the Suf proteins can be isolated with various ratios, it is thought that the active form of the SUF system has the ratio of one SufB, two SufC, and one SufD ($SufBC_2D$) (Figure 2B) (21, 22). The SufBD heterodimer interface may be the site of Fe-S cluster synthesis (22).

2.1. Sulfur. Sulfur is typically mobilized from a free cysteine (Cys) by PLP-dependent cysteine desulfurases (SufS, IscS, NifS) (13). Cysteine desulfurases form a covalent persulfide intermediate and alanine as a byproduct (23). The persulfide can subsequently be transferred to the synthesis machinery scaffold directly or through a surrogate carrier molecule (i.e. SufU or SufE) (19, 24). Thus far, the only described

persulfide sulfur carrier molecules are associated with SUF systems. Biochemical analyses suggest that these persulfide carrier proteins allow for a controlled delivery that protects the system from poisoning by oxidants, such as hydrogen peroxide, which would be deleterious to sulfur transfer (25, 26). SufU and SufE act as persulfide carriers for $SufBC_2D$ in *Bacillus subtilis* and *E. coli*, respectively. SufU was initially thought to be a scaffold protein because of its ability to bind Fe-S clusters and its homology to IscU and NifU; however, biochemical analyses of the SufS-SufU complex demonstrated a unique sulfur transfer mechanism dependent on a zinc ligand from SufU (24, 27). Although SufU and SufE primary amino acid sequences differ, they can individually act as protective persulfide carriers for $SufBC_2D$; however, a *suf* operon usually only codes for one, suggesting that this functionality may have evolved twice. Some Archaea such as *Methanococcus maripaludis* lack homologs of cysteine desulfurases. When this archaean was cultured with $^{35}S^{2-}$ there was an enrichment of $^{35}S^{2-}$ in Fe-S cluster containing proteins, but not in free Cys suggesting that sulfide, and not Cys, is the source of the sulfur for Fe-S synthesis (28).

2.2. Electrons. The *isc* operon typically encodes a [2Fe-2S] ferredoxin (Fdx), which can provide electrons for ISC directed Fe-S synthesis. Fdx interacts with IscS and reduced Fdx provides an electron to the IscS complex for sulfane (S^0) reduction (29, 30). A second reduction event is required to produce S^{2-}, which is the substrate for Fe-S cluster synthesis on IscU. NADH and $NADP^+$-ferredoxin-reductase can provide electrons for Fdx reduction *in vitro* (31). The electron donors to the scaffold proteins NifU and $SufBC_2D$ are unknown. $SufBC_2D$ co-purifies with $FADH_2$ consistent with the complex conducting redox chemistry (21). NifU, from the NIF system, contains a stable redox-active [2Fe-2S] cluster that may provide electrons for NIF-directed synthesis (32). The membrane-associated Rnf complex has a role in dinitrogen fixation in *A. vinelandii*, by donating electrons from NADH to ferredoxin using reverse electron flow and $\Delta\mu Na^+$ or $\Delta\mu H^+$ (33). *Azotobacter vinelandii rnf* mutants have a decreased capacity for dinitrogen reduction because of poor Fe-S cluster occupancy of the dinitrogenase reductase NifH (34). The *rnf* mutants also have decreased activity of the Fe-S enzyme aconitase. It is tempting to speculate that Rnf has a role in providing electrons for Fe-S cluster synthesis or repair.

2.3. Iron. The source of Fe for cluster building remains unknown. Several candidates such as CyaY and IscX have been proposed based on *in vitro* considerations, but subsequent *in vivo* investigations failed to provide supporting evidence. CyaY is the counterpart of mitochondrial frataxin. The reason frataxin/CyaY was predicted to act as an iron donor came from (i) observing iron homeostasis disturbance in mitochondria from frataxin-deficient tissues or organisms, and (ii) iron-binding to CyaY *in vitro* although with weak affinity. Frataxin, both in eukaryotes and prokaryotes, forms a tri-partite complex with cysteine desulfurase NFS1/IscS and scaffold ISU/IscU. *in vitro*, frataxin appears to have the opposite effect on Fe-S formation whether one studies the prokaryote (*i.e.* inhibition) or the eukaryote system (*i.e.* stimulation) (35). Possible explanations lie in differences in IscS (prokaryote) and NFS1 (eukaryote) cysteine desulfurases intrinsic biochemical features. In any case, studies *in vivo* in *E. coli* confirmed that CyaY is a positive effector of ISC-mediated Fe-S cluster biogenesis (36, 37).

The *E. coli isc* operon codes for IscX, which also binds Fe^{2+} with low affinity and has a role in ISC-directed Fe-S synthesis (36, 38). IscX binds to IscS at a location that overlaps the CyaY binding site (39). The presence of Fe^{2+} increases the affinity between IscX and IscU and stabilizes the complex (38). IscX associates with IscS-IscU forming a tripartite complex resulting in inhibition of cysteine desulfurase activity. Analyses using both CyaY and IscX, in conjunction with the IscU-IscS complex, found that CyaY inhibits Fe-S cluster formation on IscU, which is mitigated by the addition of IscX at low Fe concentrations (<20 μM); however, the effect of IscX is negligible at higher concentrations (40).

While evidence suggests that IscA is a Fe-S cluster carrier (discussed below), one group of researchers found that *E. coli* IscA copurified with Fe, but not sulfide [55]. Apo-IscA could be loaded with Fe^{2+} *in vitro* (K_a = 3.0 x 10^{-19} M^{-1}) and it bound one Fe per IscA. The Fe-loaded IscA could provide Fe for IscS-directed Fe-S cluster synthesis on IscU. An $IscA_{Y40F}$ variant was defective in Fe^{2+} binding but appeared to bind an Fe-S cluster. A K_a for Fe association, Fe-S cluster stability, Fe-S cluster transfer kinetics, and labile Fe and sulfide concentrations were not reported for the reconstituted $IscA_{Y40F}$ variant (41). A wild-type *iscA* allele, but not an *iscA*$_{Y40F}$ allele could complement an *iscA* mutant. Whether IscA is a *bona fide* Fe donor *in vivo* remains to be established.

2.4. Energy. The assembly of Fe-S proteins can require an input of energy. SufC, of the SUF system, has both Walker A and Walker B nucleotide-binding motifs and functions as an ATPase (42, 43). ATPase activity is stimulated by interaction with either SufD or SufB (44). The presence of SufC is necessary for SufB to interact with the sulfur transfer protein SufE (19). A conserved lysine (Lys40) in the Walker A motif is required for ATPase activity (45). The $SufC_{K40R}$ variant interacts with SufB and SufD *in vitro*, but the *sufC*$_{K40R}$ allele cannot replace *sufC*, suggesting that ATPase activity is necessary for SUF function (22). Consistent with these findings, the $SufBC_{2(K40R)}D$ variant does not assemble an Fe-S cluster *in vivo* whereas $SufBC_2D$ does (46).

