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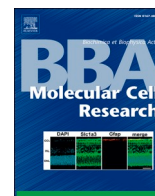
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Review

Harnessing iron-sulfur enzymes for synthetic biology

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ABSTRACT

Reactions catalysed by iron-sulfur (Fe-S) enzymes appear in a variety of biosynthetic pathways that produce valuable natural products. Harnessing these biosynthetic pathways by expression in microbial cell factories grown on an industrial scale would yield enormous economic and environmental benefits. However, Fe-S enzymes often become bottlenecks that limits the productivity of engineered pathways. As a consequence, achieving the production metrics required for industrial application remains a distant goal for Fe-S enzyme-dependent pathways. Here, we identify and review three core challenges in harnessing Fe-S enzyme activity, which all stem from the properties of Fe-S clusters: 1) limited Fe-S cluster supply within the host cell, 2) Fe-S cluster instability, and 3) lack of specialized reducing cofactor proteins often required for Fe-S enzyme activity, such as enzyme-specific flavodoxins and ferredoxins. We highlight successful methods developed for a variety of Fe-S enzymes and electron carriers for overcoming these difficulties. We use heterologous nitrogenase expression as a grand case study demonstrating how each of these challenges can be addressed. We predict that recent breakthroughs in protein structure prediction and design will prove well-suited to addressing each of these challenges. A reliable toolkit for harnessing Fe-S enzymes in engineered metabolic pathways will accelerate the development of industry-ready Fe-S enzyme-dependent biosynthesis pathways.

1. Introduction

Iron-sulfur (Fe-S) clusters are versatile cofactors that appear in a wide variety of proteins. Enzymes bearing Fe-S clusters catalyse reactions including dehydration, oxidoreduction, and hydride and methyl group transfer. Electron carrier proteins known as ferredoxins support many other cellular functions using Fe-S clusters with readily-tuneable properties. Fe-S enzymes and proteins are thus of considerable interest for synthetic biologists and metabolic engineers. Fe-S enzymes and Fe-S electron carriers appear in biosynthetic pathways that generate high-value natural products including antibiotics [1–7], antivirals [8], and vitamins [9–11], and commodities such as hydrogen [12] and bioavailable nitrogen [13]. Harnessing these pathways by expression within genetically-tractable hosts could open the door to producing antibiotics from uncultivated organisms [14], shift our economy away from reliance upon petrochemicals [15], and liberate agriculture from its dependency upon synthetic nitrogen fertilizer [16].

Unfortunately, Fe-S enzymes have a difficult reputation among bioengineers. In overexpressed biosynthetic pathways, it is often the Fe-S enzymes that form insoluble aggregates or limit product formation

[17,18]. Nevertheless, various obstacles to harnessing Fe-S enzymes have been identified and can be overcome. These success stories demonstrate that engineering metabolic pathways that rely upon Fe-S enzymes is possible provided these obstacles are anticipated and addressed. Synthetic biologists hoping to harness Fe-S enzymes must understand the fundamentals of Fe-S cluster assembly and maintenance and be familiar with the approaches specifically developed to overcome those obstacles.

This review examines the use of Fe-S enzymes and proteins in synthetic biology. As the synthetic biology field is very broad, we focus upon harnessing Fe-S enzymes for metabolic engineering: specifically, the construction of artificial biosynthetic pathways that are expressed in heterologous hosts with the aim of producing high quantities of a natural product [19–21]. Our goals are to introduce synthetic biologists to Fe-S cluster biology and to explain the aims of synthetic biology to Fe-S scientists. First, we examine what synthetic biologists require from Fe-S enzymes by reviewing what an engineered metabolic pathway must achieve before it can be considered useful for producing chemicals on a commercial scale. Conversely, we examine what Fe-S enzymes require from synthetic biologists by briefly reviewing Fe-S cluster assembly and

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delivery. Second, we discuss pitfalls encountered by Fe-S enzyme biochemists and synthetic biologists over the years and how those pitfalls have been addressed. Finally, we examine efforts to engineer heterologous nitrogenase activity as a case study to illustrate how multiple facets of Fe-S enzyme expression can be addressed in a single system. We avoid detailed explanations of individual mechanisms, pathways, and enzymes. Instead, we highlight the various tools available for enabling and improving Fe-S enzyme activity. Many of the tools useful for synthetic biologists derive from heroic work of Fe-S biochemists who overexpress active Fe-S enzymes for *in vitro* characterization. Readers interested in the fundamental science of Fe-S enzymes, the uses of metabolic engineering, and background of specific metabolic pathways will be directed towards more targeted reviews.

2. Background for synthetic biologists and Fe-S enzyme biologists

2.1. What successful metabolic engineering requires

Intrepid Fe-S enzyme biochemists have for many decades successfully cloned, overexpressed, and purified Fe-S enzymes and proteins. Nevertheless, metabolic engineers excited to produce a valuable chemical in their favourite chassis become apprehensive when they find an Fe-S enzyme in their biosynthesis pathway. This is because metabolic engineering presents challenges distinct from Fe-S enzyme overexpression. Ultimately, the goal of metabolic engineering is to engineer a microbial strain that can be used to produce a valuable chemical in large quantities and low cost. The first step towards this goal is generally straightforward: cloning and expressing a complete biosynthetic pathway in a fast-growing, genetically-tractable organism such as *Escherichia coli*. Thanks to recent progress in synthetic biology, metabolic engineers have abundant useful tools, such as codon optimization algorithms, inexpensive synthetic genes, robust DNA assembly techniques, and approaches for balancing expression levels [21]. Initial prototypes of heterologous biosynthetic pathways often produce sufficient compound for a proof-of-concept (around 1 mg product per liter of culture [22]).

Successful heterologous production of small amounts of natural product is an encouraging and satisfying first step, but is still far from engineering a practical strain. Considerable distance lies between proof-of-concept and useful biotechnology. A parallel familiar to the metalloenzyme field is the ongoing effort to install nitrogenases into cereal plants, an achievement with the potential to revolutionize agriculture. Nitrogenase was successfully expressed in *E. coli* as early as 1972 [23]. Unfortunately, even 50 years later, engineering and deploying a cereal plant capable of nitrogen fixation remains a difficult challenge [16]. Similarly, progressing from cloning and expressing a pathway that produces milligrams of product to building a pathway that produces large quantities (>10 g per liter culture) and at production rates (>2 g per liter per hour) suitable for commercialization is extremely difficult [24]. Achieving these metrics means that each enzyme must bear high metabolic flux, which often requires that every pathway enzyme exhibits reasonable turnover numbers ($k_{cat} \sim 10/s$). Pathway enzymes must be overexpressed far above concentrations found in their natural context, and all enzymes expressed must be catalytically active. Furthermore, the abundance of each enzyme within the pathway must be well-balanced with all other pathway enzymes to prevent bottlenecks. Finally, the biosynthesis pathway and any other engineered modifications must not inflict a significant fitness burden on the host cell or else selection will favour the appearance of mutant strains that have deactivated the biosynthesis pathway, thus decreasing the productivity of the culture [25]. Obtaining high concentrations of catalytically-active enzymes without compromising cellular fitness is the core challenge of engineering metabolic pathways with Fe-S enzymes.

2.2. Fe-S enzyme basics for synthetic biologists

Establishing and maintaining a pathway that can carry high flux – active enzymes with high catalytic activity and fast turnover – is difficult for enzymes that lack exotic cofactors, but is extremely demanding for Fe-S enzymes. This is because the key cofactor required for activity (Fe-S clusters) do not spontaneously appear when Fe-S enzymes are expressed; nor are Fe-S clusters freely-available metabolites floating in the cytoplasm. Instead, Fe-S clusters are assembled and delivered to Fe-S enzymes by dedicated protein networks. If the host network cannot supply sufficient Fe-S clusters, only a fraction of the overexpressed Fe-S enzymes will be catalytically active. Furthermore, Fe-S clusters are readily disabled by reactive oxidizing molecules that are continuously produced and highly abundant in growing cells. Ongoing deactivation decreases the catalytically-active fraction of Fe-S enzymes. To understand how these problems can be addressed with further engineering, synthetic biologists must understand how Fe-S enzymes acquire and lose Fe-S clusters.

