The impact of O$_2$ on Fe-S cluster biogenesis requirements of *Escherichia coli* FNR

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Summary

In this study, the function of two established Fe-S cluster biogenesis pathways, Isc (Iron sulfur cluster) and Suf (Sulfur mobilization), was compared under aerobic and anaerobic growth conditions by measuring the activity of the *Escherichia coli* global anaerobic regulator FNR. A [4Fe-4S] cluster is required for activity of FNR under anaerobic conditions. Assaying expression of FNR-dependent promoters in strains containing various deletions of the *iscSUAhscBAfdx* operon, revealed that under anaerobic conditions FNR activity was reduced by 60% in the absence of the Isc pathway. In contrast, a mutant lacking the entire Suf pathway had normal FNR activity, although overexpression of the *suf* operon fully rescued the anaerobic defect in FNR activity in strains lacking the Isc pathway. Expression of the *sufA* promoter and levels of SufD protein were upregulated 2–3 fold in Isc⁻ strains under anaerobic conditions, suggesting that increased expression of the Suf pathway may be partially responsible for the FNR activity remaining in strains lacking the Isc pathway. In contrast, use of the O$_2$-stable [4Fe-4S] cluster FNR variant, FNR-L28H, showed that overexpression of the *suf* operon did not restore FNR activity to strains lacking the Isc pathway under aerobic conditions. In addition, activity of FNR-L28H was more impaired under aerobic conditions compared to anaerobic conditions. The greater requirement for the Isc pathway under aerobic conditions was not due to a change in the rate of Fe-S cluster acquisition by FNR-L28H between aerobic and anaerobic conditions as shown by ^{55}Fe labelling experiments. Using ^{35}S-methionine pulse-chase assays, we observed that the Isc pathway, but not the Suf pathway, is the major pathway required for conversion of O$_2$-inactivated apo-FNR to [4Fe-4S]-FNR upon the onset of anaerobic growth conditions. Taken together, these findings indicate a major role for the Isc pathway in FNR Fe-S cluster biogenesis under both aerobic and anaerobic conditions.

Introduction

Fe-S clusters are cofactors of proteins that function in diverse and vital roles in multiple cellular processes such as respiration, photosynthesis, gene regulation and nitrogen fixation.[1] How

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these Fe-S cluster cofactors are synthesized and inserted into the relevant target proteins is not well understood. In *Escherichia coli*, the two gene clusters that are involved in Fe-S biogenesis are *iscSUAhscBAfdx*, encoding the proteins of the Isc pathway, and *sufABCDSE*, encoding the proteins of the Suf pathway. While characterization of strains lacking one or the other pathway has led to the conclusion that the Isc proteins function as the major housekeeping Fe-S cluster biogenesis system of *E. coli*, strains lacking both pathways are inviable[2; 3; 4], raising the question of the interplay between these two pathways. In addition, even though some Fe-S clusters are sensitive to O_2 or reactive oxygen species, a systematic comparison of the requirements of Fe-S biogenesis under aerobic and anaerobic growth conditions has not been investigated. In this study, we examine the contributions of the Isc and Suf pathways to Fe-S cluster biogenesis under aerobic and anaerobic growth conditions using the transcription factor FNR as a model system.

In the housekeeping Isc system, IscS is a cysteine desulfurase[5; 6; 7] that provides the sulfur for Fe-S cluster biogenesis. IscU serves as the scaffold upon which Fe-S clusters are assembled. [8; 9] HscB and HscA are homologs of the molecular chaperones DnaJ and DnaK, respectively. However, unlike DnaJ and DnaK, HscA and HscB are "specialized chaperones" that specifically interact with IscU.[10; 11] The roles of IscA and Fdx in Fe-S cluster assembly are less clear; however, it has been proposed that *E. coli* IscA functions as an alternate Fe-S scaffolding protein[12] or as an Fe donor for the assembly process.[13] The *E. coli* Suf Fe-S cluster biogenesis pathway includes proteins that show functional similarity to those of the Isc pathway. For example, SufS is also a cysteine desulfurase[14] and SufA exhibits sequence similarity to IscA and can function as an Fe-S cluster scaffolding protein.[15; 16] SufC has ATPase activity[17; 18] as found with HscA of the Isc system.[11] SufC also forms a complex with SufB and SufD, which further increases the cysteine desulfurase activity of SufS in the presence of SufE *in vitro*.[19] However, components of the Isc and Suf systems do not appear to be interchangeable in *E. coli* since deletions of any of the *suf* genes, with the exception of *sufA*, were shown to be synthetically lethal with the *iscS* deletion.[4; 20] Apparently, the cysteine desulfurase, SufS, cannot directly substitute for IscS and most likely requires the other Suf accessory proteins. In addition, persulfide bound IscS acts as a sulfur donor in other sulfur transferase reactions, a function apparently not present in SufS.[21] Nevertheless, the reduced activities of the Fe-S proteins glutamate synthase and succinate dehydrogenase observed in a strain lacking the Isc system could be restored when expression of the *suf* operon was elevated, suggesting that the two systems have redundant functions in overall Fe-S biogenesis.[3]

The roles of the Isc and Suf pathways have largely been examined under aerobic and oxidative stress conditions because of the extreme sensitivity of some Fe-S clusters to oxidation by oxygen, hydrogen peroxide, and superoxide.[22; 23] The involvement of the Isc proteins in Fe-S cluster biogenesis under aerobic growth conditions has been established for several Fe-S proteins.[24; 25; 26] In addition, expression of both the Isc and Suf pathways are upregulated upon exposure to hydrogen peroxide, suggesting that both pathways may contribute to Fe-S cluster assembly under oxidative stress conditions.[27] However, a comparison to anaerobic growth conditions, where clusters are expected to be more stable, has not been systematically investigated, although expression of both the *isc* and *suf* operons is known to be lower under these conditions.[28]

To address whether there are functional differences in the Isc or Suf pathways between aerobic and anaerobic conditions, we examined changes in activity of the anaerobic regulator FNR and a constitutively active mutant derivative, FNR-L28H. Under anaerobic conditions, FNR regulates the expression of genes involved in anaerobic metabolism and its activity is dependent on the presence of a [4Fe-4S] cluster, which promotes dimerization and subsequent DNA binding.[29] Under aerobic conditions, the [4Fe-4S] cluster is destroyed by O_2, leading to the formation of inactive, apo-FNR.[30; 31; 32; 33; 34] Previous studies demonstrated that IscS is

involved in Fe-S cluster assembly for FNR since FNR activity was impaired in strains lacking IscS grown under anaerobic conditions.[24] We report here the role of the other Isc proteins and the Suf system in providing Fe-S clusters for FNR under anaerobic growth conditions. To directly compare Fe-S cluster biogenesis under anaerobic and aerobic conditions, we monitored the activity of the mutant FNR-L28H, which contains an O_2-resistant [4Fe-4S] cluster35, in strains lacking Isc or Suf. In addition, we directly compared the rate of Fe-S cluster acquisition under aerobic and anaerobic conditions by measuring the steady-state rate of [55]Fe incorporation into FNR-L28H. Finally, we examined the involvement of the Isc and Suf pathways in the reactivation of apo-FNR to [4Fe-4S]-FNR upon O_2-deprivation.

Results

FNR activity is only partially decreased in anaerobic cells lacking the Isc pathway

Deletion of the genes encoding the entire Isc pathway, $\Delta iscSUAhscBAfdx$, reduced FNR activity by ~60% (Figure 1). The magnitude of the decrease in FNR activity was similar for several FNR-activated promoters analyzed (*dmsA*, *ydfZ*, and *narG*) (Figure 1) and was not due to changes in FNR protein levels in wild-type and $\Delta iscSUAhscBAfdx$ strains as shown by Western blot analysis (Figure 1d). These results confirm that the Isc pathway is the major route for FNR Fe-S biogenesis under anaerobic conditions and show that for the Isc directed portion of FNR Fe-S biogenesis, no other cysteine desulfurase substitutes for IscS in agreement with previous studies.[4; 20] Furthermore, with the exception of IscA, we found that strains lacking individual components such as IscS or IscU yielded a similar phenotype as strains lacking IscS, IscU and IscA ($\Delta iscSUA$) or HscA, HscB and Fdx ($\Delta hscBAfdx$), suggesting that all of these proteins contribute a necessary function to FNR Fe-S biogenesis.

The FNR activity (~40%) remaining in the $\Delta iscSUAhscBAfdx$ strain was shown to be dependent on the [4Fe-4S] cluster by assaying FNR variants previously shown to be specifically defective in ligating the Fe-S cluster of FNR (FNR-C23A, FNR-C122A).[29; 36; 37; 38; 39] Substitution of either FNR Fe-S cluster ligand, C23 or C122, eliminated expression of P*ydfZ* in the strain lacking the Isc pathway (Figure 2), indicating that the FNR activity present in the $\Delta iscSUAhscBAfdx$ strain still depends on the [4Fe-4S] cluster of FNR. Thus, these data show that another Fe-S cluster biogenesis pathway must be functional under anaerobic conditions.

Other known Fe-S biogenesis proteins are not necessary for FNR activity under anaerobic conditions when the Isc pathway is functional

To test whether the Suf pathway provides [4Fe-4S] clusters for FNR under anaerobic conditions, FNR activity was measured in a strain deleted for the *sufABCDSE* operon. FNR-dependent activation of the *ydfZ* (Figure 3), *dmsA*, and *narG* promoters (data not shown) in the $\Delta sufABCDSE$ strain was similar to that of the parent *suf*+ strain, indicating that the Suf pathway is not necessary for FNR Fe-S cluster assembly under anaerobic growth conditions when the Isc pathway is present. However, since previous studies have shown that SufA can functionally replace IscA[3; 4; 20], we investigated whether the function of SufA explained the minor (less than 2 fold) effect of $\Delta iscA$ strains on FNR activity. By comparing *ydfZ* expression in $\Delta iscA$ $\Delta sufA$ or $\Delta sufA$ strains, we found that only strains that lacked both IscA and SufA reduced *ydfZ* expression to levels observed with the deletion of the entire Isc pathway, indicating that SufA and IscA function interchangeably under anaerobic growth conditions (Figure 3). Furthermore, FNR activity was not decreased in strains lacking either CsdA, a third cysteine desulfurase[40], or NfuA, a protein containing an N-terminal region similar to IscA and a C-terminal region similar to that of NifU[41; 42] (Figure 3). Strains lacking IscA and NfuA or SufA and NfuA also showed no additional decrease in *ydfZ* expression compared to any of the single mutants, indicating the absence of functional redundancy that was observed between SufA and IscA (Figure 3). Finally, deletion of *ytfE*, whose gene product has recently been

implicated in Fe-S cluster assembly and/or repair[43], also did not affect FNR activity (Figure 3). Thus, in the presence of the Isc pathway, other known Fe-S assembly proteins are not necessary for FNR Fe-S cluster biogenesis under anaerobic conditions.

