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1 **The Fe-S proteome of *Escherichia coli*: prediction, function and fate.**

2

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12 **Abstract**

13 Iron-sulfur (Fe-S) clusters are inorganic ubiquitous and ancient cofactors. Fe-S bound proteins
14 contribute to most cellular processes, including DNA replication and integrity, genetic
15 expression and regulation, metabolism, biosynthesis and most bioenergetics systems. Also, Fe-
16 S proteins hold a great biotechnological potential in metabolite and chemical production,
17 including antibiotics. From classic biophysics and spectroscopy methodologies to recent
18 development in bioinformatics, including structural modeling and chemoproteomics, our
19 capacity to predict and identify Fe-S proteins has spectacularly increased over the recent years.
20 Here, these developments are presented and collectively used to update the composition of
21 *Escherichia coli* Fe-S proteome, for which we predict 181 occurrences, *i.e.* 40 more candidates
22 than in our last catalog (Py and Barras, 2010), and equivalent to 4% of its total proteome.
23 Besides, Fe-S clusters can be targeted by redox active compounds or reactive oxygen and
24 nitrosative species, and even be destabilized by contaminant metals. Accordingly, we discuss
25 how cells handle damaged Fe-S proteins, *i.e.* degradation, recycling or repair.

26 1. Introduction

27 Fe-S clusters are assemblies of iron and sulfur atoms and stand among the most frequently used
28 protein cofactors in biology [1]. Fe-S clusters arise in various forms, wherein 2, 3 or 4 iron
29 atoms are linked to sulfide ions, yielding to the typical Fe₂S₂ (rhombic), Fe₃S₄ (cuboidal) or
30 Fe₄S₄ (cubane) clusters and some atypical types such as Fe₄S₃, Fe₄S₅, Fe₈S₈ or Fe₈S₉ clusters
31 [2–6]. Fe-S clusters were discovered as devices implicated in electron transfer in enzymes
32 participating in photosynthesis and respiration, but we now know that they contribute in
33 indispensable functions to nearly all cellular processes (see below). Fe-S proteins are present
34 in both prokaryotes and eukaryotes where their pleiotropic role extends their influence onto
35 general traits such as pathogenicity, CRISPR immunity systems and antibiotic resistance in
36 bacteria, aging, cancer or ataxia in humans, plant growth and even replication of coronaviruses
37 such as the SARS-CoV-2, the causal agent of the CoVID-19 pandemic [7–10].

38 Fe-S clusters undergo one-electron redox processes and can exhibit various redox states. This
39 makes them ideal catalysts for intra- and inter- molecular electron transfer processes. Their
40 redox potential value ranges from -600 mV (S.H.E.), depending upon the chemical nature of
41 their coordination environment and electronic properties of their immediate surrounding within
42 the polypeptide. Fe-S clusters with low redox potentials can be used as catalysts for
43 thermodynamically unfavorable reactions. This is best illustrated by radical-S-
44 adenosylmethionine (SAM) enzymes, which use a Fe₄S₄ cluster to inject one electron at a low
45 potential, generating reactive free radicals exploited in a myriad of biosynthetic and metabolic
46 reactions [11–15]. Fe-S clusters can also allow access of small compounds to ferric ions with
47 strong Lewis acidity properties. Such clusters can be used in non-redox catalysis, as in
48 dehydratases [16]. Last, reversible interconversions between cluster forms, with different redox
49 states or different nuclearity, is used in Fe-S bound transcriptional regulators that function as
50 sensors of oxygen, superoxide, nitric oxide, or even in proteins controlling DNA integrity [17–
51 22].

52 As a general trend, Fe-S cluster ligation sites are composed of cysteine residues whose thiol
53 side chains provide bonding to Fe atoms or form sulfide bridges. Hence, a four Cys-containing
54 motif, although admitting uncertainties on the space between individual Cys residues, is often
55 seen as a predictor of a Fe-S cluster binding site. However, this can be misleading for two
56 reasons. First, these sites may bind other metals, such as Zinc. Second, Fe-S cluster
57 coordination with oxygen-based (aspartate, tyrosine or glutamate) and nitrogen-based
58 (histidine or arginine) residues have also been described [23]. Hence, if the richness and
59 diversity of cluster environment is an asset for biology as it allows versatility, it prevents simple

60 and straightforward Fe-S cluster signature to be applied from primary sequence analysis. In
61 this article, we review the last advances in the search, identification, and assessment tools
62 applied to Fe-S proteins with an emphasis on the *Escherichia coli* Fe-S proteome for which we
63 present an updated version of the catalog of Fe-S proteins. We also address the question of the
64 relationship between O₂ and Fe-S-based biology and provide original data that led us to
65 envision different fates for damaged Fe-S proteins.