2.2.1. How enzymes acquire Fe-S clusters

Fe-S cluster biogenesis pathways assemble Fe-S clusters from reduced sulfur and iron [30,31]. The two biogenesis pathways found throughout prokaryotes (known as *Isc* and *Suf*) have been extensively characterized [32]. The specific roles of individual proteins within these pathways closely parallel each other and have been largely identified (Fig. 1). In brief, cysteine desulfurase enzymes (*IscS* or *SufSE*) break down cysteine to obtain sulfur, which is then transferred to a scaffold protein (*IscU* or *SufBCD*) and assembled with iron into a 2Fe–2S cluster. From there, various Fe-S carrier proteins deliver the assembled clusters from the scaffold to client Fe-S apo enzymes [30,31]. Cluster delivery from Fe-S scaffold or carrier proteins into apo protein targets is often aided by dedicated chaperones, such as *HscA* and *HscB* in *E. coli* (reviewed in [33]).

Fe-S cluster assembly in eukaryotes is compartmentalized entirely within the mitochondria (reviewed in [34]). Here, an *Isc*-type Fe-S cluster biogenesis pathway follows the scheme of the prokaryotic pathway: components of Fe-S clusters are synthesized and assembled by cysteine desulfurase and scaffolding proteins and subsequently delivered to mitochondrial apo enzyme targets by Fe-S carrier proteins. However, as Fe-S clusters assembly is restricted to the mitochondria, cytosolic Fe-S proteins depend upon Fe-S cluster export from the mitochondria to the cytosol. Fe-S clusters are distributed to cytosolic apo enzyme targets by the cytosolic iron-sulfur assembly (CIA) pathway. Due to compartmentalization of metabolism and Fe-S cluster assembly, obtaining active heterologous Fe-S enzymes in eukaryotes has proven considerably more challenging than heterologous expression in prokaryotes [35].

2.2.2. How enzymes lose Fe-S clusters

Maintaining an Fe-S enzyme to carry high flux requires not only acquiring Fe-S clusters but also preserving Fe-S clusters. Fe-S clusters are highly vulnerable to oxidizing species that are abundant in growing cells (Fig. 1) [36–38]. Fe-S cluster oxidation rapidly decomposes clusters and releases free Fe, which then proceeds to generate harmful reactive species such as superoxide and hydroxyl radical [39,40]. Fe-S assembly is thus tightly controlled by various homeostatic mechanisms that integrate Fe-S cluster demand, oxygen concentration, and iron availability [41,42]. Evolution has adapted Fe-S assembly pathways to presence of molecular oxygen [43–45]. Many metalloproteins lose activity through adventitious oxidation and must be restored by enzymes dedicated to cluster reassembly [46,47].

3. What can go wrong with Fe-S enzymes and how metabolic engineers can fix it

The requirements for Fe-S cluster assembly, transfer, and

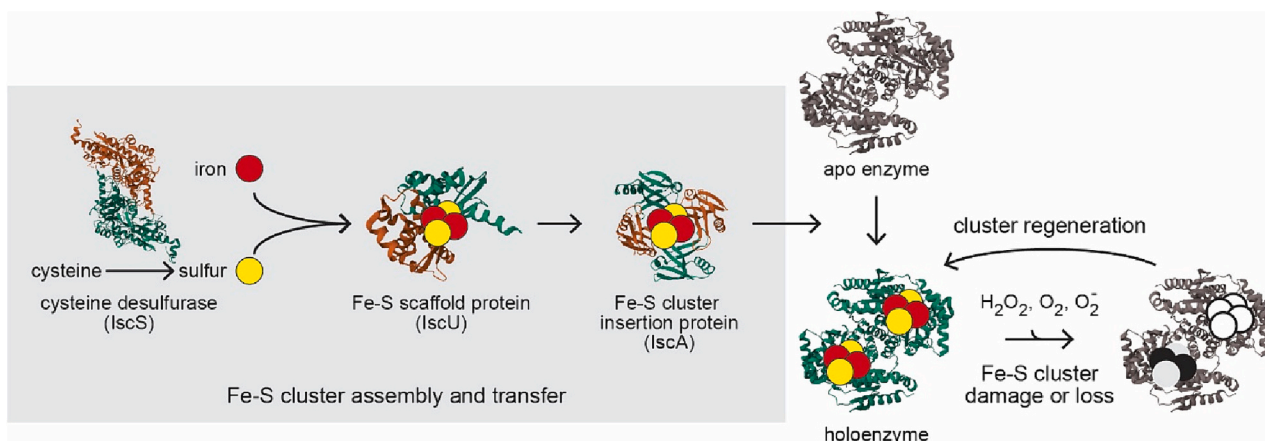


Fig. 1. *In vivo* Fe-S cluster assembly and Fe-S cluster loss. For simplicity, only proteins of the Isc Fe-S assembly pathway are depicted. Structures shown and protein data bank codes: IscS (3LVM) [26], IscU (7C8M) [27], IscA (1S98) [28], and Fe-S enzyme IspG (4S3D) [29].

maintenance summarized above present clear obstacles to using Fe-S enzymes. Fe-S enzyme biochemists and metabolic engineers have developed techniques that overcome these obstacles in the context of a specific enzyme or metabolic pathway. These techniques often prove useful for Fe-S enzymes in other pathways. Such success stories show how identifying and tackling these obstacles individually can improve Fe-S enzyme activity. We classify three distinct obstacles to the use of Fe-S enzymes in metabolic pathways: Fe-S cluster acquisition, Fe-S cluster maintenance, and Fe-S cluster reduction by electron carriers (Fig. 2).

3.1. Obstacle 1: insufficient supply of Fe-S clusters for overexpressed enzymes

3.1.1. Overexpressing Fe-S enzymes creates demand for Fe-S clusters that exceeds supply

The *in vivo* supply of Fe-S clusters often cannot keep up when Fe-S proteins are expressed to high concentrations. Indeed, overexpression of Fe-S enzymes alone causes increased expression of the native Isc Fe-S biogenesis pathway in *E. coli* [41], indicating response to increased demand for Fe-S clusters. As a consequence, a considerable fraction of the overexpressed Fe-S enzymes will not contain Fe-S clusters. The tendency of Fe-S enzyme overexpression to overwhelm host Fe-S assembly pathways was discovered in experiments with the simple Fe-S protein ferredoxin. Overexpression of ferredoxin genes from diverse species in *Escherichia coli* from a highly-active T7 phage promoter generated mixtures of apo-ferredoxins and holo-ferredoxins. Confirming that insufficient Fe-S cluster supply limited the acquisition of Fe-S

clusters, co-expression of the *isc* gene cluster together with the ferredoxin genes improved the yield of holo-ferredoxins by up to 8-fold [49]. Supplementing iron and sulfur (as cysteine) to the growth medium further improved holo-ferredoxin yield. Thus increasing the cellular capacity to supply Fe-S clusters by simply overexpressing Fe-S biogenesis pathways may increase Fe-S cluster acquisition. Co-expressing Fe-S biogenesis pathways such as the *Azotobacter vinelandii* *isc* pathway [50] together with medium supplementation is routinely used by biochemists to improve the yield of overexpressed Fe-S enzymes that are properly matured [51] (Fig. 3).

Problems with Fe-S cluster acquisition in overexpressed enzymes may also be caused by the use of a strain partially defective in Fe-S cluster assembly. The *E. coli* strain most used for enzyme overexpression, BL21, has long been known to lack a functional Suf pathway due to an inactivating mutation in SufA and SufB. Correcting the mutation, and increasing the expression of the Suf pathway, increased the yield of cluster-bearing Fe-S enzymes [52].