In the absence of the Isc pathway, higher levels of the Suf Fe-S cluster biogenesis proteins can restore FNR activity

Overexpression of the *sufABCDSE* operon (*suf*) has previously been shown to restore the defects in activities of other Fe-S proteins, e.g. glutamate synthase and succinate dehydrogenase, in strains lacking the Isc pathway.[3] These results raised the question of whether the defect in FNR activity observed in Isc⁻ strains under anaerobic conditions could also be rescued by overexpression of *suf*. To answer this question, FNR activity was measured in strains that contained a plasmid with the *sufABCDSE* operon under the control of the arabinose-inducible P_{BAD} promoter (Figure 4) and contained Δ*iscSUAhscBAfdx* or Δ*iscSUAhscBAfdx*, Δ*sufABCDSE*. As expected, the strain lacking both the Isc and Suf pathways (Δ*iscSUAhscBAfdx*, Δ*sufABCDSE*) was viable only when the suf plasmid, not the vector, was present.[2; 3; 4] Even when no arabinose was present in the growth medium, strains containing the *suf* plasmid had a 9-fold increase in SufD protein levels (measured by Western blots using antibodies to SufD; data not shown) and a ~2-fold increase in FNR activity relative to the parent *suf*⁺ strain. When 0.2% arabinose was present in the growth medium, the defect in FNR activity observed in the Isc⁻ strain was completely rescued (Figure 4) by the ~ 30-fold overexpression (data not shown) of the *suf* operon; a similar level of *ydfZ* expression was observed in the Isc⁻Suf⁻ strain upon arabinose induction of the suf plasmid. This restoration of FNR activity can be attributed to the increased levels of the Suf proteins since FNR activity was not increased in the Δ*iscSUAhscBAfdx* strain transformed with the vector alone. Taken together, these data suggest that the Suf pathway is biochemically sufficient to replace the Isc pathway in FNR Fe-S cluster assembly when expressed at elevated levels under anaerobic conditions.

Expression of the suf operon is upregulated in strains lacking the Isc pathway

Since overexpression of the Suf pathway can restore FNR activity to wild-type levels in strains completely lacking the Isc pathway, we considered the possibility that the FNR activity (40%) remaining in strains lacking the Isc pathway might result from Suf-directed Fe-S biogenesis if Suf protein levels are upregulated. Expression of the *suf* operon is known to be repressed by FNR under anaerobic conditions[44] and is activated by IscR lacking an Fe-S cluster[28]; thus a change in activity of either regulator, resulting from the absence of the Isc proteins, could increase *sufA* promoter expression. Indeed, anaerobic expression of P*sufA*::*lacZ* was ~2 fold higher in the Δ*iscSUAhscBAfdx* strain compared to that of wild-type (Figure 5). In addition, Western blot analysis using α-SufD antibodies revealed that Isc⁻ strains contained ~2–3-fold more SufD protein than Isc⁺ strains (data not shown), consistent with the observed increase in P*sufA* expression. Thus, elevated levels of the Suf proteins may explain some or all of the remaining FNR activity under anaerobic conditions in cells lacking the Isc pathway.

The requirement for the Isc pathway in FNR Fe-S cluster assembly is greater under aerobic growth conditions

A key unanswered question is whether there are differences in the requirements for the Isc and Suf pathways in FNR Fe-S cluster assembly between aerobic and anaerobic growth conditions. This question is particularly relevant since both pathways are upregulated under aerobic conditions, when some Fe-S clusters are known to be less stable. However, it is difficult to measure wild-type FNR Fe-S cluster formation under aerobic conditions because the [4Fe-4S] cluster in FNR is unstable to O_2, and the protein is predominately in the apo-form, resulting in undetectable regulation of most FNR-dependent promoters. To circumvent this problem, two approaches were used. First, we examined the aerobic activation of an unusually strong

FNR regulated promoter, that of *ydfZ*, which still has measurable, FNR-dependent activity under aerobic conditions, albeit at levels ~60-fold less than in anaerobic grown cultures. Second, we utilized an FNR mutant (FNR-L28H) in which the Fe-S cluster is resistant to disruption by O_2.[35] Therefore, [4Fe-4S]-FNR-L28H accumulates under aerobic conditions, in contrast to wild-type FNR, which would be largely in the apo-form as a result of cluster turnover.

The first approach takes advantage of the observation that aerobic activation of P*ydfZ* is still dependent on the [4Fe-4S] form of FNR (data not shown), indicating that some low level of holo-FNR is present under aerobic conditions. While aerobic *ydfZ* promoter activity was not affected in Δ*sufABCDSE*, Δ*csdA*, Δ*ytfE* or Δ*nfuA* strains (data not shown), we found that the *isc* genes were absolutely required (Figure 6a). Activity of the Δ*iscSUAhscBAfdx* strain was decreased 10-fold, the same magnitude as the *fnr* deletion strain (Figure 6a), in remarkable contrast to the just 60% reduction in *ydfZ* expression observed under anaerobic conditions (Figure 1b). Importantly, overexpression of the Suf pathway was not able to rescue the defects in FNR activity observed in the Δ*iscSUAhscBAfdx* strain (Figure 6b), in stark contrast to what was observed under anaerobic conditions (Figure 4). When expression of the *suf* operon, under the control of P$_{BAD}$, was induced with arabinose under aerobic conditions to levels similar to those under anaerobic conditions (~30 fold; data not shown), only a small increase in wild-type FNR activation of *ydfZ* was observed in either the Δ*iscSUAhscBAfdx* or Δ*iscSUAhscBAfdx*, Δ*sufABCDSE* strains (Figure 6b).

The second approach utilized a mutant of FNR containing a Leu to His substitution at position 28 (FNR-L28H) that renders the [4Fe-4S] cluster relatively resistant to destruction by O_2.[35] Furthermore, similar levels of [4Fe-4S]-FNR-L28H accumulate in aerobically and anaerobically grown cells[35; 45], thus providing a unique system to compare Fe-S biogenesis requirements under aerobic and anaerobic conditions. Similar to that observed for *narG* expression by wild-type FNR (Figure 1), FNR-L28H function was reduced ~2–3 fold under anaerobic growth conditions in the various *isc* deletion strains except the Δ*iscA* strain (Figure 7a). However, under aerobic growth conditions, FNR-L28H activity was decreased more than 20-fold in strains lacking the entire Isc pathway or only IscS, 10-fold for the strains lacking IscU or HscB/HscA/Fdx and ~3-fold in the Δ*iscA* strain. Similar to that observed for wild-type FNR, neither the Suf pathway nor the CsdA protein were required for Fe-S cluster assembly of FNR-L28H under either anaerobic or aerobic growth conditions (Figure 7b).

Taken together, these results indicate that only the Isc pathway functions in FNR Fe-S cluster synthesis under aerobic growth conditions. Furthermore, even when the Suf machinery was overexpressed under aerobic conditions to the same levels as under anaerobic conditions, it could not functionally substitute for the Isc machinery suggesting that the Suf machinery shows a difference in substrate selectivity or activity under aerobic conditions.

The rate of Fe-S cluster acquisition by FNR-L28H is similar between aerobic and anaerobic conditions

Previous studies indicated that expression of both the Isc and Suf pathways is decreased under anaerobic growth conditions[28], thus raising the question as to whether a reduction in the levels of Fe-S cluster biogenesis proteins under anaerobic conditions is rate-limiting for FNR Fe-S cluster synthesis *in vivo*. To address this issue, we developed an *in vivo* assay to directly measure the rate of [55]Fe incorporation into plasmid-derived FNR-L28H. FNR-L28H is not targeted for protein degradation[46] and so the rate of [55]Fe incorporation should reflect the rate of [4Fe-4S] acquisition by newly synthesized apo-FNR-L28H. We found that [55]Fe was incorporated into immunoprecipitated FNR-L28H in a linear fashion from steady state labelled cultures grown under either aerobic or anaerobic growth conditions (Figure 8). Furthermore, the rate of [55]Fe incorporation into FNR-L28H from equal volumes of cell culture was similar

for aerobic and anaerobic conditions (125 ± 17 and 120 ± 26 DPM ^{55}Fe ml^{-1} min^{-1} or $8.2 \pm 1.1 \times 10^9$ and $7.9 \pm 1.1 \times 10^9$ [4Fe-4S] clusters ml^{-1} min^{-1}, respectively). Since Western blot analysis revealed that there is less than a 2-fold difference in total FNR-L28H protein levels in aerobically and anaerobically grown cells (data not shown), these data suggest that the rate of Fe-S cluster acquisition by FNR-L28H was similar under both conditions. The incorporation of ^{55}Fe was specific to the cluster-containing form of FNR, since little ^{55}Fe incorporation was detected when the experiment was performed with a strain expressing FNR-L28H-C122S (Figure 8), a mutant that is unable to ligate a Fe-S cluster.[47]

The plasmid-derived levels of FNR-L28H, which were ~6–7-fold higher than chromosomally produced wild-type FNR levels[31], did not perturb normal Fe-S metabolism, as measured by changes in IscS protein levels, expressed from the *isc* operon. Western blot analysis showed no difference in IscS protein levels between the parent and plasmid containing strain and IscS levels were still oxygen regulated (data not shown), consistent with the reported ~ 7-fold increase in expression of the *isc* operon under aerobic growth conditions.[28] Taken together, these data indicate that the differences in the levels of the Isc machinery under aerobic and anaerobic conditions do not affect the rate of Fe-S cluster acquisition by FNR-L28H and supports the notion that the greater role of the Isc pathway for FNR Fe-S biogenesis under aerobic conditions likely reflects changes in the function of the Suf pathway.