66

67 **2. Experimental assessment of the presence of an Fe-S cluster in a protein**

68 Experimental methods are required to assess whether a given protein hosts an Fe-S cluster or
69 not (for review see [24, 25]). An arsenal of *in vitro* strategies is available, but they constitute a
70 long and tedious path before a conclusion can be reached. In most cases, the biochemistry
71 requires manipulation in the absence of O₂ inside glove boxes and high amounts of either the
72 as-isolated holo-form protein or an apo-form in which an Fe-S cluster is chemically or
73 enzymatically reconstituted. Once the purified holo-protein is obtained, methods for
74 quantifying iron and sulfur are the first steps to suggest the presence of a cluster. Next,
75 complementary biophysical analyses, such as spectroscopy (UV-visible, Electron
76 Paramagnetic Resonance - EPR, Resonance Raman, Mössbauer, etc.), mass spectrometry, and
77 crystallography must be used to establish the presence of a cluster and to characterize its
78 properties and chemical environment, in particular the nature of its ligands [25]. Importantly,
79 those spectroscopic techniques usually require biochemical and/or genetic manipulations and
80 are complemented by functional studies. For in-depth study of Fe-S clusters, EPR and
81 Mössbauer spectroscopy are widely used since they provide a solid characterization of the Fe-
82 S cluster type, its environment, and redox state. Mössbauer spectroscopy is the more suited
83 method since it allows characterization of iron complexes in any oxidation or spin state.
84 However, this type of spectroscopy requires ⁵⁷Fe isotope incorporation into the sample,
85 demanding careful manipulation during cell culture or protein reconstitution. EPR studies can
86 bypass this step using the natural Fe isotope, however it cannot detect silent Fe-S clusters (Spin
87 = 0). For organisms amenable to genetic manipulation, use of strains lacking specific factors
88 required for building and inserting clusters into proteins can be used to predict whether a given
89 protein would hold a cluster and to assess its functional importance for activity *in vivo* [25].

90

91 **3. Bioinformatic prediction of Fe-S cluster containing proteins**

92 *In silico* identification of Fe-S binding sites from primary sequences or structures of proteins
93 remains challenging. Yet, several bioinformatic analyses [26–28] have proven decisive in

94 allowing identification of new Fe-S binding proteins further demonstrated by experimental
95 methods, giving significant insights into the detection of metabolic pathways on poorly
96 characterized species [29–32].

97 *Estellon et al.* developed a sophisticated machine learning tool to design and assess a penalized
98 linear model to predict Fe-S proteins using primary amino acid sequences [26]. Their approach
99 was based on an ensemble of descriptors from a tailored non-redundant database of Fe-S-
100 specific HMM (Hidden Markov Model) profiles and a curated selection of Fe-S coordinating
101 domains and signatures, which were used to guide their machine learning algorithm on a large
102 training dataset of protein sequences (PDB70, a data set of sequence alignments with identity
103 <70% and using PDB structures as query excluding *E. coli* K12 sequences). This Fe-S
104 predictive model of 67 descriptors reached 87.9% of precision and 80.1% of a recall (or
105 sensitivity) on the *E. coli* K12 proteome, which are values of higher performance when
106 compared to other motif-based analysis (Prosite, Pfam, InterPRO, etc.). Despite the inability
107 of their model to detect 27 known Fe-S proteins -at the time of the publication-, the capacity
108 of their software was demonstrated by the prediction and experimental validation of YhcC and
109 YdiJ. Furthermore, the model was tested on 556 proteomes from bacterial and archaeal species
110 and predicted an averaged content of Fe-S proteins at $2.37 \pm 1.31\%$ of those prokaryotic total
111 proteomes. A related method was created by *Valasatava et al.* [27] (available in the
112 MetalPredator webserver). In this case, the search for metal-binding motifs by HMM-profiles
113 combines domain-based predictions [33] and the local nature of Minimal Function Sites [34].
114 This tool has a similar precision (85.2%) and slightly higher recall (86.5%) when compared to
115 the results of the IronSulfurProteHome [26] on the *E. coli* proteome. Validation of their work
116 was based on 3D homology on seven of their predictions.