The solution of co-expressing Fe-S biogenesis pathways together with the Fe-S enzyme of interest is readily implemented by simply cloning an Fe-S assembly pathway on a plasmid. However, uncontrolled overexpression of Fe-S biogenesis pathways may be toxic to the cell. In general, using plasmids to express pathways incurs severe fitness costs to the host, including the need for an additional antibiotic to maintain the plasmid, the use of an expensive chemical inducer to activate pathway expression, and the burden of simply maintaining and replicating a multi-copy plasmid [53]. These burdens may not matter if the only goal is to obtain sufficient Fe-S enzyme for *in vitro* characterization. However,

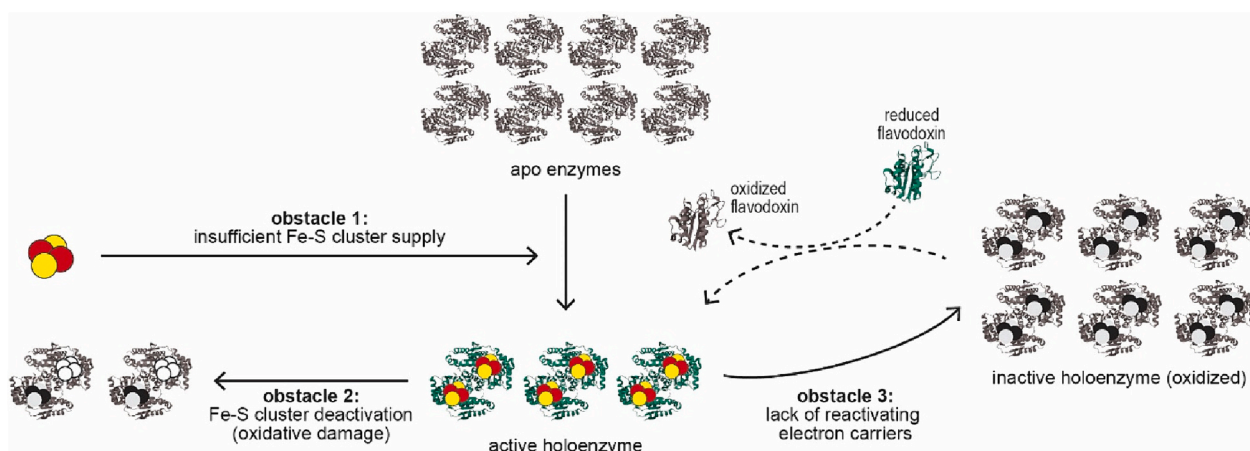


Fig. 2. Obstacles to maintaining *in vivo* activity of Fe-S enzymes. Flavodoxin structure (1AHN) [48].

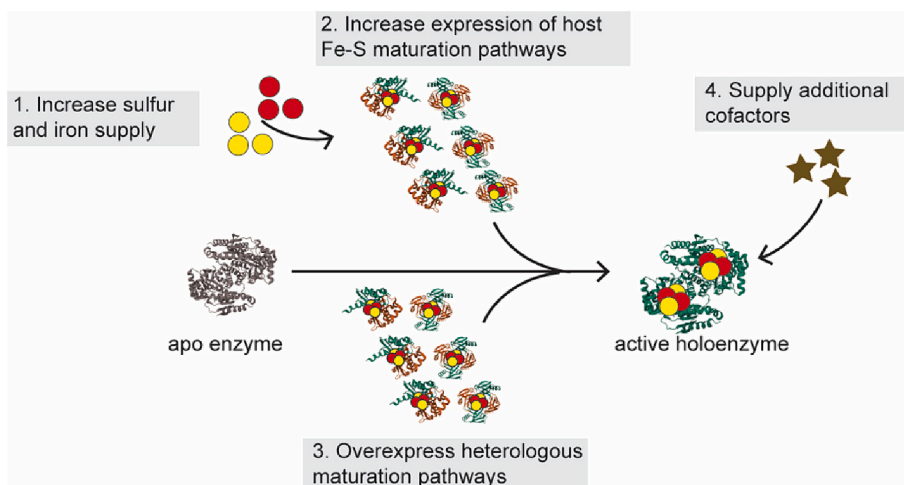


Fig. 3. General approaches for increasing supply of Fe-S clusters and other cofactors to inactive Fe-S enzymes.

cellular burdens imposed by plasmids and synthetic circuits reduce productivity of engineered metabolic pathways. These burdens can be anticipated and minimized by the use of computational and experimental approaches (reviewed in [54]). Unfortunately, the specific cellular burdens particular to Fe-S enzyme overexpression cannot presently be predicted by existing fitness burden calculators.

An alternative approach for increasing Fe-S pathway expression that avoids using plasmids is simply eliminating the transcriptional repressors that control Fe-S biogenesis in the host. Fe-S cluster assembly and delivery pathways are transcriptionally regulated according to cellular demand for Fe-S clusters [55,56]. In *Escherichia coli*, expression of Isc and Suf Fe-S pathways is controlled by the transcriptional regulator IscR. IscR is itself an Fe-S protein that inhibits expression of the Isc pathway when it is bound to an Fe-S cluster [41,56,57]. Loss of the Fe-S cluster from IscR relieves inhibition of Isc. Thus, the presence of a cluster within IscR provides an elegant signal for the level of Fe-S cluster demand within the cell. Additional regulators such as OxyR and Fur, which respond to oxidative stress and iron availability respectively, also play a role in tuning expression of Fe-S assembly pathways [42]. IscR knockouts exhibit increased expression of the Isc pathway [56]. Increasing Fe-S cluster supply via *iscR* knockout has been repeatedly used and successfully extended to improving production from multiple Fe-S-dependent biosynthesis pathways, including hydrogen, antiviral nucleotide analogues, and isoprene [8,58,59]. However, *iscR* knockouts suffer from growth defects [59], which can undermine the performance and robustness of engineered organisms. Furthermore, *iscR* knockouts exhibit decreased expression of the oxidative stress-resistant Suf pathway [60].

An elegant technique for increasing production of Fe-S clusters for overexpressed enzymes was discovered during efforts to increase biotin production in engineered *E. coli* [11]. The final step of the biotin synthesis pathway is catalysed by the radical SAM enzyme BioB, which is also the bottleneck enzyme of the overall pathway: biotin pathway overexpression leads to accumulation of the BioB substrate dethiobiotin [61]. BioB overexpression is also toxic to the *E. coli* host [11]. Mutant strains more resilient to BioB overexpression were isolated and their genomes were sequenced, which revealed two point mutations in the *iscR* gene that target the Fe-S binding motif of IscR. Proteomic analysis revealed increased expression of the Isc pathway, an indication that BioB overexpression depletes the Fe-S cluster pool. Interestingly, strains bearing *iscR* mutations showed increased expression of proteins from both Isc and Suf pathways. The strains with the *iscR* mutations were able to express BioB to twofold higher concentrations, resulting in a twofold increase in biotin production. The authors also tested the effects of the mutations on production of two vitamins whose pathways also rely upon

Fe-S cluster enzymes (thiamine and lipoic acid), and found that the *iscR* mutations substantially increased titers of these compounds as well. The effects of the discovered *iscR* mutations on vitamin production compared favourably to simple *iscR* deletion, likely because *iscR* deletion increases expression of the Isc pathway but does not increase Suf pathway expression. Furthermore, the *iscR* mutations also raised vitamin titers well above those achieved by individually overexpressing Isc and Suf pathways. These results suggest that even when transcriptional repression is completely relieved by *iscR* deletion, the capacity of host pathways to provide clusters may still prove insufficient for overexpressed enzymes. Furthermore, simultaneous expression of Isc and Suf pathways by the *iscR* mutants may prove more effective than overexpressing either pathway individually.

3.1.2. Host Fe-S cluster assembly pathways may be incompatible with heterologous Fe-S enzymes

Importing biosynthesis pathways from other organisms requires heterologous expression. *E. coli* is certainly capable of providing Fe-S clusters to many heterologous Fe-S enzymes, and overexpression of *E. coli* or *A. vinelandii* Fe-S biogenesis pathways has repeatedly been used to increase yields of heterologous holo Fe-S enzymes. These results imply that Fe-S cluster biogenesis pathways are readily compatible with heterologous Fe-S enzymes. Early studies also indicate a significant degree of interspecies compatibility: the *E. coli* Isc and Suf pathways can be replaced with Fe-S assembly pathways from the ϵ -Proteobacteria *Helicobacter pylori* (NifSU) [62], as well as the Suf operon from the Firmicutes species *Enterobacter faecalis* [63]. But is it reasonable to expect the *E. coli* Isc or Suf pathways to deliver Fe-S clusters to Fe-S enzymes from any species?