Reactivation of apo-FNR to [4Fe-4S]-FNR requires the Isc pathway

A recent study demonstrated that the population of apo-FNR present in aerobic cells is reactivated to [4Fe-4S]-FNR upon a shift to anaerobic growth conditions.[46] To determine what Fe-S biogenesis machinery is necessary to mediate this process, we measured apo-FNR conversion to [4Fe-4S]-FNR in cells lacking either the Isc or Suf pathway. Reactivation of apo-FNR was determined by monitoring the FNR degradation rate via ^{35}S-methionine pulse-chase assays since apo-FNR is targeted for degradation, whereas [4Fe-4S] cluster-containing FNR is resistant to proteolysis. In this assay, an aerobically grown culture is pulse-labelled with ^{35}S-methionine for one minute, excess unlabelled methionine is added, and the amount of immunoprecipitated ^{35}S-met-FNR is quantified over time. As observed previously for the wild-type strain[46], FNR was degraded under aerobic growth conditions, but upon shifting the culture to anaerobic growth conditions at 120 minutes following addition of the methionine chase, degradation of FNR was impaired (Figure 9). As suggested previously[46], these data indicate that the existing apo-FNR present in aerobic cells acquires a [4Fe-4S] cluster upon the switch to anaerobic growth conditions and thus escapes protein degradation. Similar results were observed in the Δsuf strain, suggesting that reactivation of apo-FNR to [4Fe-4S]-FNR does not require the Suf pathway. In contrast, the labelled population of apo-FNR in cells lacking the Isc proteins continued to decrease after switching the culture to anaerobic growth conditions. These data indicate that the Isc machinery is necessary for the reactivation of apo-FNR that previously lost its cluster. Thus the Isc pathway functions in *de novo* Fe-S biogenesis of FNR and in rebuilding Fe-S clusters in O$_2$-inactivated FNR protein.

Discussion

This study examined the roles of the Isc and Suf pathways in FNR Fe-S cluster biogenesis. Our findings demonstrate that the Isc pathway is the major pathway for FNR Fe-S biogenesis under both aerobic and anaerobic conditions. However, the ability of the alternative Fe-S biogenesis pathway, Suf, to support FNR Fe-S biogenesis was inhibited by aerobic growth conditions even though Suf functions in the biogenesis of other Fe-S proteins under aerobic conditions.[4; 48] These results open new areas of inquiry regarding how Fe-S proteins are targeted to either the Isc or Suf pathways during acquisition of Fe-S clusters and the role of O$_2$ in regulating usage of these pathways.

Aerobic growth conditions increase the demand for Fe-S biogenesis

The demand for overall Fe-S biogenesis is expected to be greater under aerobic growth conditions because the general rate of Fe-S cluster turnover is increased under these conditions. For example, it has been reported that the rate constants with which Fe-S clusters of dehydratases react with superoxide and hydrogen peroxide are extremely high under aerobic conditions: 3×10^6 M^{-1} s^{-1} and 4×10^3 M^{-1} s^{-1}, respectively.[22; 23] Although *E. coli* synthesizes enzymes such as superoxide dismutase, catalase, and peroxidase to combat these oxidants, it is predicted that even micromolar concentrations of superoxide and hydrogen peroxide are sufficient to destroy the FeS clusters of some classes of Fe-S proteins.[22; 23] Thus, under aerobic growth conditions, Fe-S clusters are most likely continually being destroyed by these oxidants, which would increase the demand for Fe-S biogenesis or repair. In this case, the Isc machinery and/or the Suf pathway would be expected to provide Fe-S clusters to a greater population of substrate proteins, providing a rationale for the increased expression of both the *suf* and *isc* operons under aerobic conditions as compared to anaerobic conditions.[28]

The major role of the Isc pathway for FNR Fe-S biogenesis under both aerobic and anaerobic conditions further supports the housekeeping function described for the Isc pathway

Previous studies have shown that the Isc pathway is required for the activity of many Fe-S proteins although a systematic investigation of all the pathway components has not been undertaken.[24; 25; 26] Our studies show that all of the Isc components, with the exception of IscA, are necessary for FNR Fe-S cluster assembly under both aerobic and anaerobic conditions. In particular, we found that HscB and HscA are as important for Fe-S cluster assembly under anaerobic conditions as IscS and IscU, despite the fact that many obligate anaerobic microbes lack *hscB*- and *hscA*-like genes.[3]

In addition, we found that the rate of FNR Fe-S cluster biosynthesis is the same under both aerobic and anaerobic conditions suggesting that the Isc pathway is not limiting for FNR Fe-S cluster synthesis, which supports its role as the housekeeping Fe-S biogenesis pathway. Consistent with this notion, there was no further increase in FNR-L28H activity when the levels of the Isc proteins were increased (data not shown). Thus, our results show that, even for an abundant protein like FNR, the Isc machinery is present at sufficient levels to accommodate Fe-S biogenesis. The Isc machinery was also found to promote synthesis of Fe-S clusters in O_2-inactivated FNR, suggesting that the determinants used for targeting FNR to the machinery are similar for both the apo-form of FNR generated from cluster loss and newly synthesized FNR.

Under anaerobic conditions, usage of Fe-S biogenesis pathways may be more promiscuous

While the Isc pathway is the major pathway for FNR Fe-S cluster synthesis under anaerobic conditions, analysis of strains lacking the Isc pathway under anaerobic conditions revealed an additional Fe-S cluster insertion activity(s). Our finding that the Suf pathway, when overexpressed, could functionally replace the Isc pathway under anaerobic conditions suggests that the Suf pathway may be responsible for FNR Fe-S cluster assembly in the absence of the Isc pathway. However, since strains lacking both pathways are not viable[2; 3; 4], it is experimentally difficult to resolve this question. For similar technical reasons, we were unable to determine whether these minor Fe-S insertion activities are always active in anaerobic cells or are implemented only in the absence of the Isc pathway. Whatever the explanation, these minor activities are not sufficient under aerobic conditions since we found that only the Isc pathway was utilized for FNR Fe-S cluster assembly in the presence of O_2. Given the large number of anaerobic respiratory enzymes that contain Fe-S clusters, it will be important to determine whether the utilization of the Fe-S biogenesis pathways is similar to FNR. As far as we are aware, a systematic study has not been carried out to investigate the magnitude of the defects in activity of other anaerobic Fe-S enzymes in strains lacking the Isc pathway.

Why does the Suf pathway not function in FNR Fe-S cluster synthesis under aerobic conditions?

We were surprised to find that under aerobic growth conditions, overexpression of the Suf pathway to the same level as under anaerobic conditions does not restore FNR Fe-S biogenesis. It seems less likely that the inability of the Suf pathway to restore Fe-S cluster synthesis is due to a decrease in the specific activity of the Suf pathway under aerobic conditions since this pathway has been shown previously to be active under these growth conditions as well as under conditions of oxidative stress.[4; 48] Rather, it seems more likely that, under aerobic conditions, FNR no longer competes efficiently with other substrates to engage the Suf machinery (Figure 10). Aerobic and oxidative stress conditions are known to damage Fe-S clusters, thus increasing the demand for Fe-S cluster synthesis, and accordingly, more specific substrates to compete with FNR. Since FNR is an abundant protein at several thousand molecules per cell, our result suggests that other Fe-S protein substrates must interact with the Suf pathway with greater affinity than FNR, thereby limiting FNR cluster acquisition. Regardless of the specific mechanism, these results raise the question of how apoproteins are targeted to the Isc pathway versus the Suf pathway. Further experiments will be necessary to determine if substrate proteins contain specific targeting sequences that direct them to particular Fe-S biogenesis pathways and/or if there are other intrinsic differences in the activities of the Suf and Isc pathways. In addition, this study shows that growth under aerobic conditions is a sufficient perturbant to study differences in Fe-S biogenesis pathway function.

Differences in the roles of the IscA homologs between aerobic and anaerobic conditions

Although previous studies have demonstrated that IscA can serve as an Fe-S cluster scaffolding protein *in vitro*[12], we found that IscA was not necessary for FNR Fe-S cluster assembly under either aerobic or anaerobic growth conditions. Rather, we found that SufA can functionally replace IscA under anaerobic growth conditions for FNR Fe-S cluster assembly. We assume that this is also true under aerobic conditions, but we could not test this genetically because the plating efficiency of a ΔiscA ΔsufA strain was reduced 100-fold under aerobic conditions and we were unable to obtain linear growth in liquid cultures to assay FNR activity. We were also surprised that strains lacking both SufA and IscA are viable under anaerobic conditions (albeit with a 2-fold slower growth rate on glucose minimal media; data not shown), since deletion of both the entire *isc* and *suf* operons is lethal under anaerobic conditions (data not shown). This suggests that either the function of IscA and SufA is not absolutely required for Fe-S cluster biogenesis under anaerobic conditions or that the third IscA homolog, ErpA, functions under these conditions.[49] ErpA is essential for growth by either aerobic or anaerobic respiration but not fermentation, because it has a specialized role in maturation of Fe-S proteins required for synthesis of precursors of quinones, required for respiration. Since ErpA is not required for growth under the anaerobic fermentative conditions used in this study, and *erpA* is expressed under these conditions[49], it is possible that ErpA could support both growth and partial activity of FNR in the strain lacking IscA and SufA. The more dramatic phenotype of strains lacking IscA and SufA under aerobic conditions is consistent with a recent report by Lu *et al*[20] and underscores a critical role of all IscA homologs under aerobic growth conditions.

In summary, we propose that the specificity and/or activity of the Suf and Isc Fe-S biogenesis pathways may be optimized to respond to both the particular environmental conditions that can damage Fe-S clusters and to changes in the levels of specific substrates. Overall, such a strategy would allow cells to maintain the function of Fe-S proteins even when cells find themselves in a hostile environment. Future work will address whether the properties of the Isc and Suf pathways acquired by studying FNR as a model, extend to other Fe-S proteins.