In the ISC system, the scaffold IscU interacts with a Hsp70-like chaperone (HscA) and a J-protein co-chaperone (HscB) (47). Hsp70 chaperones have an ATP binding domain and a protein substrate-binding domain. Biochemical and biophysical studies found that interactions between IscU and HscAB aid in building the Fe-S cluster on IscU and/or the transfer of the Fe-S cluster from holo-IscU to a target apo-protein (48). The proposed role of the co-chaperone HscB is to escort IscU to HscA-ATP and promote ATP hydrolysis (49). After ATP hydrolysis, HscB is released, because it has low affinity for HscA-ADP, and IscU is prompted to deliver the Fe-S cluster to an apo-protein or Fe-S cluster carrier protein (48). ADP release by HscA induces conformational changes that promote IscU release prompting a new cycle of chaperone-mediated Fe-S cluster synthesis and transfer (50, 51). The roles of HscAB were recently reviewed (52).

3. Delivery of iron-sulfur clusters to target apo-proteins.

After Fe-S cluster construction on a scaffolding protein, it is passed to a client apo-protein forming the holo-protein. *In vitro* evidence for a direct Fe-S cluster transfer from a scaffold to an apo-protein was demonstrated, but *in vivo* observations stress the essential role of carriers and cast doubt on the physiological relevance of the direct scaffold-apo-protein connection (53-56).

Fe-S cluster carriers have been shown to bind [4Fe-4S] clusters, [2Fe-2S] clusters, or both (57). It is thought that the carriers typically deliver the Fe-S cluster they are provided; however, the Fe-S carrier IscA (A-type carrier) from *Azotobacter vinelandii* can

bind both $[4Fe\text{-}4S]^{2+}$ and $[2Fe\text{-}2S]^{2+}$ clusters and can convert the $[2Fe\text{-}2S]^{2+}$ form to the $[4Fe\text{-}4S]^{2+}$ form using a two-electron reductive coupling (57). It was proposed that the $[4Fe\text{-}4S]^{2+}$ form could cycle back to the $[2Fe\text{-}2S]^{2+}$ form by dioxygen catalyzed cleavage, but this has not been experimentally demonstrated. Whether these cluster dynamics have a physiological role is unknown.

The number of carriers varies within different bacterial species. For instance, *E. coli* synthesizes at least six carriers (the A-type carriers (IscA, SufA, ErpA) and NfuA, GrxD, Mrp) while *Bacillus subtilis* and *Staphylococcus aureus* only have two characterized carriers (SufA and Nfu). Strains lacking one or more Fe-S cluster carrier can often maintain the assembly of Fe-S proteins, suggesting that, in general, the carriers have a degree of functional overlap (53, 58). Note, however, that *erpA* is essential under aerobic growth in *E. coli* (9).

The number of targets exceeds the number of carriers raising questions about the dynamic and specificity of the delivery network. While *in vitro* transfer assays have provided evidence that carriers can transfer clusters to apo-proteins, they did not reveal substantial insights into substrate specificity (55). In *E. coli*, the six carriers are not synthesized to the same levels, and expression varies upon growth state and condition, suggesting that genetic control is key for orchestrating functional redundancy (59). We briefly discuss some of these intermediate actors below.

3.1. A-type carriers. A-type carriers are predicted to bind Fe-S clusters using three cysteine ligands. An X-ray structure of *Thermosynechococcus elongatus* IscA ligating a [2Fe-2S] cluster is illustrated in Figure 3A (60). The three cysteinyl ligands (C37, C101, and C103) to the Fe-S cluster are highlighted. Phylogenetic analysis allowed researchers to divide the A-type carriers (ATC) into two groups: ATC-I and ATC-II (53). Further functional studies showed that ATC-I proteins directly interact with the apo-proteins, while the ATC-II proteins associate with the scaffold (53). In *E. coli*, ErpA belongs to the ATC-I family and IscA and SufA are of the ATC-II family. ErpA interacts physically with apo-proteins, whereas SufA and IscA, to an appreciable degree, do not. This suggests that the ATC-II carriers pass their Fe-S clusters to ATC-I for delivery to their final destinations (61). However, this simple view, mostly derived from normal growth conditions, might change under stress conditions. Indeed, SufA is predicted to interact

directly with targets in such conditions, while ErpA could also interact with another type of carrier, NfuA, to form a stress-resistant heteromer (61). Fe-S clusters on holo-ErpA, holo-IscA, and holo-SufA are equally stable in the presence of dioxygen; however, the addition of the Fe-S cluster carrier NfuA to holo-ErpA nearly doubled the half-life of the ErpA Fe-S cluster (61). These data suggest that NfuA and ErpA may work in conjunction to form an oxidant-resistant Fe-S cluster delivery system. Altogether, the associations between NfuA and ErpA, as well as ErpA and apo-target proteins, supports the hypothesis that, under balanced aerobic conditions, ErpA conducts the last step of Fe-S cluster delivery in *E. coli*. As a matter of fact, with the exception of SoxR, *in vivo* maturation of all target proteins studied depended upon ErpA (see below) (62).

3.2. Nfu-type carriers. The C-terminal domain of the *A. vinelandii* NifU Fe-S cluster scaffold is referred to as Nfu (Figure 3B). The Nfu domain was demonstrated to bind and transfer a $[4Fe\text{-}4S]^{2+}$ cluster to an apo-protein (63). An alternate protein, named NfuA, contains a C-terminal domain with homology to the Nfu-domain of NifU, and an N-terminal domain with homology to an A-type carrier (Figure 3C). The NfuA A-type domain is referred to as “degenerated” because it lacks a cysteine required for Fe-S cluster ligation (64, 65). The C-terminal Nfu domain, but not the “degenerated” A-type domain, binds a $[4Fe\text{-}4S]^{2+}$ cluster and can transfer this cluster to apo-proteins. The Fe-S cluster bound by holo-NfuA is more stable in the presence of dioxygen than that of holo-A-type carriers suggesting a role for NfuA under oxidative stress conditions (61). Consistent with this hypothesis, strains lacking *nfuA* are defective in maturating Fe-S proteins facing oxidative stress (64, 65). Both the Nfu and A-type domains are necessary for NfuA function *in vivo* (64, 65). The A-type domain was demonstrated to function in targeting NfuA to apo-proteins (66, 67).

Three additional bacterial proteins consisting solely of a Nfu domain have been described. Nfu was necessary for the proper maturation of the Fe-S protein photosystem complex I (PS1) in *Synechococcus* and the maturation of several Fe-S proteins in *Staphylococcus aureus* and *Helicobacter pylori* (58, 68, 69). Holo-Nfu is a dimer with a bridging $[2Fe\text{-}2S]^{2+}$ or $[4Fe\text{-}4S]^{2+}$ cluster. Holo-Nfu could activate apo-PS1 and apo-aconitase. A *S. aureus nfu* mutant had decreased virulence in murine models of infection

and decreased survival in neutrophils. A *H. pylori nfu* mutant was defective in colonizing murine stomachs.

