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Regulation of iron-sulfur cluster homeostasis through transcriptional control of the Isc pathway by [2Fe-2S]-IscR in *Escherichia coli*

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SUMMARY

Fe-S clusters are essential across the biological world, yet how cells regulate expression of Fe-S cluster biogenesis pathways to cope with changes in Fe-S cluster demand is not well understood. Here, we describe the mechanism by which IscR, a [2Fe-2S] cluster-containing regulator of *Escherichia coli*, adjusts the synthesis of the Isc Fe-S biogenesis pathway to maintain Fe-S homeostasis. Our data indicate that a negative feedback loop operates to repress transcription of the *iscRSUA-hscBA-fdx* operon, encoding IscR and the Isc machinery, through binding of [2Fe-2S]-IscR to two upstream binding sites. IscR was shown to require primarily the Isc pathway for synthesis of its Fe-S cluster, providing a link between IscR activity and demands for Fe-S clusters through the levels of the Isc system. Surprisingly, the *isc* operon was more repressed under anaerobic conditions, indicating increased Fe-S cluster occupancy of IscR and decreased Fe-S cluster biogenesis demand relative to aerobic conditions. Consistent with this notion, overexpression of a Fe-S protein under aerobic conditions, but not under anaerobic conditions, led to derepression of P_{iscR}. Together, these data show how transcriptional control of *iscRSUA-hscBA-fdx* by [2Fe-2S]-IscR allows *E. coli* to respond efficiently to varying Fe-S demands.

Keywords

IscR; Fe-S biogenesis; Isc; Suf; autoregulation

INTRODUCTION

Iron-sulfur (Fe-S) proteins are nearly ubiquitous across all domains of life and carry out many important cellular functions such as respiration, photosynthesis, nitrogen fixation, and

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gene regulation (Beinert, 2000, Fontecave, 2006, Kiley and Beinert, 2003). In *Escherichia coli*, the majority of Fe-S cluster biogenesis under non-stress conditions is proposed to be catalyzed by the highly conserved, housekeeping Isc (iron-sulfur cluster) pathway, encoded by the *iscRSUA-hscBA-fdx* (*isc*) operon (Johnson *et al*., 2005, Py and Barras, 2010, Py *et al*., 2011). The current model for Isc-mediated Fe-S biogenesis in *E. coli* is that IscS, a cysteine desulfurase, provides the sulfur to build a transient Fe-S cluster on the scaffold protein IscU. HscB and HscA form a complex with IscU to apparently facilitate transfer of a Fe-S cluster from IscU to a subset of apo-proteins. Alternatively, IscA, an A type carrier, promotes cluster transfer by delivering the Fe-S cluster from the scaffold to target apo-proteins. Other genes in the operon encode a [2Fe-2S] ferredoxin (Fdx) and the transcription factor IscR. Although transcription of the *isc* operon is repressed by IscR (Schwartz *et al*., 2001), a [2Fe-2S] cluster-containing protein encoded by the first gene of this operon, a detailed understanding of how this autoregulatory mechanism occurs and ultimately how Fe-S biogenesis is regulated *in vivo* remains to be elucidated.

While IscR was first discovered for its role in regulating transcription of the Isc system (Schwartz *et al*., 2001), subsequent genome-wide transcription profiling data revealed that IscR controls the expression of more than 40 genes in *E. coli* (Giel *et al*., 2006).Among these genes are those encoding for anaerobic respiratory Fe-S enzymes (periplasmic nitrate reductase, hydrogenases-1 and −2) and additional proteins with roles in Fe-S formation, including ErpA, NfuA, and the Suf system. IscR also directly activates the expression of the alternate Fe-S biogenesis pathway encoded by the *sufABCDSE* (*suf*) operon (Nesbit *et al*., 2009, Yeo *et al*., 2006), which includes proteins that exhibit functional similarity to those of the Isc pathway (Py and Barras, 2010, Py *et al*., 2011, Johnson *et al*., 2005). However, Suf is proposed to function primarily under oxidative and nitrosative stress and iron-limiting conditions (Justino *et al*., 2005, Mukhopadhyay *et al*., 2004, Outten *et al*., 2004, Zheng *et al*., 2001). Nevertheless, the transcription profiling results implicated a broader role for IscR in sensing the cellular Fe-S status and thus controlling the expression of the Isc and Suf systems accordingly.

Further analysis of promoters directly regulated by IscR revealed two classes of DNA target sites for IscR site-specific binding, denoted as Type 1 and Type 2 sites (Giel *et al*., 2006). The nucleotide sequences vary between Type 1 (Giel *et al*., 2006) and Type 2 (Nesbit *et al*., 2009) sites, and it is not yet clear how a dimer of IscR distinguishes between them (Nesbit *et al*., 2009).Of the IscR-dependent promoters identified thus far, most contain a single Type 1 or Type 2 site (Giel *et al*., 2006). In contrast, DNase I footprinting has revealed that IscR binds to three individual sites within the *iscR* promoter region (P_{iscR}) (Giel *et al*., 2006). One region of protection, which encompasses nucleotides −67 to −14 relative to the +1 transcription start site, includes two Type 1 IscR binding sites: site A (−65 to −41) and site B (−40 to −16) (Giel *et al*., 2006). The remaining IscR site, located from +9 to +26, does not share sequence similarity with other known IscR binding sites and is referred to as P_{iscR} site C (Giel *et al*., 2006). The contribution of each site to repression of the *isc* operon *in vivo* is not known.

A previous study provided important insight that repression of the *isc* operon by IscR may allow cells to homeostatically regulate the synthesis of the Isc Fe-S biogenesis pathway

(Schwartz *et al*., 2001). In this model, IscR [2Fe-2S] cluster occupancy would be a reporter of the cellular Fe-S status, tuning synthesis of the Isc pathway in response to changing demands for Fe-S biogenesis. In support of the model, the presence of [2Fe-2S]-IscR was demonstrated *in vivo* by Mössbauer spectroscopy and repression of P_{iscR} was shown to require [2Fe-2S]-IscR, since clusterless IscR mutants failed to repress P_{iscR} (Fleischhacker *et al*., 2012, Yeo *et al*., 2006). In addition, strains lacking IscS and HscA showed decreased IscR repression, indicating a role of the Isc pathway in maintaining the [2Fe-2S] form of IscR (Schwartz *et al*., 2001). Nevertheless, the effect of other Isc proteins or the Suf pathway on [2Fe-2S]-IscR activity has not been examined. In addition, whether the decrease in *in vivo* repression observed with the clusterless mutants can be explained by a lack of DNA binding is of particular interest because IscR target promoters containing Type 2 sites (such as those in the *sufABCDSE, napFDAGHBC*, *hyaABCDEF*, and *hybOABCDEFG* operons) do not require the [2Fe-2S] cluster form of IscR either for binding DNA *in vitro* (Nesbit *et al*., 2009) or for transcriptional regulation *in vivo* (Nesbit *et al*., 2009, Yeo *et al*., 2006). Together, the unique features of the [2Fe-2S] cluster-dependent and -independent modes of IscR regulation have led to the proposal that conditions that increase the Fe-S demand will lead to decreased [2Fe-2S]-IscR levels, de-repression of the *isc* operon, elevated apo-IscR levels, and accordingly, increased regulation of promoters with Type 2 sites by apo-IscR. Such differences in [2Fe-2S] occupancy of IscR may explain why the *isc* operon is expressed at higher levels under aerobic conditions (Giel *et al*., 2006) since the demand for Fe-S biogenesis is expected to be greater because of the O_2 sensitivity of some Fe-S clusters (Imlay, 2006, Imlay, 2008). Thus, determining whether the [2Fe-2S] cluster occupancy of IscR differs between aerobic and anaerobic conditions is an important question to address.

In this study, we dissected the mechanism by which IscR negatively regulates P_{iscR} to better understand how cells maintain Fe-S homeostasis. We first established roles for the three predicted IscR binding sites in repressing P_{iscR} transcription *in vivo*. The requirement of the [2Fe-2S]-containing form of IscR for site-specific binding *in vitro* and regulation of P_{iscR} under aerobic and anaerobic conditions *in vivo* was analyzed using IscR mutants that cannot ligate an Fe-S cluster but are otherwise functional. We report the role of other Isc pathway components and the Suf system in providing Fe-S clusters to IscR under aerobic and anaerobic growth conditions. To address whether differences in [2Fe-2S] cluster occupancy for IscR exist between aerobic and anaerobic growth, an *in vivo* titration experiment was performed to determine the level of IscR protein required for P_{iscR} repression for both conditions. Finally, to examine whether IscR responds to cellular demands for Fe-S clusters, we tested whether overexpression of an Fe-S protein affected transcription from P_{iscR}. Together, our data indicate that [2Fe-2S]-IscR regulates expression of the Isc pathway via a negative feedback mechanism based on the cellular Fe-S demand.

RESULTS

The two Type 1 sites play a role in negative autoregulation of the *iscR* promoter

To examine the contribution of each of the three IscR binding sites (A, B, and C; Fig. 1A) within the *iscR* promoter region to negative autoregulation, mutations in each individual site

were constructed and their effect on IscR binding was determined. Since sites A and B are representative of the Type 1 IscR binding site, the first AT of each site, which are among the most highly conserved bases within the Type I site (Giel *et al*., 2006), were substituted with GC (Fig. 1A). Using a wild-type DNA fragment for comparison (Fig. 1B), DNase I footprinting showed that when site A was mutated, anaerobically isolated [2Fe-2S]-IscR no longer protected the region (−67 to −41 bp relative to the +1 *iscR* transcription start site) encompassing site A, but still protected the regions (−40 to −14 bp and +9 to +26 bp) encompassing sites B and C in a protein concentration-dependent manner (Fig. 1C). In an analogous fashion, the DNA fragment containing the mutated site B showed IscR-mediated protection in the regions encompassing sites A and C, but not site B (Fig. 1D). Site C is not as well-conserved among enterobacteria and does not resemble other known IscR binding sites (Giel *et al*., 2006). Nevertheless, upon mutating the base pairs located at positions +9, +10, +12, and +14 within this site (Fig. 1A), protection of site C from DNase I cleavage was eliminated, while there was no effect on IscR binding to sites A and B (Fig. 1E). Thus, our data demonstrate that mutation of sites A, B, and C within P_{iscR} decreases *in vitro* binding of IscR to each individual site but does not affect IscR binding to the remaining intact sites.

