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Review

Genetic approaches of the Fe–S cluster biogenesis process in bacteria: Historical account, methodological aspects and future challenges ☆

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ABSTRACT

Since their discovery in the 50's, Fe–S cluster proteins have attracted much attention from chemists, biophysicists and biochemists. However, in the 80's they were joined by geneticists who helped to realize that *in vivo* maturation of Fe–S cluster bound proteins required assistance of a large number of factors defining complex multi-step pathways. The question of how clusters are formed and distributed *in vivo* has since been the focus of much effort. Here we review how genetics in discovering genes and investigating processes as they unfold *in vivo* has provoked seminal advances toward our understanding of Fe–S cluster biogenesis. The power and limitations of genetic approaches are discussed. As a final comment, we argue how the marriage of classic strategies and new high-throughput technologies should allow genetics of Fe–S cluster biology to be even more insightful in the future. This article is part of a Special Issue entitled: Fe/S proteins: Analysis, structure, function, biogenesis and diseases.

1. Introduction

Genetic approaches define functional loci (coding or control regions) by their alteration and infer, from the consequences, the role of those loci in the parental strain. What made this intrinsically indirect approach so successful in deciphering cellular mechanisms and processes? First, genetic analyses provide understanding of processes as they occur *in vivo*, thereby including the constraints and complexities of the living cell. Second, genetic selections have the unsurpassed capacity to unearth the unexpected as it relies on selection of extremely rare events. Alternatively, screening protocols can be rewarding but their detection capacity is logs less than selection. Third, genetic approach provides an unbiased way of thinking about a process as it requires no *a priori* knowledge on how newly discovered genes will influence the processes under study.

The objective of the present review is to recognize the impact genetics has had in our current understanding of Fe–S cluster biogenesis. With due apologies to many colleagues, we do not intend to cover all of what has been achieved but rather present the different approaches that were used by bacterial geneticists in describing their principle objectives, noting their strengths and limitations. We will describe how the application of classical concepts of genetics with new integrated high-throughput approaches for rapid phenotypic screening, genome sequencing and engineering, will offer efficient ways to tackle Fe–S cluster biology. Last, we do not provide background on Fe–S cluster biogenesis as many excellent dedicated articles are to be found in the present special issue, which the reader might want to consult first.

2. The early days: from enzyme activity to gene identification

The specific power of bacterial genetics derives from the ability to mutagenize cells, to analyze very large numbers of events and to perform selections on large populations. In 1971, Streicher and collaborators sought to identify *Klebsiella pneumoniae* mutants lacking nitrogenase activity [31]. Because nitrogenase activity was thought to depend upon two polypeptides, the expectation was that mutations would cover a region of DNA of 2–3 kb in size. Mutants unable to use N_2 as their sole source of nitrogen were obtained after penicillin enrichment of nitrosoguanidine-treated cells incubated under N_2 atmosphere. Then, nitrogenase-deficient mutants were isolated after screening for a difference in colony size and pigmentation on minimal agar medium under N_2 atmosphere. Several loci were identified and mapped close to the *his* locus by P1 transduction. In the author's words, "a cluster of *nif* genes, perhaps representing the long awaited *nif* operon, has been mapped near *his* on the *Klebsiella* chromosome." Such a cluster was eventually transferred to *Escherichia coli*, which endowed it with nitrogen fixing capability [10]. Clearly from the size of the DNA piece required, the apparent number of *nif* genes identified exceeded the two required for the sole synthesis of the nitrogenase enzyme and this opened the way towards identifying ancillary factors involved in regulation, maturation or localization of nitrogenase. Years later, a protein with cysteine desulfurase activity, was purified from *Azotobater vinelandii*, its N-terminal sequence defined and used to design oligonucleotide primers for PCR to probe

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an *A. vinelandii* genomic library [40]. After cloning and sequencing of a 2.5 kb DNA fragment, the *iscS*, encoding the cysteine desulfurase, *iscU*, and *iscA* genes were identified [40]. These genes were also present in *E. coli* and this set the ground for the discovery of the ISC system. It took additional time to verify that *isc* genes were required for general Fe–S cluster biogenesis whereas orthologs NifS and NifU were specific for maturation of nitrogenase (see below), highlighting the crucial importance of the initial screen or selection procedure used.