We evaluated this question with a systematic complementation assay. *E. coli* strains lacking conditionally-essential Fe-S enzymes (NadA and IspG) were complemented using orthologs drawn from a pool representative of known prokaryotic diversity. Growth (successful complementation) indicates that the foreign ortholog recovered activity, and therefore is able to acquire an Fe-S cluster from the *E. coli* Fe-S biogenesis pathway [64]. We were immediately struck by the incredible versatility of the *E. coli* pathway: roughly one-third of all NadA orthologs tested recovered growth of *E. coli* Δ nadA. Interestingly, the probability of a NadA ortholog recovering activity in *E. coli* correlated with its phylogenetic distance from *E. coli* NadA. Many inactive orthologs recovered activity when we co-expressed a heterologous Fe-S pathway (Suf from *Bacillus subtilis*), suggesting that compatibility between Fe-S assembly pathways and Fe-S enzymes is certainly not universal. The versatility of the *E. coli* Fe-S pathways may not actually be so surprising given that the native *E. coli* pathway routinely supplies >100

Fe-S enzymes with different structures and functions. Nevertheless, the *E. coli* pathways are not universally compatible, or may not be most efficient at delivering clusters to all Fe-S enzymes. In these cases, over-expressing a heterologous Fe-S pathway may broaden the compatibility of the chassis organism with heterologous Fe-S enzymes (Fig. 3).

Expressing Fe-S enzymes in eukaryotes faces considerably greater challenges. These challenges largely stem from the compartmentalization of Fe-S cluster assembly within the mitochondria, whereas heterologous proteins are typically expressed within the cytosol. Fe-S clusters are trafficked to the CIA machinery, which involves at least 8 proteins, and from there delivered to cytosolic apo client proteins. Attempts to express prokaryotic Fe-S enzymes within the cytosol of *Saccharomyces cerevisiae* have mostly failed (as comprehensively reviewed in [35]), suggesting that the CIA pathway does not recognize prokaryotic enzymes as clients. Expressing prokaryotic Fe-S assembly pathways within the cytosol has not been successful at reviving prokaryotic enzymes, which may be due to the oxidizing environment of the cytosol. However, expression of heterologous Fe-S enzymes within the mitochondria has repeatedly proven successful, indicating that the mitochondrial Fe-S cluster assembly pathway is at least somewhat compatible with prokaryotic Fe-S enzymes. For pathways that can be restricted to the mitochondria, this is not necessarily a problem. For pathways with cytosolic proteins or cytosolic substrates, a functioning Fe-S enzyme in the mitochondria may not be so useful.

Recently, a study identified sequence similarities in CIA client Fe-S enzymes [65]. The C-termini of multiple cytosolic and nuclear Fe-S enzymes in yeast and humans feature a high occurrence of hydrophobic residues, which the authors demonstrated was required for native yeast proteins to associate with the CIA machinery and acquire Fe-S clusters. Appending the C-terminal motif to non-client proteins increased their recruitment to CIA proteins and increased the activity of a prokaryotic Fe-S enzyme expressed in the cytosol. This is a promising development that demonstrates how heterologous Fe-S enzymes might be functionally expressed within the eukaryotic cytosol. However, maintaining Fe-S clusters within cytosolic proteins faces further challenges, such as the oxidizing milieu of the cytoplasm and absence of specific electron transfer proteins (including 2Fe—2S ferredoxins) that may be needed for activity.

3.1.3. Some metalloenzymes require specific cofactors or assembly pathways

Enzymes and protein complexes that require unique cofactors may require more complex maturation pathways beyond Isc and Suf for to function *in vivo*. The pathways that supply additional cofactors, or that are required to fully assemble the metal cofactor must be co-expressed with the structural components of the enzyme. Although this imposes additional engineering and metabolic burdens, this is a solvable problem, as many examples exist of expressing cofactor-dependent Fe-S enzymes. For instance, many radical SAM methyltransferases require the cofactor cobalamin (vitamin B12), which is not synthesized by most chassis organisms. Engineered expression of the highly complex cobalamin synthesis pathways in chassis organisms is generally low-yielding [66]. Fortunately, cobalamin added to the growth medium is readily taken up by *E. coli* and other chassis organisms. Overexpressing cobalamin import pathways such as the *E. coli* *btu* operon massively increases yields of functional enzyme for both *in vitro* characterization [67] and *in vivo* activity [64].

In sulfur-insertion reactions, the Fe-S cluster may donate a sulfur to the substrate to create the product, thus decomposing as part of the catalytic cycle. The lipoate synthesis enzyme LipA, a radical SAM enzyme, catalyses the insertion of a sulfur atom to an 8-carbon fatty acid, creating a C—S bond. Early biochemical studies concluded that one of the two Fe-S clusters donated a sulfur atom and was destroyed in the process, suggesting that the enzyme is single-turnover. However, addition of the Fe-S cluster carrier protein NfuA (or the Fe-S scaffold protein IscU) rapidly restored the Fe-S cluster and allowed additional reactions

[68]. The LipA reaction proved catalytic in the presence of excess NfuA. Excess NfuA did not show evidence of biphasic kinetics, indicating that the LipA reaction was not limited by the Fe-S cluster re-assembly by NfuA. This finding is encouraging, as it suggests that Fe-S enzymes that appear to be single-turnover *in vitro* (and thus extremely difficult to harness for metabolic engineering), such as BioB, might prove to be catalytic when provided with the proper protein cofactor that can restore the Fe-S cluster.

Hydrogenases interconvert molecular dihydrogen with protons and electrons and are attractive for their potential use as generating biohydrogen. Comprehensive reviews specific to engineering hydrogen production in various microbes are available [12,69–72]. Here, we briefly describe only the hydrogenase maturation pathway and its successful use in generating functional hydrogenases. Two families of hydrogenases, the FeFe and NiFe variants, are known [73]. Both enzymes feature 4Fe—4S clusters that feed electrons to their core metal cofactors. The metal cofactors in turn feature unusual ligands (cyanide ion and carbon monoxide) whose synthesis, assembly, and insertion into apo enzymes rely upon specific pathways [74]. The FeFe hydrogenase assembly pathways rely upon Fe-S cluster-bearing radical SAM enzymes for ligand synthesis and cofactor assembly. Interestingly, NiFe hydrogenases do not require Fe-S clusters for cofactor assembly, and use a Ni-bearing metallochaperone for cofactor maturation [75].

Despite the apparent complexity of hydrogenase cofactor assembly, hydrogenases and their maturation pathways can be successfully expressed in heterologous hosts. Simple co-expression of maturation pathway genes together with structural genes from a single synthetic operon resulted in successful production of functional FeFe hydrogenase from *Clostridium acetobutylicum* [76]. Deletion of *iscR* increased production of functional hydrogenase, and increased hydrogen in partially aerobic environments (but interestingly did not increase expression or H₂ accumulation in anoxic environments) [58]. Overexpression of the *E. coli* Isc pathway boosted expression of NiFe hydrogenase from *Shewanella oneidensis* as well [77]. An FeFe hydrogenase from *Chlamydomonas reinhardtii* was also successfully expressed in *E. coli* together with corresponding maturation factors [78]. Active NiFe hydrogenases from anaerobic hyperthermophilic archaea were successfully expressed in *E. coli*. Surprisingly, the maturases of the *E. coli* NiFe hydrogenase proved sufficient to mature the *P. furiosus* hydrogenase, with the added requirement of only a heterologous protease required for maturation [79].

3.2. Obstacle 2: Fe-S clusters are easily inactivated by abundant and ubiquitous oxidizing species

The reactivity of Fe-S clusters enables catalytic versatility but also facilitates undesirable reactions. Fe-S clusters readily decompose when exposed to oxidizing agents such as oxygen, hydrogen peroxide, and superoxide [40]. Thus, aerobic conditions may decrease Fe-S enzyme activity by decomposing Fe-S clusters in overexpressed enzymes and by limiting the availability of Fe-S clusters within the cell. This is inconvenient for metabolic engineering because oxic conditions are both convenient and useful to speed the growth of chassis organisms, which accelerates the design-build-test engineering cycle needed to improve engineered pathways. Oxygen sensitivity of Fe-S enzymes has been blamed for difficulties in engineering the deoxy-xylulose phosphate (DXP) pathway [80], microbial hydrogen production [81], and nitrogen fixation [82]. Low-potential clusters involved in generating radicals for hydride transfer or methyl transfer reactions may be especially vulnerable to oxidation.

