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Coordinate Regulation of the Suf and Isc Fe-S Cluster Biogenesis Pathways by IscR Is Essential for Viability of *Escherichia coli*

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Fe-S cluster biogenesis is essential for the viability of most organisms. In *Escherichia coli*, this process requires either the housekeeping Isc or the stress-induced Suf pathway. The global regulator IscR coordinates cluster synthesis by repressing transcription of the *isc* operon by [2Fe-2S]-IscR and activating expression of the *suf* operon. We show that either [2Fe-2S]-IscR or apo-IscR can activate *suf*, making expression sensitive to mainly IscR levels and not the cluster state, unlike *isc* expression. We also demonstrate that in the absence of *isc*, IscR-dependent *suf* activation is essential since strains lacking both the Isc pathway and IscR were not viable unless Suf was expressed ectopically. Similarly, removal of the IscR binding site in the *sufA* promoter also led to a requirement for *isc*. Furthermore, *suf* expression was increased in a Δ*isc* mutant, presumably due to increased IscR levels in this mutant. This was surprising because the iron-dependent repressor Fur, whose higher-affinity binding at the *sufA* promoter should occlude IscR binding, showed only partial repression. In addition, Fur derepression was not sufficient for viability in the absence of IscR and the Isc pathway, highlighting the importance of direct IscR activation. Finally, a mutant lacking Fur and the Isc pathway increased *suf* expression to the highest observed levels and nearly restored [2Fe-2S]-IscR activity, providing a mechanism for regulating IscR activity under stress conditions. Together, these findings have enhanced our understanding of the homeostatic mechanism by which cells use one regulator, IscR, to differentially control Fe-S cluster biogenesis pathways to ensure viability.

Iron-sulfur (Fe-S) clusters are ubiquitous cofactors of proteins whose functions are fundamental to a wide range of biological processes, including respiration, photosynthesis, DNA repair, and regulation of gene expression (1–3). Given their significant roles, a major objective has been to understand how Fe-S clusters are assembled and how cellular Fe-S cluster homeostasis is maintained in response to environmental cues, such as O_2 or reactive oxygen species (ROS), which can destabilize Fe-S clusters (4–6). In *Escherichia coli*, the majority of Fe-S cluster biogenesis is catalyzed by proteins of the housekeeping Isc pathway, encoded by the *iscRSUA-hscBA-fdx* (*isc*) operon (7–9). The *isc* operon also encodes IscR, a [2Fe-2S] transcription factor that modulates expression of the Isc pathway according to the cellular needs for Fe-S cluster biogenesis. Upon acquisition of a [2Fe-2S] cluster from the Isc machinery, the cluster-containing form of IscR can directly bind the *iscR* promoter (P_{iscR}) to repress transcription. Conditions that increase the Fe-S cluster demand (e.g., growth in the presence of O_2) are predicted to also increase competition between IscR and other substrate proteins for the Isc machinery such that derepression of *isc* occurs (10). Thus, *isc* expression is coupled to Fe-S demand through levels of the IscR [2Fe-2S] cluster. In this study, we investigate the mechanism by which IscR coordinates regulation of the Isc pathway to the Suf pathway, the second Fe-S cluster biogenesis pathway encoded by the *sufABCDSE* (*suf*) operon of *E. coli*.

While *isc* and *suf* are both direct targets of the IscR regulon and are expressed in response to similar signals (11–15), IscR controls expression of these two operons by different mechanisms: IscR represses *isc* expression, whereas IscR activates transcription of *suf* (14–18). Furthermore, the [2Fe-2S] cluster is not required for IscR to activate *suf* (14, 15, 18). Oxidative stress conditions increase *suf* and *isc* expression, which was attributed to a shift from the cluster-containing form to the apoprotein form of IscR (14, 15), although whether apo-IscR and [2Fe-2S]-IscR function similarly in activating *suf* has not been tested *in vivo*. The differential regulation of *isc* and *suf* by IscR is in part due to the ability of IscR to recognize two classes of DNA sites. For instance, P_{iscR} contains two type 1 IscR binding sites, each of which requires [2Fe-2S]-IscR for high-affinity binding (10). In contrast, DNase I footprinting results have shown that IscR binds up to three regions within P_{sufA}, with the strongest binding region located at bp −60 to −26 relative to the transcription start site (TSS), which encompasses a type 2 IscR binding site (designated region 1) (Fig. 1) (14, 16). Previous studies have demonstrated that a clusterless IscR variant (IscR-C92A/C98A/C104A) binds this site with high affinity *in vitro* and is sufficient to activate *suf* expression both *in vivo* and *in vitro* (14, 18). In addition, this type 2 IscR binding site overlaps the binding site for the Fe^{2+}-sensing transcription factor Fur (Fig. 1), and binding of these two transcription factors to P_{sufA} *in vitro* is mutually exclusive (15). The other two IscR binding regions have not yet been tested for their role in *suf* regulation *in vivo*; only weak binding of region 3 was observed by high IscR concentrations *in vitro*, and integration host factor (IHF), which activates *suf* in conjunction with OxyR in response to peroxide stress, exhibits stronger binding affinity than does IscR for region 2 (11, 12, 14, 15). While definitive roles for IscR binding regions 2 and 3 remain to be established, deletion of bp −56 to −51 within the type 2 site

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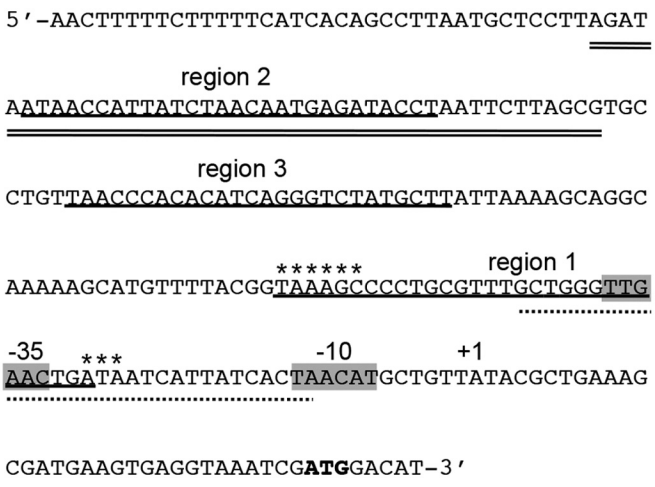

FIG 1 Diagram of the *sufA* promoter region showing nucleotides −200 to +40 relative to the transcription start site (+1). The three regions previously shown to be protected from DNase I cleavage by IscR (underline) are indicated, as are the regions protected by Fur (dotted line) and IHF (double underline). The *sufA* start codon is in bold, and shaded are the putative −35 and −10 promoter elements. Marked with asterisks are nucleotides of the IscR and Fur binding sites that were deleted or mutated in this study.

of region 1 is sufficient to eliminate IscR binding to this site *in vitro* and IscR-dependent activation of *suf* both *in vitro* and *in vivo* (16).