3.3. Mrp-type carriers. Genetic studies identified *apbC*, encoding a member of the Mrp class, as necessary to maturate the Fe-S enzymes ThiH and/or ThiC in *Salmonella enterica* (70). Biochemical studies demonstrated that ApbC can bind and effectively transfer Fe-S clusters to apo-proteins (71). ApbC is a dimer and each monomer contains two cysteines separated by two amino acids (C-X-X-C motif). These cysteines are thought to provide four ligands, two from each monomer, for ligation of a [4Fe-4S] cluster that bridges the dimer interface. Mrp proteins contain Walker A and B ATP hydrolysis motifs. An $ApbC_{K116A}$ variant was defective in ATP hydrolysis and inactive *in vivo*. The addition of ATP did not accelerate ApbC-directed cluster transfer *in vitro* and the $ApbC_{K116A}$ variant proficiently transferred Fe-S clusters. These data led to the hypothesis that ATP hydrolysis is required for loading ApbC with an Fe-S cluster [85]. ApbC was required for growth on the carbon source tricarballylate presumably because it functions in assembling the Fe-S enzyme tricarballylate reductase (TcuB) (72). The absence of ApbC could be bypassed by increasing expression of *iscU*, or by decreasing tricarballylate influx, and thereby preventing tricarballylate accumulation which inhibits isocitrate dehydrogenase (73, 74).

3.4. Monothiol glutaredoxins. As the name suggests, monothiol glutaredoxins lack the traditional dithiol C-X-X-C motif and instead have a C-G-F-S motif. The *E. coli grxD* encodes a monothiol glutaredoxin (75). Combining a *grxD* mutation with an *iscU* mutation resulted in synthetic lethality, suggesting that GrxD functions in conjunction with the SUF machinery to assembly Fe-S proteins (*E. coli* must have functional SUF or ISC for viability (7)). GrxD purified from *E. coli* contained a [2Fe-2S] cluster. To chemically reconstitute an Fe-S cluster on apo-GrxD the reaction mixture required glutathione (GSH) (76). GrxD binds a [2Fe-2S] cluster that bridges a homodimer interface using one cysteine ligand from each monomer and GSH thiolates provide two additional ligands (77). Holo-GrxD homodimer can transfer an Fe-S cluster to apo-Fdx forming the [2Fe-2S] holo-Fdx. GrxD was recently shown to cooperate with NfuA in the maturation of the Fe-S enzyme MiaB (78).

E. coli BolA is an ortholog of *Saccharomyces cerevisiae* Fra2, which forms a heterodimer with a monothiol glutaredoxin to bind a [2Fe-2S] cluster [89, 92]. When purified from *E. coli*, BolA co-purifies with GrxD and vice versa. An Fe-S cluster could not be reconstituted on BolA; however, an Fe-S cluster could be reconstituted on the BolA-GrxD heterodimer and the cluster could be transferred to apo-Fdx (76). *E. coli bolA* and *grxD* mutants do not phenocopy one another suggesting that they can also function independently.

4. Auxiliary factors utilized in iron-sulfur protein maturation.

Several loci have been identified that function in the assembly of Fe-S proteins but are not considered part of the core Fe-S cluster biosynthetic apparatus. These factors are not typically found within operons encoding for the core ISC, SUF, or NIF machineries.

4.1. SufT. The *sufT* gene is often associated with *suf* operons (defined by having *sufB* and *sufC*) in bacterial and archaeal genomes (79). Typically, SufT proteins, such as those encoded by *S. aureus* and *B. subtilis*, are composed entirely of a domain of unknown function 59 (DUF59). Larger proteins containing a DUF59 domain have roles in Fe-S cluster assembly, including the eukaryotic cytosolic Fe-S cluster assembly (CIA) factor CIA2, which functions in the maturation of nuclear and cytosolic Fe-S proteins (80-82). *S. aureus* strains lacking SufT have decreased activities of Fe-S enzymes during conditions requiring a high demand for Fe-S clusters. The phenotypes associated with the Δ*sufT* and Δ*nfu* mutations were synergistic (79, 83). Moreover, overproduction of Nfu mitigated the phenotypes of the Δ*sufT* strain. These data suggest that SufT functions in Fe-S carriage and has some degree of functional overlap with Nfu; however, Fe-S cluster binding by SufT remains elusive. SufT was reported to be essential in *Mycobacterium tuberculosis* (84).

4.2. Low molecular weight (LMW) thiols. The role of LMW thiols in Fe-S protein assembly is likely multifaceted and they could function in all four steps of Fe-S protein assembly: biogenesis, trafficking, assembly, and repair. In eukaryotes, glutathione (GSH) has been associated with the synthesis and trafficking of Fe-S clusters from the mitochondrion to the cytosol (85, 86). In bacteria, genetic and biochemical studies

demonstrated a role for GSH in assembling Fe-S proteins, in addition to its roles in maintaining proper intracellular redox (87). GSH can act as an Fe buffer by binding non-incorporated cytosolic Fe (88, 89). GSH can also provide electrons to reduce Fe^{3+} to Fe^{2+} (90). GSH, in conjunction with monothiol glutaredoxins, delivers Fe-S clusters to the apoprotein targets (77, 91, 92). GSH can bind and deliver Fe-S clusters *in vitro*, but the *in vivo* relevance of this chemistry is unknown (93). GSH can also reduce oxidized protein cysteine residues before Fe-S cluster insertion.

Many microorganisms, including *S. aureus*, do not produce GSH but instead produce the LMW thiol bacillithiol (BSH) (94). A *S. aureus* strain defective in producing BSH exhibits phenotypes similar to cells lacking Fe-S cluster carriers including decreased activities of Fe-S dependent enzymes (95). The phenotypes of a BSH minus strain were suppressed by multicopy expression of *sufA* or *nfu*, but not by overexpression of the SUF system. These data suggest that the phenotypes of a BSH minus strain were not the result of faulty *de novo* Fe-S synthesis, but rather defective assembly or repair of Fe-S proteins (96). A strain lacking BSH did not appear to suffer from decreased ROS metabolism, but a protective role for BSH in buffering against metal ion poisoning of Fe-S enzymes or the maturation machinery has not been ruled out (97).

4.3. Folic acid-binding protein (YgfZ). *E. coli* YgfZ (COG0354) is a homolog of yeast Iba57p, which is a mitochondrial protein that participates in the assembly of mitochondrial Fe-S proteins (98). A Δ*ygfZ* mutant strain has decreased activities of selected Fe-S enzymes including MiaB and is sensitive to ROS stress (99). YgfZ binds tetrahydrofolate (THF) and an *E. coli* strain lacking the ability to synthesize folate has similar MiaB activity as a Δ*ygfZ* mutant suggesting that folate, as well as YgfZ, is utilized in assembling some Fe-S proteins. The COG0354 proteins are paralogous to enzymes that utilize THF to accept formaldehyde units leading to the hypothesis that YgfZ functions to remove one-carbon units that deleteriously affect the functions of Fe-S proteins (100).

4.4. Repair of iron clusters (RIC) proteins. The *E. coli ytfE* encodes a RIC protein that has increased expression during nitric oxide stress (101). The expression of *ytfE* is directly controlled by NsrR, which directly responds to nitric oxide ($NO^{\bullet}$) levels (102, 103). An *E. coli ytfE* mutant is sensitive to $NO^{\bullet}$ or H_2O_2 stress and Fe-S enzymes have decreased activities after cell-free extracts from the *ytfE* mutant are treated with hydrogen

peroxide (H_2O_2) or $NO^{\bullet}$. Importantly, damaged Fe-S proteins had a slower rate of repair in the *ytfE* mutant (104). YtfE, and its homologs, are di-Fe hemerythrin-like proteins (105, 106). The Fe atoms of holo-YtfE are labile and can be used as an Fe source for Fe-S cluster synthesis *in vitro* (107). These findings resulted in renaming these proteins as "repair of iron clusters (RIC)". The mechanism by which RIC proteins may repair damaged Fe-S clusters is unknown. YtfE interacts with the Fe scavenger Dps *in vivo*, and their corresponding genes have genetic interactions. These findings led to the hypothesis that Dps may be providing Fe to YtfE to be used for the repair of damaged Fe-S clusters (108). In *S. aureus*, *ytfE* (*scdA*) and *dps* protect against H_2O_2 damage and are both transcriptionally regulated by SrrAB which responds to electron flux through respiratory pathways (109, 110).