3. The recent days: from gene inactivation to integrative physiology

Forward genetics became popular when the nucleotide sequence of genomes was made available and genes could be inactivated systematically with no *a priori* knowledge on their function. Later, after genomic sequencing became a routine procedure, several groups embarked on constructing genome-wide collection of single-gene deletion mutants. A frequently used library is the *E. coli* KEIO collection that offers deletion/substitution by a kanamycin resistance cassette of 3 985 genes, out of 4288 genes in *E. coli* K12 [1]. Using high-throughput-based methods, such a collection can be screened against large sets of different culture conditions and the cellular role of the inactivated gene can be inferred from the phenotypic pattern. For instance, a mutant exhibiting altered sensitivity to oxidative or nitrosative stresses, poor growth under iron limitation or with TCA-dependent carbon sources might be a good gene candidate for Fe–S cluster biogenesis. Unfortunately such an approach failed to be useful in the case of Fe–S cluster biogenesis genes, because of gene redundancy (see below).

Another way to exploit such a mutant collection is to screen for mutants altering a given activity, if an efficient screening protocol is available. For instance, Esaki and collaborators searched for mutations that would compromise activity of dihydropyrimidine dehydrogenase (DPD), a heterotetrameric protein (PreT/PreA) containing four [4Fe–4S] clusters [15,16]. DPD catalyzes the reduction of uracil (and also thymine) to its 5,6-dihydro-derivatives (DHU). The reverse reaction allows uracil produced from DHU by DPD to be converted to nucleobases by the pyrimidine metabolic pathway. Thus, a DPD-deficient strain grown in a medium containing radiolabeled DHU shows reduced incorporated radioactivity into DNA. Forty-nine mutants exhibiting reduced radioactivity incorporated into DNA were identified, which included *isc* mutants as expected [16]. Other strains included mutations in *pdxH* and *atpABCEFGH*, which affect synthesis of PLP and ATP, respectively, both required in the functioning of ISC system. Others such as that inactivating *feoAB* are of high interest as they affect iron homeostasis and might provide us with some clues about the iron source for Fe–S cluster biogenesis.

Similar general screens of the Keio bank for widely different phenotypes or activities have very often revealed a role for Fe–S cluster biogenesis factors. This was the case in screening for antibiotic sensitivity, motility or even for lambda phage infection [18,22,26,33]. Although these general approaches do not provide us with new insight on how Fe–S cluster biogenesis is carried out or regulated, they illustrate the central role of Fe–S clusters in cell physiology and pave the way towards an integrated view of Fe–S cluster homeostasis at the cellular level.

4. Genetic study of essential functions

By definition, an essential gene cannot be inactivated and will escape all loss of function mutant selection/screen protocols. Hence, the geneticist will deduce a gene is essential from the inability to isolate a mutated variant. To back up this reasoning however, additional evidence is necessary and several genetic strategies have been developed to demonstrate the essentiality of a gene. This issue was often the case in the study of Fe–S cluster biogenesis study because it is an essential process.

One strategy relies on genetic linkage and was employed by Outten *et al.* [27] and Trotter *et al.* [35]. Basically, the question is to test whether a mutation X can be co-transferred with a selectable marker M in a given recipient strain. Usually, in *E. coli*, the selectable marker M is a cassette conferring resistance to an antibiotic and the transfer is mediated by P1 transduction, which can carry over 100 kb DNA. Thus if M and X are less than 100 kb apart, they will be co-transduced at a certain frequency, the value of which is independent of the recipient strain used. However, the case might arise that the frequency of co-transduction will be undetectable when using a particular recipient strain (strain containing a mutation Y), i.e. in this very recipient, none of the transductants selected to acquire the M marker got the X allele as well. If so, the usual interpretation is that the mutation Y prevented stable acquisition of X because the presence of both mutations Y and X is lethal. This will be reported as a case of synthetic lethality between Y and X and be interpreted as the two cognate wild type (Y_{wt} and X_{wt}) genes being functionally redundant and essential. Likewise, deletions of the *suf* operon (or of individual genes except *sufA*) could not be transferred into strain containing a deletion of the *isc* operon (or of individual *isc* genes but *iscA*). This approach has been widely used for decades in genetic mapping and gene analysis and proven to be highly reliable with a few exceptions in which local chromosomal structure might distort the relationship between gene distance and gene co-transfer frequency.