The increased expression of the *suf* operon under oxidative stress and Fe-limiting conditions led to the proposal that the Suf pathway functions primarily under stress conditions (11–15, 19, 20). Indeed, deletion of *suf* was previously shown to have no effect on [2Fe-2S]-IscR activity under nonstress conditions (10). Nevertheless, the Suf pathway includes proteins that exhibit functional similarity to those of the Isc pathway (8, 9). For example, the cysteine desulfurases IscS and SufSE provide sulfur to the respective scaffold proteins, IscU and SufB, upon which Fe-S clusters are transiently assembled. ATP-hydrolyzing proteins, such as HscBA or SufC, facilitate transfer of the cluster directly to apoproteins or to the A-type carrier proteins IscA and SufA. The A-type carrier proteins can also transfer clusters to specific apoproteins. Furthermore, in the absence of the Isc pathway, Suf appears to meet the minimal cellular Fe-S cluster requirements since a Δ*iscSUA-hscBA-fdx* mutant is viable, whereas a strain lacking both the Isc and Suf pathways is inviable (11, 21, 22).

Since the Suf pathway can support cellular Fe-S cluster biogenesis and viability of a Δ*iscSUA-hscBA-fdx* mutant, we hypothesized that the increase in *suf* expression observed previously in the absence of Isc was necessary to meet the cellular Fe-S demands (23). Since IscR levels also increase in mutant strains lacking components of the Isc pathway (10), we address here whether this increase in *suf* expression specifically relies on IscR-dependent activation and is essential for viability. Using genetic analyses, we examined the viability of mutants lacking the Isc pathway and either IscR or an intact type 2 IscR binding site within P_{sufA}. The role of Fur derepression in maintaining viability of a Δ*iscSUA-hscBA-fdx* mutant was also evaluated. Using β-galactosidase assays, we examined the sensitivity of P_{sufA}-*lacZ* expression to elevated IscR protein levels and addressed whether equal levels of wild-type IscR and a clusterless IscR mutant (IscR-C92A) activate *suf* to the same extent. Finally, we measured how [2Fe-2S]-IscR activity is affected by the increased *suf* expression observed in a Δ*fur* Δ*iscSUA-hscBA-fdx* double mutant. Together, our findings emphasize the significance of the role of IscR in differential regulation of the Isc and Suf pathways, thus providing a mechanism by which *E. coli* can maintain Fe-S homeostasis.

MATERIALS AND METHODS

Strain construction. Strains and plasmids described in this work are listed in Table 1, and sequences of primers used are available upon request. In-frame deletions of *iscR*, *iscU*, *iscSUA-hscBA-fdx*, *iscRSUA-hscBA-fdx*, *iscRSUA*, *lacY*, and *fur* were constructed by replacing the respective BW25993/pKD46 coding region(s) with *cat* or *kan* flanked by FLP recognition target (FRT) sites from plasmid pKD32 or pKD13, respectively, as previously described (24). Transduction with P1 *vir* was used to move the *cat* or *kan* allele to the appropriate strain backgrounds (25). In some cases, the Cm^r or Kan^r cassette was removed by transforming strains with pCP20, encoding FLP recombinase, and screening for chloramphenicol (Cm) or kanamycin (Kan) sensitivity (24). All gene deletions were confirmed by colony PCR and DNA sequencing.

To construct the P_{sufA} (bp −200 to +40)-*lacZ* fusion in which $^{-26}$ATA^{-24} relative to the TSS was changed to $^{-26}$TAT^{-24} or in which $^{-50}$C was changed to G, QuikChange (Stratagene) was first used to introduce either mutation into pPK6837, generating pPK10888 and pPK11183, respectively. A *lacI-kan*-P_{sufA}-*lacZ* fragment was PCR amplified from each vector, recombined onto the chromosome in the *lac* promoter region of BW25993/pKD46 and PK11102 as previously described (24), and transduced into appropriate strain backgrounds using P1 *vir*.

Mutations within the native *sufA* promoter were made in several steps. First, *cat* was PCR amplified from pKD32 using primers containing XbaI and BamHI restriction sites and was cloned into the same sites of pET11a to form pPK8626. A fragment containing bp −200 to +385 relative to the *sufA* TSS was PCR amplified from the MG1655 chromosome with primers containing XbaI and SphI restriction sites and cloned into the same sites of pPK8626 (forming pPK8643) so that the direction of transcription for *sufA* and *cat* was in the opposite orientation. QuikChange (Stratagene) was used to disrupt IscR binding to the *sufA* promoter region by deleting bp −56 to −51 or by replacing the C at bp −50 with a G, forming pPK8644 and pPK11186, respectively; similarly, the Fur binding site was disrupted ($^{-26}$ATA^{-24} changed to $^{-26}$TAT^{-24}) to form pPK10874. Finally, to replace the native *sufA* promoter with *cat-sufA* or its derivatives, the constructs were PCR amplified using primers containing ends with homology to this region of the chromosome, electroporated into BW25993/pKD46, and selected for Cm^r. After verification by DNA sequencing, the *cat-sufA* allele (designated *zdh-3632::cat*) or its mutant derivatives were transduced into appropriate strain backgrounds using P1 *vir*.