Fortunately for metabolic engineers, the oxygen sensitivity of Fe-S enzymes observed *in vitro* does not necessarily correspond to oxygen sensitivity *in vivo*. Many Fe-S enzymes that are deactivated within seconds of oxygen exposure when purified are able to maintain vigorous activity in cells growing in oxic environments [83]. This is a consequence of evolved defence mechanisms that reduce oxidative damage to

valuable metal cofactors. Transition from anaerobic to aerobic environments increases transcription of *isc* and *suf* pathways [84], and increases expression of oxygen-resistant Fe-S carrier proteins [85].

The structures of Fe-S cluster enzymes themselves have been adapted to environmental oxygen [83]. In our wide screen of interspecies compatibility, NadA phylogeny clustered into two groups, which were distinguished mainly by originating from either aerotolerant organisms or obligate anaerobes [64]. Orthologs of NadA from the oxygen-sensitive clade were recovered when our cells were grown in anoxic conditions. A phylogenetic analysis of an Fe-S cluster enzyme from the MEP pathway, IspH, identified structural adaptations specific to homologs found within anaerobic and aerobic bacteria. IspH orthologs from aerobic bacteria feature a cluster of aromatic residues surrounding the Fe-S cluster, which were demonstrated to protect the cluster from oxidation [86]. These evolved protective mechanisms, which act by sterically shielding the metal cofactors, may however reduce the turnover rate of the enzyme [83] and thus decrease the productivity of engineered pathways. For instance, the *E. coli* aconitase expressed during exponential phase (AcnB) is solvent-exposed and highly oxygen sensitive but is also far more catalytically active than the oxygen-resistant variant aconitase expressed during stress (AcnA) [87]. Some oxygen-sensitive enzymes also rely upon helper proteins or enzymes that restore reactivity, such as ferredoxins that reduce cofactors that are adventitiously oxidized [88]. Co-expressing these salvaging proteins or pathways may help maintain reactivity and efficient turnover in over-expressed Fe-S enzymes [64] (Fig. 4).

Synthetic biologists could thus take advantage of natural diversity and select orthologs from aerotolerant organisms rather than obligate anaerobes. Hydrogen production in aerobic conditions was improved by using an oxygen-tolerant NiFe hydrogenase from *Hydrogenovibrio marinus* [89]. The study of structural modifications that stabilize oxygen-sensitive reactive sites inspired the rational engineering of an NiFe hydrogenase from *Desulfovibrio fructosovorans* [90]. While this mutant is reactivated more readily after oxygen exposure than the wild-type enzyme, the production of hydrogen is impaired. The use of proteins that actively shield reactive centres during oxygen exposure can also preserve activity of oxygen-sensitive enzymes (Fig. 4). Co-expression of an oxygen-protective protein from *A. vinelandii* prevents oxidation of nitrogenase and allows nitrogen production in microaerobic environments [91].

Expressing prokaryotic Fe-S enzymes and pathways in eukaryotes is difficult due to the oxidizing environment of the cytoplasm. One solution is to express enzymes within cellular compartments with diminished oxygen concentration, such as the mitochondria [92] (Fig. 4). This

can be accomplished by appending targeting sequences to the proteins to be expressed. As we discuss further below, this overall approach appears promising in engineering nitrogenase expression in eukaryotes.

3.3. Obstacle 3: many Fe-S enzymes require specific electron transfer proteins

Fe-S cluster redox reactions involve electron transfer from an Fe-S cluster to a substrate or to an enzyme cofactor such as cobalamin. In many cases, the catalytic cycle does not return the electron to the cluster; thus, electrons must be supplied from an external source for every catalytic cycle. Electrons may also be lost from reduced clusters or cofactors via adventitious oxidation, thus requiring regeneration (Fig. 5). Electrons for reduction reactions are often obtained from carrier proteins such as ferredoxins or flavodoxins. In turn, these carrier proteins acquire electrons from flavodoxin/ferredoxin oxidoreductases.

The need for an electron transfer step as part of a reaction mechanism may not be apparent to a non-expert seeking to transplant a biosynthesis pathway into a new host. This is especially the case for radical SAM enzymes that use Fe-S clusters to catalyse methyl transfer or hydride abstraction. These reactions modify unreactive substrates and therefore require a highly reactive species from SAM, specifically a 5-deoxyadenosyl radical. These radicals are obtained by transferring a single electron from an Fe-S cluster to SAM [93,94]. The reduction potentials of soluble electron carriers NAD(P)H are not low enough, and so electron carriers with suitably low reduction potentials must be used. These carriers are frequently small proteins known as flavodoxins or ferredoxins, which feature an electron-carrying cofactor (a flavin or Fe-S cluster, respectively). Without carrier proteins, enzyme activity will be diminished or eliminated entirely. Electron carrier proteins also provide electrons for Fe-S cluster assembly [95,96].

3.3.1. Fe-S enzymes require electron transfer proteins with compatible reduction potentials

All cells possess electron transfer proteins that are often capable of transferring electrons to heterologous enzymes. Electron transfer proteins are versatile: the *E. coli* flavodoxin FldA serves many client enzymes within the host, including MethH, BioB, IspG, and IspH [97–99]. The acyl lipid desaturase of *B. subtilis* accepts electrons from two different flavodoxins or a ferredoxin [100]. This promiscuity may be sufficient to demonstrate some level of activity in an engineered host, but may also obscure the need for specific electron transfer proteins that are more effective.

Electron carrier specificity arises from two properties: reduction potential and structural complementarity (ability to bind target proteins). The reduction potentials of electron carrier proteins vary over a wide range [101]. As electrons travel towards electron acceptors with decreasing reduction potential (increasing E), any Fe-S or cofactor-bearing enzyme requires electron carrier proteins with greater reduction potential (lower E). Supplying electron carriers with suitable reduction potentials can mean the difference between turnover numbers on the order of 1/h versus 1/min or 1/s. For instance, *in vitro* activity of the cobalamin-dependent rSAM methyltransferase Fom3 increased substantially when titanium (III) citrate (−800 mV vs NHE) was used instead of *E. coli* flavodoxin (−433 mV) [6]. Co-expressing compatible electron carrier proteins can increase productivity by many fold (10-fold higher titers in the case of tryptophan methyltransferase TsrM) [64] (Fig. 5). Note that compatible electron carriers alone may not be sufficient, as the carriers must be supplied by compatible flavodoxin/ferredoxin oxidoreductases that generate low-potential electrons. This is illustrated by the use of a pyruvate:flavodoxin/ferredoxin-oxidoreductase in conjunction with a 4Fe–4S ferredoxin from *Clostridia pasteurianum* to increase hydrogen production from the native *E. coli* pathway. Co-expression of the ferredoxin and oxidoreductase in *E. coli* BL21 increased hydrogen gas titers by at least 9-fold [102].

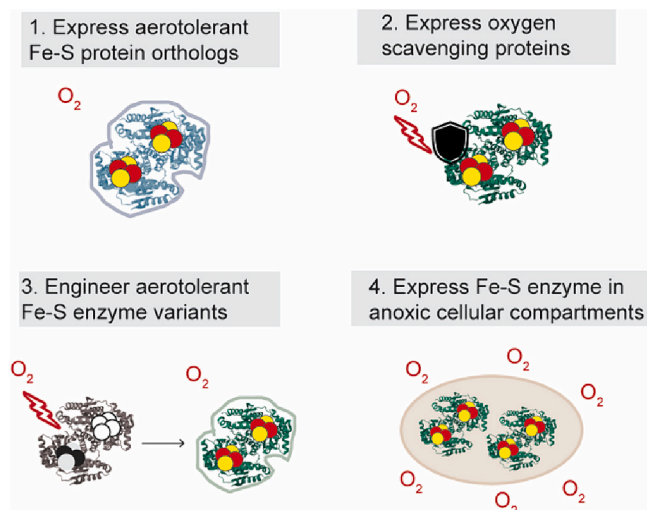


Fig. 4. Approaches for circumventing the oxygen sensitivity of Fe-S enzymes.