The next question is how to study essential genes, as one cannot delete them. A classic way is to construct conditional allele. In strain containing such alleles, the mutant phenotype is expressed solely under restrictive — and possibly, transient — conditions, while the viability of the strain is maintained under so-called permissive conditions. Given the essentiality of Fe–S cluster biogenesis for most organisms, such genetic approaches were used broadly to construct conditional alleles.

Another strategy rests on conditional expression of an otherwise wild type allele. This was used by the group of Dennis Dean in studying *A. vinelandii* that contains an *isc* and *nif* operon. A strain was constructed which carried two separate copies of the contiguous and cotranscribed *nifUS* genes, one at its normal genomic context and under its normal endogenous regulatory circuit, and the other at a different site and with its expression under a sucrose inducible promoter (P_{scrX}) [11]. In the presence of sucrose, i.e. permissive conditions, mutations were introduced in the endogenous *nifUS* genes and the resulting strains tested for growth under nitrogen-fixing conditions. In the absence of sucrose, strains deleted either for the *nifU* or the *nifS* genes could not grow under nitrogen-fixing conditions. Next, attempts were made to introduce mutations in the *isc* operon but were unsuccessful even in the presence of sucrose. These results indicated that levels of NifU and NifS reached with the sucrose-controlled alleles were sufficient to provide enough Fe–S clusters for nitrogenase maturation but could not fulfill the overall cellular demand for other Fe–S cluster proteins. Essentiality of the Isc system and the specificity of the Nif system were inferred [19]. Moreover, these experiments provided the authors with tools to investigate the interplay between the two systems, if any, *in vivo* (see below).

Another genetic strategy is to use genetic by-pass. This was first developed with the study of ErpA, an A-type carrier. ErpA was identified on the basis of its sequence similarity with IscA and SufA. At first recombining an *erpA* allele inactivated by insertion of a kanamycin resistance cassette in the *E. coli* chromosome yielded strains that had undergone duplication of a large chromosomal region (>13 kb in size) such that they carried both the inactivated and the wild type copies of the *erpA* gene [20]. This indicated that *erpA* gene might be essential (this is a cautionary tale of how compensating secondary mutations can easily be picked up when dealing with essential processes). A way to demonstrate that *erpA* was essential, and to assess its contribution and importance was to design a by-pass thanks to serendipity and evolution. In *E. coli*, the two Fe–S containing protein IspG and IspH are essential for growth as they catalyze the synthesis of isopentenyl phosphate (IPP), a precursor of a myriad of secondary metabolites as well as a key component for cell envelope biosynthesis (Fig. 1). This so-called 2-C-methylerythritol-4-phosphate (MEP) pathway is present in most prokaryotes whereas most eukaryotes synthesize IPP from mevalonate

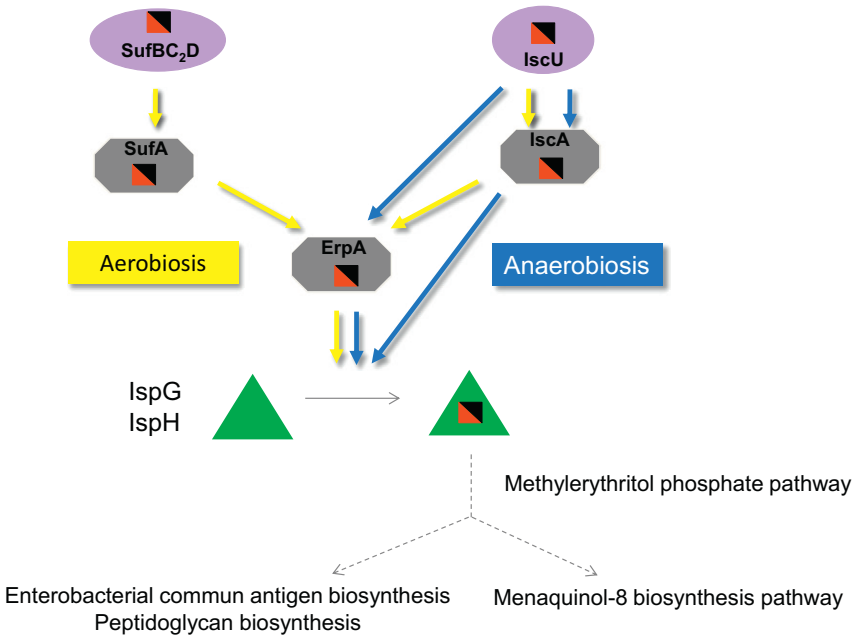

Fig. 1. A model of Fe–S cluster delivery from scaffolds to apotargets. This illustrates the redundancy between the ATCs (grey octagons) for maturation of IspG/H (green triangles), two proteins containing a [4Fe–4S] cluster (red/black squares) under different environmental growth conditions (aerobiosis: yellow arrows; blue arrows: anaerobiosis). The IspG/H enzymes participate to the production of isoprenoids, *via* the methylerythritol-4-phosphate (MEP) pathway, which are precursors of components for the cell envelope biosynthesis (dotted arrows).