Construction of strains to evaluate the effects of various wild-type IscR or IscR-C92A protein levels on P_{sufA} (bp −200 to +40)-*lacZ* expression was also performed in several steps. First, FRT-*cat*-FRT-*araC*-P_{BAD} was PCR amplified from pPK9127 with primers homologous to the region upstream of *iscS* on the chromosome and electroporated into MG1655 containing pKD46, and colonies were selected for Cm^r. Following removal of the Cm^r cassette by transforming pCP20 (24), the *araC*-P_{BAD} allele was moved by P1 *vir* transduction into a recombineering strain (PK9659) containing λcI857 Δ(*cro-bioA*) and *gua-26*::Tn*10* at 30°C and colonies were screened for guanine prototrophy and Tet^s. The *bla*-P_{tac}-*iscR* region from pPK9156 (wild-type *iscR*) or pPK9157 (*iscR*-C92A) was amplified and electroporated into the above strain derivative to replace bp −100 to +28 of P_{iscR} relative to the TSS with *bla*-P_{tac}. After verification by DNA sequencing, the *bla*-P_{tac}-*iscR-araC*-P_{BAD}-*iscS* or *bla*-P_{tac}-*iscR* (C92A)-*araC*-P_{BAD}-*iscS* allele was transduced into appropriate strain backgrounds and selected for Ap^r with 10 mM arabinose to derepress P_{BAD}-*iscSUA*.

β-Galactosidase assays. Strains were grown aerobically by shaking at 250 rpm or anaerobically in screw-cap tubes, to an optical density at 600

TABLE 1 Strains and plasmids used in this work

Strain or plasmid	Relevant genotype or phenotype	Reference or source
Strains		
BW25993	*lacI*^{q} *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{L478}$	24
PK9659	*gua-26*::Tn*10* λcI857 Δ(*cro-bioA*)	This study
MG1655	λ$^-$ F$^-$ *rph-1*	Laboratory stock
PK11102	MG1655 Δ*lacY* + pKD46	This study
PK11135	PK11102 (bp −200 to +40 relative to TSS[a]) P$_{sufA}$-*lacZ*	This study
PK11285	PK11135 but with mutation in P$_{sufA}$-*lacZ* ($^{-26}$ATA^{-24} relative to TSS changed to $^{-26}$TAT^{-24})	This study
PK11136	PK11135 + pACYC*lacI*^{q}	This study
PK11290	PK11285 + pACYC*lacI*^{q}	This study
PK11052	PK11136 *bla*-P$_{tac}$-*iscR-araC*-P$_{BAD}$-*iscS*	This study
PK11053	PK11136 *bla*-P$_{tac}$-*iscR*(C92A)-*araC*-P$_{BAD}$-*iscS*	This study
PK11291	PK11290 *bla*-P$_{tac}$-*iscR-araC*-P$_{BAD}$-*iscS*	This study
PK11292	PK11290 *bla*-P$_{tac}$-*iscR*(C92A)-*araC*-P$_{BAD}$-*iscS*	This study
PK6879	MG1655 (bp −200 to +40 relative to TSS) P$_{sufA}$-*lacZ*	16
PK6880	PK6879 Δ*iscR*	16
PK10864	PK6879 Δ*fur*	This study
PK10865	PK6880 Δ*fur*	This study
PK11045	PK6879 Δ*iscSUA-hscBA-fdx*	This study
PK11046	PK11045 Δ*fur*	This study
PK10899	PK6879 but with mutation in P$_{sufA}$-*lacZ* ($^{-26}$ATA^{-24} relative to TSS changed to $^{-26}$TAT^{-24})	This study
PK11001	PK10899 Δ*fur*	This study
PK11050	PK10899 Δ*iscSUA-hscBA-fdx*	This study
PK11204	PK6879 but with mutation in P$_{sufA}$-*lacZ* ($^{-50}$C relative to TSS changed to G)	This study
PK7722	MG1655 (bp −393 to +90 relative to TSS) P$_{sufA}$-*lacZ*	16
PK7723	PK7722 Δ*iscR*	16
PK11099	PK7722 Δ*fur*	This study
PK11100	PK7723 Δ*fur*	This study
PK11092	PK7722 Δ*iscSUA-hscBA-fdx*::*cat*	This study
PK11094	PK11092 Δ*fur*	This study
PK6364	MG1655 λP$_{iscR}$-*lacZ*	17
PK6512	PK6364 Δ*iscR*	10
PK10022	PK6364 Δ*iscSUA-hscBA-fdx*	This study
PK10826	PK6364 Δ*fur*	This study
PK10832	PK10826 *zdh-3632*::*cat*	This study
PK10833	PK10832 but Δ bp −56 to −51 relative to *sufA* TSS	This study
PK10834	PK10832 Δ*iscSUA-hscBA-fdx*::*kan*	This study
PK10028	PK6364 *zdh-3632*::*cat*	This study
PK11225	PK10028 but $^{-50}$C relative to *sufA* TSS changed to G	This study
PK10882	PK10028 but $^{-26}$ATA^{-24} bp relative to *sufA* TSS changed to $^{-26}$TAT^{-24}	This study
PK10883	PK10882 Δ*iscSUA-hscBA-fdx*	This study
PK10029	PK10028 but Δ bp −56 to −51 relative to *sufA* TSS	This study
PK10813	PK6364 + pGS0164	This study
PK10824	PK10813 Δ*iscRSUA-hscBA-fdx*::*cat*	This study
PK10830	PK10813 Δ*iscRSUA*::*cat*	This study
PK10814	PK6364 + pBAD/Myc-*hisC*	This study

TABLE 1 (Continued)

Strain or plasmid	Relevant genotype or phenotype	Reference or source
Plasmids		
pGS0164	*sufABCDSE* cloned into pBAD/Myc-*hisC*	48
pBAD/Myc-hisC	Ap^r	Invitrogen
pACYC*lacI*^{q}	*lacI*^{q} in pACYC184 Tet^r	49
pKD46	Ap^r	24
pCP20	Ap^r	24
pET11a	Ap^r	50
pPK6837	bp −200 to +40 relative to *sufA* TSS cloned into pPK7035	16
pPK10888	pPK6837 but $^{-26}$ATA^{-24} relative to *sufA* TSS changed to $^{-26}$TAT^{-24}	This study
pPK11183	pPK6837 but $^{-50}$C relative to *sufA* TSS changed to G	This study
pKD32	FRT-*cat*-FRT	B. L. Wanner
pKD13	FRT-*kan*-FRT	B. L. Wanner
pPK8626	*cat* from pKD32 in XbaI and BamHI sites of pET11a	This study
pPK8643	bp −200 to +385 relative to *sufA* TSS cloned into XbaI and SphI sites of pPK8626	This study
pPK8644	pPK8643 but Δ bp −56 to −51 relative to *sufA* TSS	This study
pPK11186	pPK8643 but $^{-50}$C relative to *sufA* TSS changed to G	This study
pPK10874	pPK8643 but $^{-26}$ATA^{-24} relative to *sufA* TSS changed to $^{-26}$TAT^{-24}	This study
pPK9127	FRT-*cat*-FRT-*araC*-P$_{BAD}$	10
pPK9156	*bla*-P$_{tac}$-*iscR*	30
pPK9157	*bla*-P$_{tac}$-*iscR*(C92A)	30