117 The search of Fe-S binding sites using 3D protein structures has been limited by the number of
118 structures or models available. However, a very recent study from *Wehrspan et al.* [28]
119 elaborated a new ligand-search algorithm to identify Fe-S cluster or Zn binding sites exploiting
120 the novel AlphaFold2 structure database [35, 36]. Their method is based first in the compilation
121 of a repertoire of all sidechain or backbone atoms that could potentially coordinate Fe-S
122 cofactors or Zn ions in the AlphaFold2 database, which are later grouped and used as potential
123 binding regions to be examined using a standard single-linkage clustering algorithm (within 8
124 Å) to superimpose each ligand type (Fe_2S_2 , Fe_3S_4 or Fe_4S_4) and their coordinating atoms in all
125 possible combinations. Next, those solutions are evaluated using a root-mean-squared
126 deviation (RMSD) of the ligands and checked for steric clashes. Results with poor RMSD
127 scores or steric clashes are removed, the remaining combinations filtered, the one with the

128 lowest RMSD retained and the ligand is placed into the structure. This study identified
129 thousands of potential Fe-S clusters in the proteomes of 21 organisms listed in the AlphaFold2
130 structures. When this analysis was applied to the *E. coli* proteome, it obtained a recall rate of
131 74%, which is lower than the sensitivity of *Valasatava et al.* (86.5%) or *Estellon et al.* (80.1%),
132 however it found a good overlap on the predicted Fe-S proteins or the false positives predicted
133 by those two previous studies. Moreover, this tool was able to calculate if some proteins are
134 more likely to accommodate Fe₂S₂, Fe₃S₄ or Fe₄S₄ clusters. Interestingly, the 3D scan made by
135 this work predicted that YjiM, YcbX, CyuA (YhaM) and PreT (YeiT) are indeed Fe-S
136 containing proteins, which were classified as false positives by *Estellon et al.* Likewise, it
137 agreed with *Valasatava et al.* that seven of their false positives could coordinate Fe-S clusters.
138 In addition, this work could place Fe₄S₄ clusters on the structural models of the newly
139 characterized DppD or on the *E. coli* U32 protease homologs that have been experimentally
140 confirmed (UbiU, UbiV and TrhP) or strongly suspected (RlhA) as Fe-S proteins [15, 37, 38].
141 Interestingly, none of these computational tools predicted MnmA as a Fe-S binding protein, an
142 issue that remains controversial [35, 36]. Noteworthy, no bias or specific enrichment in a
143 functional class of Fe-S protein or type of Fe-S binding motif, was found to be associated with
144 one particular algorithm. It is clear that precision and sensitivity of any of those *in silico*
145 approaches are still limited, due to the strength of their descriptors and conditioned to the
146 curation and size of the datasets used for training and testing of the algorithms. Thus, those
147 predictive methods will only improve as long as the factors involved in the Fe-S cluster binding
148 and assembly -such as plasticity, abundance and nature- are elucidated by experimental and
149 functional studies.

150

151 **4. The *E. coli* Fe-S proteome: an update**

152 In the specific interest to establish the *E. coli* Fe-S proteome, we used two validated
153 bioinformatic tools to predict a list of Fe-S bound candidate proteins: MetalPredator and
154 IronSulfurProteHome websites [26, 27]. For *E. coli*, those tools predicted 141 iron-sulfur
155 cluster containing proteins and we added 21 proteins, for which evidences of Fe-S binding were
156 recently published. Furthermore, 19 additional proteins were predicted by 3D modelling using
157 AlphaFold database [28]. This yielded to 181 entries represented in Figures 1 and 2. For each
158 protein, type of evidence, either computational (Figure 1A) or biophysical (Figure 1B),
159 supporting the notion that it is a Fe-S bound protein is given in Supp Table 1. Roughly half of
160 the 181 *E. coli* Fe-S proteins have been validated as such by biophysical methods (Figure 1B).