Materials and Methods

Construction of strains

The strains and plasmids used in this study are listed in Table 1. An in frame $\Delta iscS$ deletion strain was constructed by replacing the coding region of *iscS* (codons 2-404) with a Cm^R resistance cassette flanked by FRT (FLP recognition target) sites from plasmid pKD32 in strain BW25993 / pKD46 as described.[50] In frame $\Delta iscU$ (codons 2-128), $\Delta iscA$ (codons 2-107), $\Delta iscSUA$ (codon 2 of *iscS* through codon 107 of *iscA*), $\Delta hscBAfdx$ (codon 2 of *hscB* through codon 111 of *fdx*), $\Delta iscSUAhscBAfdx$ (codon 2 of *iscS* through codon 111 of *fdx*), $\Delta sufA$ (codons 2-122), $\Delta sufABCDSE$ (codon 2 of *sufA* through codon 138 of *sufE*), $\Delta nfuA$ (codons 2-191), $\Delta ompT$ (codons 2-317), $\Delta lacY$ (codons 2-417), $\Delta iscR$ (codons 2-120)[51] and $\Delta csdA$ (codons 2-401) deletion strains were constructed in a similar manner; in some cases the coding regions were replaced with the Kn^R cassette (instead of Cm^R) flanked by FRT sites from plasmid pKD13.[50] Transduction with P1 *vir* was used to move the Cm^R or Kn^R alleles to strains containing the *dmsA* (PK3292), *ydfZ* (PK8202), *sufA* (PK6879, PK6898), or *narG* (RZ7350, PK910) promoter-*lacZ* fusions to produce the corresponding strain derivatives listed in Table 1. In strains containing one or more *isc* gene deletions, it was confirmed by Western blot analysis that the Cm^R or Kn^R alleles did not decrease expression of downstream genes within the *isc* operon. In some cases, the Cm^R cassette was deleted by transforming strains with pCP20 encoding FLP recombinase[50] and screening for Cm sensitivity. All gene deletions were confirmed by colony PCR.

β-galactosidase assays

Levels of β-galactosidase were measured in strains containing *dmsA*, *ydfZ*, *narG*, and *sufA* promoter-*lacZ* fusions as described.[52]; *dmsA* and *narG* encode for a subunit of dimethyl sulfoxide reductase and nitrate reductase, respectively[53; 54]; *ydfZ* encodes for a putative selenium-binding protein.[55] Cultures were grown aerobically or anaerobically to an OD_{600} of ~0.2 in M9 minimal medium with 0.2% (w/v) glucose, 10 μM ferric ammonium citrate, 2 μg ml^{-1} thiamine, and 0.2 μM ammonium molybdate as previously described.[56] Final concentrations of nicotinic acid (12.5 μg ml^{-1}), KNO_3 (20 mM), chloramphenicol (20 μg ml^{-1}), or ampicllin (50 μg ml^{-1}) were added to the medium where indicated. To terminate cell growth and any further protein synthesis, either chloramphenicol (final concentration 20 μg ml^{-1}) or tetracycline (final concentration 10 μg ml^{-1}) was added, and cells were placed on ice until assayed for β-galactosidase activity.[52] β-galactosidase assays were repeated at least three times and error bars represent the standard errors of triplicate samples. The β-galactosidase activity was normalized to account for the difference in cell number per ml of culture as determined via viable plating assays.[31] At an OD_{600} of 0.4, aerobic and anaerobic cultures contained $(2.6 \pm 0.2) \times 10^8$ and $(4 \pm 0.3) \times 10^8$ cells ml^{-1}, respectively. Therefore, β-galactosidase activity was normalized by multiplying the aerobic values by a factor of 1.5.

Western blots

Western blots using α-FNR antibodies or α-IscS antibodies were performed as previously described.[31] Western blots using α-SufD antibodies were carried out in a similar manner except that the α-SufD antibodies were precleared using a SufD$^-$ cell lysate[57] and HRP-conjugated goat anti-rabbit secondary antibodies (Santa Cruz Biotechnology) were used. After treating the blots with Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer), chemiluminescence was quantified using a UVP Laboratory Products Epi Chem II Darkroom and Molecular Dynamics Image Quant software.

[35]S-methionine pulse-chase assays followed by immunoprecipitation of FNR

[35]S-methinonine pulse-chase assays followed by immunoprecipitation of FNR were carried out as previously described.[46]

[55]Fe labelling followed by immunoprecipitation of FNR-L28H under non-denaturing conditions

An overnight culture of a strain expressing FNR-L28H or FNR-L28H-C122S from a plasmid (PK8436 or PK8499, respectively) was grown aerobically in M9 minimal medium containing final concentrations of 0.2% (w/v) glucose, 2 μM ferric ammonium citrate, 2 μg ml^{-1} thiamine, 0.2 μM ammonium molybdate, and 20 μg ml^{-1} chloramphenicol. The overnight culture was diluted 1:100 into fresh media, which in addition to containing 2 μM ferric ammonium citrate, also contained 2.6 μCi of [55]Fe]ferric ammonium citrate (specific activity 93.76 mCi/mg) per milliliter of culture; the final Fe concentration was 2.5 μM. ([55]Fe]ferric ammonium citrate was obtained by treating 5 mCi of [55]Fe]ferric chloride in 0.5 M HCl (GE Healthcare) with 100 μl of 0.3 M potassium citrate and 20 μl of 14.5 M ammonium hydroxide.) The culture was sparged with either an aerobic or anaerobic gas mixture as previously described.[56] At an OD_{600} of ~0.2, aliquots (1 ml) of the culture were collected at various times by centrifugation (2 min at 13,600 $\times g$) in Eppendorf microfuge tubes. The cell pellets were washed once in 1 ml of fresh media containing 2 μM of unlabelled ferric ammonium citrate, centrifuged (2 min at 13,600 $\times g$), and frozen on a dry ice/ethanol bath. For immunoprecipitation of [55]Fe radiolabelled FNR-L28H (or FNR-L28H-C122S), cell pellets were thawed and lysed using the EasyLyse Bacterial Protein Extraction Solution (Epicentre) by resuspending each pellet in 200 μl of a mixture containing 5 mM Tris-HCl [pH 7.5], 0.5% Triton X-100, 2 mM $MgCl_2$ plus 0.2 μl of Epicentre Enzyme mix. After incubating the samples at room temperature for 5 min, the samples were centrifuged (2 min at 13,600 $\times$ g) and the supernatant was transferred to 200 μl of a solution containing pre-cleared α-FNR antibodies[57] in 50 mM Tris-HCl [pH 7.9], 10% glycerol, 500 mM NaCl, 1% Triton X-100 that were pretreated with 10 μl of UltraLink Protein A beads (Pierce). (The antibodies were pretreated with Protein A beads by incubating for one hour at room temperature with gentle rocking.) After gently rocking the samples at room temperature for one hour, the beads were pelleted, washed twice with 50 mM Tris-HCl [pH 7.9], 10% glycerol, 500 mM NaCl, 1% Triton X-100, and resuspended in 50 μl of double-distilled water. The samples were transferred as a slurry of beads to scintillation vials containing 3 mls of BioSafe II scintillation cocktail (Research Products International) and counted for 1 min on a Packard Tri-Carb 2100TR Liquid Scintillation Analyzer. Counting regions A and B were set at 0–5 keV and 5–180 keV, respectively. Using the previously determined counting efficiencies for [55]Fe in the two counting regions (9.5% in region A, 1.2% in region B), the total DPM for [55]Fe was determined. The number of [4Fe-4S] clusters was determined from the DPM by dividing by the specific activity of added [55]Fe [1.905×10^{-8} dpm [55]Fe molecule^{-1}], multiplying by 5 for the dilution with unlabelled Fe and dividing by 4 for the number of Fe per cluster.

Acknowledgements

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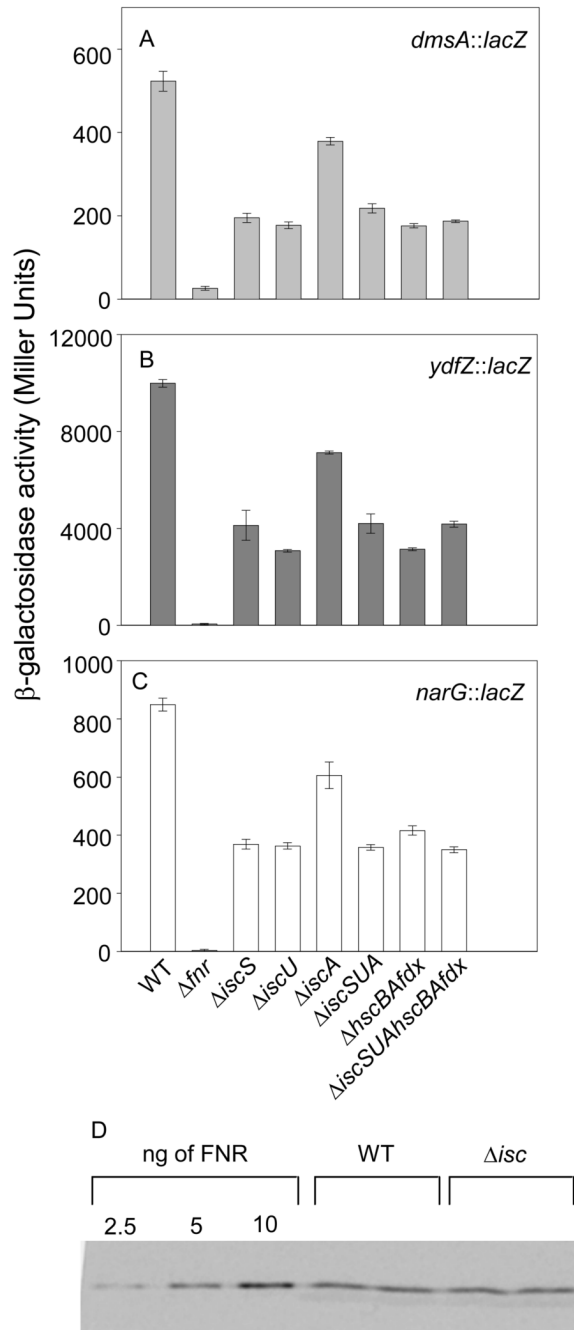
Figure 1.
FNR-dependent activation of the A) *dmsA*, B) *ydfZ*, and C) *narG* promoters in anaerobically grown strains containing various deletions of the *iscSUAhscBAfdx* operon measured by β-galactosidase assays. Strains were grown anaerobically in M9 minimal glucose medium containing nicotinic acid (12.5 μg ml^{-1}). Strains containing the P*narG*::*lacZ* fusion (C) were also grown in the presence of 20 mM KNO$_3$. The error bars represent the standard errors of triplicate samples. D) Western blot analysis of FNR protein assayed from anaerobically grown wild-type and Δ*iscSUAhscBAfdx* (Δ*isc*) strains. The lanes represent the signals obtained from duplicate samples of 250 μl of cells (WT or Δ*isc*) grown to an OD$_{600}$ of 0.4 and purified FNR protein (2.5 ng, 5 ng and 10 ng). Quantification of the FNR protein bands from three