[a] TSS, transcription start site.

nm (OD_{600}) of 0.1 to 0.2 at 37°C in LB; M9 minimal medium containing 0.2% glucose, 1 mM $MgSO_4$, 2.5 μg ml^{-1} ferric ammonium citrate, 2 μg ml^{-1} thiamine, 0.02% ammonium molybdate, 12.5 μg ml^{-1} nicotinic acid, and 0.1 mM $CaCl_2$; or morpholinepropanesulfonic acid (MOPS) minimal medium containing 20 mM arabinose and 0.2% Casamino Acids (26). When indicated, medium was additionally supplemented with 10 μg ml^{-1} tetracycline (Tet) and various concentrations of isopropyl-β-D-thiogalactopyranoside (IPTG) (0 to 640 μM). For all assays, Tet, Cm, or spectinomycin (Sp) was added to culture samples at final concentrations of 10, 20, or 100 μg ml^{-1}, respectively, to terminate further protein synthesis and cell growth, and cells were kept on ice until assayed for β-galactosidase activity as previously described (27). Due to aerobic and anaerobic cell count differences for cultures grown in M9 minimal medium (28), the Miller units from aerobic cultures were multiplied by 1.5. Assays were repeated at least three independent times, and error bars represent the standard errors of three biological replicates.

Western blot analysis. Strains were grown as described for β-galactosidase assays, and IscR or SufD levels were measured by Western blotting assays as described previously (18, 23).

RESULTS

IscR-mediated activation of *suf* is essential for viability when the Isc pathway is absent. To address if the viability of a Δ*iscSUA-hscBA-fdx* mutant relies on IscR-dependent activation of *suf*, we used P1 *vir* transduction to introduce into this strain a deletion within P$_{sufA}$ (Δ bp −56 to −51 relative to the TSS) that eliminates IscR binding and, accordingly, transcription activation (16). No colonies were recovered when the strain was plated aerobically

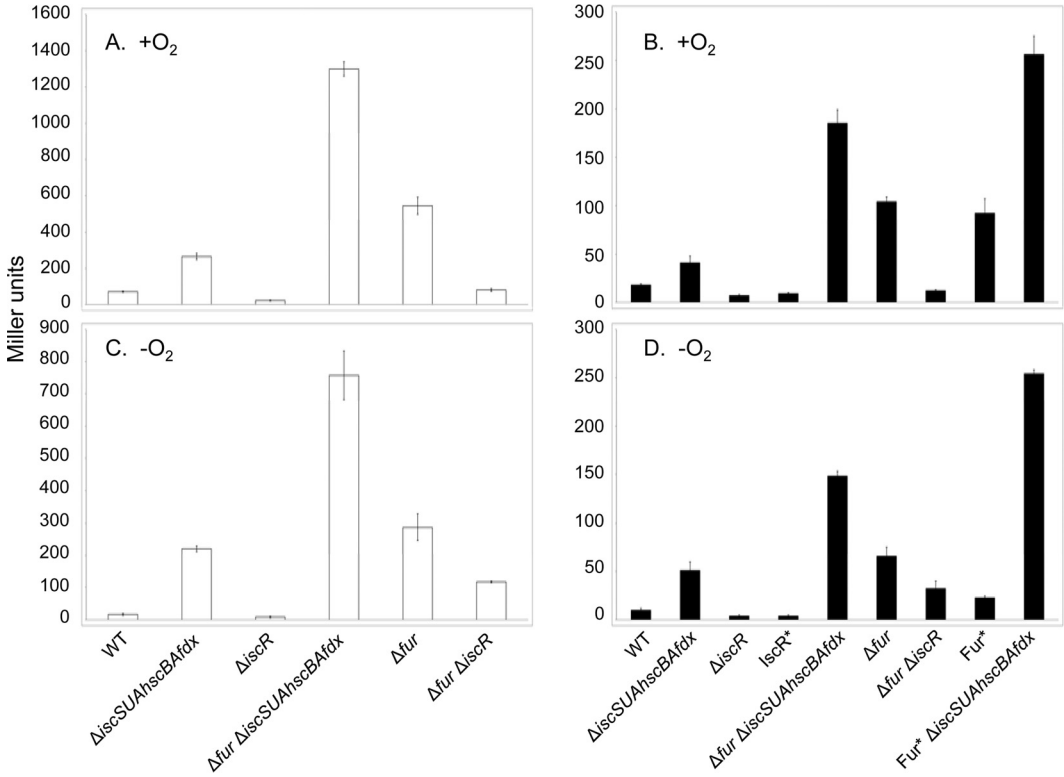

FIG 2 β-Galactosidase activity (Miller units) measured in wild-type or mutant strains containing a P_{sufA}-*lacZ* fusion encompassing either bp −393 to +90 (white bars) or bp −200 to +40 (black bars) relative to the *sufA* TSS. Cultures were grown under aerobic (A and B) or anaerobic (C and D) conditions in LB. IscR* indicates that the IscR type 2 binding site within P_{sufA}-*lacZ* was mutated ($^{-50}$C changed to G); Fur* indicates that the Fur binding site was mutated ($^{-26}$ATA^{-24} changed to $^{-26}$TAT^{-24}). WT, wild type.

despite the fact that introduction of the same mutation into a wild-type strain occurred with the expected frequency. This was also the case when we introduced a more conservative disruption of the IscR binding site within P_{sufA} ($^{-50}$C changed to G) that eliminates IscR-dependent *suf* activation *in vivo* (Fig. 2B and D). Furthermore, no colonies were recovered upon introducing Δ*iscSUA-hscBA-fdx* or Δ*iscU* alleles into a strain already containing a mutated version of P_{sufA}. When transductants were plated under anaerobic conditions in which the need for Fe-S biogenesis should be minimized, some colonies were isolated and confirmed to have the desired mutations; however, these isolates were likely suppressor mutants, since they exhibited heterogeneous colony sizes and occurred at a low frequency. Since strains lacking both Isc and Suf pathways are not viable, the simplest interpretation of these results is that IscR-mediated upregulation of *suf* in the Δ*iscSUA-hscBA-fdx* mutant is necessary for cell viability.