161 *E. coli* makes use of Fe-S proteins in most, if not all, processes such as DNA replication, repair
162 and transcription, RNA modification and translation, amino acid, vitamin and metabolite
163 biosynthesis, and bioenergetics (Figure 2). Respiration is the most represented cellular process
164 as *E. coli* allocates 28% of its Fe-S proteome to respiratory pathways (Figure 2). Biosynthesis
165 of metabolites and cofactors, including Fe-S themselves, constitute the second most populated
166 group. As expected, Fe-S bound proteins allow stress sensing and adaptation. Interestingly, a
167 few Fe-S proteins are predicted to be involved in transport. Interestingly, there are 15 Fe-S
168 proteins for which no functional prediction, neither from homology search or genetic screen
169 was found in the literature. Attribution of function for these proteins might unearth activities
170 of interest.

171

172 **5. Fe-S & Oxygen**

173 Besides their intrinsic redox chemical properties, the reason why Fe-S clusters were retained
174 in such a wide range of proteins might be related to the geological conditions prevailing at the
175 onset of life, namely abundance of Fe and S and anoxic atmosphere. The Earth's Great
176 Oxygenation Event, probably due to the activity of Fe-S containing photosynthesis apparatus,
177 caused shortage in bioavailable iron as it precipitated from the soluble ferrous form into
178 insoluble ferric form. Besides, Fe²⁺ can act as a catalyst for production of reactive oxygen
179 species (ROS), via Fenton reaction, which destabilize Fe-S cluster. Therefore, the long-
180 admitted view is that sophisticated Fe-S cluster biogenesis machineries emerged to build and
181 insert Fe-S clusters into proteins [39–41]. Indeed, Fe-S cluster-based biology and aerobic life
182 might be seen as mutually exclusive, unless a great investment was put to control Fe-S
183 biogenesis and its use in the cell. Support for this view was put forward by *Andreini* and
184 collaborators in a bioinformatic analysis of Fe-S proteins predicted content of prokaryotic
185 genomes [42]. A set of 434 prokaryotes genomes, including 18 genomes from obligate aerobes,
186 29 from obligate anaerobes, 214 from aerobes, 130 from aerotolerant anaerobes and 43 from
187 facultative anaerobes was analyzed for occurrence of Fe-S proteins as predicted by
188 bioinformatic protocols. Remarkably, there are more predicted Fe-S proteins per genome from
189 anaerobes than aerobes, even when corrected with genome size. In aerobes the number of
190 predicted Fe-S proteins correspond to less than 3% of the total predicted proteome whereas in
191 genomes of anaerobes (obligate, facultative, aerotolerant) predicted Fe-S proteins amount to
192 more than 3% of the total predicted proteins. Accordingly, we observe that *E. coli* Fe-S
193 proteome is 4% and some recent *in silico* studies in archaea suggest that in methanogens and
194 alkanotrophs 5-10 % of the proteome corresponds to Fe-S proteins [29]. Both aerobes and

195 anaerobes exhibit shared family of Fe-S proteins involved in energy production and conversion,
196 amino acid metabolism, nucleotide and coenzyme metabolism, and Fe-S biogenesis.
197 Interestingly, the number of paralogs involved in energy production and conversion is much
198 higher in anaerobes than in aerobes, presumably reflecting the multiplicity of potential electron
199 acceptor chemical recruited for anaerobic respiratory chains instead of the unique O₂ in aerobic
200 ones. Another important observation was that the number of proteins using Fe₂S₂ cluster, which
201 are more resistant to O₂ and less demanding in iron, appeared to increase in aerobes vs
202 anaerobes. Hence, this study stands as a validation of the expected negative interaction between
203 O₂ and Fe-S-based biology. It also provides a rationale of why organisms developed strategy
204 to mitigate deleterious consequences of enhanced O₂ level in the atmosphere after emergence
205 of photosynthesis, either by reducing the use of Fe-S cluster containing proteins, or by evolving
206 dedicated assisting biogenesis machineries.