Physiological, genetic, and biochemical data suggest that a *ytfE* mutant has more $NO^{\bullet}$-induced damage and reduced activity of the Fe-S cluster utilizing transcription factor NsrA. Structural data show that YtfE has a hydrophobic channel where $NO^{\bullet}$ could access the Fe ions and the Fe atoms have been shown to ligate $NO^{\bullet}$ (106, 111). These data support the hypothesis that YtfE functions in S-trans-nitrosylation or the removal of $NO^{\bullet}$ from nitrosylated proteins (112). However, YtfE was not able to release $NO^{\bullet}$ from nitrosylated fumarase, a [4Fe-4S] requiring dehydratase. YtfE contributes to *Y. pseudotuberculosis* and *Haemophilus influenzae* pathogenesis (113, 114).

5. Regulation of Iron-Sulfur Cluster Synthesis

Fe-S cluster biosynthesis is controlled by regulators that sense environmental conditions potentially adverse for Fe-S cluster assembly such as Fe limitation, or oxidative and nitrosative stress, which affect the stability and integrity of the cofactors. Integrating these stimuli with Fe-S synthesis ensures demand is met and fitness is maintained.

5.1. The situation in *E. coli*: adapting to fluctuating conditions and switching between machineries.

In *E. coli*, Fe-S cluster biosynthesis is achieved by using two types of machineries, ISC and SUF, which permit maturation of the same set of apo-proteins under a wide

386 breadth of growth conditions. Particularly, genetic control circuits occur that endow *E. coli*
387 with the capacity to synthesize one or the other machinery in different growth conditions,
388 and thereby have the Fe-S cluster biogenesis capacity match the Fe-S demand,
389 regardless of the growth conditions. Two transcriptional regulators, IscR and Fur, and a
390 non-coding RNA, RyhB, are key actors in orchestrating this adaptative response, as all
391 three control, directly or indirectly, expression of the *isc* and *suf* operons.

392 5.1.1. IscR-mediated Fe-S cluster homeostasis control. IscR is a transcription
393 factor (TF) that belongs to the Rrf2 family of winged helix-turn-helix TFs. It hosts a [2Fe-
394 2S] cluster, which allows sensing aerobiosis, oxidative stress, iron limitation, and possibly
395 reactive nitrogen species. Mutagenesis and structural studies have identified residues
396 Cys92, Cys98, Cys104, and His107 as Fe-S cluster ligands (Figure 4A) (115, 116). A His
397 ligand is uncommon, and this might render the cluster labile and sensitive to stress
398 signals. It is particularly useful for the IscR regulator since its activity is not influenced by
399 the oxidative state of its cluster but is strictly dependent on the presence/absence of the
400 cluster (116, 117). IscR is found in the apo- and holo-forms and both types can have
401 regulatory functions (118).

402 Two types of binding sites, type 1 and 2, are found within IscR-regulated
403 promoters. Holo-IscR binds type 1 sites and the holo- and apo-forms bind the type 2 sites
404 (Figure 4B) (115). The type 1 inverted repeat sequence is well conserved and is mainly
405 found upstream genes encoding Fe-S building proteins (the *isc*, *erpA*, and *nfuA* loci)
406 (119). The type 2 sequence is an imperfect palindrome and highly degenerated, which
407 causes large variations in binding affinities between operator regions (119). Interestingly,
408 while the type 1 sequence containing promoters are all repressed by IscR, genes
409 preceded by a type 2 sequence can be either repressed or activated by IscR. This does
410 not correlate with the position of the IscR binding site as shown with the *hyaA* and the
411 *sufA* genes. Both promoters exhibit a type 2 sequence within their -35 consensus
412 promoter sequence, yet show opposite expression patterns as *hyaA* expression is
413 repressed by holo-IscR whereas *sufA* expression is activated by apo-IscR (119).

414 In *E. coli*, *iscR* is the proximal gene in the *isc* operon and is separated from the
415 next gene (*iscS*) by an unusually long untranslated region that is targeted by RyhB, a
416 non-coding RNA (see below). Under balanced conditions, IscR is maturated by the ISC

machinery and holo-IscR acts as a repressor of its own expression, as well as the expression of downstream *isc* genes, until equilibrium shifts towards a low level of apo-IscR. At this point, repression is alleviated, more IscR is synthesized, and the ISC machinery is produced, resulting in an increased level of holo-IscR in the cell. A feedback loop is then set up and holo-IscR represses its own expression as well as those of the following *isc* genes. Upon an increase in the cellular demand for Fe-S cluster synthesis (iron limitation, oxidative stress), there is a competition between apo-protein substrates and newly synthesized apo-IscR for the ISC machinery. Then, apo-IscR accumulates, and this form activates the expression of the *suf* operon (120). In summation, IscR represses transcription of *isc* and activates *suf* transcription in its Fe-S-bound and unbound forms, respectively, directly connecting both cell's Fe-S cluster biogenesis capacity and Fe-S cluster demand (Figure 4C).

Evidence has been provided that the efficiency of ISC proteins, in particular IscU, would be lowered under stress conditions, opening the possibility that the contribution of the ISC machinery declines under such conditions and the cell would rather switch from ISC to SUF, rather than cumulating both (25). Importantly, IscR appears to be a poor substrate for the SUF system, and therefore, IscR is likely to remain mostly in its apo-form if the cell thrives under stress conditions (54). The iron responding Fur-RhyB genetic circuit also favors such a switch (see below).

5.1.2. Iron-mediated control of Fe-S biogenesis by Fur and RyhB. Iron availability is sensed by the transcriptional regulator Fur, which represses synthesis of the non-coding RNA, RyhB, among others. Because both Fur and RyhB regulate the expression of the *isc* and *suf* operons, directly or indirectly, they are likely to contribute to the switch between the ISC and SUF machineries as a mode of adaptation to iron bioavailability. Holo-Fur acts as a repressor of *suf* operon transcription. Under iron limiting conditions, the Fe^{2+} cofactor of Fur is lost, and repression is alleviated, providing an opportunity for IscR-dependent *suf* operon expression (121). Meanwhile, *ryhB*, which is also repressed by holo-Fur, is expressed and targets the intergenic region between *iscR* and *iscS*, causing translation inhibition of the downstream *iscSUA* genes, and probably mRNA decay whereas a stem-loop structure forms enabling stabilization of the upstream *iscR* messenger moiety (Figure 4C) (122). Under these conditions, apo-IscR accumulates and

activates expression of the *suf* operon. Consequently, iron limitation enhances expression of the *suf* operon by both alleviating Fur repression and favoring apo-IscR activation, while expression of the *iscSUA* is shut off by RyhB-mediated translation inhibition and possibly poor activity of the encoded IscU scaffold protein. It should be noted that a recent study suggests that Fur senses iron homeostasis by binding a [2Fe-2S] cluster instead of Fe^{2+} as it is currently suggested (123). If this observation were to be confirmed *in vivo*, it would link the Fur repressing activity to both iron availability and Fe-S biogenesis, in which case the interplay between IscR and Fur would be an important issue to decipher *in vivo*.