One prediction of this hypothesis is that a strain lacking IscR, in addition to the Isc machinery, should not be viable. Using the Datsenko and Wanner method for creating in-frame deletions of chromosomal genes (24), Δ*iscRSUA-hscBA-fdx* and Δ*iscRSUA* derivatives of the recombineering strain BW25993 were constructed. However, introduction of these alleles into the wild-type *E. coli* strain MG1655 via P1 *vir* transduction was unsuccessful the strain was when plated aerobically. Given that only suppressor mutants appeared to be recovered when the strain was plated anaerobically, it is likely that the BW25993 strain derivatives also had unlinked suppressor mutations. Indeed, the BW25993 derivatives grew aerobically in LB with a doubling time similar to that of the wild-type MG1655 strain (~30 min), as opposed to a longer doubling time typically associated with mutants lacking *iscS* (~90 min; data not shown) (29).

Taken together, our findings suggest that deletion of *iscR* and the remaining *isc* genes is lethal, most likely because the levels of Suf are not sufficient to support the cellular need for Fe-S cluster biogenesis. In support of this notion, the Δ*iscRSUA-hscBA-fdx* and Δ*iscRSUA* alleles were successfully moved by P1 *vir* transduction into a wild-type strain containing a plasmid (pGS0164) expressing *suf* from the arabinose-inducible P_{BAD} promoter and grown in the presence of arabinose (1 mM); Western blot analysis showed that at this concentration of arabinose, SufD levels are increased ≥10-fold over chromosomal levels (data not shown). In contrast, transductants were not recovered when arabinose was absent and/or when only the vector control was present. Thus, it appears that elevated expression of the Suf proteins can restore Fe-S cluster biogenesis, and thus viability, in the Δ*iscRSUA-hscBA-fdx* and Δ*iscRSUA* mutants.

The increased expression of *suf* in the absence of the Isc pathway requires IscR. To determine the extent to which *suf* expression is increased in the absence of the Isc pathway under conditions similar to those carried out for the transductions, we measured β-galactosidase activity from a chromosomal *sufA* promoter (bp −393 to +90)-*lacZ* fusion in wild-type and Δ*iscSUA-hscBA-fdx* strains grown aerobically and anaerobically in LB (Fig. 2A and C). In the Δ*iscSUA-hscBA-fdx* strain, aerobic and anaerobic *suf* expression increased 4- to 13-fold relative to the wild type, respectively. This was also the case when β-galactosidase activity

was measured from a shorter *sufA* promoter (bp −200 to +40)-*lacZ* fusion that lacks the upstream OxyR binding site (Fig. 2B and D) (11, 12). Because a previous study established that under aerobic and anaerobic conditions IscR is elevated to the same high level when negative autoregulation is mostly eliminated by the absence of the Isc pathway (10), we reasoned that increased IscR protein levels may be responsible for the increased *suf* expression observed in the absence of the Isc pathway. Indeed, Western blot analysis revealed that under the growth conditions used in this assay, aerobic and anaerobic IscR protein levels are ~4- and 23-fold higher in the Δ*iscSUA-hscBA-fdx* strain than in the wild type, respectively (see Fig. S1 in the supplemental material), indicating a similar loss of negative autoregulation. Together, these findings demonstrate that the increased expression of *suf* in a mutant lacking the Isc pathway correlates with increased IscR protein levels.

Fur derepression is not sufficient for viability when the Isc pathway and IscR-dependent activation of *suf* are eliminated. Lee et al. previously demonstrated that *suf* transcription is repressed by Fur primarily by antagonizing IscR binding to P_{sufA} (15). Therefore, we also addressed whether eliminating Fur-dependent repression resulted in levels of *suf* expression sufficient to maintain viability when Suf was the sole Fe-S biogenesis pathway. Despite the role of Fur in repressing *suf* in the absence of IscR, no colonies or only suppressor mutants were recovered when Δ*iscRSUA-hscBA-fdx* or Δ*iscRSUA* alleles were introduced into a Δ*fur* strain. Similar results were observed when the Δ*iscSUA-hscBA-fdx* or Δ*iscU* alleles were moved by P1 *vir* transduction into a Δ*fur* strain also containing the mutated *sufA* promoter defective in IscR binding. Finally, mutating the Fur binding site within P_{sufA} ($^{-26}$ATA^{-24} relative to the TSS was changed to $^{-26}$TAT^{-24}), which decreased Fur repression of *suf* (Fig. 2B and D), also had no effect on the ability to recover Δ*iscRSUA-hscBA-fdx* mutants.

We then assayed *suf* expression to determine the effect of Fur under our growth conditions. We observed that under both aerobic and anaerobic conditions, Fur repressed expression of P_{sufA} (bp −393 to +90)-*lacZ* and P_{sufA} (bp −200 to +40)-*lacZ* as expected, since β-galactosidase activity was ~6- to 17-fold lower in the wild-type strain than in a Δ*fur* mutant (Fig. 2). In the absence of Fur, the increase in *suf* expression could be attributed to both an increase in basal promoter activity and IscR-dependent activation (Fig. 2; compare wild-type, Δ*iscR*, Δ*fur*, and Δ*fur* Δ*iscR* strains). Thus, *suf* expression in the wild-type strain reflects contributions from both IscR activation and Fur repression. Surprisingly, under aerobic conditions, wild-type expression levels are nearly equivalent to the basal activity of the *suf* promoter, which we found is insufficient for viability in the absence of the Isc pathway. However, in strains lacking the Isc pathway, Fur repressed *suf* expression only ~4-fold, presumably because increased IscR levels are able to compete with Fur for its binding site. Taken together, these data show that in the absence of the Isc pathway, *suf* expression is higher than wild-type levels, which can be explained by both increased IscR activation and diminished Fur repression, providing sufficient *suf* expression to support viability.