(MVA). Of interest was the fact that the MVA-dependent pathway includes no Fe–S cluster containing enzymes. Hence, if the *erpA* mutation was lethal because of a reduced pool of IPP following an inefficient maturation of IspG or IspH proteins, then we reasoned the problem might be solved by expressing the eukaryotic MVA-dependent pathway in the *E. coli erpA* mutant. In their study on IPP biosynthesis, Campos et al. [8] had already constructed a chimeric operon encoding the eukaryotic MVA-dependent pathway under the control of a pBAD promoter. As predicted this MVA-dependent pathway was able to rescue an *erpA* mutant if both MVA and arabinose were present in the medium [20]. This genetic by-pass was subsequently exploited to demonstrate the essentiality of several Fe–S cluster biogenesis encoding genes or combination thereof, demonstrating that death of *isc suf* mutant under aerobic conditions and in rich medium is due to a too low level of IPP [35,36].

5. Genetic approaches to study redundancy

5.1. Revealing the possible

Because Fe–S cluster biogenesis is an essential process, it is not surprising that organisms have evolved several redundant ways to carry on such a task. Redundancy renders interpretation difficult as it can mask deleterious effect of some mutations. The conditional alleles described above were remarkable tools to sort out neutrality from redundancy. For instance, following their study on *A. vinelandii* briefly presented above, Dean and colleagues engineered another *nifUS*-conditional operon that allowed high level of NifU and NifS to be synthesized (using an arabinose inducible promoter). They observed that an *iscU* mutant could now be rescued by arabinose-induced *nifU* gene [11]. Hence this study highlighted potential redundancy between the two scaffolds IscU and NifU, but only if the latter is overexpressed. Evidently, the question raised was whether this has any significance physiologically, in other words, whether such high level will ever been produced by the bacterium under natural conditions. This concern is legitimate but should not overshadow the added value of such approach, which can reveal potential capacities of some components while pointing the incapacity of others. For instance, regardless of its level of production, NifS was unable to substitute for IscS, revealing differences in the *in vivo* specificities of two highly related enzymes [11].

5.2. Redundancy varies with growth conditions

Textbooks teach us that phenotype is the product of genotype and environment. Studies of Fe–S cluster biogenesis provide a remarkable illustration of how varying growth conditions might change phenotypes of mutants. The genes of concern here are those involved in the trafficking step, *erpA*, *iscA* and *sufA*, referred to as the A-type carriers. The already mentioned *erpA* mutant is non-viable under aerobic conditions because it does not contain enough IPP, as a consequence of deficient maturation of IspG/H proteins [20] (Fig. 1). In contrast, *iscA* or *sufA* mutants do not show any gross phenotype. However, combining *iscA* and *sufA* yielded a non-viable strain, which, like *erpA*, could be rescued by the MVA-dependent pathway [36]. This implied that all three, *iscA*, *sufA* and *erpA* genes were necessary for IspG/H maturation. This implied also that *iscA* and *sufA* were fulfilling redundant functions, different from that of *erpA*. With the additional help of phylogenomic studies, IscA and SufA were proposed to act upstream of ErpA in a sequential Fe–S cluster transfer relay that eventually reached the apotargets IspG/H (Fig. 1). However, when strains were incubated under anaerobiosis, the requirement for "redundant factors" was no longer the same. Under anaerobiosis, an *erpA* mutant was viable, indicating that Fe–S clusters could still reach IspG/H (Fig. 1) [36]. Presumably, the absence of ErpA could be compensated by IscA (or SufA) or the cluster could go directly from the ISC system to the apotargets by-passing ATCs. Moreover, an *iscA sufA* mutant was found viable, again in contrast to what has been observed under aerobiosis, again suggesting that either ATCs are of no use in these conditions or that ErpA compensated for the absence of IscA. That the latter was true was demonstrated by the fact that an *erpA iscA* mutant was not viable. This meant that under anaerobiosis, IscA and ErpA are functionally redundant while they have unique task under aerobiosis (Fig. 1). Redundancy is supposed to reflect shared biochemical features and this is what biochemical studies have indeed shown concerning the ATCs. But clearly this does not suffice to explain the *in vivo* behavior of the mutants reported above and other aspects, specific to the *in vivo* situation might have to be considered.