5.1.3 Switching the Fe-S cluster synthesis machineries under redox stress. The OxyR transcriptional regulator activates the expression of *suf* genes in response to H_2O_2 (Figure 4C). The OxyR binding site is located far upstream from the *sufA* promoter (operon *sufABCDSE*), and the OxyR-dependent activation requires the binding activity of IHF to bring the OxyR site closer to the -10 and -35 promoter elements (124). OxyR and apo-IscR mediated activations have been found to be additive (120). Thus, because oxidative stress could well favor the shift from holo-IscR to apo-IscR, it is possible, that under such conditions, *E. coli* cumulates synthesis of both ISC, following alleviation of IscR repression, and SUF, following activation by OxyR. However, oxidative stress is known to lower iron bioavailability (by oxidizing Fe^{2+} to Fe^{3+}). This could alleviate holo-Fur-dependent repressions, leading to *ryhB* expression and subsequently *isc* repression, thereby preventing the accumulation of both systems. Moreover, IscU activity was reported to be altered by oxidative stress (25). Thus, under oxidative stress, a genetic switch, like that observed under iron limiting conditions, might prevail. Expression of the *suf* operon is also repressed by the [4Fe-4S] cluster containing transcription factor NsrR under normal growth conditions. Under nitrosative stress, the Fe-S cluster is lost, and NsrR-dependent repression is alleviated (125). Again, because IscR could shift from holo to apo under such redox stress conditions, a shift between the machinery could take place.

5.1.4. Switching between machineries promotes antibiotic tolerance. One phenotypic consequence of the stress-controlled switch between the ISC and SUF systems is enhanced resistance to aminoglycoside antibiotics (126). The uptake of aminoglycosides is dependent upon the proton motive force (PMF), and therefore, the

bactericidal activity of these antibiotics is proportional to respiration efficiency. Mechanistic causes of the aminoglycoside tolerance resulting from iron limitation are: (i) down and up regulation of ISC and SUF, respectively, by the IscR- and Fur/RyhB-dependent controls described above, (ii) inefficient maturation of Respiratory Complexes I and II by the SUF system resulting in decreased respiratory efficiency, and (iii) Fur/RyhB-dependent downregulation of Complex I and II synthesis (126, 127).

Another illustration of the link between switching machinery and antibiotic tolerance is demonstrated in the case of fluoroquinolones. Exposing *E. coli* to phenazine methosulfate (PMS), a redox cycling compound that causes oxidative stress and NAD(P)H exhaustion, yielded an enhanced tolerance to norfloxacin, a DNA gyrase inhibitor (62). Under PMS exposure, *E. coli* switches to the SUF system, which can target Fe-S clusters to the transcriptional activator SoxR. The Fe-S cluster bound to SoxR gets oxidized and allows SoxR to activate *soxS* transcription. SoxS then activates expression of *acrAB,* encoding an efflux pump, which exports fluoroquinolones out of the cell.

5.2. A feed forward loop mediated by IscR and RyhB.

Acting at both transcription and translation initiation permits finely tuned gene expression. An example is given by the dual control afforded by IscR and RyhB on the expression of *erpA* in *E. coli*. As mentioned above, *E. coli* synthesizes multiple Fe-S carriers and whether they have a degree of functional redundancy has been a matter of debate. Transcription of *erpA* is repressed by holo-IscR while *erpA* mRNA translation is negatively regulated by RyhB (59). These data led to the hypothesis that ErpA is synthesized neither under Fe replete conditions (repression by IscR) nor Fe limitation conditions (inhibition by RyhB). This double control allows ErpA synthesis within a window of intermediate Fe concentrations. The added value of this double control is that ErpA is synthesized under conditions in which neither of the two other A-type carriers, SufA and IscA, are fully synthesized. This control ensures a continuing presence of at least one carrier throughout fluctuating iron concentrations (59).

5.3. IscR as a sensor of the anaerobic/aerobic switch.

Regulation of gene transcription often involves multiple transcriptional regulators, which might compete (or synergize) for closely located operator sites and modify the importance of each other's influence. The influence of IscR on alternate regulators is well documented by an unexpected role of IscR in cell-to-cell variability during the shift from dioxygen respiration to trimethyl amine oxide (TMAO) respiration. The genes that encode TMAO reductase are under the transcriptional control of the TorT/TorS/TorR three-component regulatory system (128, 129). In the presence of dioxygen, *torT/S* expression is repressed by IscR and the level of TorT/S is so low that a stochastic effect prevails leading to cell-to-cell variability in TMAO reductase synthesis. In contrast, under anoxic TMAO-respiring conditions, IscR titers decrease, and *torT/S* expression is derepressed. Levels of TorT/S are now high enough to cancel any effect from stochasticity in gene expression. Therefore, IscR is determining in this “regulated stochasticity” by acting upstream in the cascade, controlling the level of TorT/S, and mediating the oxygen regulation of cell-to-cell variability (130).

5.4. The role of IscR in pathogenic bacteria

IscR is widely conserved and was studied in several bacteria including the pathogens *Erwinia chrysanthemi* (18), *Pseudomonas aeruginosa* (131), *Burkholderia mallei* (132), *Vibrio vulnificus* (133), *Salmonella enterica* (134), and *Yersinia pseudotuberculosis* (135). Because Fe-S-based biology is central to cellular bioenergetics and metabolism, it is expected to be important for bacterial fitness and multiplication within its host. Moreover, both iron limitation and oxidative stress are conditions met by pathogens during host colonization suggesting that IscR may be instrumental in coordinating adaption.

Less expected, however, was that IscR would directly control the synthesis of key virulence determinants as it was reported in both *S. enterica* and *Yersinia pseudotuberculosis*. Both pathogens rely on type 3 secretion systems (T3SS) utilized to inject effectors in the host cells and IscR controls synthesis of T3SS in both bacterial species. *S. enterica* synthesizes two T3SS, referred to SPI1 and SPI2. SPI1 is required for the passage of the bacterium across the epithelial border while SPI2 is required to establish a *S. enterica*-containing vacuole in macrophages. The *spi1* locus includes *hilD*,

encoding a major virulence regulator, which controls its own synthesis and that of effectors. A type 2 IscR binding site is present upstream of *hilD* and IscR binding was proposed to interfere with HilD positive autoregulation, thereby lowering virulence (134). Consistently, an *iscU* mutant, which has a high level of apo-IscR, exhibited reduced invasion capacity in epithelial cells and attenuated virulence in a murine model of infection. Conversely, an *iscR* mutant was hyper-invasive in HeLa cells (134). In *Y. pseudotuberculosis* IscR binds a type 2 motif within the promoter of a gene encoding the transcription factor LcrF. The *lcrF* gene is located in the virulence plasmid pYV that also encodes a T3SS. LcrF regulates transcription of the T3SS secreted effectors genes, and thereby, virulence. IscR was essential for T3SS-dependent secretion and an *iscR* mutant was deficient in colonization of the Peyer's patches, spleen, and liver in murine models (135). In *V. vulnificus* IscR directly activates expression of the *vvhBA* genes encoding a cytolysin in response to host-derived signals such as nitrosative stress and iron starvation (136). In *E. coli* some fimbriae genes are directly regulated by IscR, such as *cfaA* and *fimE* (137, 138). And lastly, IscR has been shown to coordinate oxidative stress resistance during pathogenesis in *Pseudomonas aeruginosa* and *Xanthomonas campestris* (131, 139).