Fur binds to P_{sufA} with higher affinity than does holo- or apo-IscR *in vivo*. Because IscR levels and Fe-S cluster occupancy vary depending on the genetic backgrounds used in the previous experiment, we constructed strains where *iscR* was ectopically expressed to systematically examine the effect of IscR levels on Fur repression of *suf*. This was done by comparing β-galactosidase activity from P_{sufA} (bp −200 to +40)-*lacZ* to a derivative construct in which the Fur binding site within P_{sufA} was mutated [P_{sufA} (Fur*)-*lacZ*] in strains containing chromosomally encoded *iscR* and *iscSUA-hscBA-fdx* under the control of P_{tac} and P_{BAD}, respectively. Under aerobic and anaerobic growth conditions, P_{sufA}-*lacZ* expression increased in an IPTG dose-dependent manner (Fig. 3). However, even in the presence of 320 μM IPTG, which produced ~3- to 50-fold more IscR protein than wild-type levels under aerobic and anaerobic conditions, respectively (see Fig. S4 in the supplemental material), β-galactosidase activity from P_{sufA}-*lacZ* never reached levels that occurred in the absence of Fur repression, e.g., P_{sufA} (Fur*)-*lacZ* (Fig. 3). Thus, consistent with previous *in vitro* studies (15), the higher binding affinity of Fur cannot be outcompeted by physiological changes in IscR levels.

We extended this analysis to compare the effects of IscR cluster occupancy on Fur repression of *suf* by comparing wild-type IscR to IscR-C92A, the variant lacking a Fe-S cluster, as a mimic for the apoprotein form of IscR that is generated in strains lacking the Isc pathway. Recent studies made use of similar strains to demonstrate that, while repression of the *iscR* promoter requires [2Fe-2S]-IscR (see Fig. S2 in the supplemental material), [2Fe-2S]-IscR is an inefficient repressor of transcription from the *hyaA* promoter (see Fig. S3) (10, 30). In contrast, IscR-C92A is a robust repressor of P_{hyaA}, which, like P_{sufA}, contains a type 2 IscR binding site (30). Thus, to compare the abilities of holo-IscR and apo-IscR to activate *suf*, wild-type IscR or IscR-C92A was produced from P_{tac} and P_{sufA}-*lacZ* and P_{sufA} (Fur*)-*lacZ* expression was measured. We observed that wild-type and apoprotein (IscR-C92A) forms of IscR behaved similarly in activating P_{sufA}-*lacZ* (Fig. 3) even under anaerobic conditions, where wild-type IscR is predominantly in the [2Fe-2S] cluster form. This was the case over the entire range of protein concentrations tested, including when levels of wild-type IscR and IscR-C92A (~80 μM IPTG) were comparable to chromosomal IscR levels under aerobic conditions (see Fig. S4). In the case where Fur binding to P_{sufA} was disrupted, providing a measure of only IscR activation, anaerobic β-galactosidase activity was slightly increased (≤2-fold) in the strain expressing IscR-C92A compared to that expressing wild-type IscR (Fig. 3). Thus, P_{sufA} is unlike P_{hyaA} in that both cluster-containing and apoprotein forms of IscR are sufficient to regulate *suf*. Furthermore, these *in vivo* findings are consistent with previous *in vitro* experiments demonstrating that [2Fe-2S]-IscR and IscR-C92A both activate *sufA* transcription *in vitro* (31).

IscR-dependent *suf* activation can partially restore holo-IscR activity when the Isc pathway is absent. Since our data show that IscR-dependent *suf* activation is increased in the absence of Fur (Fig. 2), we tested whether this induction resulted in increased [2Fe-2S]-IscR activity in a strain also lacking the Isc pathway. Using aerobically or anaerobically grown cultures, expression of P_{iscR}-*lacZ*, which requires the [2Fe-2S] cluster-containing form of IscR for repression, was measured. As observed previously, there was a 4- to 9-fold defect in P_{iscR}-*lacZ* repression in the Δ*iscSUA-hscBA-fdx* mutant under aerobic and anaerobic conditions in glucose minimal medium, respectively (Fig. 4) (10). Upon introduction of the Δ*fur* allele into the Δ*iscSUA-hscBA-fdx* strain, however, there was a 2-fold increase in P_{iscR}-*lacZ* repression under both growth conditions. Similar results were observed when the Fur binding site within P_{sufA} was mutated in the Δ*iscSUA-hscBA-fdx* strain (Fig. 4) and when the cultures were grown in LB (data not