We might speculate that IscA is less efficient than ErpA and that under aerobiosis the overall Fe–S cluster demand, e.g. the sum of the number of proteins waiting for their clusters and of the stability of the holoforms, might be higher. In these conditions, the contribution of ErpA would be essential for fulfilling the demand. In contrast, under anaerobiosis, the overall demand might be low because of enhanced stability of the matured proteins and under such condition, IscA might be as efficient as ErpA. This genetic study brought to light the complexity of the Fe–S cluster trafficking step within the cellular context.

5.3. Specificity beyond redundancy

The conclusion that the two ISC and SUF systems are redundant roots in the synthetic lethality of an *isc suf* double mutant. However, assays for Fe–S dependent enzyme activities suggested that there might be some level of substrate specificity between the two systems. This was nicely illustrated by constructing a strain that synthesizes only the SUF system under the control of the pBAD promoter. This strain was extremely inefficient, even when the SUF system was overproduced, to mature the multi-subunit Complex I (or Nuo complex) that contains 9 Fe–S clusters. The consequences were that the proton motive force (p.m.f.) was highly reduced and the strain exhibited enhanced resistance to aminoglycosides, whose uptake is p.m.f. dependent [12]. When the ISC system was reintroduced, the strain recovered wild type level of p.m.f. and sensitivity to aminoglycosides [12]. Hence, for reasons yet to be deciphered at the molecular level, it appears that the SUF system is less efficient in maturing Nuo than the ISC system. This was also observed for FNR, the transcriptional sensor of the anaerobic/aerobic switch, which appeared matured only by the ISC system under aerobiosis [23].

6. Genetic interactions: an old trick in vogue again

Genetic interactions are part of the arsenal of genetic approaches since the early 1900's when the term “epistasis” was coined by William Bateson to describe conditions wherein the action of one locus masked allelic effect at an another locus. This approach was recently revived by high throughput analysis and availability of comprehensive single gene knock-out mutant collection. Negative/aggravating genetic interactions are identified when the combinations of mutations cause cell death (synthetic lethality) or retard growth (synthetic sickness). In most cases, negative interactions are due to mutations in genes acting in parallel redundant pathways. Conversely, positive genetic interactions correspond to cases where the double mutant is healthier than the single parental mutant strains. Positive interactions are expected to reveal buffering (also referred to as alleviating or suppressing) mechanisms in the cell against genetic perturbations.

E. coli genetic interactions were analyzed by Emili and colleagues on a genome-wide scale [2,3,7]. The approach, termed *E. coli* synthetic genetic array (eSGA) used robotic automation to construct *E. coli* double mutants by conjugation between high frequency recombination (Hfr) strains as donors, and F− strains as recipients. In the Hfr strains the transmissible low-copy episome fertility factor (F), which promotes DNA transfer via conjugation into an F− bacterial cell, is integrated into the chromosome. Chromosomal DNA transfer occurs in a unidirectional manner starting with loci adjacent to the episome origin of transfer, until mating is interrupted. Once inside the recipient, the donor DNA is integrated into the recipient chromosome by homologous recombination. In a series of experiments, the donor was an *E. coli* Hfr Cavalli strain containing an *isc* mutation (deleted and replaced by a chloramphenicol (Cm^R) resistance cassette), and it was mated with a genome-wide high-density arrayed KEIO collection. Growth defects or advantages, if any, of the trans-conjugants were detected by colony imaging. All the individual *isc* mutations resulted in aggravating interaction when combined with mutations in the *suf* genes. An exception was the *iscA* mutation, which showed aggravating interaction only with the *sufA* mutation, consistent with previous work (detailed above). Negative interactions were observed between the *isc* genes and several other genes such as *grxD*, *ygfZ*, *ravA* and *viaA*. These negative interactions suggested that the SUF pathway might recruit GrxD, YgfZ, and RavA/ViaA under certain conditions and/or for maturating certain Fe–S cluster proteins. Interestingly, there is additional evidence that these four proteins have a role in Fe–S cluster biogenesis. GrxD is a homodimeric monothiol glutaredoxin that binds and transfers a Fe–S cluster to an apotarget *in vitro*, and interacts with the Fe–S cluster carrier, NfuA, and the Fe–S cluster protein MiaB [5,39]. YgfZ was proposed to play a role in Fe–S cluster biogenesis or repair during oxidative stress based on its impact on Fe–S cluster protein activities under plumbagin stress [37]. The RavA ATPase and its putative cofactor ViaA were proposed to facilitate maturation of the Nuo complex [38]. Negative gene interactions were also observed between the *isc* genes and genes involved in heme cytochrome maturation (*ccmC*), ubiquinone biosynthesis (*ubiH*), and folate biosynthesis (*folB*). These negative genetic interactions might reflect a burden for a cell to have multiple mutations in different components of the membrane electron transfer chain.