5.5. Regulation of Fe-S biogenesis by SufR.

SufR, first described in Cyanobacteria, is another Fe-S biogenesis-dedicated transcriptional regulator (140). Interestingly, while Cyanobacteria have the two main Fe-S biogenesis machineries, ISC and SUF (and sometimes the NIF system dedicated to nitrogenase maturation), the IscR regulator only regulates transcription of the *isc* locus. The expression of the *suf* locus is under the transcriptional control of its own regulator, SufR. SufR belongs to the DeoR family of helix-loop-helix regulators. Its DNA binding domain is located in the N-terminal portion of the protein and it has a non-conventional Fe-S binding site in the C-terminal portion (C-X_{12}-C-X_{13}-C-X_{14}-C) where a [4Fe-4S] cluster is coordinated (141). Holo-SufR is a repressor of the *suf* locus, thereby down-regulating its own expression. It binds a perfect palindromic sequence (CAAC-N6-GTTG) that is highly conserved in the promoter regions of *suf* loci in Cyanobacteria (141). SufR regulatory activity is sensitive to redox stress, oxidative stress, and iron starvation (140,

141). It is interesting to note that the SUF system appears to be the most important in Cyanobacteria and all the genes of the *suf* locus are essential. This could be why a dedicated regulator controls *suf* expression. Most of the Gram-positive bacteria possess only the SUF system; however, SufR seems to be under-represented with only two examples described in Actinobacteria: *Mycobacterium tuberculosis* and *Streptomyces avermitilis* (142, 143). How the *suf* locus is regulated in most of the Gram-positive bacteria lacking SufR is unknown.

Overall, IscR, and to a lower extent SufR, appear to have primary functions as regulators of Fe-S biosynthesis. IscR is conserved among the bacterial species producing an ISC machinery and coordinates Fe-S biosynthesis with other cellular functions including pathogenesis. In contrast, SufR was only found in Cyanobacteria and some Actinobacteria and is dedicated to regulating the *suf* locus. Both regulators coordinate Fe-S biogenesis to Fe-S bioavailability, and they are assisted in this task by stress specific regulators such as Fur for iron availability, OxyR for oxidative stress, and NsrR for nitrosative stress.

6. Iron-sulfur protein assembly as an anti-pathogen target.

The susceptibility of bacteria to host-distributed chemicals such as copper (Cu) ions, ROS, and RNS, which act, in part, to poison the Fe-S cluster requiring proteins, implies that higher eukaryotes have evolved to prevent bacterial growth by targeting Fe-S protein assembly (5, 144, 145). High-density transposon screens or directed mutagenesis studies suggest that the assembly of Fe-S proteins is essential for many human bacterial pathogens (6). Importantly, microbes synthesize Fe-S clusters using machineries that are functionally similar but biochemically distinct from the machineries used by higher eukaryotes. Bacteria defective in maturating Fe-S proteins have decreased virulence or fitness in models of infection (6, 58, 146). An inability to assemble Fe-S proteins affects numerous metabolic pathways resulting in metabolic chaos. These facts imply that Fe-S protein assembly is a viable target for antimicrobial therapy.

As an example, a small molecule called ‘882 decreased the activity of aconitase *in vivo*, but not *in vitro* (147). A “pull-down” assay using immobilized ‘882 as bait found that

it associates with SufBCD. SufC associated with ‘882 with a K_d for ‘882 of ~3 μM. These data led to the hypothesis that ‘882 inhibited Fe-S protein assembly by inhibiting Suf-dependent Fe-S cluster synthesis. The Suf system has also been proposed to be a target for other non-bacterial pathogens including *Toxoplasma gondii* and *Plasmodium falciparum* (148). For the latter, the molecule D-cycloserine, which can form a covalent adduct with PLP, can inhibit the cysteine desulfurase SufS resulting in growth inhibition (149).

7. Future directions.

New Fe-S cluster assembly factors are continually being discovered lending support to the hypothesis that additional factors exist and that our current knowledge is incomplete. To move forward, we need to broaden our approaches by using newly available techniques and expand the organisms studied. Studies using *E. coli* and *A. vinelandii* have provided the bulk of the information about how bacteria assemble Fe-S proteins. These Gram-negative organisms are relatively unique in the fact that they have more than one biosynthetic system, which are, for the most part, functionally redundant. In contrast, very few studies have been conducted on Fe-S cluster assembly in Gram-positive bacteria, which typically encode only one Fe-S cluster biosynthesis system.

Several questions remain about Fe-S protein maturation and its regulation. The Fe and electron donors for Fe-S cluster synthesis and repair remain elusive. We also do not fully understand the mechanism by which Suf synthesizes Fe-S clusters, or the functions of many factors utilized for maturating Fe-S proteins. We need to increase our understanding of how the Fe-S cluster assembly machinery is integrated with metabolic pathways that require Fe-S proteins. It is not well understood if Fe-S cluster carriers transfer Fe-S clusters to all apo-targets with the same efficacy or if there is an apo-protein hierarchy driven by carrier specificity. Understanding this integration will provide insights into metabolite balance and the consequences of decreasing metabolic flux through a pathway that requires an Fe-S protein since it is a costly process for the cells (150). This knowledge will not only be important for medicine and the development of specific anti-pathogen targets, but also for scientists using organisms to conduct green chemistry.

633 Inefficient Fe-S protein maturation, such as in organisms engineered to produce biofuels,
634 fix dinitrogen, or generate secondary metabolites, could decrease the yields and the
635 efficiency of desired processes, ultimately decreasing profits and productivity (151).
636

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Figure Legends.

Figure 1. General mechanism of bacterial Fe-S protein assembly. Monoatomic Fe^{2+} and S^0 are combined with electrons on a proteinaceous molecular scaffold forming an Fe-S cluster. The Fe-S cluster is transferred to one or more carrier proteins before being transferred to an apo-protein forming a holo-protein. Reactive oxygen species (ROS) can either damage the Fe-S cluster, which can subsequently be repaired, or destroy it, resulting in apo-protein formation.

Figure 2. Iron-sulfur cluster synthesis. Panel A. Structure of IscU from *Thermus thermophiles* (PDB: 2qq4). The grey ball is a Zn(II) ion and the three ligating cysteines are highlighted. Panel B. The structure of $SufBC_2D$ from *Escherichia coli* (PDB: 5awf). SufC is shown in green and SufB and SufD are shown in purple and tan, respectively. Panel C. Working models for ISC and SUF-directed iron-sulfur protein maturation in *Escherichia coli*.