independently grown samples (data not shown) showed that wild-type and Δ*iscSUAhscBAfdx* strains contained 34 ± 2 and 35 ± 2 pg of FNR protein / μl of cells at OD_{600} of 0.4, respectively.
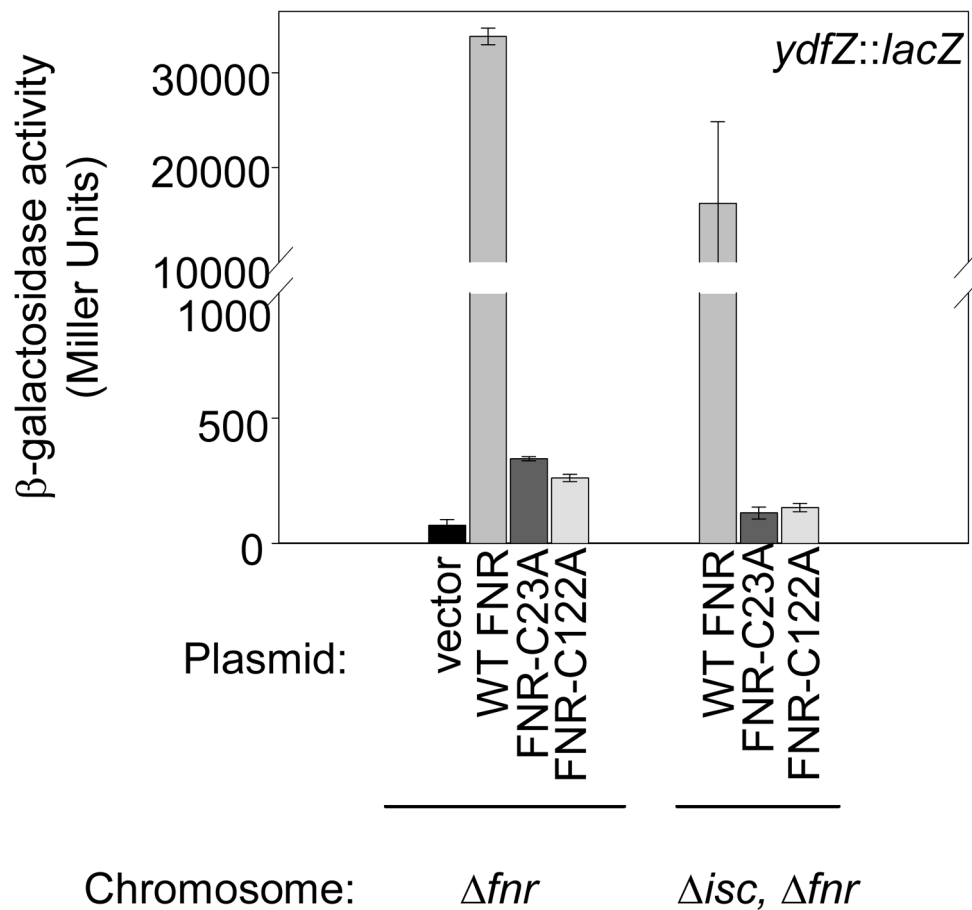
Figure 2.
Anaerobic expression of the *ydfZ* promoter in Δ*fnr* and Δ*fnr*, Δ*iscSUAhscBAfdx* strains expressing either wild-type FNR, FNR-C23A or FNR-C122A from the vector pACYC184. Strains were grown under anaerobic conditions in M9 minimal glucose medium containing chloramphenicol (20 μg ml^{-1}) and nicotinic acid (12.5 μg ml^{-1}).
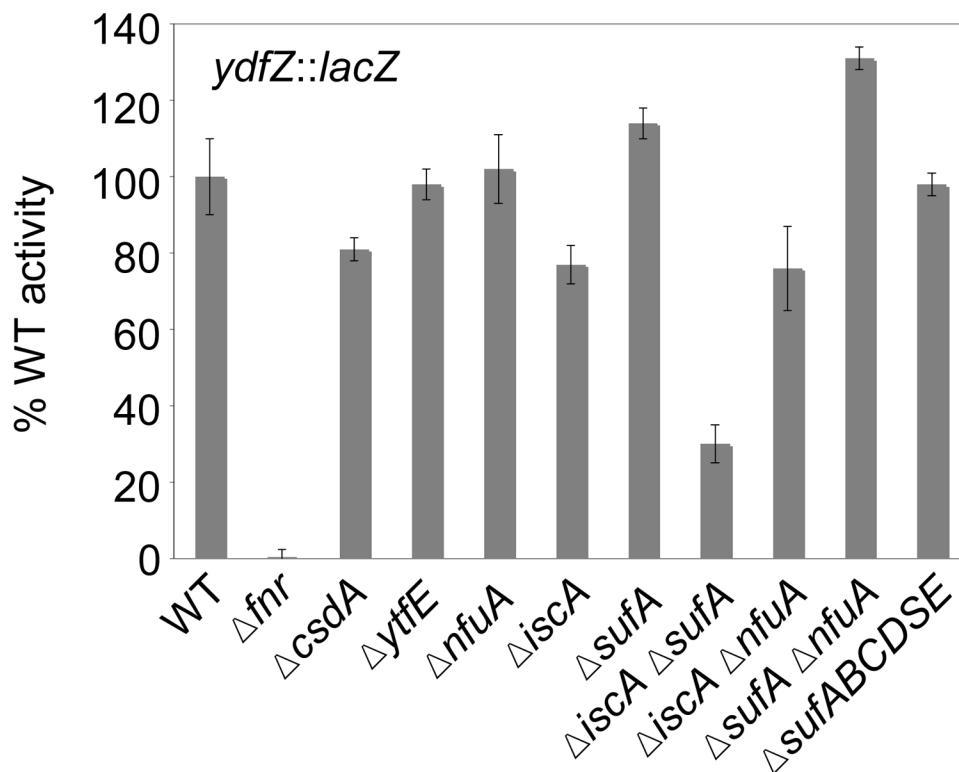
Figure 3.
β-galactosidase activity from the FNR-activated *ydfZ* promoter in anaerobically grown strains containing single or double gene deletions of *fnr*, *csdA, ytfE*, *nfuA*, *iscA*, and *sufA* or deletion of the entire the *sufABCDSE* operon. Strains were grown in M9 minimal glucose medium containing nicotinic acid (12.5 μg ml^{-1}). β-galactosidase activities are normalized to FNR activity in the WT strain (100%).
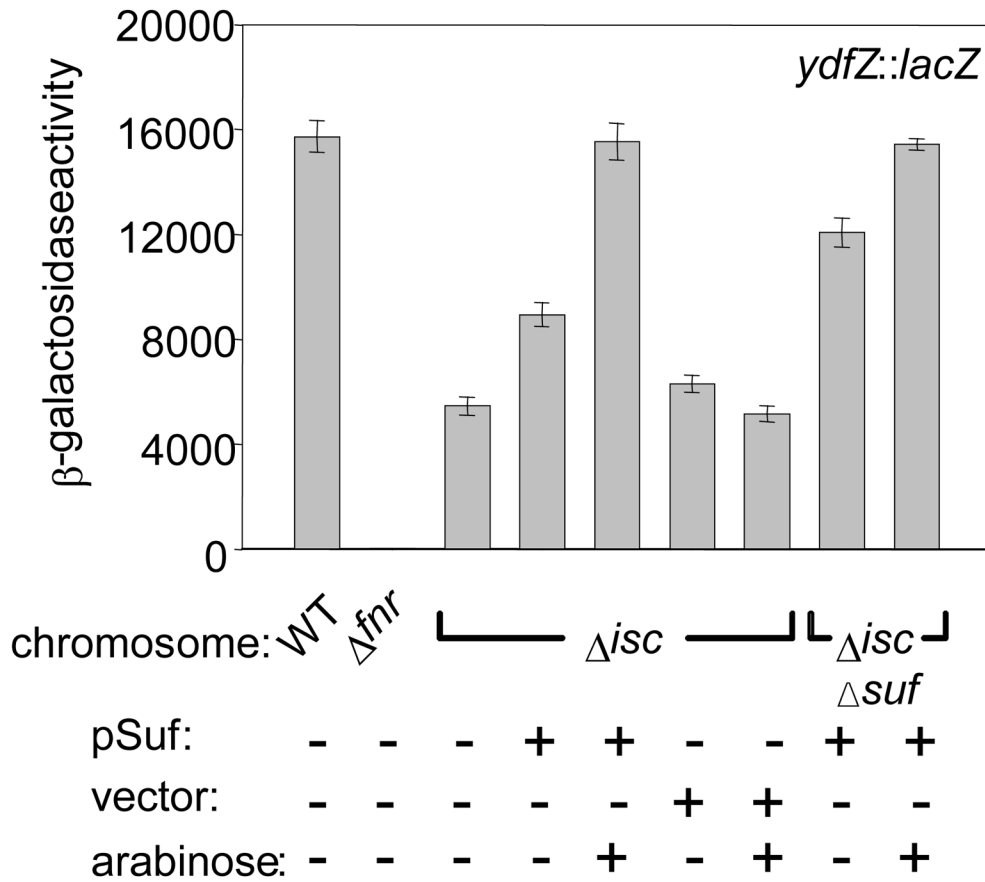
Figure 4.
β-galactosidase activity from the *ydfZ* promoter in anaerobically grown wild-type, Δ*fnr*, and
Δ*iscSUAhscBAfdx* (Δ*isc*), and Δ*iscSUAhscBAfdx*, Δ*sufABCDSE* (Δ*isc*, Δ*suf*) strains. The
Δ*iscSUAhscBAfdx* strain derivatives contained either the plasmid expressing the *sufABCDSE*
operon from the arabinose-inducible P_{BAD} promoter (pSuf), the vector alone, or no vector. All
cultures were grown anaerobically in M9 minimal glucose medium containing nicotinic acid
(12.5 μg ml^{-1}). The plasmid-containing strains were also grown in the presence of ampicillin
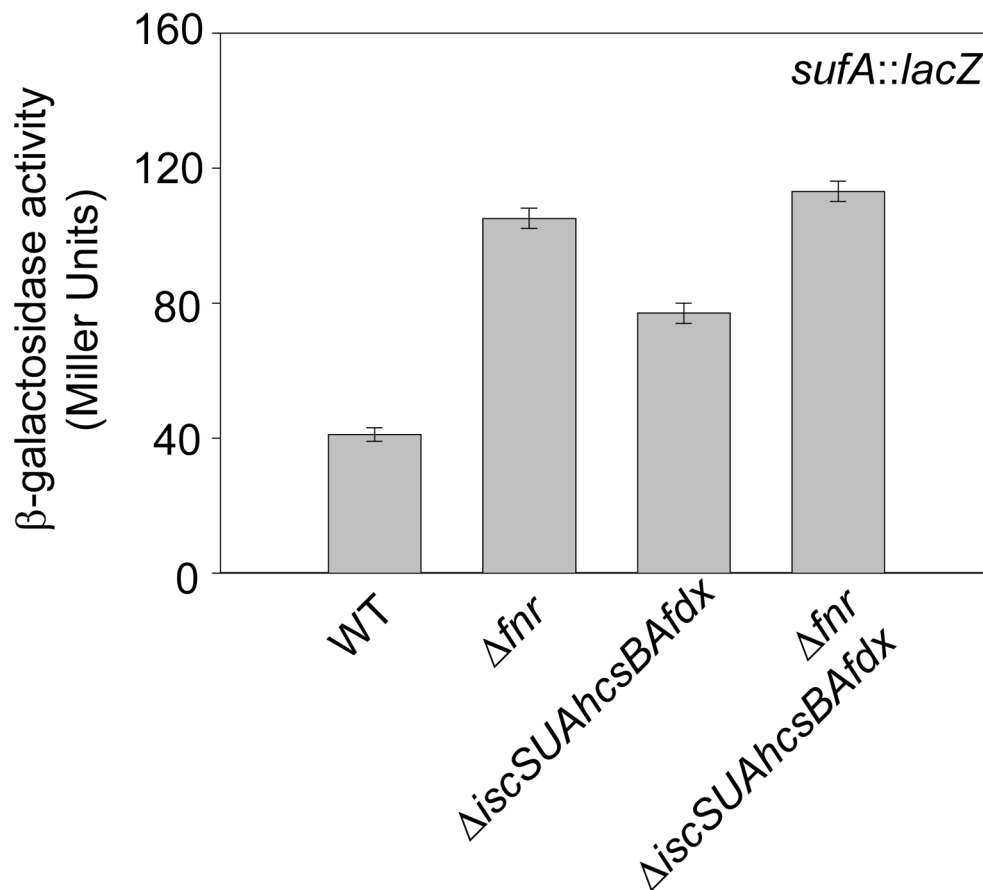(final concentration of 50 μg ml^{-1}) and, where indicated, 0.2% arabinose.