To conclude, genetic interactions on a large scale have provided a global map of relationships between many cellular pathways and the Fe–S cluster biogenesis process, the molecular basis of these interactions remained to be elucidated.

7. The suppressor-based strategies

Suppressors are secondary mutations that revert the mutated phenotype to, or close from, wild type in the presence of the primary initial mutation. Just like the primary mutation helped in identifying a component or a pathway, identification of a suppressor might reveal new components. This is the basic line to account for the geneticist's hunger for suppressors and the successful use of this approach throughout the years.

7.1. Second-site suppressors to identify new systems

A most illuminating illustration of the strength of the suppressor approach was the identification of the second general Fe–S cluster biogenesis system, SUF [32]. The SUF system has since been the focus of multiple studies as it plays key roles both in bacteria and plant and might actually be much more widely distributed than the ISC system. A Δ*isc* mutant possesses reduced capacity to synthesize nicotinic acid, vitamin B1 and several branched amino acids. Takahashi and Tokumoto selected five phenotypic revertants that could grow on minimal medium. Because they arose at low frequency (10^{-6} to 10^{-7}) and because the primary mutation was a deletion of the *isc* operon, these revertants ought to originate from a single second site mutation. The independently isolated suppressor mutations were localized by constructing genomic libraries of the revertants, and screening for clones that would phenotypically complement the nicotinic acid auxotrophy and the slow growth phenotype of the Δ*isc* mutant. Sequencing of the complementing DNA regions led to the discovery of the *suf* operon. The suppressor mutations were all localized in the promoter region of the *suf* operon and led to its overexpression. In retrospect, at least two mutations can easily be molecularly understood since they altered the binding site of the Fur repressor, e.g. insertion of A or T at −51 within the fur box at −51. The mode of action of the three others (IS insertion at positions nt −181 and nt −128 from the transcription start site, C to T base substitution at position −149) to activate *suf* expression remains unexplained but might be associated with a local modification of the structure of the promoter. In the wt strain, inactivation of the wild type *suf* locus had no effect on succinate dehydrogenase activity, suggesting that either this operon was cryptic under natural conditions or that consequences of its deletion were masked by the presence of the *isc* operon. By using the plasmid shuffling approach, described above, the authors showed that a functional redundancy between *isc* and *suf*

was the reason for the absence of phenotype associated with Δ*suf* mutation [32,34]. However, this raised the question of why the functional redundancy did not similarly mask the phenotype of Δ*isc* mutation in the first place. Subsequent studies, in particular by Kiley's lab showed that it was because the *suf* operon was indeed poorly expressed under the growth conditions used. Surprisingly, the primary deletion had taken away the *iscR* gene, shown to be required for expression of the *suf* operon, thus how this could be by-passed by the suppressor mutations remains to be explained. Additional reasons to account for the dissymmetric phenotype between Δ*isc* and Δ*suf* strains might be related to some level of substrate specificity as presented above.

Clearly, there is no better illustration of the power of genetics as the work described above that started by isolating a spontaneous pseudorevertant growing on a Petri dish to discover a highly conserved key system in a wide array of living organisms. A tedious part of secondary-site suppressors analysis has for long been the localization of the suppressor mutations and in the evoked case, a genomic bank of the pseudorevertant has to be constructed and screened. Moreover, expression of a mutated locus from a plasmidic copy could have led to a negative trans-dominant effect and precluded identification of the mutated locus (see below). However, this localization step would be avoided nowadays as new genomic sequencing techniques could identify mutations in less than a day. Hence, hunt for second site suppressors should be high again on the list of future analysis.