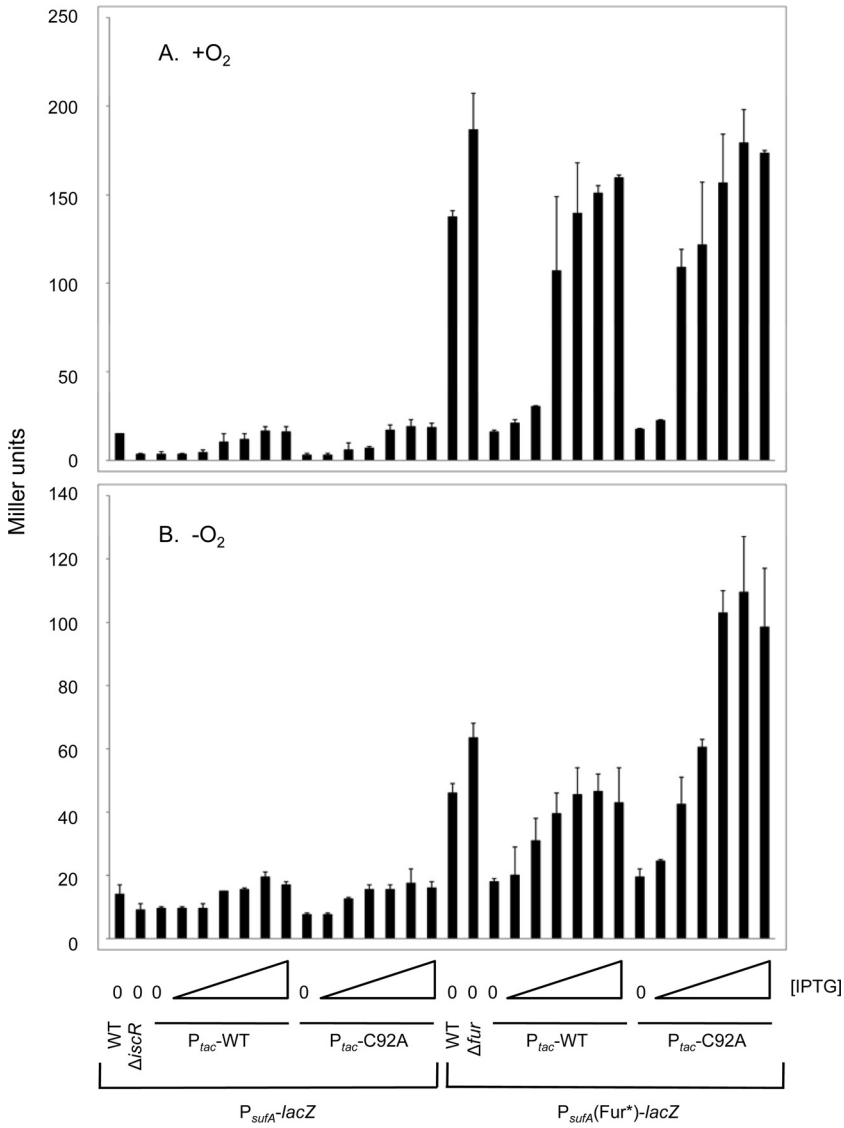

FIG 3 β-Galactosidase activity (Miller units) from P_{sufA} (bp −200 to +40)-*lacZ* or its derivative [P_{sufA} (Fur*)-*lacZ*] in which the Fur binding site is mutated ($^{-26}$ATA^{-24} changed to $^{-26}$TAT^{-24}) in strains expressing chromosomally encoded wild-type *iscR* (PK11052 and PK11291, respectively) or the apoprotein derivative *iscR*-C92A (PK11053 and PK11292, respectively) from the P_{tac} promoter. For comparison, β-galactosidase activities from wild-type (PK6879 and PK10899), Δ*iscR* (PK6880), and Δ*fur* (PK11001) strains are shown. Cultures were grown aerobically (A) and anaerobically (B) in MOPS minimal medium containing arabinose and Casamino Acids. The P_{tac}-containing strains were also grown in the presence of Tet and various concentrations of IPTG (0, 20, 40, 80, 160, 320, or 640 μM). WT, wild type.

shown). Western blot analysis revealed that under aerobic growth conditions, SufD levels are at least 2-fold higher in the Δ*fur* Δ*iscSUA-hscBA-fdx* mutant than in the Δ*iscSUA-hscBA-fdx* parent (data not shown). Thus, the corresponding ~2-fold increase in P_{iscR}-*lacZ* repression observed in the Δ*fur* Δ*iscSUA-hscBA-fdx* mutant suggests that the increased levels of Suf machinery can provide IscR with [2Fe-2S] clusters.

Interestingly, we observed a small defect (≤2-fold) in [2Fe-2S]-IscR-dependent repression of P_{iscR}-*lacZ* in the Δ*fur* strain (Fig. 4), consistent with IscR protein levels being ~2- to 3-fold elevated in this mutant relative to the wild type (see Fig. S1 in the supplemental material). This defect may be attributed to the fact that Fur directly represses expression of the small RNA RyhB, which targets specific mRNAs for degradation, including the *iscSUA-hscBA-fdx* transcript (32). Therefore, in the Δ*fur* mutant, levels of the Isc machinery may not be sufficient to fully mature IscR, yet corresponding upregulation of the Suf pathway by increased levels of apo-IscR could at least partially account for IscR [2Fe-2S] cluster assembly. Together, these findings reinforce the connection between the role of Fur in Fe homeostasis and Fe-S cluster biogenesis (11, 15, 32).

DISCUSSION

The findings presented in this study have extended our knowledge of how Fe-S cluster homeostasis is maintained in *E. coli*. We have shown that in the absence of the Isc pathway, Fe-S cluster biogenesis and, hence, viability depend on activation of the *suf* operon by IscR. The increased IscR protein levels present in a Δ*iscSUA-*

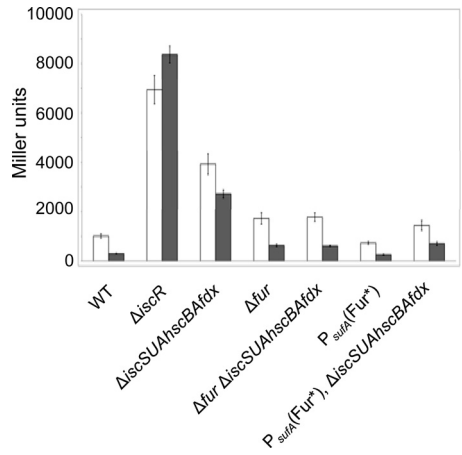

FIG 4 β-Galactosidase activity (Miller units) from P_{iscR}-*lacZ* in aerobic (white bars) and anaerobic (gray bars) strains grown in M9 glucose medium. P_{sufA} (Fur*) indicates that the chromosomal *sufA* promoter contains a mutation within the Fur binding site ($^{-26}$ATA^{-24} changed to $^{-26}$TAT^{-24}). WT, wild type.

hscBA-fdx mutant are critical for this regulation; we demonstrated that by elevating the cellular concentration of IscR, a corresponding increase in *suf* expression occurs even when Fur is present. Furthermore, both the apo- and holoprotein forms of IscR are capable of activating *suf*. Together, these findings highlight the role of IscR as the master regulator of Fe-S cluster biogenesis due to the ability of IscR to differentially regulate the Isc and Suf pathways. In addition, our data reaffirm earlier studies (11, 15, 19, 32) indicating that Fur is integrated into this homeostatic model.

IscR maintains Fe-S cluster homeostasis in the absence of the Isc pathway. Our data indicate that the elevated levels of apo-IscR in a mutant lacking the Isc pathway maintain cellular Fe-S cluster homeostasis by promoting transcription of the *suf* operon. Thus, any alteration that may lead to increased IscR levels, such as an increase in the Fe-S demand via oxidative stress or Fe limitation, would lead to an increase in *suf* expression. The significance of this regulatory mechanism is emphasized by the results of previous studies demonstrating that under a variety of stress conditions, the Isc pathway is unavailable or inactive to provide Fe-S clusters (20, 32). For instance, under Fe-limiting conditions, the small regulatory RNA RyhB targets the *iscSUA-hscBA-fdx* transcript for degradation. In contrast, the *iscR* transcript remains relatively stable, presumably due to a secondary structure within the transcript that shields it from degradation (32). Thus, this differential regulation could allow for IscR-mediated upregulation of Suf under conditions when Fe is limiting. While further studies are required to decipher why the Suf system may be better equipped than Isc under conditions of Fe limitation, it has been proposed that Suf may be more efficient in cluster assembly and in acquiring Fe when it is scarce (33–35).