We determined the effects of the mutated IscR sites on *in vivo* P_{iscR} repression via β-galactosidase assays using aerobically or anaerobically grown strains with chromosomal P_{iscR} -*lacZ* fusions containing the mutations in sites A, B, or C (Fig. 2). As expected from previous results (Giel *et al*., 2006), IscR repressed P_{iscR} expression more under anaerobic (17-fold) than aerobic (6-fold) growth conditions. Nevertheless, under both growth conditions, mutation of either site A or B caused a 3- to 4-fold loss in repression compared to the intact promoter, indicating a defect in P_{iscR} repression. When sites A and B were both mutated, repression was eliminated, similar to that observed in a strain lacking IscR. Surprisingly, mutation of site C had no effect on P_{iscR} expression. Furthermore, mutation of site C did not result in any further significant defect in P_{iscR} repression when sites A or B were also mutated. Thus, our data suggest that both Type 1 sites A and B are necessary for full IscR-mediated repression of P_{iscR} *in vivo*. Since sites A and B overlap sequences (−65 to −16 bp) of the −35 and extended −10 promoter elements required for σ^{70} binding (Record *et al*., 1996) and a region potentially encompassing an UP element important for recognition by αCTD (Gourse *et al*., 2000), it appears that IscR binding of both Type 1 sites is required to fully prevent RNA polymerase from binding P_{iscR}.

IscR requires its Fe-S cluster to repress expression from the *iscR* promoter *in vivo* under aerobic and anaerobic conditions

To establish the extent of the IscR [2Fe-2S] cluster requirement for negative autoregulation under both aerobic and anaerobic growth conditions, we measured P_{iscR} repression in strains expressing IscR mutants that are defective in cluster binding. These mutants contain one or more alanine substitutions of three of the four IscR [2Fe-2S] cluster ligands (C92, C98, C104) (Fleischhacker *et al*., 2012) and were previously shown to contain no detectable Fe-S cluster upon isolation (Nesbit *et al*., 2009). Under either aerobic or anaerobic conditions, strains containing chromosomally encoded IscR-C92A, IscR-C98A, IscR-C104A, or IscR-C92A/C98A/C104A exhibited a nearly complete defect in P_{iscR}-*lacZ* repression (Fig. 3), indicating that under both growth conditions, IscR requires its [2Fe-2S] cluster to repress

P_{iscR}. Measurements of IscR protein in these strains showed that each of the IscR variants was present at levels ~2-fold and ~8-fold greater than wild-type IscR under aerobic and anaerobic conditions, respectively, (Table 2) (Nesbit *et al*., 2009), indicating that the loss in P_{iscR} repression did not result from a defect in protein accumulation. Furthermore, the same mutants were previously shown to be otherwise functional at a subset of IscR-regulated promoters that contain the Type 2 IscR binding motif and do not require the Fe-S form of IscR for binding (Nesbit *et al*., 2009). Taken together, our data support the notion that IscR requires its [2Fe-2S] cluster to negatively regulate P_{iscR} under both aerobic and anaerobic conditions and this occurs by [2Fe-S]-IscR binding to the two Type 1 sites within the *iscR* promoter region.

[2Fe-2S]-IscR binds with greater affinity to the P_{iscR} sites A and B *in vitro* than does apo-IscR

Previous *in vitro* studies demonstrated that [2Fe-2S]-IscR and IscR-C92A/C98A/C104A bind with equal affinity to P_{hyaA} (Nesbit *et al*., 2009), which contains a Type 2 IscR binding site. However, the above *in vivo* data raise the question as to whether [2Fe-2S]-IscR binds with higher affinity than apo-IscR to the Type 1 sites within P_{iscR}. To address this question, fluorescence anisotropy assays were performed under anaerobic conditions with DNA fragments containing either site A or B from P_{iscR} (Fig. 4). Curve fitting the fraction bound with the Hill equation indicates that the apparent K_d of monomeric [2Fe-2S]-IscR is 93 ± 5 nM for P_{iscR} site A and 112 ± 2 nM for P_{iscR} site B (Fig. 4C). Since the purified wild-type IscR used in this experiment was ≥50% occupied with [2Fe-2S] clusters, it is likely that the actual K_d values for P_{iscR} sites A and B may be lower than the apparent K_d values determined. In contrast, K_d values could not be determined for IscR-C92A/C98A/C104A because saturation was not obtained even at the highest protein concentration tested (1.0 μM)(Fig. 4B). Thus, our data suggest that [2Fe-2S]-IscR has a much higher binding affinity for the P_{iscR} A and B sites than does clusterless IscR. Furthermore, the observed difference in [2Fe-2S]-IscR and clusterless IscR binding affinity for the two P_{iscR} Type 1 sites provides an explanation as to why the [2Fe-2S] cluster is required for IscR to repress P_{iscR} *in vivo*.

IscR requires a functional Isc system to fully repress P_{iscR} *in vivo*

P_{iscR} repression requires [2Fe-2S]-IscR and is decreased in strains lacking *iscS* or *hscA* (Schwartz *et al*., 2001), supporting the model that the Isc machinery synthesizes the [2Fe-2S] cluster for IscR. However, since *iscS* and *hscA* are reported to have roles beyond Fe-S cluster assembly (Py and Barras, 2010, Johnson *et al*., 2005), we characterized the role of other members of the Isc pathway in supplying IscR its [2Fe-2S] cluster. Strains lacking individual components such as IscS, IscU, or Fdx yielded a similar defect in repression as the strain lacking the entire pathway (Δ*iscSUA-hscBA-fdx*), suggesting that these proteins are necessary for the biogenesis of [2Fe-2S]-IscR (Fig. 5). Strains lacking IscA had a smaller effect (~2-fold) on IscR activity (Fig. 5).Quantitative Western blots revealed that under aerobic growth conditions, levels of IscR are increased about 4- and 2-fold in Δ*iscU* and Δ*iscA* strains, respectively (data not shown), indicating that IscR protein was present but unable to fully repress P_{iscR}. Finally, we found that elimination of the Isc pathway or individual components showed a similar defect in P_{iscR} repression under both aerobic and anaerobic growth conditions (Fig. 5). Interestingly, these results contrast those of a previous

study that revealed that the [4Fe-4S] cluster-containing transcription factor FNR had a more stringent requirement for the Isc pathway under aerobic conditions than anaerobic conditions (Mettert *et al*., 2008).

Other Fe-S biogenesis proteins are not necessary for [2Fe-2S]-IscR activity when the Isc pathway is functional

We also examined whether other Fe-S biogenesis proteins contribute to IscR cluster assembly since a small amount of [2Fe-2S]-IscR activity remained in the strain lacking the Isc pathway (Fig. 5). Under either aerobic or anaerobic conditions, deletion of the *suf* operon had no effect on P_{iscR} repression (Fig. 5), indicating that the Suf pathway plays no apparent role in IscR function under nonstress conditions when the Isc system is present. In a strain lacking NfuA, an IscA/NifU-like protein that facilitates Fe-S cluster transfer (Bandyopadhyay *et al*., 2008, Angelini *et al*., 2008)and whose expression is repressed by IscR (Giel *et al*., 2006), there was a small defect (∼2-fold) in IscR repression, but only under anaerobic conditions (data not shown). In addition, P_{iscR} repression was not affected in strains lacking CsdA, a cysteine desulfurase (Trotter *et al*., 2009), or YtfE, a protein implicated in Fe-S cluster assembly or repair (Justino *et al*., 2009)(data not shown). Finally, deletion of the genes encoding IscX, PepB, and SseB, which are located downstream of *iscRSUA-hscBA-fdx* and are repressed by IscR (Giel *et al*., 2006), were also tested for their effects on IscR function, although only IscX has been suggested to have a possible function in Fe-S biogenesis (Pastore *et al*., 2006, Shimomura *et al*., 2005). Only the strain lacking IscX showed any defect, slightly less than a 2-fold effect under either aerobic or anaerobic conditions (Fig. 5). Thus, in the presence of the Isc pathway, other Fe-S assembly proteins do not have a major effect on IscR-dependent repression of P_{iscR}, thus supporting our model that the Isc pathway provides the [2Fe-2S] cluster required for IscR to fully repress P_{iscR} expression under aerobic and anaerobic conditions.

In the absence of the Isc machinery, elevated expression of the Suf pathway can fully restore [2Fe-2S]-IscR activity

Unlike regulation of P_{iscR}, regulation of the *suf* operon by IscR does not require IscR to ligate a [2Fe-2S] cluster (Nesbit *et al*., 2009, Yeo *et al*., 2006). Thus, we considered the possibility that upregulation of the Suf machinery by apo-IscR and subsequent transfer of a Suf-generated [2Fe-2S] cluster to IscR may explain the residual [2Fe-2S]-IscR-dependent activity measured in the Δ*iscSUA-hscBA-fdx* strain. To address whether IscR could serve as a substrate for the Suf machinery, P_{iscR} expression was measured in strains that contained the Δ*iscSUA-hscBA-fdx* or Δ*iscSUA-hscBA-fdx* Δ*sufABCDSE* alleles and a plasmid with the *suf* operon under control of P_{BAD}. As expected, the strain lacking both the Isc and the Suf pathways was viable only when the *suf* plasmid, not the vector, was present since deletion of both pathways is lethal (Py *et al*., 2011). Furthermore, when 10 mM arabinose was present in the growth medium to induce *suf* operon expression, strains containing the *suf* plasmid had P_{iscR} activity similar to that of the wild-type strain under aerobic and anaerobic growth conditions (Fig 6). This activity correlated with at least 30-fold higher levels of SufD protein compared to that of the wild-type strain as determined by Western blot analyses (data not shown). Thus, the Suf pathway, when expressed at elevated levels, appears to be able to

substitute for the Isc pathway by inserting a [2Fe-2S] cluster into IscR, and this may account for the residual [2Fe-2S]-IscR activity measured in the strain lacking the Isc pathway.