7.2. Second site suppressors to study structure function relationships

Molecularly speaking, identification of the *suf* operon, as described above, was due to its overexpression under normally repressing conditions. A similar case was offered to us by searching for suppressors of the *erpA iscA* mutant. This lethal gene combination is rendered viable by the presence of the MVA-dependent pathway (see above). Suppressors were selected on plates lacking both arabinose and MVA [36]. Remarkably, all the suppressor mutations exerted their effect via an increased expression of the *sufA* gene. This provided another case that, *in vivo*, one barrier to full functional redundancy lies in the control of gene expression. Moreover, two independently isolated mutations were found to modify a His to Asn change at position 107 within the IscR amino acid sequence. Because this allelic variant was required for *sufA* to be expressed, and since *sufA* expression is activated by apo-IscR, the His-107 position was proposed to favor shift towards apo-IscR enhanced pool. Subsequent biochemical and structural characterization of IscR by the Kiley's group established the His 107 position as a ligand for the Fe–S cluster. Although not unique to IscR, use of a His residue as a ligand constitutes an atypical ligation mode that might be linked to its function as a sensor of Fe–S cluster homeostasis [13]. This illustrates how suppressor can also be extremely rewarding in providing insight in structure function relationships prevailing in protein or RNA.

7.3. Multicopy suppressors

Gene dosage has frequently been exploited to select for genetic interactions between alleles of the same gene or genes with related functions. This approach became popular in the 80's with the use of high-copy number plasmid in routine molecular genetics protocols. In essence, the idea is that higher-than-normal level of a gene product (protein or RNA) might mimic a gain of function mutation and allows it to phenotypically complement, in a non-allelic mode, the defects caused by a mutation. A few examples are given thereafter. In *Salmonella enterica*, plasmid driven overexpression of *iscU* suppressed a Fe–S defect associated with a mutation in the *apbC* gene (also called *mrp* in *E. coli*), whose function in Fe–S cluster biogenesis is unclear [6]. This multicopy suppression effect supported the notion that ApbC was involved, possibly as a scaffold, in the maturation of TcuB, a 4Fe–4S protein catalyzing tricarballylate metabolism in *S. enterica* [6]. Also, a high level of expression of *sufA*, but not that of *iscA* suppressed the lack of *erpA*, indicating that beyond their overall functional redundancy, some specificities differentiate the two ATCs IscA and SufA [36]. Also *erpA* could act as a multicopy suppressor of *iscA sufA* mutations indicating that Fe–S transfer might occur directly from the ISC machinery to ErpA, given this later to be at high level in the cell [36]. This illustrates how this approach, by alleviating gene regulation, can reveal potential pathways followed by Fe–S clusters *in vivo*, and by inference provide insight the influence fluctuating environmental conditions bear on this step.

7.4. Search for trans-dominant negative mutations

In the 80's, Herskowitz described the use of trans dominant mutation as a new strategy for assigning a function to genes [14]. Initially, this type of mutation was called 'antimorphs' by Muller [25]. A mutation can be 'dominant' because its phenotype is manifested in the presence of the wt allele. The mutated allele "dominates" the wt allele because overproduction of the variant has a negative effect on the process under study. Such variants can be obtained with proteins that have multiple functional sites, which can be mutated independently: for example sites of oligomerization, substrate binding, catalysis or targeting signal. As described below, this approach was elegantly used in probing the function of the U-type scaffold.

After it was realized that Fe–S cluster biogenesis required ancillary factors *in vivo*, the concept of a Fe–S scaffold was put forward [9]. The proposal was that a protein called scaffold provides the environment needed for Fe and S to get together and form a transient Fe–S cluster, the nature of which would be identical to that eventually observed in the matured target. The scaffold was predicted to be IscU in the ISC system and use of a trans-dominant negative allele provided decisive support for this model [29]. IscU proteins contain a conserved Asp residue at position 39. The prediction was that a "transient" cluster could be trapped by substituting Asp 39 by a Cys residue. The Asp → Ala version was also constructed as a control. It turned out that when overexpressed, the Asp → Cys mutant had no phenotype whereas the Asp → Ala was a loss of function mutation, i.e. it failed to complement an *iscU* mutant. The key observation though was that the Asp → Ala mutation caused cells to grow much slower when expressed in trans with the wild type allele in the genome, i.e. it acted as a trans-dominant negative mutation. Biochemical characterization revealed that the IscU D39A was in its 2Fe–2S form trapped in a complex with IscS. This suggested that the dominant negative phenotype was due to the incapacity of IscU to release the bound Fe–S cluster. The IscU D39A was eventually exploited for biochemical and crystallographic analyses [21]. This study was the first to give full credence to the concept of scaffold *in vivo*, as it had been possible to isolate a mutation that separated Fe–S cluster building and Fe–S cluster release functions.