Figure 5.
Anaerobic β-galactosidase activity from the *sufA* promoter in strains lacking *fnr*, the
iscSUAhscBAfdx operon (Δ*isc*), or both. Cultures were grown anaerobically in M9 minimal
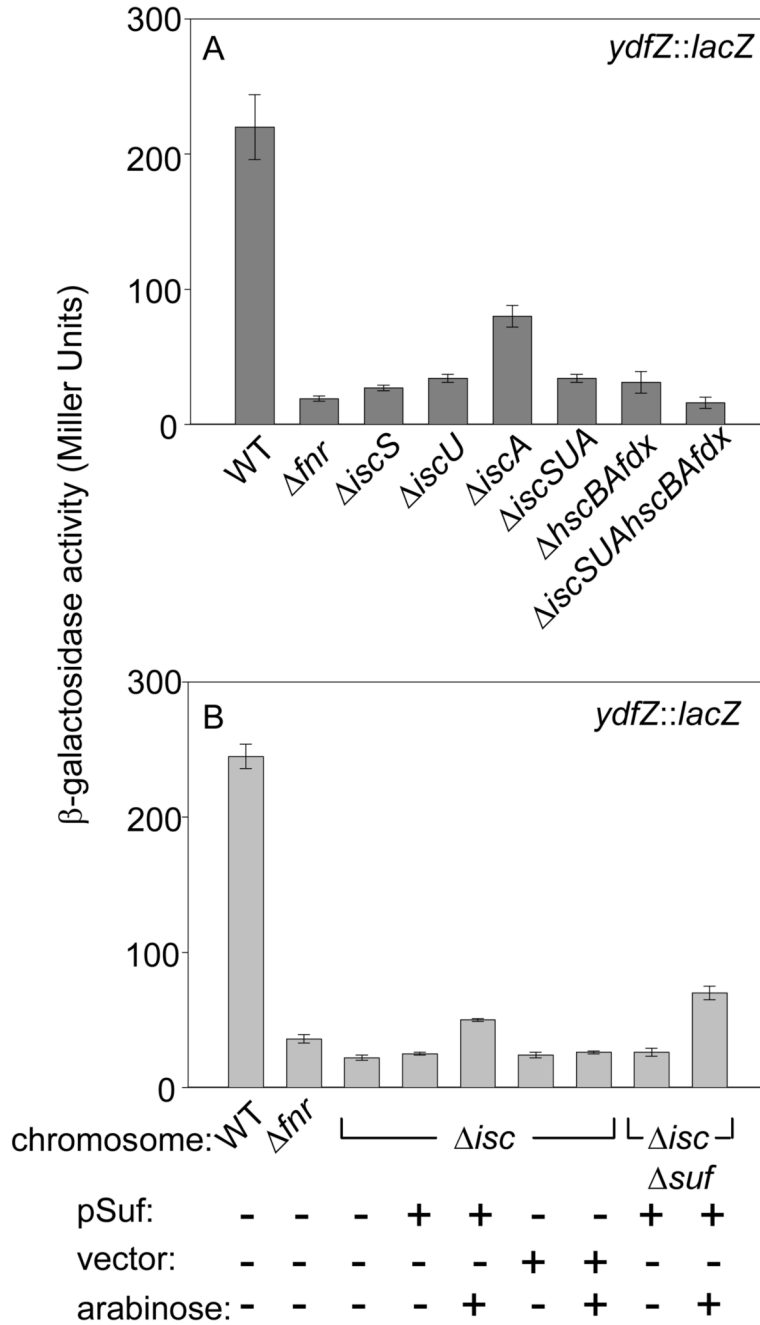glucose medium containing a final concentration of nicotinic acid (12.5 μg ml^{-1}).