207

208 **6. What to do with damaged Fe-S proteins: to repair, recycle or degrade them?**

209 The capacity of Fe-S clusters to detect O₂, ROS or reactive nitrogen species (RNS) has its
210 drawbacks, as Fe-S clusters can be altered or degraded by such compounds, which might lead
211 to inactivation and destabilization of the hosting polypeptide. One question is to know whether
212 all Fe-S proteins are similarly destabilized in the face of ROS or RNS. The answer is evidently
213 negative since alteration of Fe-S cluster will depend upon its location into the structure of the
214 hosting polypeptide. Well buried, solvent inaccessible clusters are likely to be more stable. On
215 the contrary, well exposed cluster might be more prone to targeting by toxics and ensuing
216 alteration.

217 This was well studied with members of the dehydratase family, such as aconitase or fumarase.
218 The nature of the ligands holding the cluster can also be a determining factor for its stability.
219 The case of fumarase had been investigated in detail [43]. Fumarases contain Fe₄S₄ bound via
220 a CX_nCXXC motif. Like all dehydratases, cluster in the *E. coli* fumarase exhibits a labile
221 exposed catalytically active Fe atom, which is freed upon oxidation of the cluster, yielding to
222 an inactive Fe₃S₄-bound enzyme⁴⁴. In contrast, the *Bacteroides thetaiotaomicron* fumarase
223 harbors a CX_nC motif, which cannot be reactivated when oxidized by H₂O₂. This is due to the
224 generation of radical species that carbonylate the peptide chain [44]. Thus, composition of the
225 ligating motif itself could be essential for Fe-S proteins in the resistance to oxidative
226 environments. Besides, the number of ligand cysteines could also be important for tolerance
227 to oxygen attack, as reported in the case of the six cysteines liganded Fe₄S₃ cluster of
228 hydrogenase from *Ralstonia eutropha* [6].

229 Another example of Fe-S cluster targeted by environmental redox conditions is given by the
230 transcriptional regulator Fnr that controls anaerobiosis/aerobiosis switch [45–47]. The Fe₄S₄
231 cluster integrity of Fnr controls the monomer-dimer equilibrium and eventually the expression
232 of hundreds of genes. Briefly, its Fe₄S₄-bound homodimeric form binds to operator regions of
233 genes, repressing those involved in aerobic metabolism and activating those required for
234 anaerobic metabolism. Exposure of the cluster to O₂ leads to the Fe₄S₄ and subsequent
235 conversion to Fe₃S₄ and Fe₂S₂ causing the dissociation of the dimer in inactive apo-monomers.
236 Another question concerns the fate of the “damaged” (or oxidized) Fe-S proteins. Anew,
237 studies with Fnr as a model provided important insights. Indeed, it was found that in aerobic
238 condition, apo-Fnr was degraded by the chaperone/protease ClpXP [45, 48, 49] but it was also
239 shown that reconversion of apo-Fnr to Fe₄S₄-Fnr is possible upon switch to anaerobic growth
240 [49]. This perfectly illustrates how cells handle “damaged” Fe-S protein, either degrading them
241 or reactivating them. This last possibility suggests that apo-forms of some proteins are stable
242 long enough to engage in a new cycle of maturation via their recognition by ISC or SUF
243 machineries. This view is consistent with an early study aiming at investigating whether SUF
244 was a repairing system [50]. Interestingly, besides Fnr, other Fe-S proteins (AcnB, IscU, IscR,
245 LipA and MoaA) have been identified as substrates of ClpXP in *E. coli* [51, 52], which may
246 suggest that this protease could be involved in a broader spectrum in the homeostasis of the Fe-
247 S proteome. Whether ClpXP acts on these Fe-S proteins specifically when they are damaged
248 remain to be established.

249 A distinct possibility put forward for long, is the existence of dedicated factors that would
250 “repair” damaged clusters. Interestingly, Clp might play a role in the repairing of damaged Fe-
251 S proteins by helping iron release from Dps sequestration [53]. YtfE might also be such a repair
252 factor acting by re-metalating clusters having lost Fe atoms [54] like those invoked in the
253 progressive dismantlement of Fnr or in the case of dehydratases. For instance the oxidatively-
254 damaged cluster Fe₃S₄ of the *E. coli* fumarase can be reactivated if an iron source is provided
255 [55].

256 Furthermore, studies in members of the radical-SAM family have enlarged our view of protein
257 factors needed for the repair of their cannibalized Fe-S clusters. The NfuA carrier protein has
258 been endowed with the capacity to regenerate the Fe₄S₄ cluster in the lipoyl synthase (LipA),
259 which sulfur atom has been sacrificed during catalysis [56].