Figure 3. Iron-sulfur cluster carriage. Panel A. structure of the A-type carrier IscA from *Thermosynechococcus elongatus* (PDB: 1x0g) with [2Fe-2S] cluster bound. Panel B. Structure of Nfu from *Staphylococcus epidermidis* (PDB: 1xhj). The cysteine thiols that are proposed iron-sulfur cluster ligands are highlighted. Panel C. A schematic representation of iron-sulfur cluster scaffolds and carriers.

Figure 4. Regulation of iron-sulfur cluster synthesis in *Escherichia coli*. Panel A. An X-ray structure of apo-IscR monomer with the proposed Fe-S cluster ligands (C92A, C98A, C104A, and H107) are highlighted in red (PDB: 4hf1). Note that in this IscR variant the ligating cysteines have been changed to alanines. Panel B. An X-ray structure of dimeric apo-IscR bound to the *hya* promoter which is a type 2 binding site (PDB: 4hf1). Each monomer is differently colored (blue and pink). Panel C. Model for the regulation of ISC and SUF expression in *Escherichia coli.*

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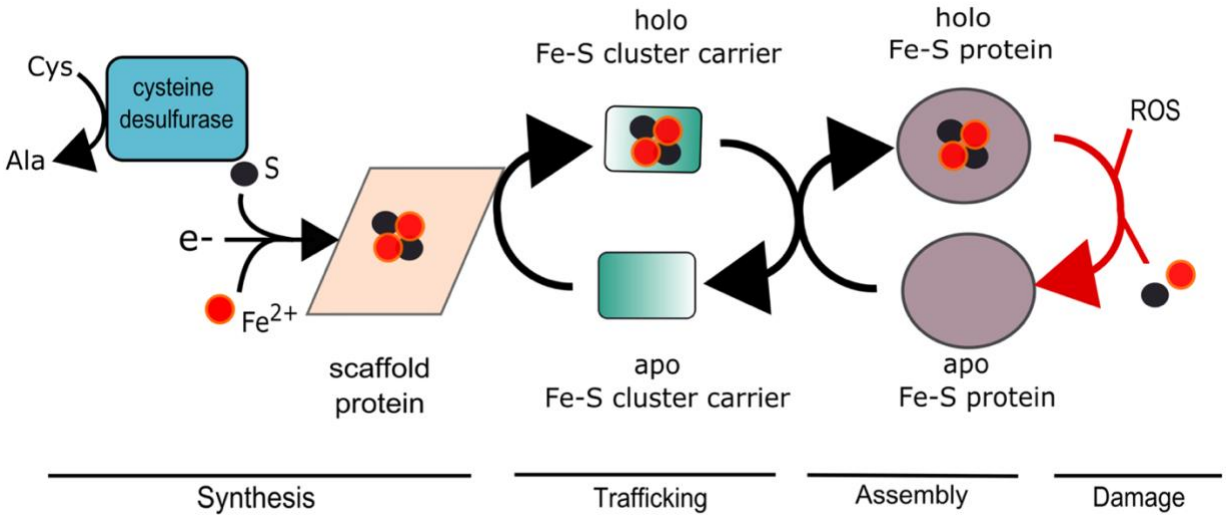

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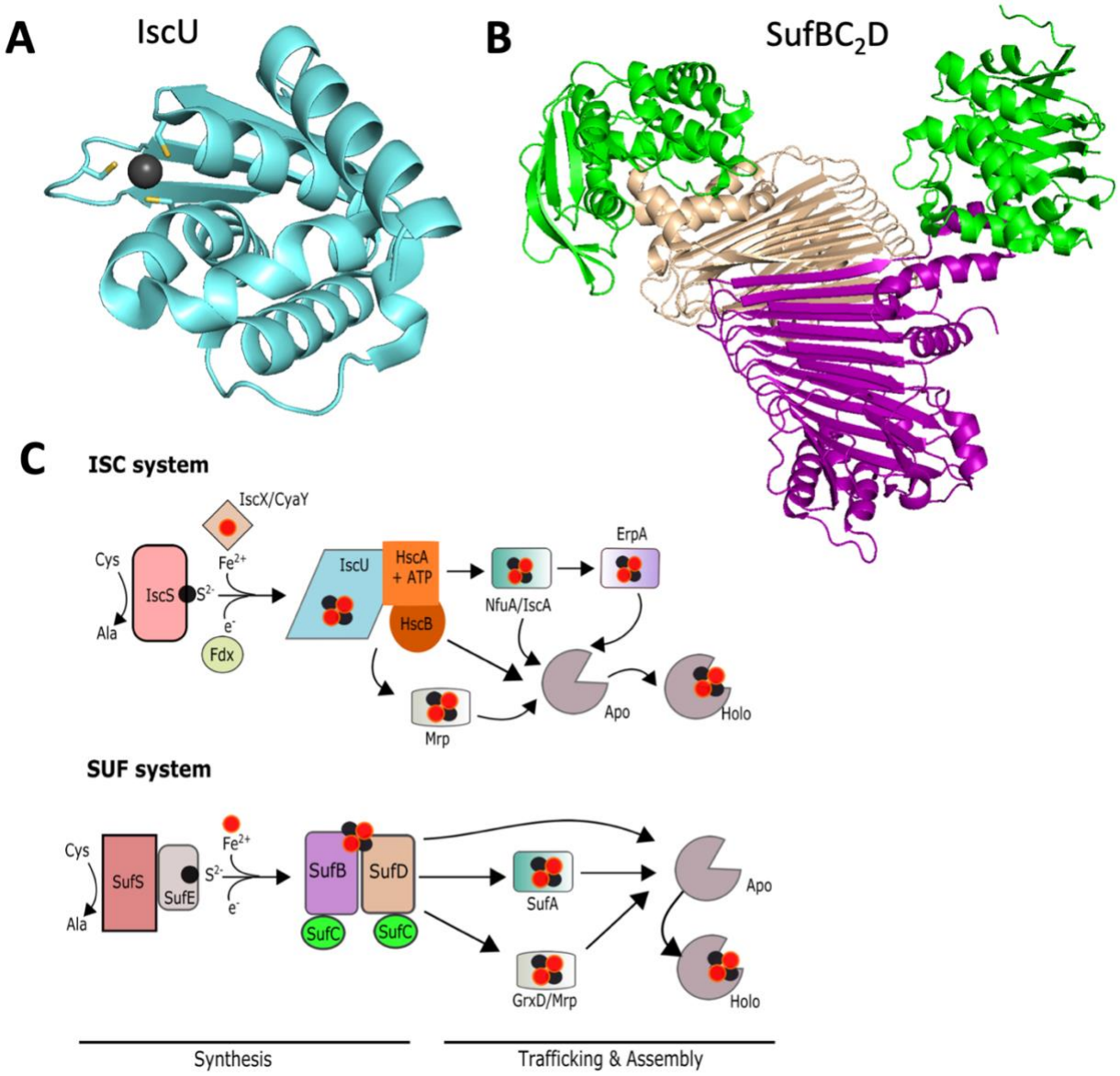