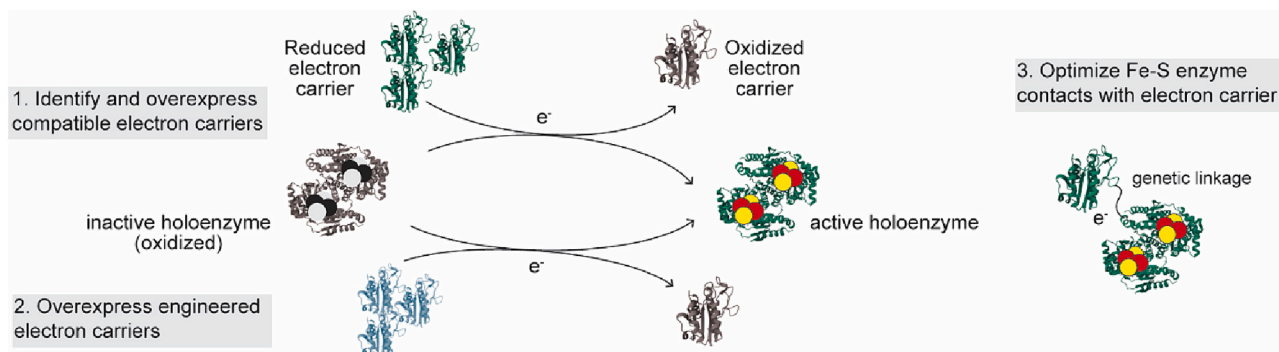


Fig. 5. Methods for maintaining Fe-S enzymes in active state using electron carriers.

3.3.2. Structural complementarity of electron carrier protein with client enzyme

Even if electron carriers with suitable reduction potentials are provided, the electron carrier protein may not bind to client enzymes due to lack of shape or electrostatic surface complementarity with client enzymes [103–105]. Structural incompatibility, which prevents protein-protein interactions needed for electron transfer, may make the difference between an active enzyme and an inactive enzyme. As we observed in our systematic screen of Fe-S enzyme compatibility, the vast majority of heterologous IspG orthologs failed to complement growth of *E. coli* Δ ispG [64]. Activity of many IspG orthologs could be recovered if compatible electron transfer proteins were known or could be identified within the genomes of the original hosts by screening. This indicates that many IspG orthologs could not be supported by the host *E. coli* flavodoxin FldA, despite FldA exhibiting equivalent or lower reduction potential than cognate electron transfer proteins that recovered complementation by the IspG orthologs. Using cognate electron donors may also further improve activity of redox-active Fe-S enzymes. Overexpression of cognate electron carriers together with flavo/ferredoxin reductases has increased titers of derivatives of the IspG-dependent DXP pathway from 1.7 to 3-fold in several systems [59,106,107].

3.3.3. Finding suitable electron carriers by genome mining

The unpredictable requirements of Fe-S enzymes for electron carrier proteins with specific features (reduction potential or structural complementarity) can pose a challenge for engineered pathways. Prokaryotic genomes contain multiple electron carrier proteins with varying numbers of client enzymes [105], each with specific roles in host metabolism [104]. Identifying compatible electron transfer proteins needed for Fe-S enzymes is not straightforward. For some of these electron carrier proteins, client enzymes may be inferred by genome context, such as co-occurrence with genes encoding catalytic proteins within a gene cluster. However, many electron transfer protein genes (such as *E. coli* fldA) occur as monocistronic operons lacking genomic context that might hint at their role in the cell.

What can synthetic biologists do when faced with a genome containing multiple electron transfer proteins? This problem of genome mining for electron transfer proteins is familiar to the cytochrome P450 field [108]. Simply cloning each individual electron carrier and co-expressing with the pathway of interest is a brute force method that may work. Genome mining can be better informed if the requirements of the Fe-S enzyme of interest are considered. For instance, cobalamin-dependent rSAM methyltransferases feature extremely low reducing potentials [6,109]. The various families of electron transfer proteins (flavodoxins, 2Fe–2S, and 4Fe–4S ferredoxins) each have overlapping but distinct reduction potential ranges [105], with 4Fe–4S ferredoxins exhibiting the lowest potentials. Therefore, screening for a low-potential electron carrier should begin with the 4Fe–4S ferredoxins within the genome of the original host. In our efforts to improve the activity of the methyltransferase TsrM, we identified three 4Fe–4S ferredoxins in the

genome of *Streptomyces laurentii*. Co-expression of each individual ferredoxin with TsrM identified one ferredoxin (Fdx1) able to increase 2-methyltryptophan titers by about 10-fold [64]. Unlike the other two ferredoxins, Fdx1 exhibited both lower reducing potential than the TsrM Fe-S cluster and demonstrated measurable binding affinity to TsrM.

3.3.4. Improving enzyme performance by engineering electron transfer protein and interaction partners

Electron transfer proteins have been subjects of tinkering for many decades. Structural and electrostatic determinants of electron carrier reduction potential have been extensively examined through mutagenesis (reviewed in [110]). *De novo*-designed ferredoxins capable of electron transport *in vivo* have even been generated [111]. More recently, artificial chimaeric ferredoxins with a range of reduction potentials have been assembled from two parental ferredoxins. Most of the ferredoxin chimaeras were able to support electron transfer from a ferredoxin reductase to a client enzyme (sulfite reductase) [112]. Interestingly, most chimaeras featured reduction potentials below the parental ferredoxins. Binding specificities between electron transfer proteins and client enzymes can also be tweaked with targeted mutagenesis, as demonstrated using a ferredoxin and a hydrogenase [78]. Creating protein fusions between enzymes and electron carriers has also been used to improve hydrogen productivity by 4-fold. This approach however often requires screening to optimize the length of the linker peptide between enzyme and carrier protein [113,114].

3.3.5. Turning a ferredoxin into a chemical-activated electrical switch

One of the most remarkable examples of ferredoxin engineering is the creation of a chemical-sensitive molecular switch. Atkinson et al. [115] used a synthetic *E. coli*-based complementation assay using ferredoxin-dependent sulfur assimilation to identify functional (2Fe–2S) ferredoxins [116]. The authors next used multiple sequence alignments and the complementation assay to identify sites in the ferredoxin structure able to tolerate large protein insertions without compromising electron transfer function. Synthetic peptide motifs that stably associated were next fused to each ferredoxin half, and each half was separately expressed from titratable promoters. Ferredoxin assembly (and thus sulfur assimilation and cell growth) required simultaneous expression of both halves, showing that each half was able to assemble into a functional ferredoxin. Finally, the authors inserted proteins that change conformation upon binding specific small molecules between the two halves of the ferredoxin (such as the ligand-binding domain of human estrogen receptor). These fusions were constructed such that the conformational change triggered by the chemical would join the two ferredoxin halves and enable electron transfer. In the absence of the chemical, the ferredoxin remained unassembled and nonfunctional. The authors successfully demonstrated that the protein fusions enabled a chemically-activated switch that enables electron transfer to the sulfur assimilation pathway. The split ferredoxins were effective in both activating transcription of a synthetic circuit and an engineered allosteric

(post-translational) switch.

4. The grand Fe-S enzyme case study: heterologous N₂ fixation with nitrogenase

Most of the nitrogen used by life on Earth has been produced from non-reactive nitrogen gas by the metalloenzyme nitrogenase. As nitrogen availability limits food production, traditional agriculture supplied nitrogen to cereal plants by crop rotation with plants that host nitrogen-fixing bacteria. The slow production of bioavailable nitrogen limited human population until the development of the Haber-Bosch process, a means for producing ammonia from atmospheric nitrogen using high temperature and pressure. While the Haber-Bosch process has been credited with securing the food supply, it incurs serious environmental damage, such as enormous energy investments and fertilizer runoff [117]. These problems could be mitigated by engineering nitrogenase expression directly into cereal plants. Engineering nitrogen fixation in cereal plants is one of the promises of genetic engineering recognized in the 1970's. Indeed, nitrogen fixation by a heterologous host was achieved without modern molecular biology tools [23]. Successful transfer of the *nif* gene cluster *E. coli* from *Klebsiella pneumoniae* (now *K. oxytoca*) via bacterial conjugation established the possibility of heterologous nitrogen fixation. However, as metabolic engineers appreciate, there is a vast distance between a proof-of-concept and producing sufficient product for an application.