Figure 6.
Activation of the *ydf*Z promoter by wild-type FNR in aerobically grown cultures as measured by β-galactosidase assays. A) Aerobic FNR activity in strains containing various deletions of the Δ*iscSUAhscBAfdx* operon. B) Aerobic FNR activity in wild-type, Δ*fnr*, and Δ*iscSUAhscBAfdx* (Δ*isc*), and Δ*iscSUAhscBAfdx*, Δ*sufABCDSE* (Δ*isc*, Δ*suf*) strains. The Δ*iscSUAhscBAfdx* strain derivatives contained either the plasmid expressing the *sufABCDSE* operon from the arabinose-inducible P$_{BAD}$ promoter (pSuf), the vector alone, or no vector. All cultures were grown aerobically in M9 minimal glucose medium supplemented with nicotinic acid (12.5 μg ml^{-1}). The plasmid-containing strains were also grown in the presence of ampicillin (final concentration of 50 μg ml^{-1}) and, where indicated, 0.2% arabinose.
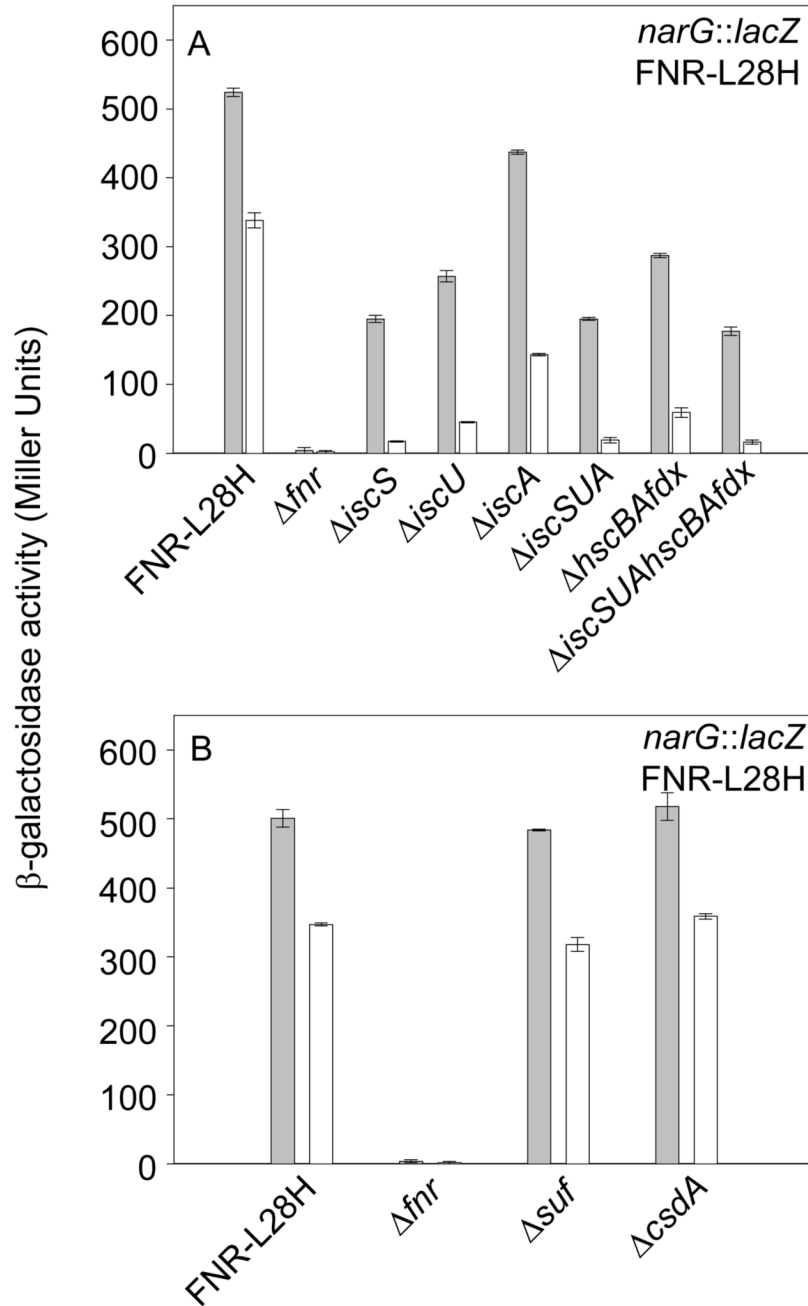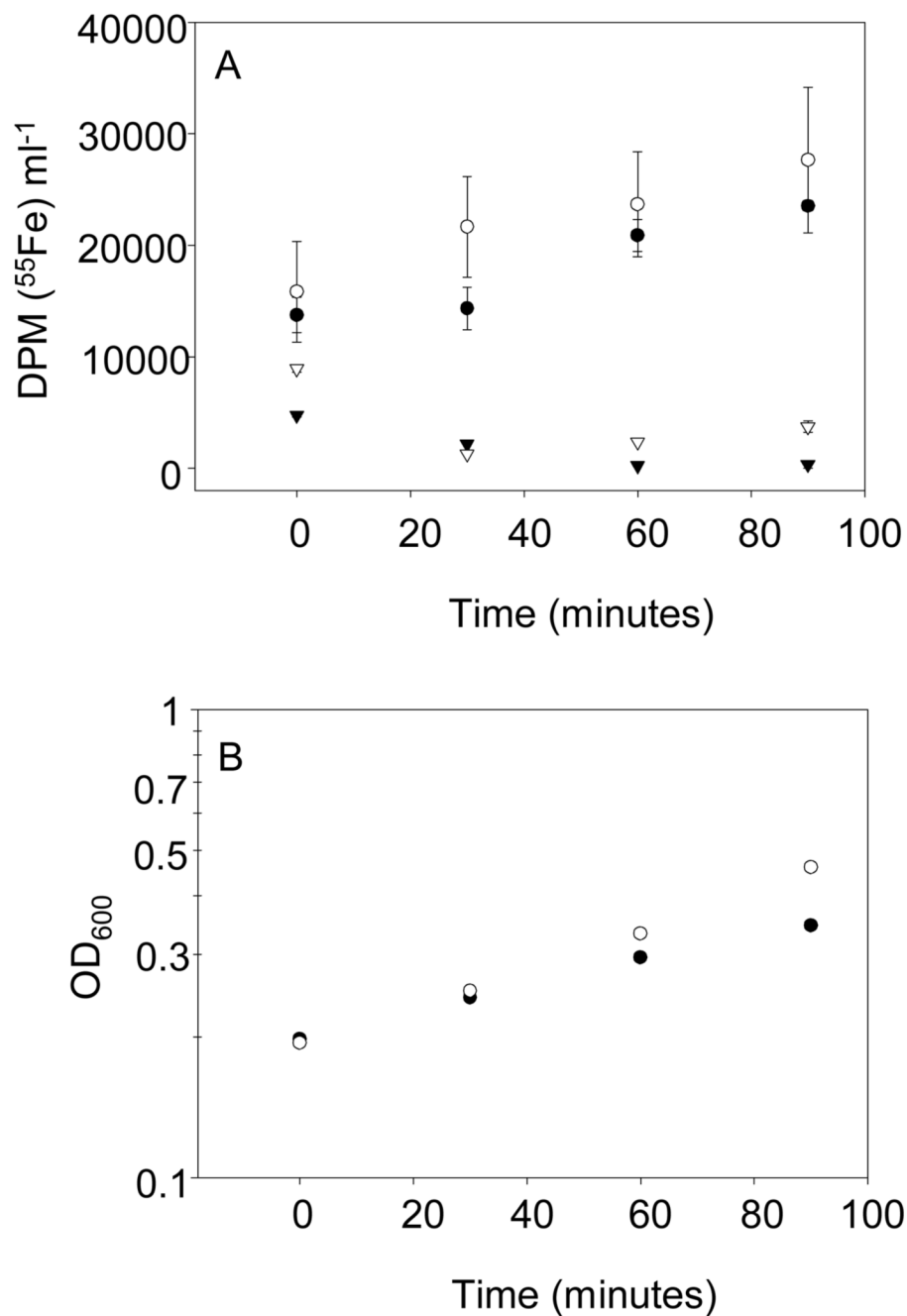
Figure 7.
Activity of the mutant FNR-L28H, which contains an O_2-stable [4Fe-4S] cluster, in anaerobic (gray bars) and aerobic (white bars) cultures grown in M9 minimal glucose medium containing a final concentration of 20 mM KNO_3. All strains lacked the wild-type chromosomal copy of *fnr* but expressed FNR-L28H in single copy from the chromosome except Δ*fnr*. A) Activation of P*narG* by FNR-L28H in strains containing various deletions of the *iscSUAhscBAfdx* operon. Cultures also contained nicotinic acid (12.5 μg ml^{-1}) in the medium. B) Activation of P*narG* by FNR-L28H in strains lacking the *sufABCDSE* operon (Δ*suf*) or *csdA*.

Figure 8.
A) *In vivo* incorporation of ^{55}Fe into [4Fe-4S] cluster-containing FNR-L28H (circles) and clusterless FNR-L28H-C122S (triangles). pACYC184-derived FNR-L28H and FNR-L28H-C122S were immunoprecipitated from 1 ml aliquots of aerobically (open symbols) or anaerobically (closed symbols) grown cultures. The total DPM per ml for ^{55}Fe was calculated as described in the Methods. The error bars represent the standard errors of triplicate samples. B) Growth measured by the optical density at 600 nm, of the aerobically (open circles) or anaerobically (closed circles) ^{55}Fe-labelled cultures expressing FNR-L28H.
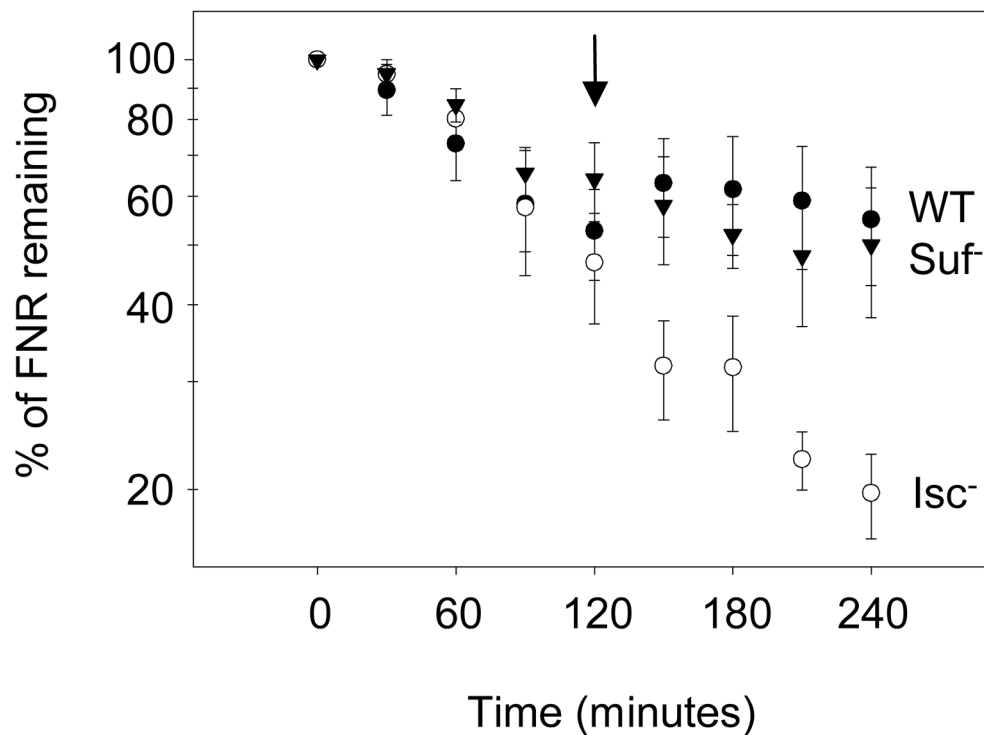
Figure 9.
Reactivation of apo-FNR to [4Fe-4S]-FNR upon switching aerobic cultures to anaerobic growth conditions. Methionine was added after 1 min of labelling of aerobically grown cells with ^{35}S-methionine, followed by immunoprecipitation of FNR. At 120 minutes after adding the unlabeled methinonine, wild-type (filled circles), Isc$^-$ (open circles) or Suf$^-$(filled triangles) cultures were switched to anaerobic growth conditions (indicated by the arrow). The amount of ^{35}S-met-FNR remaining over time was calculated by normalizing to the amount of ^{35}S-met-FNR present upon addition of the unlabeled methionine at time zero.
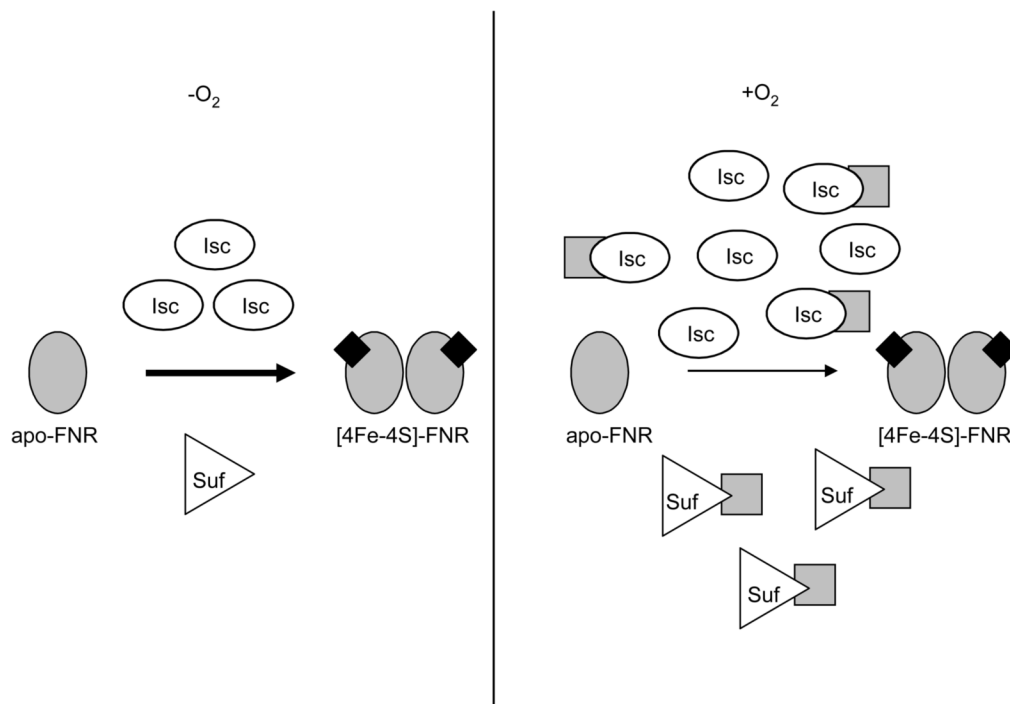
Figure 10.
Model for FNR Fe-S cluster biogenesis requirements under aerobic and anaerobic conditions. Under both conditions, monomeric, apo-FNR acquires Fe-S clusters (represented as black diamonds) primarily from the Isc pathway. Under anaerobic conditions, Suf can provide up to 40% of Fe-S clusters for FNR when the Isc pathway is absent. Although expression of both the Isc pathway and the Suf pathway is increased under aerobic conditions compared to anaerobic conditions, Suf does not appear to provide Fe-S clusters to FNR in the absence of Isc. Furthermore, the demand for FNR Fe-S cluster biogenesis via the Isc pathway is greater under aerobic conditions compared to anaerobic conditions. This increased demand for Fe-S cluster biogenesis may be due to the higher rate Fe-S cluster turnover under aerobic conditions, and thus increased levels of apo-protein substrates (gray squares).