8. Conclusion

Fe–S cluster biology has received much attention from biochemists, chemists, spectroscopists, and from geneticists afterwards. We listed an impressive, though not exhaustive, series of different genetic studies, which provided a great deal of new information towards understanding of Fe–S cluster biogenesis *in vivo*.

Second-site suppressors have long been a rewarding strategy for revealing functional overlaps between different cellular components or pathways. Most of the time such mutations alter substrate specificity, regulation, or restore interaction between partners. We saw a few examples of the two former but none of the latter type. The reason is trivial: genetics of Fe–S cluster biogenesis has been resting on deletion as primary mutation. Now that structural information is available for many Fe–S cluster biogenesis factors, site-directed mutagenesis on specific positions could be made such as they could ease search for secondary extragenic suppression. Such an approach will undoubtedly be informative in probing protein/protein interactions that underlie the organization and the functioning of these machineries.

Trans-dominant negative mutations have been highly beneficial for the understanding of several multi-protein complexes involved in various biological processes, from transcription to secretion. We described how the sole IscU D39A mutation became both a tool for biochemistry and a direct proof of the scaffold hypothesis. This should encourage new trans-dominant negative allele to be sought for to investigate dynamics of Fe–S cluster biogenesis machineries.

Conditional mutations are efficient to study essential processes and we described some of these cases. An exploitation, which was not presented, lies in its power to order steps in sequential multi-step processes. As a matter of fact, use of conditional inducible promoters along with ^{55}Fe-labeling and immuno-precipitation was efficiently used Mühlenhoff et al. to define steps in the mitochondrial ISC Fe–S cluster biogenesis pathway [24]. Surprisingly enough given their experimental amenability, this was not used for studying bacteria. In the same vein, the utility temperature sensitive (ts) alleles have in ordering multi-step processes was totally overlooked despite such alleles being well known to facilitate association between genes and steps to be defined.

It is common knowledge that a geneticist's talent lies in designing selection procedures. Again the identification of the SUF system following a selection procedure rests the best illustration of the strength of this type of approach. However, it is a fact that dedicated selection procedures were rarely used in the field of Fe–S cluster genetics. Most of the studies identified *isc* and *suf* genes on the basis of genome analysis and comparison, and phenotypic characterization rested on hypersensitivity to stress-inducing chemicals (H_2O_2, 2,2′ dipyridyl, cobalt, etc.). Others used screening procedures by taking advantage of Fe–S cluster dependent metabolic pathways. For instance, search for mutations interfering with thiamine biosynthesis and tricarballylate catabolism pathways in *S. enterica,* led to the identification of accessory proteins involved in Fe–S cluster biology, such as RseC, ApbE, ApbC/Mrp and Yggx [4,6,28,30]. Although they might not reach the capacity of selection that can detect an event at $<10^{-9}$, it is fair to say that current development of high throughput phenotypic and robotic analyses can now permit to envision larger and more efficient screening procedures that can somehow compensate for the lack of selection. In this regard, the engineering of a Fe–S biosensor, based on the split human Grx2-GFP protein, was an interesting step forward to provide an easy detectable output to be used in high-throughput screening of large mutant collection, but this tool awaits to be exploited [17].

The field of Fe–S cluster biology has been moving extremely fast thanks to collaborative multi-disciplinary efforts between chemistry, enzymology, structural biology, biophysics, genetics and physiology. Genetics has joined and contributed very efficiently to these joined projects by keeping the *in vivo* concern always high in the picture. Even more could be expected as a significant panel of very useful approaches and protocols are still to be used. Along with other disciplines, new genomics and system-levels based investigations will undoubtedly provide new hypothesis to be tackled by classic reductionist approaches. The overall outcome will be a multi-level multi-scale understanding of Fe–S cluster biology, from atomic resolution to cellular physiology integration.

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