Assembling active nitrogenases in heterologous hosts is an enormous challenge. The problems discussed in this review are well-known among nitrogenase researchers, as all three obstacles described apply to nitrogenases. First, assembly of nitrogenase cofactors (which are assembled from Fe-S clusters by Fe-S enzymes) requires dedicated pathways that in turn depend upon host Fe-S biogenesis pathways. Second, nitrogenases, nitrogenase reductases, and enzymes of cofactor assembly pathways are extremely sensitive to oxygen. Finally, the reduction of nitrogen to ammonia requires low-potential electrons, which must be continuously supplied to the nitrogenase reductase enzyme by specific electron carriers. As described below, significant advances have been made along each of these fronts. We note that these efforts also illustrate how fundamental research benefits from tools developed for synthetic biology. Installing nitrogenases into chassis organisms provides a platform for testing various components of the maturation pathway, an approach that can provide valuable insight into nitrogenase maturation and function within the native hosts [91,118–121].

4.1. Obtaining Fe-S clusters for synthesizing nitrogenase cofactors in heterologous hosts

Nitrogenase maturation is highly complex (reviewed in [122]). The nitrogenase gene clusters (*nif*) of *A. vinelandii* and *K. oxytoca* consist of 16 and 20 genes, respectively [123,124]; however only three genes encode the nitrogenase and nitrogenase reductase complex. Included in the *A. vinelandii* and *K. oxytoca* pathways are cysteine desulfurase and Fe-S cluster scaffold proteins *nifS* and *nifU* [125]. The presence of Fe-S cluster assembly proteins NifS and NifU within the *nif* clusters implies that nitrogenase cluster assembly requires a dedicated Fe-S assembly pathway. However, nitrogenase activity was successfully reconstituted in *E. coli* using a minimal 9-gene *nif* gene cluster lacking *nifS* and *nifU* [126]. This supports the notion that the nitrogenase assembly may not necessarily require specialized Fe-S assembly pathways [127]. Nevertheless, heterologous nitrogenase activity was only 10 % of activity obtained from the native host (*Paenibacillus*). Nitrogenase activity in *E. coli* could be partly restored to up to 50 % of *Paenibacillus* levels by co-expressing either the *Paenibacillus suf* pathway or *nifSU* from *K. oxytoca*, suggesting that the native *E. coli* Isc pathway does not produce sufficient Fe-S clusters for nitrogenase maturation. We suspect that the presence of *nifS* and *nifU* within the *A. vinelandii* and *K. oxytoca* gene clusters may help to boost overall Fe-S cluster synthesis simultaneously with

nitrogenase expression, coordinating Fe-S cluster supply and demand.

Expressing nitrogenase within mitochondria is another front in the engineering of nitrogen-fixing cereal plants. The mitochondria is a logical compartment to express nitrogenase enzymes, as Fe-S assembly takes place within the mitochondria. However, it is not certain that mitochondrial Fe-S pathways are capable of supporting nitrogenase enzymes. A major advance on this front was the successful expression of the *A. vinelandii* nitrogenase reductase enzyme NifH within *S. cerevisiae* mitochondria [82]. The authors further tested whether co-expression of maturation enzymes NifU, NifS, and NifM are required for NifH activity. Interestingly, only NifM, a proline cis-trans isomerase, proved essential for NifH activity. As co-expressing the *A. vinelandii* Fe-S assembly proteins NifU and NifS within the mitochondria proved unnecessary, the mitochondrial Fe-S assembly pathway is apparently able to supply NifH with Fe-S clusters.

Successes with obtaining functional NifH within mitochondria did not extend to other Fe-S enzymes within the nitrogenase maturation pathway. The *A. vinelandii* nitrogenase cofactor is a 7 iron, 9 sulfur, 1 carbon, 1 molybdenum cofactor (FeMo-co). A precursor to this cofactor (NifB-co) is produced by the Fe-S enzyme NifB, a radical SAM enzyme bearing two [4Fe-4S] clusters [128]. Expression of functional NifB within mitochondria proved unsuccessful [129]. A group therefore sought a strategy to successfully produce the FeMo-co adduct in *S. cerevisiae* mitochondria [130]. Two combinatorial libraries consisting of genes from a variety of organisms were assembled: one library consisting of various maturation proteins, with one library consisting of 28 *nifB* orthologs. Co-expression of Fe-S assembly proteins NifU and NifS and a ferredoxin from *A. vinelandii* together with NifB orthologs from archaea successfully generated NifB capable of assembling NifB-co. This work also reveals that NifB apparently depends upon specific Fe-S assembly proteins NifU and NifS, as well as specific electron transfer proteins as discussed below. This study also highlights the potential of mining the enormous diversity of prokaryotic organisms for orthologs that are able to function within heterologous hosts [118].

4.2. Keeping oxygen away from nitrogenase

The sensitivity of nitrogenases to oxygen is an intimidating obstacle to heterologous expression in plant tissues. In nitrogen-fixing organisms, oxygen sensitivity is circumvented by separating respiration from nitrogen fixation via heterocysts in cyanobacteria [131], and expression of oxygen-scavenging proteins by the plants that host nitrogen-fixing bacteria [132]. Surprisingly, the obligate aerobe *A. vinelandii* fixes nitrogen despite the presence of oxygen. Several proteins have been identified that protect the nitrogenase from dissolved oxygen, such as the Shethna protein II (FeSII), which responds to oxygen exposure by switching nitrogenase into an oxygen-resistant inactive conformation [133]. More recently, a comprehensive transcriptome analysis of *A. vinelandii* in aerobic nitrogen-fixing conditions identified a protein of unknown function (NafU). An *A. vinelandii* Δ *nafU* strain exhibited reduced nitrogenase activity at ambient oxygen levels relative to the wild-type strain [91]. The potential of NafU for heterologous hosts was further demonstrated by co-expressing *nafU* with the *A. vinelandii* nitrogenase pathway in *E. coli*, which increased nitrogenase activity in aerobic conditions by nearly 10-fold. The authors speculate that NafU, which appears to be a membrane-bound enzyme, may maintain nitrogenase activity by decreasing intracellular oxygen levels.

In addition to its ability to support Fe-S cluster delivery, the low-oxygen environment of mitochondria makes it highly attractive as a potential host for nitrogenase expression in eukaryotes. The suitability of mitochondria for hosting nitrogenase enzymes is supported by encouraging work demonstrating that the mitochondria can host the highly oxygen-sensitive nitrogenase reductase NifH [82]. Particularly encouraging is successful expression within aerobically-grown yeast, as this implies that aerobically-grown eukaryotic cells may prove suitable for nitrogen fixation. Importantly, this work also demonstrated that the

cytosol is not friendly for nitrogen fixation.

4.3. Connecting nitrogenases with the cellular electricity network

Nitrogenases require low-potential electron carriers to reduce the nitrogenase cofactor. As nitrogenases are found in a diverse array of bacteria from various environmental niches, many different sources of low potential electrons are used to power nitrogen reduction, even within the same organism [134]. Many gene clusters encoding nitrogenases also include specific electron transport systems, such as the pyruvate-ferredoxin reductase NifJ and its cognate ferredoxin NifF from *K. oxytoca*. The observation that the minimal *Paenibacillus nif* gene cluster generated functional nitrogenase within *E. coli* indicates that *E. coli* electron transfer proteins are able to supply electrons to nitrogenase. However, nitrogenase activity was improved by co-expressing electron transfer systems and components from various organisms with the *Paenibacillus nif* gene cluster [120]. Co-expressing the *Paenibacillus* oxidoreductase PfoAB with a cognate flavodoxin, FldA, achieved the best improvement (4-fold). This result illustrates the need for screening electron transfer proteins for optimizing enzyme activity.