It has also been shown that the Isc pathway can be inactivated by submicromolar levels of H_2O_2, most likely through oxidation of Fe-S clusters formed on the IscU scaffold protein (20). In contrast, the Suf pathway, which is upregulated by both apo-IscR and OxyR during H_2O_2 stress, remains functional (11–15, 20). While it is not yet clear how the Suf pathway is resistant to damage caused by H_2O_2, findings have also shown that the Suf pathway is functional during conditions of cobalt and copper toxicity (36–40). Thus, the ability of a common regulator, IscR, to coordinate transcription of both the Isc and Suf pathways by derepression and activation, respectively, is a vital homeostatic mechanism that ensures cell survival under a variety of growth conditions. Recently, a genome-wide genetic interaction screen revealed possible functional associations between components of the Suf pathway and the RavA-ViaA ATPase complex, in addition to products of the *ynjABCDEFI* genes that currently lack annotations to specific cellular pathways (41). Further studies are needed to establish what role these additional factors may play in the regulation of Suf and in maintaining Fe-S cluster homeostasis.

The Suf pathway cannot fully compensate for the Isc pathway. Despite the ability of the Suf pathway to operate under Fe limitation and oxidative stress, the elevated levels of Suf machinery present in a Δ*iscSUA-hscBA-fdx* mutant do not appear to fully compensate for the loss of the Isc pathway. For example, only residual Fe-S cluster-dependent activities were detected for IscR, fumarate nitrate regulator (FNR), NsrR, glutamate synthase, isopropylmalate isomerase, NADH dehydrogenase I, and succinate dehydrogenase in strains lacking a functional Isc pathway (10, 20, 23, 29, 42–44). Furthermore, while ectopic expression of the Suf pathway could restore cluster-containing activities for some substrate proteins (IscR, NsrR, and FNR) (10, 23, 43), even high levels of the Suf machinery could not efficiently mature NADH dehydrogenase and succinate dehydrogenase in a Δ*iscUA* mutant (42). Together, these studies bring to light how not all Fe-S proteins are matured to the same level in the absence of the Isc pathway. While the Suf pathway may be more efficient at Fe-S cluster biogenesis than the Isc pathway in times of stress, Suf may meet only the minimal requirements for survival under laboratory conditions by providing clusters to essential Fe-S proteins. In contrast, it appears that the broadened specificity of the Isc pathway makes this system ideal as the housekeeping Fe-S cluster biogenesis pathway. Given the challenges associated with being exposed to a range of environmental conditions, it makes physiological sense why *E. coli* employs two pathways to carry out the essential cellular process of Fe-S cluster biogenesis.

Additional regulation of the Suf pathway. In our attempts to construct strains lacking the Isc pathway and either IscR or its target type 2 site within P_{sufA}, we occasionally recovered suppressor mutants. Thus, we propose that the Δ*iscRSUA-hscBA-fdx-iscX* mutant isolated in a previous study also likely contained suppressor mutations (44). Further analysis of the suppressors isolated in our study revealed that a subset contained SufD protein levels at least 3-fold higher than that of a wild-type strain (data not shown), suggesting that their viability depended on upregulation of Suf. This finding led us to question the mechanism by which this upregulation occurred since IscR was either not present or not able to activate *suf*. We did not find evidence of insertion elements ~4,200 to 2,100 bp upstream of the *sufA* TSS, nor did we find any unexpected base substitutions within P_{sufA} of the suppressor mutants. In cases where *iscR* was present, we found no mutations within the gene that may render IscR able to bind the P_{sufA} variant containing a mutation within the type 2 IscR binding site. Although we did not specifically address whether the difference in activity for the short and long P_{sufA} promoter-*lacZ* fusions was due to the presence or absence of the OxyR binding site, OxyR is not presumed to be active under the growth conditions used for the strain constructions, and we found no mutations within *oxyR* that may have resulted in a constitutively active regulator (45). Fur-

thermore, previous chromatin immunoprecipitation with microarray technology (ChIP-chip) experiments have reported that the nitric oxide (NO)-responsive transcriptional repressor NsrR binds to the *sufA* promoter region (46); however, we did not observe any change in P_{sufA} (bp −200 to +40)-*lacZ* activity in a strain lacking *nsrR* when grown in minimal glucose medium (data not shown). Together, these observations suggest that there may be an additional regulator working in *trans* to upregulate expression of the Suf pathway by an unknown mechanism.

In addition to IscR, Fur is a key player in maintaining Fe-S cluster homeostasis. Not only do our data underscore IscR's critical role in adjusting expression of the Isc and Suf pathways to meet the cellular Fe-S cluster demand, they also support previous findings demonstrating that Fur plays an important role in maintaining Fe-S homeostasis. Under nonstress conditions, Fur antagonizes IscR binding to P_{sufA}, thus keeping *suf* expression low under conditions when the Isc system is functional (15). In contrast, under Fe-limiting conditions, loss of the Fur Fe^{2+} cofactor eliminates Fur binding, allowing apo-IscR to fully activate *suf* expression (11, 15). In addition, because Fur directly represses transcription of RyhB (32), Fur indirectly affects the stability of the *iscSUA-hscBA-fdx* transcript, thus promoting Fe-S cluster biogenesis via the Suf pathway when Fe is limiting. Finally, since Fur regulates expression of Fe uptake pathways (47), the amount of Fe available for building Fe-S clusters is ultimately dictated by Fur. This in turn provides a way to reestablish use of the housekeeping Isc pathway following adaptation to stress. Therefore, *E. coli* employs two major global regulators, IscR and Fur, to integrate Fe availability with Fe-S cluster homeostasis.

In summary, our data provide additional insight into the mechanisms by which IscR coordinates regulation of the Isc and Suf pathways. We observed that either holo-IscR or apo-IscR promotes *suf* transcription, making *suf* expression sensitive to primarily IscR protein levels rather than both protein levels and the cluster state, as observed with *isc* or *hyaA* expression. Because of the *isc* negative feedback mechanism, IscR levels are an accurate indicator of the activity of the Isc pathway, thus providing a mechanism to coordinate *suf* expression to Isc pathway activity. This flexibility in IscR-dependent regulation of *suf* is important for maintaining Fe-S cluster homeostasis under a variety of stress conditions.

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