260

261 **7. Perspectives**

262 Since their discovery [1], Fe-S proteins have been studied by relying on a wide array of
263 approaches, from chemistry to genetics via biochemistry, structural biology and spectroscopy.
264 This has provided us with a deep insight in (i) the mechanism underlying reactivity of Fe-S
265 proteins, (ii) the role played by the cluster, (iii) the contribution of Fe-S proteins to cellular
266 homeostasis. The next step will be to apply high-throughput techniques aiming at getting an
267 integrated description of the Fe-S proteome dynamics *in vivo*. Thermo proteome profiling
268 methodology already contributed to a whole cell view by reporting the effect of genetic
269 alteration of the ISC system [57]. Another methodology is chemoproteomic [37]. This
270 technique is based in tracking Fe-S cysteine ligands by LC-MS/MS coupled to the labeling
271 using iodoacetamide-alkyne derivative (termed isoTOP-ABPP and ReDiME). Its recent use to
272 follow changes in Fe-S proteome as a response to iron-depletion or mutations in the ISC system
273 opens highly promising possibility to an *in vivo* integrated description of cell response and
274 adaptation to environmental challenges and genetic disorders. We recently showed that
275 heterologous expression of some active Fe-S proteins requires the heterologous co-expression
276 of the native Fe-S cluster assembly machinery [58]. Deciphering the recognition mode between
277 machineries and targets might help in optimizing and broadening heterologous expression of
278 Fe-S containing proteins, a goal of considerable interest in biotechnology.

279 Bioinformatic approaches have provided new vision regarding distribution of Fe-S proteins
280 and their evolution. Yet, predicting whether a given polypeptide will host a cluster is still
281 limited by the wide diversity of motifs allowing Fe-S binding. Another structural feature that
282 might be used is the intrinsic information of Fe-S polypeptide to be recognized by Fe-S
283 maturation factors. Indeed, studies in eukaryotes have unearthed the so-called LYR motif,
284 which endows a subset of Fe-S proteins with the capacity to interact with Hsc20 ISC machinery
285 component. Future studies should aim at investigating whether similar type of “recognition
286 sequence” occurs and permits interaction between apo-targets and machineries in prokaryotes.

287

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292

293 **Data Availability Statement**

294 *The data underlying this article are available in the article and in its online supplementary*
295 *material.*