IscR [2Fe-2S] cluster occupancy is greater under anaerobic growth conditions

In the wild-type strain, repression of P_{iscR} was ∼3–5-fold greater under anaerobic conditions than aerobic conditions despite the fact that there is ∼10-fold less IscR protein under anaerobic conditions (Figs. 2, 3, and 5, Table 2). Since *in vitro* DNA binding by [2Fe-2S]-IscR is not sensitive to the O_2-dependent change in the [2Fe-2S] cluster oxidation state (Fleischhacker *et al*., 2012), a simple interpretation of our *in vivo* results is that the fraction of IscR containing a [2Fe-2S] cluster may be higher under anaerobic conditions. This, in turn, would lead to more P_{iscR} repression and less IscR protein. This notion was tested by measuring the levels of IscR protein required for P_{iscR} repression under both growth conditions. A strain containing chromosomally-encoded *iscR* and *iscSUA-hscBA-fdx* under control of P_{tac} and P_{BAD}, respectively, was grown in medium containing 20 mM arabinose and various concentrations of IPTG (0–640 μM). We subsequently measured P_{iscR}-*lacZ* repression which, according to our *in vivo* data, requires the [2Fe-2S] form of IscR, and the amount of IscR present at each IPTG concentration was quantified by Western blots. Under both growth conditions, the amount of P_{iscR} repression increased with increasing levels of IscR; however, more IscR protein was needed to fully repress P_{iscR} under aerobic conditions compared to anaerobic conditions (Fig S1). In fact, half-maximal P_{iscR} repression required ∼9-fold more IscR protein aerobically than anaerobically (955 ± 93 nM vs. 107 ± 14 nM monomeric IscR). Assuming that the concentration of [2Fe-2S]-IscR is equivalent to the IscR protein concentration required for 50% repression, our data indicate that under aerobic conditions IscR is at least 9-fold less occupied with [2Fe-2S] clusters than under anaerobic conditions. Thus, we conclude that the difference in the amount of P_{iscR} repression under aerobic and anaerobic conditions can be explained by O_2-dependent changes in the cluster occupancy of IscR.

The *iscR* promoter is derepressed in response to increased Fe-S demand

Because the Isc requirement for biogenesis of the [4Fe-4S] cluster of FNR is greater under aerobic than anaerobic conditions (Mettert *et al*., 2008), we considered the possibility that acquisition of the [2Fe-2S] cluster by IscR is reduced under aerobic conditions because of the competition with other substrates for the Isc machinery. Such a competition mechanism could explain how IscR senses the cellular Fe-S demands and correspondingly adjusts expression of the Isc pathway. If IscR senses the cellular Fe-S status through its [2Fe-2S] cluster, then conditions that increase the demand for Fe-S clusters are predicted to affect [2Fe-2S]-IscR activity. This notion was tested by measuring P_{iscR}-*lacZ* activity upon overexpression of the [4Fe-4S] protein FNR from P_{tac}. Since FNR does not regulate P_{iscR} (Kang *et al*., 2005, Giel, 2007), we expected that changes in *iscR* transcription may result from the competition between FNR and IscR for the Isc machinery. Furthermore, because the [4Fe-4S] cluster of FNR is not stable under aerobic conditions (Khoroshilova *et al*., 1997), we also overexpressed the mutant protein FNR-L28H, whose [4Fe-4S] cluster is more resistant to destruction by oxygen (Bates *et al*., 2000). Under aerobic conditions, P_{iscR} was derepressed within 20 minutes of addition of IPTG to a final concentration of 100 μM to the strain containing either the P_{tac}-*fnr* or the P_{tac}-*fnr*-L28H expression plasmid but not the

vector control (Fig. 7A). Furthermore, no derepression was observed for the P_{tac}-*fnr* strain when no IPTG was added (data not shown). Thus, under aerobic conditions, an increase in the Fe-S cluster demand via overexpression of FNR relieves repression of the *isc* operon, presumably due to the competition between FNR and IscR for acquiring Fe-S clusters from the Isc machinery.

In contrast, derepression of P_{iscR} was not observed when FNR was overexpressed under anaerobic conditions (Fig. 7B), despite the accumulation of FNR protein to similar levels as under aerobic conditions (data not shown). This suggests that the Isc machinery can meet an increase in Fe-S demand under anaerobic conditions such that no competition between FNR and IscR was detected. Thus, the Isc pathway seems to be more buffered against changes in Fe-S demand under anaerobic conditions, likely due to less general cluster turnover under these conditions. In summary, this apparent difference in the cellular Fe-S demand between aerobic and anaerobic conditions highlights the significance of negative feedback regulation of the *isc* operon.

DISCUSSION

The findings of this study have revealed new mechanistic insight as to how IscR regulates transcription of P_{iscR} to maintain Fe-S cluster homeostasis in *E. coli*. We have defined the requirement for the two Type 1 IscR binding sites within P_{iscR} for full repression of the *isc* operon and have shown that these sites are preferentially bound by the [2Fe-2S] form of IscR. Examination of P_{iscR} repression under aerobic and anaerobic growth has revealed that the increased repression of *isc* under anaerobic conditions is due to increased IscR [2Fe-2S] cluster occupancy under these conditions. Furthermore, decreased *isc* repression under aerobic conditions suggests that there may be more competition between IscR and substrate proteins for the Isc machinery when O_2 is present. Consistent with this notion, overexpression of an Fe-S protein under aerobic conditions, but not under anaerobic conditions, led to derepression of P_{iscR}. In particular, our results shed new insight on how IscR responds to the presence of O_2 to satisfy the cellular need for Fe-S cluster biogenesis.

IscR binding sites A and B are critical for IscR-mediated repression of P_{iscR}

Of the three IscR binding sites present within P_{iscR} (Giel *et al*., 2006), we found that only the two adjacent Type 1 IscR binding sites, A and B, are responsible for P_{iscR} repression *in vivo* under standard growth conditions. The requirement for the [2Fe-2S] form of IscR for repression also correlated well with the increased affinity of [2Fe-2S]-IscR for either site A or B. Nevertheless, the *in vivo* concentration of the clusterless mutant IscR-C92A/C98A/C104A is sufficiently high (≥17 μM; Table 2) such that some binding of the mutant might be expected even though its binding characteristics *in vitro* were poor. Perhaps an additional mechanism exists *in vivo* to prevent repression of P_{iscR} by IscR apo-protein. Because apo-IscR binds to many locations on the *E. coli* chromosome (Myers and Kiley, unpublished), one possibility is that apo-IscR is bound at other higher affinity sites across the genome. Thus, like many transcription factors, regulation of promoters by IscR is likely dependent on a hierarchy of binding affinities.

Since both IscR sites A and B span the region that RNA polymerase binds, the mechanism of IscR repression can be simply explained by promoter occlusion. Indeed, DNase I footprinting experiments at wild-type P_{iscR} showed that addition of IscR at the same time as RNAP resulted in a protection pattern virtually identical to the one for IscR alone (Giel, 2007), indicating that binding of IscR to P_{iscR} occludes RNAP. Since most IscR-regulated promoters contain just a single IscR binding site (Giel *et al*., 2006), perhaps the presence of multiple IscR binding sites within the P_{iscR} region may be important to extend the dynamic range to regulate Fe-S biogenesis *in vivo*. While our footprinting experiments indicate that [2Fe-2S]-IscR could bind independently to these two sites, the use of linear DNA in these experiments does not rule out the possibility that some protein-protein interactions occur when present on supercoiled chromosomal DNA.

The Isc pathway is the major system for IscR [2Fe-2S] cluster biogenesis

Deletion of the *iscSUA-hscBA-fdx* operon resulted in decreased P_{iscR} repression, suggesting that IscR primarily receives its [2Fe-2S] cluster from the Isc pathway. When the Isc pathway was absent, however, a small amount of [2Fe-2S]-IscR activity remained, suggesting that IscR could acquire [2Fe-2S] clusters from another source. Similar results were also observed for the transcription factor FNR and the enzymes isopropylmalate isomerase, NADH dehydrogenase I, and IspG/H, which all require Fe-S clusters for function (Mettert *et al*., 2008, Jang and Imlay, 2010, Vinella *et al*., 2009). Since apo-IscR is sufficient to activate P_{sufA} transcription (Nesbit *et al*., 2009, Yeo *et al*., 2006), we propose that in the Δ*iscSUA-hscBA-fdx* strain, upregulation of the Suf pathway by apo-IscR may explain the residual [2Fe-2S]-IscR activity. Consistent with this notion, *sufA* promoter expression and SufD protein levels were elevated 2- to 3-fold in a mutant strain lacking the Isc pathway compared to the wild-type strain (Mettert *et al*., 2008). Furthermore, our studies indicate that IscR can in fact serve as a substrate for the Suf machinery in the Δ*iscSUA-hscBA-fdx* mutant when the *suf* operon is overexpressed. Since the Isc system has been shown to be present but non-functional during H_2O_2 stress (Jang and Imlay, 2010), the ability of an Isc substrate protein to also receive Fe-S clusters from the Suf pathway would be particularly advantageous for *E. coli* under conditions of oxidative stress.