Table 1

E. coli strains and plasmids used in this work.

Construct	Relevant genotype	Reference / Source
Strains		
MG1655	F$^-$, λ$^-$, *rph*-1	This laboratory
PK4811	MG1655 but Δ*fnr*ΩSp^r / Sm^r	This laboratory
PK8431	PK4811 but Δ*ompT*::*kan*	This study
PK8436	Same as PK8431 but with	This study
	pPK434	
PK8499	Same as PK8431 but with pPK437	This study
WO19	Δ*sufABCDSE19*::*kan*	Storz/Imlay laboratories
WO231	MG1655 Δ*sufD*	F.W. Outten
JOEY18	MG1655 Δ*ytfE*::*kan*	S. Spiro
RZ4500	MG1655 but *lacZΔ145*	58
PK3292	RZ4500 but λPC25	24
PK3293	PK3292 but Δ*fnr*ΩSp^r / Sm^r	59
PK7692	PK3292 but Δ*iscS*::*cat*	This study
PK7694	PK3292 but Δ*iscU*::*kan*	This study
PK7696	PK3292 but Δ*iscA*:: *kan*	This study
PK7680	PK3292 but Δ*iscSUA*::*cat*	This study
PK7690	PK3292 but Δ*hscBAfdx*::*cat*	This study
PK7682	PK3292 but Δ *iscSUAhscBAfdx*::*cat*	This study
PK6463	PK3292 but Δ*csdA*::*cat*	This laboratory
PK6465	PK3292 but Δ*sufABCDSE19*:: *kan*	This study
RZ7350	RZ4500 but *narG234*::MudI1734	60
PK8245	RZ7350 but Δ*iscS*::*cat*	This study
PK8246	RZ7350 but Δ*iscU*::*cat*	This study
PK8247	RZ7350 but Δ*iscA*::*cat*	This study
PK8248	RZ7350 but Δ*iscSUA*::*cat*	This study
PK8249	RZ7350 but Δ*hscBAfdx*::*cat*	This study
PK8250	RZ7350 but Δ*iscSUAhscBAfdx*::*cat*	This study
PK8474	RZ7350 but Δ*sufABCDSE*::*cat*	This study
PK8479	RZ7350 but Δ*csdA*::*cat*	This study
RZ8480	RZ7350 but Δ*fnr*ΩSp^r / Sm^r	39
PK910	RZ8480 but λ*fnr*-L28H	47
PK8228	PK910 but Δ*iscS*::*cat*	This study
PK8229	PK910 but Δ*iscU*::*cat*	This study
PK8230	PK910 but Δ*iscA*::*cat*	This study
PK8231	PK910 but Δ*iscSUA*::*cat*	This study
PK8232	PK910 but Δ*hscBAfdx*::*cat*	This study
PK8233	PK910 but Δ*iscSUAhscBAfdx*::*cat*	This study
PK8478	PK910 but Δ*sufABCDSE*::*cat*	This study
PK8482	PK910 but Δ*csdA*::*cat*	This study

Construct	Relevant genotype	Reference / Source
PK8202	MG1655 (−233 to +13 relative to translational start site) P*ydfZ*::*lacZ*	This study
PK8222	PK8202 but Δ*iscS*::*cat*	This study
PK8223	PK8202 but Δ*iscU*::*cat*	This study
PK8224	PK8202 but Δ*iscA*::*cat*	This study
PK8225	PK8202 but Δ*iscSUA*::*cat*	This study
PK8226	PK8202 but Δ*hscBAfdx*::*cat*	This study
PK8227	PK8202 but *iscSUAhscBAfdx*::*cat*	This study
PK8428	Same as PK8227 but with pGS0164	This study
PK8445	Same as PK8227 but with pBADmychisC	This study
PK8405	PK8202 but Δ*nfuA*	This study
PK8407	PK8202 but Δ*ytfE*	This study
PK8476	PK8202 but Δ*sufABCDSE*::*cat*	This study
PK8605	PK8476 but Cm^S	This study
PK8607	PK8605 but with pGS0164	This study
PK8611	PK8607 but Δ *iscSUAhscBAfdx*::*cat*	This study
PK8481	PK8202 but Δ*csdA*::*cat*	This study
PK8835	PK8202 but Δ*sufA*	This laboratory
PK8836	PK8202 but Δ*sufA* Δ*nfuA*	This laboratory
PK8865	PK8202 but Δ*sufA* Δ*iscA*	This laboratory
PK8544	PK8202 but Δ*iscA* Δ*nfuA*::*cat*	This laboratory
PK8203	PK8202 but Δ*fnr*ΩSp^r / Sm^r	This study
PK8457	PK8203 but Δ*ompT* Δ*lacy*	This study
PK8601	PK8457 but with pPK434	This study
PK8234	Same as PK8203 but with pRZ7411	This study
PK8235	Same as PK8203 but with pACYC184	This study
PK8240	Same as PK8203 but with pPK6928	This study
PK8241	Same as PK8203 but with pPK6929	This study
PK8259	PK8203 but Δ*iscSUAhscBAfdx*	This study
PK8270	Same as PK8259 but with pRZ7411	This study
PK8271	Same as PK8259 but with pACYC184	This study
PK8272	Same as PK8259 but with pPK6928	This study
PK8273	Same as PK8259 but with pPK6929	This study
PK6879	MG1655 (−200 to +40 relative to transcriptional start site) P*sufA*::*lacZ*	28
PK8452	PK6879 but Δ*iscSUAhscBAfdx*::*cat*	This study
PK6898	PK6879 but Δ*fnr*ΩSp^r / Sm^r	This laboratory
PK8453	PK6898 but Δ*iscSUAhscBAfdx*::*cat*	This study
BW25993	*lacI*^{q} Δ *lacZ*$_{WJ16}$ *hsdR514*Δ*araBAD*$_{AH33}$Δ *rhaBAD*$_{LD78}$	50
Plasmids		
pBAD/Myc-hisC	Ap^r	Invitrogen
pGS0164	pBAD/Myc-hisC but with *sufABCDSE* in NcoI and EcoRI sites	19
pRZ7411	Cm^r, HindIII-BamHI of *fnr*, -521 to +1115 of *fnr* in pACYC184	39

Construct	Relevant genotype	Reference / Source
pACYC184	Cm^r	61
pPK6928	Same as pRZ7411 but *fnr*-C23A	46
pPK6929	Same as pRZ7411 but *fnr*-C122A	46
pCP20	Ap^r	50
pKD46	Phage λ *gam-bet-exo* genes under P*araB* control	B. L. Wanner
pKD32	FRT-*cat*-FRT	B. L. Wanner
pKD13	FRT-*kan*-FRT	50
pPK434	HindIII-BamHI of *fnr*-L28H (−521 to +1115 of *fnr*-LH28) in pACYC184	This laboratory
pPK437	Same as pPK434 but with *fnr*-L28H-C122S	This laboratory