296

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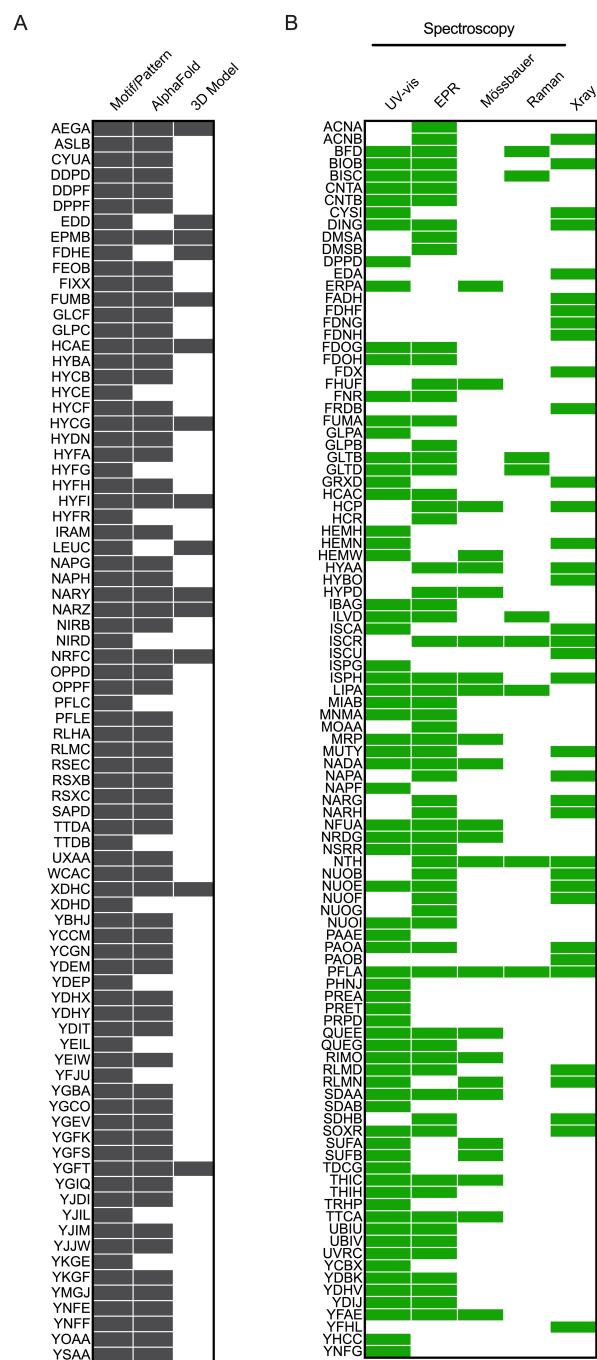
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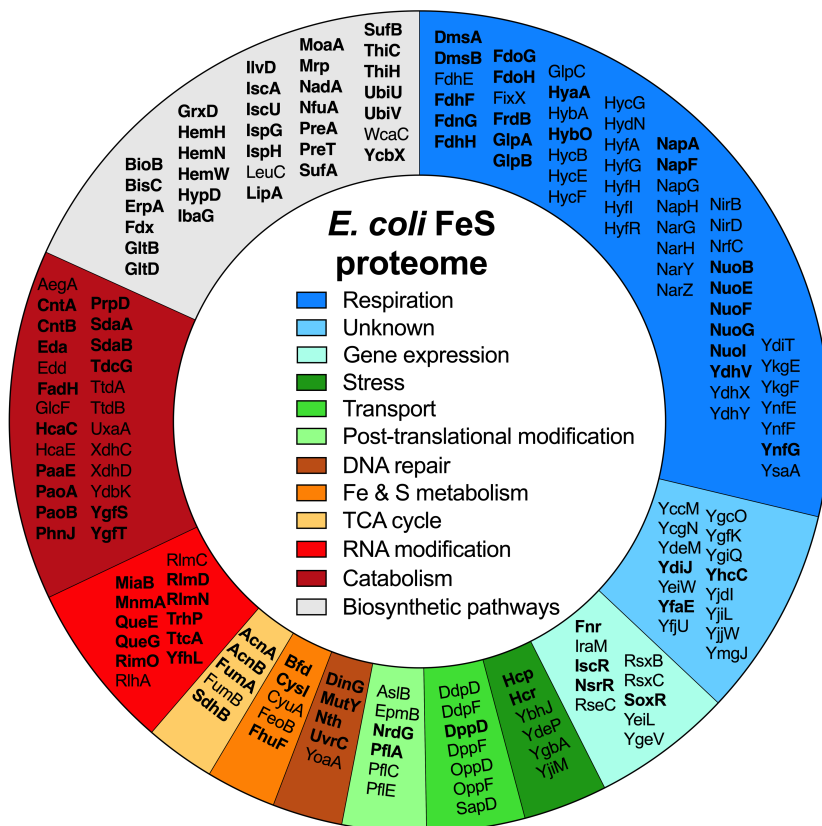
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441 **Figure 1. Characterization of *E. coli* Fe-S proteome.** A. Left panel: List of Fe-S proteins
 442 predicted by bioinformatic tools only, *i.e.* not experimentally assessed yet. Motif/Pattern stands
 443 for primary sequence motif and pattern search [26, 27] or dedicated sequence alignment (see
 444 Supp Table 1), AlphaFold [28] and 3D model obtained from structural analysis of homologous
 445 holo-proteins (see Supp Table 1). B. Right panel: List of Fe-S proteins characterized by
 446 biophysical approaches such as UV-visible (UV-vis), Electron Paramagnetic Resonance
 447 (EPR), Mössbauer and Resonance Raman spectroscopy and X-Ray crystallography (Xray) (see
 448 Supp Table 1).



449

450 **Figure 2. The Fe-S proteome of *E. coli*.** Distribution of the *E. coli* Fe-S proteins according to
 451 their association with a specific biological process. Fe-S proteins characterized by biophysical
 452 approaches are in bold. Fe, iron; S, sulfur; TCA, Tricarboxylic Acid Cycle. Details about all
 453 listed Fe-S proteins are in Supp Table 1.