IscR senses and responds to the different Fe-S demands that exist between aerobic and anaerobic growth conditions

Our finding that IscR is more sensitive to changes in Fe-S demand under aerobic conditions makes physiological sense. Considering that greater than 150 Fe-S proteins exist in *E. coli* (Py *et al*., 2011) and that some Fe-S clusters are sensitive to O_2 and/or reactive oxygen species (Imlay, 2006, Imlay, 2008), it is likely that Fe-S clusters are continually being damaged or destroyed during aerobic growth, thereby increasing the levels of substrate proteins that need Fe-S biogenesis or repair. As a result, there would be more competition between IscR and substrate proteins for the Isc machinery under aerobic conditions than anaerobic conditions. In support of this notion, we showed that aerobic but not anaerobic overexpression of either wild-type [4Fe-4S]-FNR or the [4Fe-4S]-FNR-L28H mutant derivative that contains an O_2-resistant cluster (Bates *et al*., 2000) relieved P_{iscR} repression. Why IscR may not compete as well with other substrate proteins is not known but may be related to the fact that IscR binds its [2Fe-2S] cluster with an atypical ligation scheme of

three cysteines and one histidine (Fleischhacker *et al*., 2012) perhaps making it a poor substrate for the Isc machinery.

In sum, we propose that under aerobic conditions, the sensitivity of some Fe-S clusters to O_2 and/or reactive oxygen species results in a higher rate of cluster turnover, which in turn leads to increased synthesis of the *isc* operon to meet the demands for Fe-S biogenesis. In contrast, under anaerobic conditions, the Isc machinery appears to satisfy the Fe-S demand more efficiently due to decreased general cluster turnover, and thus less competition among substrate proteins, under these conditions (Fig. 8). However, we cannot rule out the possibility that additional mechanisms may also contribute to regulating Isc Fe-S cluster biogenesis under anaerobic growth. Taken together, the different needs for Isc Fe-S biogenesis under aerobic and anaerobic conditions emphasize the important role of IscR as an Fe-S sensor, enabling *E. coli* to respond appropriately to environments of varying O_2 tension.

EXPERIMENTAL PROCEDURES

Bacterial strain and plasmid construction

Strains and plasmids described in this work are listed in Table 1 and sequences of primers used are available upon request. The λ-P_{iscR}-*lacZ* reporter fusion (−476 to +74 bp relative to the IscR start codon) (Schwartz *et al*., 2001) is located within the λ*att* site, whereas the P_{iscR}-*lacZ* reporter fusion (−428 to +144 bp relative to the IscR start codon) replaces the wildtype *lac* promoter and was constructed by a method previously described (Giel *et al*., 2006, Kang *et al*., 2005). In the latter case, a *lacI-kan*-P_{iscR}-*lacZ* fragment from pPK8508 was PCR-amplified and recombined onto the chromosome in the *lac* promoter region of BW25993/pKD46. P_{iscR}-*lacZ* fusions containing mutations within P_{iscR} were recombined onto the chromosome in the same manner after introducing mutations into pPK8508 using QuikChange (Stratagene). The resulting *kan*-promoter-*lacZ* constructs were transduced into the appropriate strain backgrounds using P1*vir* and confirmed by colony PCR and DNA sequencing.

In frame deletions of *iscR*, *iscSUA*, *iscSUA-hscBA-fdx*, *iscS*, *iscU*, *iscA*, *iscX*, *pepB*, *csdA*, and *lacY* were constructed by replacing the coding region(s) with a Cm^R or Kan^R cassette flanked by FLP recognition target (FRT) sites from plasmid pKD32 or pKD13, respectively, as described previously (Datsenko and Wanner, 2000). Transduction with P1 *vir* was used to move the *cat* or *kan* allele to the appropriate strain backgrounds. In some cases, the Cm^R or Kan^R cassette was removed by transforming strains with pCP20, encoding FLP recombinase (Datsenko and Wanner, 2000) and by screening for Cm or Kan sensitivity. All gene deletions were confirmed by colony PCR. Previously constructed alleles encoding IscR mutants in which cysteine residues were substituted with alanine (Nesbit *et al*., 2009)were transduced into PK6364 using P1 *vir* and selecting for Kan^R. The Kan^R cassette was subsequently removed by transforming strains with pCP20. The following existing alleles were also transduced into appropriate strain backgrounds using P1*vir*: :*sseB* with a Tn5KAN2 insertion at position 150, *fdx*: :*kan*, Δ*sufABCDSE*: :*kan*, and Δ*ytfE*: :*kan*.

Construction of *bla*-P_{tac}-*iscR*-*cat*-*araC*-P_{BAD}-*iscSUA* on the chromosome of strain PK9520 was performed in several steps. First, a MG1655 recombineering strain (PK9120) was constructed by transducing *nadA*::*Tn10* [λ*cI857*Δ(*cro*-*bioA*)] from DY329 into PK8039 using P1 *vir* and selecting for Tet^R at 30°C to form PK9110. The *nadA*::*Tn10* was removed by transducing to nicotinic acid prototrophy using P1 *vir* MG1655 and screening for Tet^S at 30°C to make PK9116. Colonies retaining the biotin requirement were then transduced with P1 *vir mutS104*::*mini-Tn10* from CSH115 to form PK9120. Next, FRT-*cat*-FRT was PCR-amplified from pKD32 and cloned into the SphI site of pBAD/Myc-hisC to form pPK9125. FRT-*cat*-FRT-*araC*-P_{BAD} was amplified and inserted between *iscR* and *iscS* in pPK9112, using the EcoRI site 6 nt after the *iscR* stop codon to form pPK9127. To obtain FRT-*cat*-FRT-*araC*-P_{BAD} upstream of *iscS* on the chromosome, this construct was amplified with primers containing ends with homology to this region of the chromosome, electroporated into PK9120, and selected for Cm^R. The *bla*-P_{tac} region from pPK9003 was amplified and electroporated into the above PK9120 derivative (PK9133) to replace −100 to +28 bp of P_{iscR} (relative to the +1 transcription start site) with *bla*-P_{tac}, forming PK9520. After verification by DNA sequencing, *bla*-P_{tac}-*iscR*-FRT-*cat*-FRT-*araC*-P_{BAD}-*iscS* was transduced into PK8151 using P1 *vir* and selected for Cm^R and Ap^R with 10 mM arabinose to derepress P_{BAD}-*iscSUA*. Finally, the strain was transformed with pACYC$lacI^Q$ to form PK9523.

Wild-type *fnr* and *fnr*-L28H were cloned into pDHB60 by digesting pPK823 or pPK1868, respectively, with HindIII and XbaI, followed by ligation of these fragments into the HindIII and XbaI sites of pDHB60. PK6364 was transformed with pACYC$lacI^Q$-CAM, and the resulting strain was transformed with pDHB60, pPK7332 (*fnr*), or pPK7307 (*fnr*-L28H).

β-galactosidase assays

Strains were grown aerobically by shaking at 250 rpm to an OD_{600} ∼0.2 or anaerobically in screw-capped tubes to an OD_{600} ∼0.1 at 37° C in MOPS minimal medium (Neidhardt *et al*., 1974) supplemented with 0.2% glucose (w/v) or M9 minimal medium (Miller, 1972) containing 0.2% glucose (w/v), 1mM $MgSO_4$, 2.5 μg ml^{-1} ferric ammonium citrate, 2 μg ml^{-1} thiamine, and 0.02% ammonium molybdate (w/v). As indicated in the figure legends, media were also supplemented with 12.5 μg ml^{-1} nicotinic acid and 2 μg ml^{-1} thiamine for some experiments. For the FNR overexpression experiment, strains were grown under aerobic or anaerobic conditions by sparging (Sutton and Kiley, 2003). For all β-galactosidase assays, Cm or Tet were added to culture samples at final concentrations of 20 or 10 μg ml^{-1}, respectively, to terminate cell growth and any further protein synthesis; cells were placed on ice until assayed for β-galactosidase activity as previously described (Miller, 1972). Because of aerobic and anaerobic cell count differences, the Miller units from aerobically grown strains were multiplied by 1.55, similar to previous studies (Mettert and Kiley, 2007). Assays were repeated at least three independent times and the standard errors for data plotted as “Fold Repression” were calculated using a propagation of standard error formula (Ku, 1966).

For the FNR overexpression experiment, strains were grown in M9 minimal medium (described in the above paragragh) containing 50 μg ml^{-1} Ap, and 20 μg ml^{-1} Cm under

aerobic or anaerobic conditions by sparging (Sutton and Kiley, 2003). FNR expression was induced by adding a final concentration of 100 μM IPTG at OD_{600} of 0.2. Samples were removed at various time points and processed as above, and β-galactosidase assays were performed in triplicate for each time point. The experiment was repeated on at least two separate occasions.

Western blot analysis

Strains were grown as for β-galactosidase assays, and IscR, SufD, or FNR levels were measured by Western blots as described previously (Nesbit *et al*., 2009, Mettert *et al*., 2008, Sutton *et al*., 2004). IscR was quantified using isolated proteins as standards, followed by imaging as described (Nesbit *et al*., 2009, Mettert *et al*., 2008). The cytoplasmic concentration of IscR (μM) was calculated using the molecular weight determined for monomeric IscR (17,336 Da) (Schwartz *et al*., 2001), the number of cells per ml of culture sampled as previously determined via viable cell counts (Mettert *et al*., 2008), and the estimated cell volume of $\sim 1 \times 10^{-15}$ L (Kubitschek, 1990).