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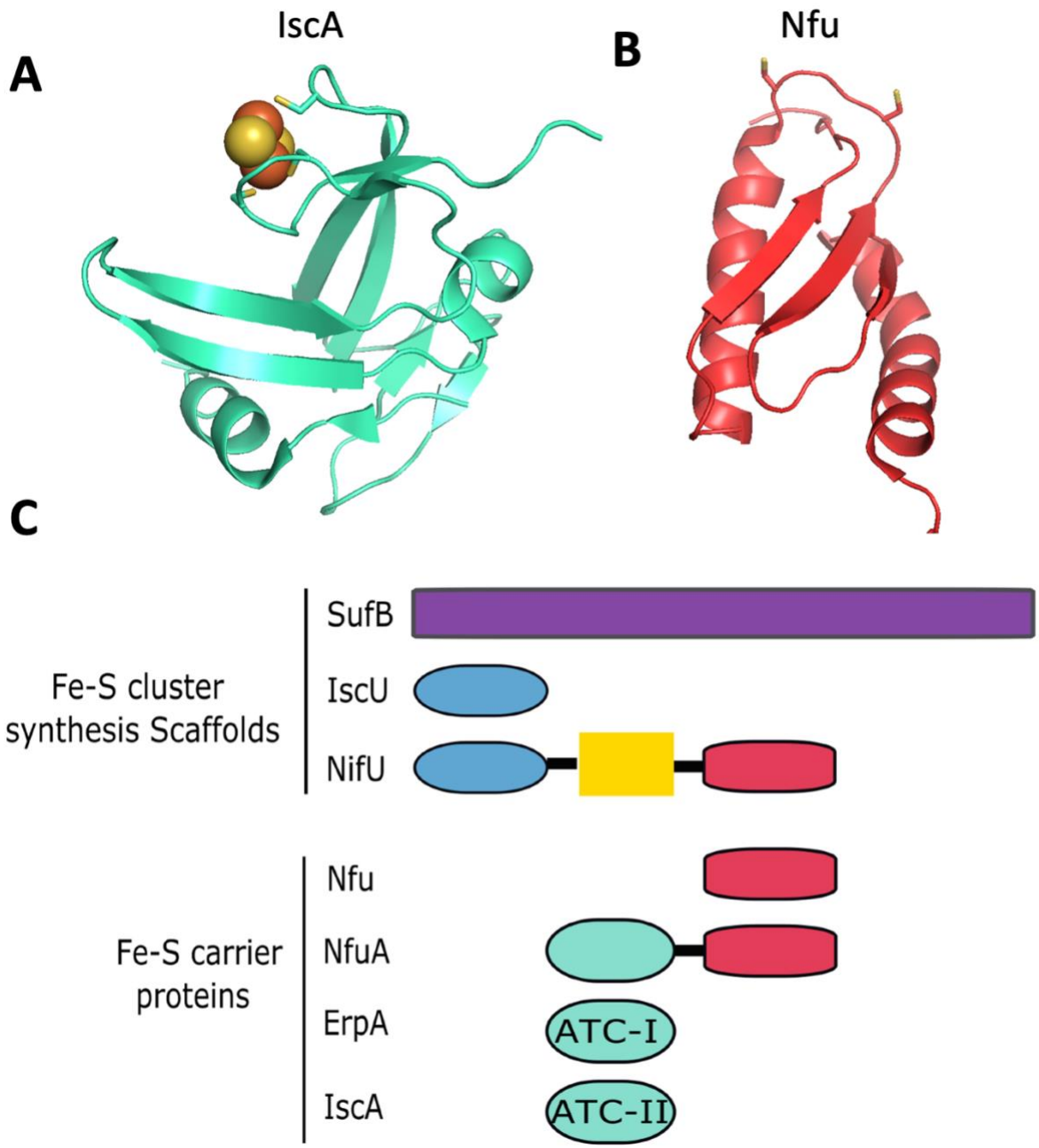
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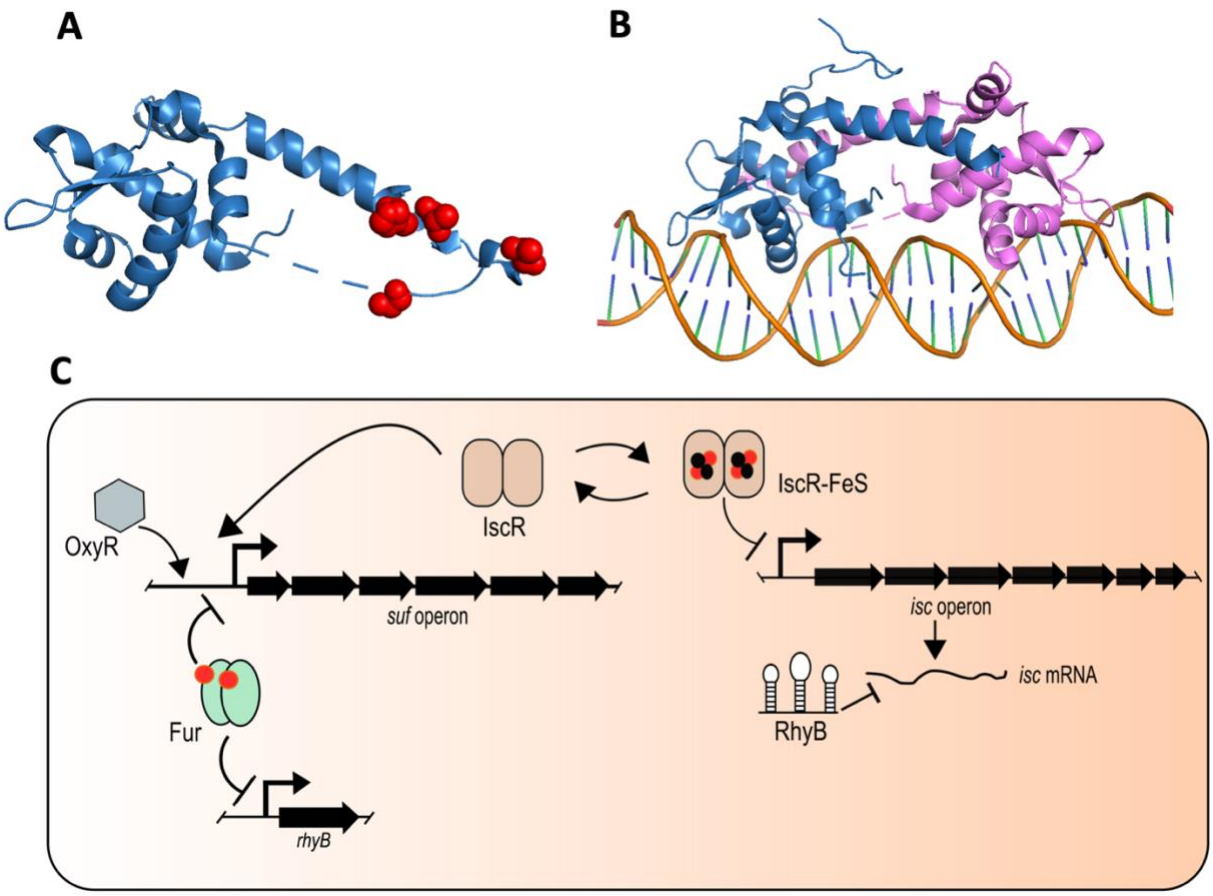
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