Going a step further, another group used *E. coli* as a chassis to test compatibility between both molybdenum and iron-only nitrogenases and several electron transport chains found within plant organelles [135]. Several transport chains from plastids and chloroplasts were identified that could power nitrogen reduction. However, the mitochondrial transport chain tested was ineffective, presenting a barrier to expressing nitrogen fixation within mitochondria. The authors then showed that a hybrid electron transport chain consisting of a mitochondrial adrenodoxin oxidoreductase with cyanobacterial ferredoxins could support nitrogenase activity. Interestingly, the most activity was consistently obtained when using hybrid electron chains consisting of an oxidoreductase and an electron carrier from different organisms, some of which reached levels obtained from the native NifJ/NifF electron transport network. Demonstrating the importance of providing cognate electron transfer proteins in heterologous hosts, obtaining functional NifB in yeast mitochondria required co-expressing the *A. vinelandii* ferredoxin FdxN in addition to other maturation factors [130,136].

5. Prospects of Fe-S proteins in synthetic biology

The steady progress surmounting obstacles in the path towards heterologous nitrogen fixation is encouraging, and highlights the usefulness of existing synthetic biology tools in developing solutions to the challenges posed by Fe-S enzyme expression. Looking ahead, we anticipate the application of protein structure predictions to Fe-S protein biology, and the further blossoming of the exciting new field of bioelectronics, in which Fe-S proteins will play a key role. Here we survey these exciting developments.

5.1. Using protein structure prediction to solve problems with in vivo Fe-S enzyme activity

The development of highly accurate protein structure prediction has accelerated biology. While the original iteration of AlphaFold [137] or its cofactor binding prediction derivative AlphaFill [138] does not include Fe-S clusters in its structural predictions, a recent study demonstrated that Fe-S binding sites (i.e. cysteine side chains that coordinate clusters) are often accurately modelled [139]. This is because AlphaFold2 is informed by solved structures of cluster-bearing Fe-S proteins.

Several of the obstacles encountered in harnessing Fe-S enzymes in metabolic pathways might be more efficiently addressed using protein structure prediction. For instance, as summarized above, many Fe-S enzymes have adapted to the presence of oxygen by modifying the structure of the protein surrounding the cluster in order to reduce solvent exposure, and thus vulnerability to oxidizing species [83,86]. Given

a sizeable library of enzyme orthologs from a variety of species, structure predictions might help identify orthologs that may be more stable when expressed in oxic conditions. Such an approach might prove to be more effective than inferring oxygen sensitivity from the habitat of the organism from which the ortholog is obtained. As we found with NadA, oxygen sensitivity of the source organism proved only weakly effective at predicting complementation of *E. coli* Δ nadA in aerobic conditions [64]. There are many examples of oxygen-sensitive and oxygen-resistant orthologs of several Fe-S enzymes, such as IspH, fumarase, and aconitase, which could be used to test this approach.

Another potential application of structure prediction is identifying electron transfer proteins within a prokaryotic genome that are best at supporting catalytic activity with a given Fe-S enzyme. Given the wealth of information on electron transfer protein reduction potentials (recently collected into a searchable database [140]), properties that restrict the range of client enzymes for electron transfer proteins (reduction potential and structural compatibility) are well-suited for the new generation of predictive tools. A recent machine learning approach applied to predicting reduction potential of flavodoxins achieved a mean prediction error of 36 mV [141]. Predicting protein binding affinity of electron transfer proteins for client Fe-S enzymes given protein structures might be accomplished using protein-protein docking tools [142]. A potential complication with this approach is that Fe-S enzymes may undergo structural changes upon substrate binding [29,143,144], and binding of flavodoxins/ferredoxins can induce difficult-to-predict structural changes. However, accurately predicting suitable interactions between electron transfer proteins and client Fe-S enzymes would greatly facilitate their use in engineered metabolic pathways. Such a tool might be capable of predicting the distinct metabolic roles of individual electron transfer proteins in native host based upon genome sequences.

5.2. De novo engineering of Fe-S proteins

De novo protein design presents an avenue for engineering Fe-S proteins and enzymes with novel functions. Recent research in this field, enabled by advances in computational modelling and artificial intelligence, allow the design of key structural elements from scratch [145]. New computational protein design tools such as the RoseTTAFold-based diffusion model, RFdiffusion, enables researchers to create proteins that show high affinity for a target of interest [146,147]. This may allow synthetic biologists to design proteins, such as reducing partners or oxygen-protective proteins, that specifically interact with an Fe-S enzyme of interest. The upcoming extension of RFdiffusion to incorporate ligands will enable modelling of precise protein-ligand interactions [148], as well as the design of Fe-S enzymes with defined substrate binding profiles and customized reduction potentials. These opportunities will advance our understanding of Fe-S protein structure-function relationships and pave the way for developing Fe-S enzymes with designed properties such as new-to-nature catalytic properties.

5.3. Fe-S proteins from analogue to digital: Electromicrobial production and bioelectronics

The rapidly-growing field of bioelectronics focusses on engineering and integrating living organisms into electronic circuits. Connecting cellular sensing with electronic devices via engineered electron transfer pathways has the potential to develop novel tools for a broad range of applications, including electromicrobial production (using electrochemistry to directly drive microbial production of chemicals, also called microbial electrosynthesis). At the heart of this research lie biological electron carriers (catalogued as “living electronic components” [149]), which includes Fe-S proteins. Here, we briefly review these two promising directions.

5.3.1. Powering metabolic pathways with renewable electricity

Coupling non-biological energy production such as solar and wind energy with microbial growth and chemical production (including microbial CO₂ capture) has theoretical efficiency advantages over feeding harvested plant biomass to engineered microbes [150], which may prove advantageous especially for producing commodity chemicals at low cost. However, the challenge lies in delivering electrical energy to microbial metabolism and biosynthesis. Many biological pathways for harnessing electrons in various forms exist [151], and multiple approaches for harnessing these pathways are being pursued (extensive reviews are available [152,153]) which have been compared and critically evaluated [154]. The ubiquity of Fe-S proteins within electron transfer pathways (especially ferredoxins, but also including catabolic pathways for C₁ substrates [155]) indicate that Fe-S clusters will play an important role in this exciting branch of metabolic engineering.

5.3.2. Plugging biological sensors into electronic devices

The immense value of environmental sensing has most recently been demonstrated by wastewater surveillance networks that detect diseases such as COVID-19 [156]. However, the responsiveness of such monitoring platforms are slowed by their reliance upon sample processing steps such as RNA extraction and RT-qPCR. Designing *in situ* sensors that combine the specificity of biological detection with the speed of electronic communication would vastly improve our ability to surveil our environment for pathogens and pollutants. The rapidly-growing field of bioelectronics seeks to merge biological specificity and function with electronic devices. Electron carrier proteins (such as ferredoxins) are a natural bridge between biological systems and electronics.

Taking advantage of existing pathways for extracellular electron transfer, chassis microbes have been engineered to couple biological processes to electronic devices [157]. These microbes can be integrated into devices that harness the diverse sensing capabilities found in biology. However, early implementations used transcriptional responses to drive expression of proteins that generate electrogenic responses and exhibited response times that are far too slow for real-time sensing. An impressive recent advance harnessed ferredoxins that fold into functional forms in response to chemical inputs [115]. These ferredoxins were used to bridge sensing and response components that communicated a biological input directly to an external electronic device [158]. Because the ferredoxin folds quickly and reversibly upon binding of its chemical trigger, the devices achieved detection times of <5 min. The engineered microbes were encapsulated into devices that were used to detect environmental contaminants from actual environmental samples.

6. Conclusion

The strength of the synthetic biology field is its focus on making biology easier to engineer [159]. Harnessing Fe-S enzymes into high-yielding biosynthesis pathways remains a considerable challenge. However, synthetic biology has successfully overcome the challenges that arise from messy biological systems, and recent breakthroughs in protein structure prediction have (nearly) solved the insolvable. We remain confident that focussed efforts attacking the challenges described in this review will accelerate metabolic engineering with Fe-S enzymes. We look forward to reading about the development of a host organism capable of fully supplying any level of Fe-S cluster demand without harming fitness, protein design approaches that ensure that expressed Fe-S enzymes remain active, and programs capable of identifying and designing electron transport proteins needed for Fe-S enzyme activity.

CRedit authorship contribution statement

Helena Shomar: Writing – review & editing, Writing – original draft.
Gregory Bokinsky: Conceptualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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