Protein purification

Purifications of [2Fe-2S] cluster-containing wild-type IscR (Giel *et al*., 2006) and IscR-C92A/C98A/C104A (Nesbit *et al*., 2009) were performed and the protein concentration and iron and sulfide content were determined as previously described (Beinert, 1983, Kennedy *et al*., 1984, Khoroshilova *et al*., 1995). For all *in vitro* experiments, wild-type IscR was ≥50% occupied with [2Fe-2S] clusters. For simplicity, all protein concentrations herein are reported as monomers, although IscR is mainly a dimer in solution (Nesbit *et al*., 2009).

DNase I footprinting assays

Mutations within P_{iscR} were introduced into pPK6511 using QuikChange (Stratagene), generating pPK8515, pPK6806, and pPK8547, and DNA fragments containing the wild-type or mutated *iscR* promoter region were isolated from their respective plasmids after digestion with XbaI and HindIII. Klenow fragment (NEB) and [α-^{32}P]dCTP were used to label the XbaI end of the fragment (comprised of bases −161 to +38 relative to the P_{iscR} transcription start site). Assays were performed as described (Giel *et al*., 2006) except the incubation time of protein with DNA prior to the addition of DNase I was 10 minutes.

Fluorescence anisotropy

DNA binding isotherms were generated under anaerobic conditions by measuring changes in fluorescence polarization when IscR bound dsDNA as described (Nesbit *et al*., 2009), and assays were repeated on three independent occasions. Briefly, 30-mer dsDNA contained the following Type 1 sites (underlined): P_{iscR} site A (5'-AAATACCCGACTAAATCAGTCAAGTAAATA-3') or P_{iscR} site B (5'-AAATAGTTGACCAATTTACTCGGGAATGTC-3'). [2Fe-2S]-IscR (20 to 718 nM) or IscR-C92A/C98A/C104A (35 to 1000 nM) was incubated with 5 nM Texas Red labeled-DNA, 40 mM Tris-Cl (pH 7.9), and 150 mM KCl for 10 min at room temperature under anaerobic conditions. For wild-type IscR, the fraction bound was determined as previously described (Nesbit *et al*., 2009).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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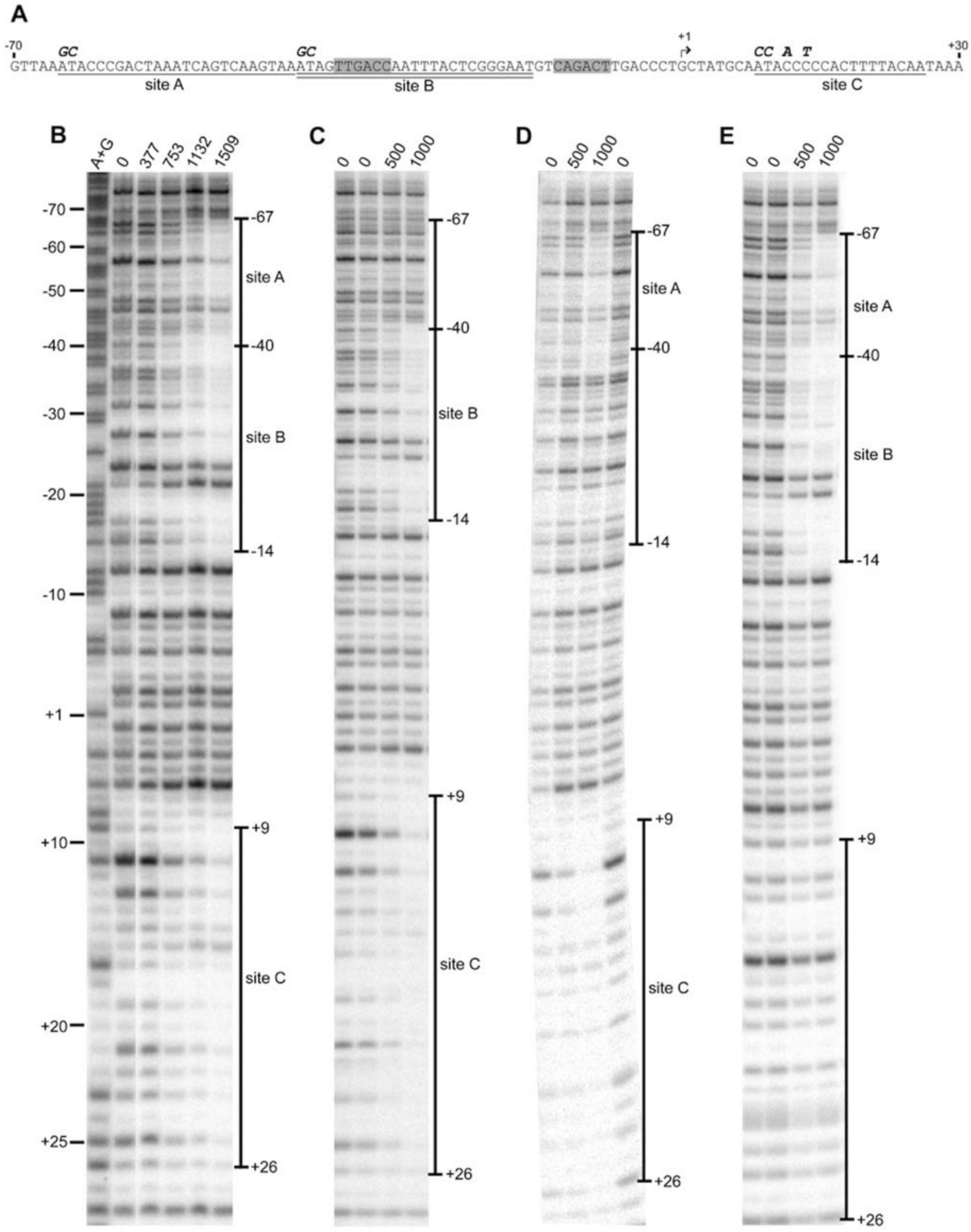

Fig. 1.

In vitro binding of anaerobically purified [2Fe-2S]-IscR to wild-type and mutated IscR binding sites within P_{iscR}. **A)** The IscR binding sites A (underlined), B (double underlined), C (underlined), the transcriptional start site (bent arrow), the −35 and −10 promoter elements (shaded),and the bases substituted in this study (bold italics) are indicated. DNase I footprints of IscR bound to DNA fragments containing the wild-type P_{iscR} region (panel **B**) or promoter regions with mutations in sites A, B, or C (panels **C, D**, and **E**, respectively). The amount of IscR protein (nM) present in each reaction and the extent of the footprint

relative to the +1 transcription start site are denoted. Samples were electrophoresed with Maxam-Gilbert (A + G) ladders. The lane order in panel B was digitally rearranged for presentation purposes.

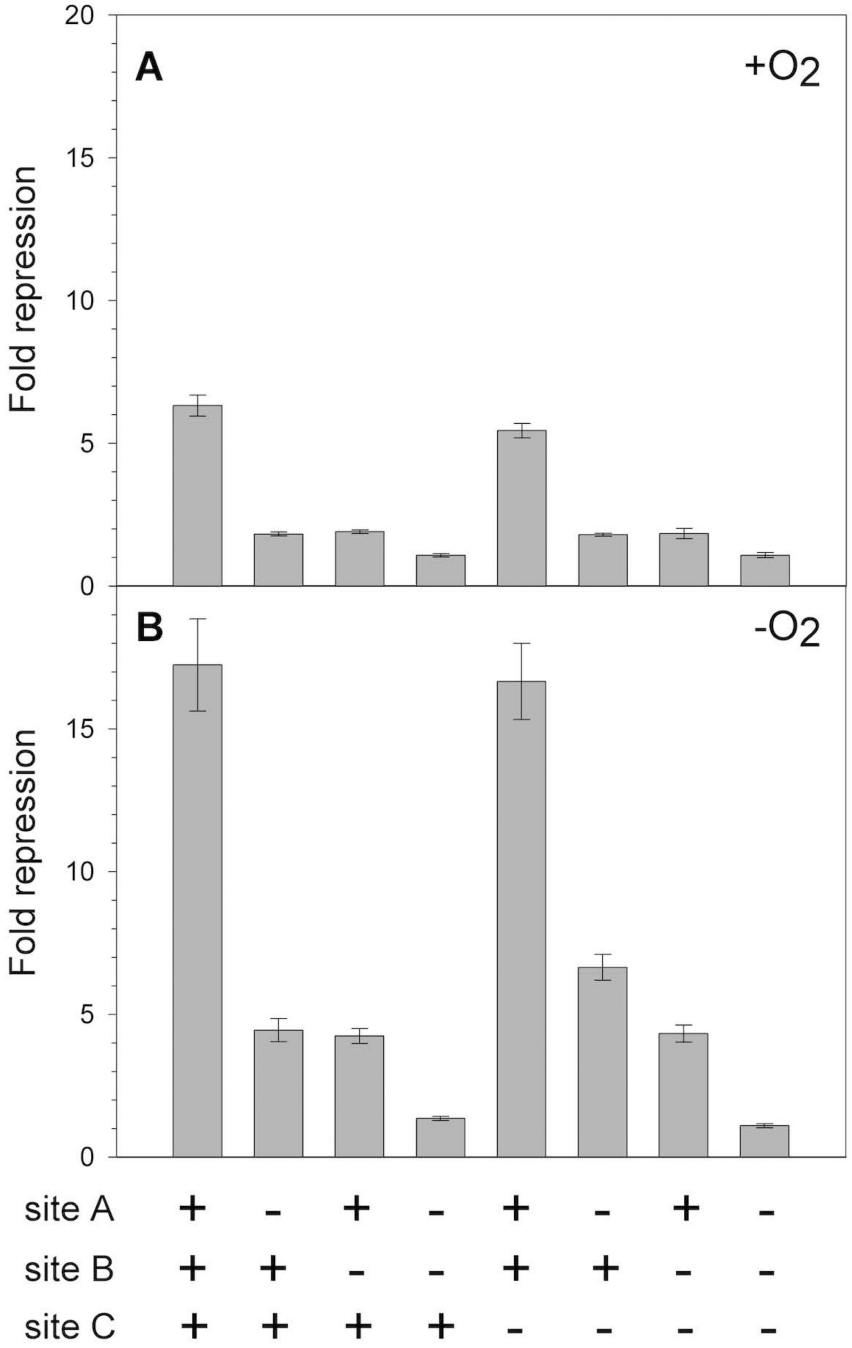

Fig. 2.

In vivo effects of mutations in IscR binding sites A, B, and C within P_{iscR}. β-galactosidase activity from wild-type (PK7571) or mutated (PK8521, PK8528, PK8582, PK8551, PK8801, PK8808, PK8820) P_{iscR}-*lacZ* fusions (recombined at the chromosomal *lac* region) was measured in cells grown under **A)** aerobic or **B)** anaerobic conditions in MOPS minimal media with glucose (0.2%). The amount of IscR-dependent repression (Fold repression) was determined by dividing the β-galactosidase activity present in the strain lacking IscR (PK7572) by the β-galactosidase activity measured for each strain. The presence (+) or

absence (−) of wild-type IscR binding sites A, B, and C in the P_{iscR}-*lacZ* fusion is indicated below the figure. Error bars represent the propagation of standard errors for three biological replicates.

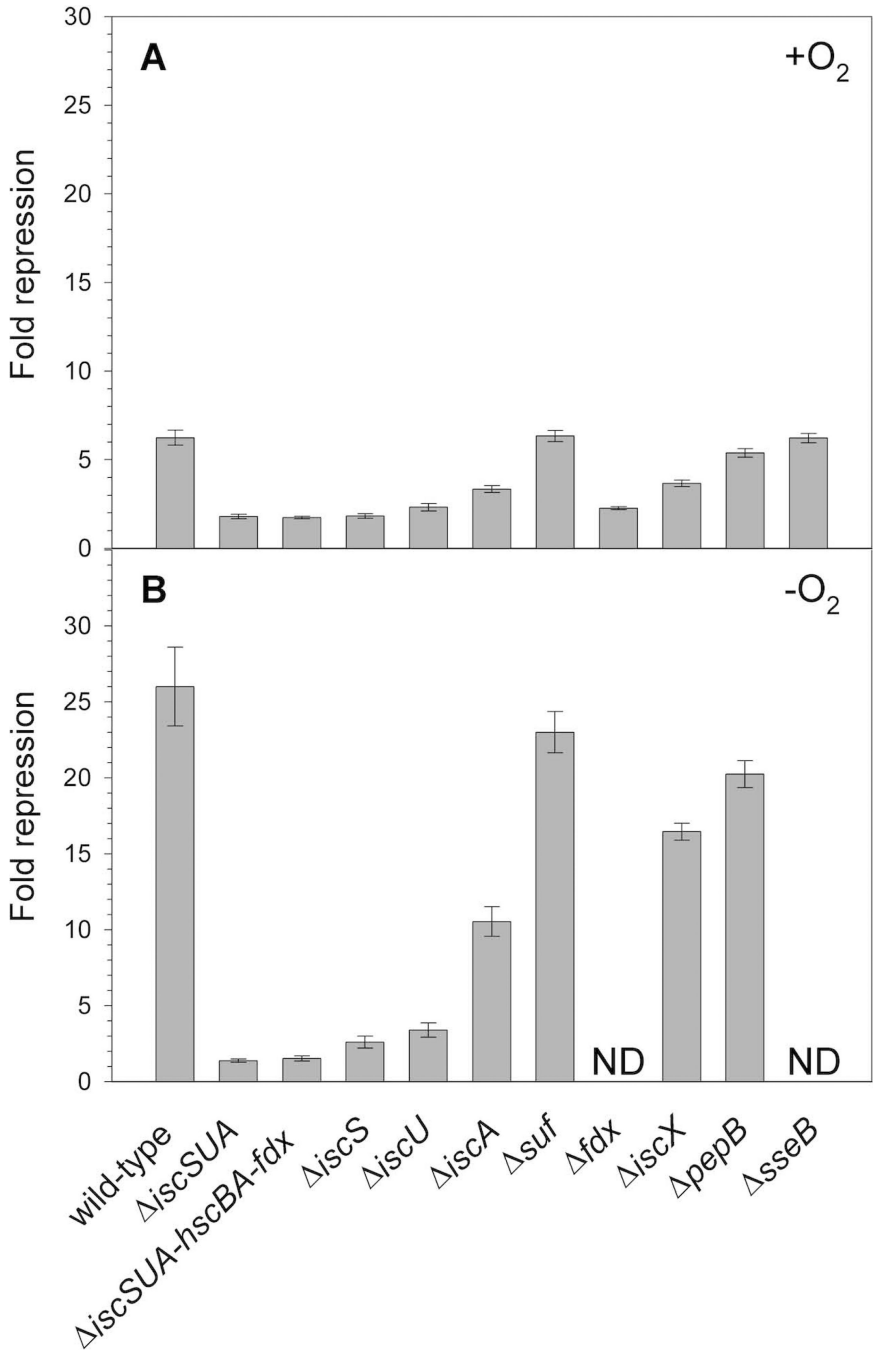

Fig. 3.

β-galactosidase activity from P_{iscR} fused to *lacZ* integrated at the λ*att* site was determined in strains containing wild-type IscR (PK6364) or chromosomal mutants defective in [2Fe-2S] cluster binding [IscR-C92A (PK7854), IscR-C98A (PK7855), IscR-C104A (PK7856), and IscR-C92A/C98A/C104A (PK7898)]. Strains were grown under **A)** aerobic or **B)** anaerobic conditions in MOPS minimal media containing glucose (0.2%). Fold repression was determined by dividing the β-galactosidase activity present in the strain lacking IscR

(PK6512) by the β-galactosidase activity measured for each strain and error bars represent the propagation of standard errors for three biological replicates.

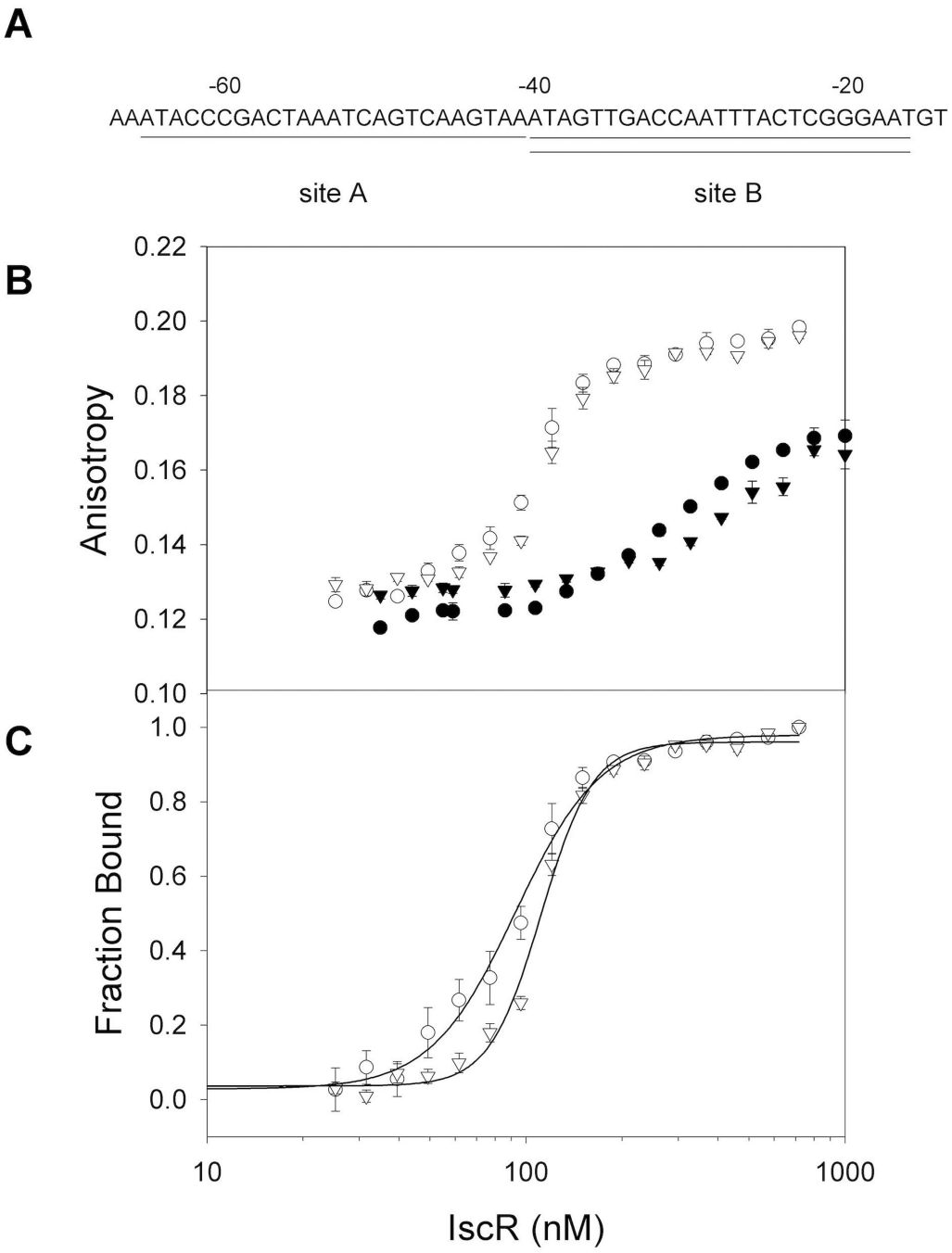

Fig. 4.

Binding isotherms of [2Fe-2S]-IscR and apo-IscR for the two Type 1 sites within the P_{iscR} region. **A)** Sequences of IscR binding sites A (underlined) and B (double underlined) within P_{iscR}. Numbers indicate the distance relative to the +1 transcription start site. **B)** DNA binding isotherms of wild-type [2Fe-2S]-IscR (open symbols) and the clusterless mutant protein IscR-C92A/C98A/C104A (closed symbols) measured as a change in anisotropy under anaerobic conditions. Both forms of IscR protein were incubated with 5 nM fluorescently labeled DNA containing either site A (circles) or site B (triangles) in 40 mM

Tris-Cl (pH 7.9) and 150 mM KCl. **C)** Fraction bound corrected for fluorescence quenching of labeled P_{iscR} sites A or B bound by [2Fe-2S]-IscR as a function of protein concentration. Error bars represent the standard errors of triplicate experiments.

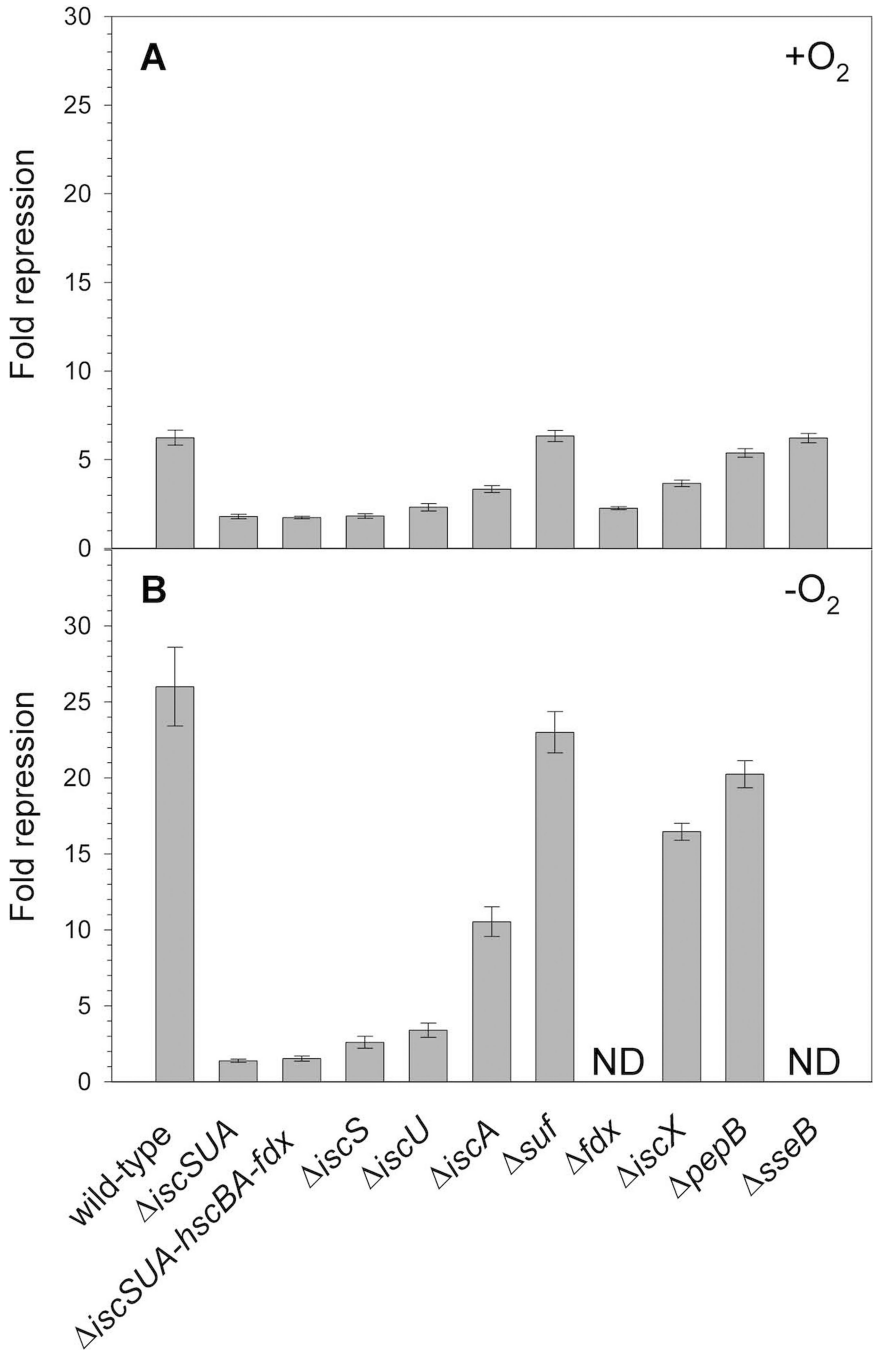

Fig. 5.

β-galactosidase activity was measured in wild-type (PK6364) or mutant (PK8120, PK8122, PK7783, PK6828, PK7759, PK6564, PK6826, PK6848, PK7540, PK7751) strains containing P_{iscR} fused to *lacZ* integrated at the λ*att* site. Cultures were grown under **A)** aerobic or **B)** anaerobic conditions in MOPS minimal media supplemented with glucose (0.2%); for the wild-type, Δ*iscSUA*, Δ*iscSUA-hscBA-fdx*, Δ*iscS*, Δ*iscU*, Δ*iscA* strains, media were also supplemented with nicotinic acid (12.5 μg ml^{-1}), and thiamine (2 μg ml^{-1}). Fold repression was determined by dividing the β-galactosidase activity present in the strain

lacking IscR (PK6512), grown in the presence or absence of nicotinic acid and thiamine, by the β-galactosidase activity measured for each strain and error bars represent the propagation of standard errors for three biological replicates. ND, not determined.

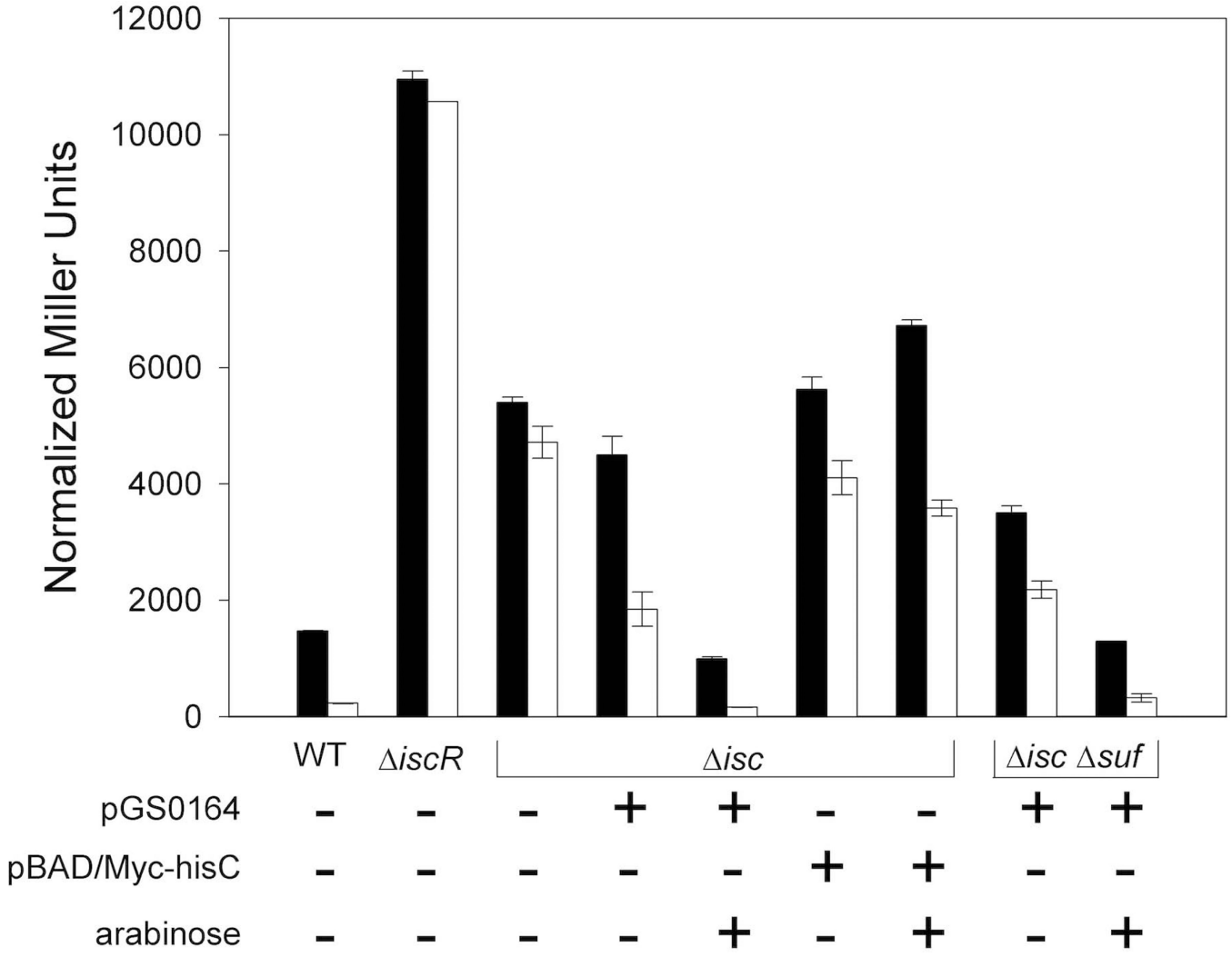

Fig. 6.

β-galactosidase activity from λ-P_{iscR}-*lacZ* was determined in aerobically (black) or anaerobically (white) grown wild-type (PK6364), Δ*iscSUA-hscBA-fdx* [Δ*isc* (PK8122, PK8614, PK8615)], and Δ*iscSUA-hscBA-fdx*Δ*sufABCDSE* [Δ*isc*Δ*suf* (PK8618)] strains. The Δ*iscSUA-hscBA-fdx* strain derivatives contained either the plasmid expressing the *sufABCDSE* operon from P_{BAD} (pGS0164), the vector alone (pBAD/Myc-hisC), or no vector. All cultures were grown in M9 minimal media containing glucose (0.2%), nicotinic acid (12.5 μg ml^{-1}), and thiamine (2 μg ml^{-1}). The plasmid-containing strains were also grown in the presence of Ap (50 μg ml^{-1}) and where indicated, arabinose (0.2%). Error bars represent the standard errors of three biological replicates.

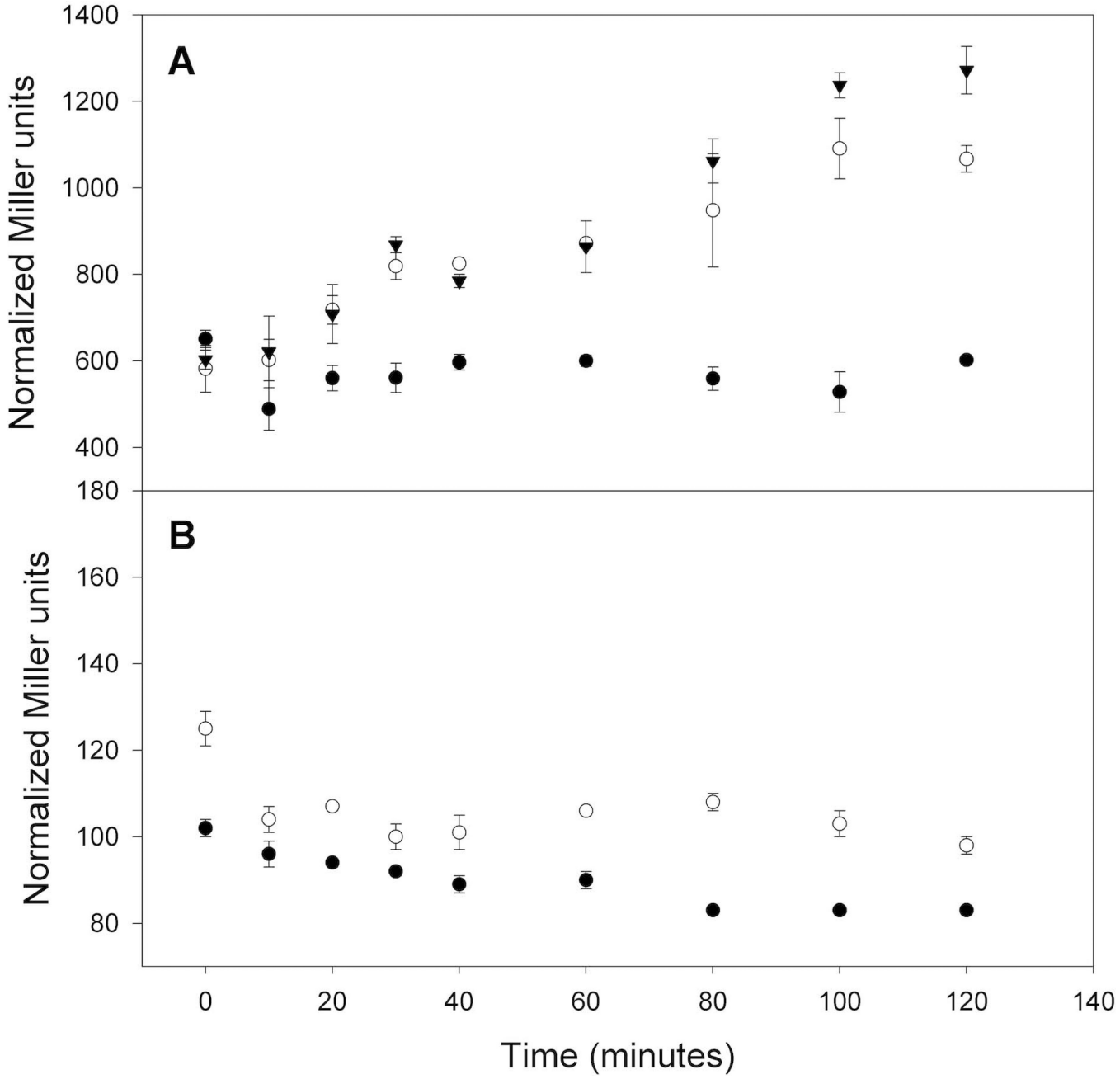

Fig. 7.

Activity from P_{iscR}-*lacZ* integrated at the λ*att* site was measured in strains (PK7329, PK7333, PK7328) containing plasmids with IPTG-inducible wild-type *fnr* (open circles), IPTG-inducible *fnr*-L28H (triangles) or the vector control (closed circles). Strains were grown under **A)** aerobic or **B)** anaerobic conditions in M9 minimal media containing glucose (0.2%), Ap (50 μg ml^{-1}), and Cm (20 μg ml^{-1}). When cells reached an OD_{600} of 0.2 (0 minutes), IPTG was added to a final concentration of 100 μM, samples were taken at various time points, and β-galactosidase activity (Miller units) was assayed and normalized for cell

number as explained in Experimental Procedures. Error bars represent the standard errors of three biological replicates.

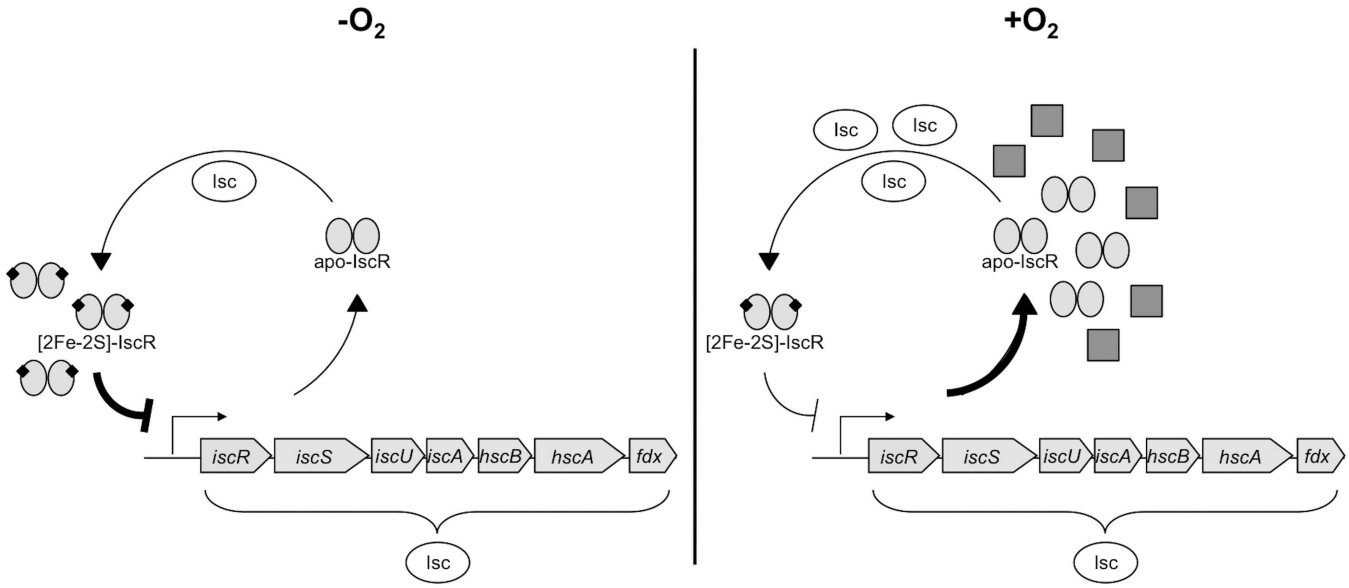

Fig. 8.

A model for the differential demand in Isc-mediated Fe-S cluster biogenesis between aerobic and anaerobic growth conditions is shown. In the presence of O_2, the need for Fe-S clusters is predicted to be high due to increased rates of general Fe-S cluster turnover under aerobic conditions, and thus elevated levels of apoprotein substrates (gray squares). As a result, there is competition between these substrates and IscR in acquiring Fe-S clusters from the Isc pathway, resulting in low IscR [2Fe-2S] cluster occupancy and thus less repression of *iscR-SUA-hscBA-fdx*. Under anaerobic conditions, the general rate of cluster turnover is decreased compared to aerobic conditions. The low demand for Fe-S biogenesis in the absence of O_2 leads to increased IscR [2Fe-2S] cluster occupancy, and in turn, more repression of the Isc pathway.

Table 1

Strains and plasmids used in this work.

Strain	Relevant Genotype or Phenotype	Source
MG1655	λ^- F^- *rph*-1	Laboratory stock
PK8039	MG1655 *yhgI-lacZ*	(Giel *et al*., 2006)
PK9110	PK8039 *nadA*::*Tn10*λ*cI857* Δ(*cro-bioA*)	This study
PK9116	PK9110 but *nadA*$^+$, Tet^S	This study
PK9120	PK9116 *mutS104*::*mini-Tn10*	This study
PK9133	PK9120 *cat*-FRT-*araC*-P_{BAD}	This study
PK9520	PK9133 *bla*-P_{tac} replacing −100 to +28 bp of P_{iscR} relative to the +1 transcription start site	This study
PK6364	MG1655 λ-*iscR'-lacZ*	(Schwartz *et al*., 2001)
PK6512	PK6364 Δ*iscR*	This study
PK8120	PK6364 Δ*iscSUA*::*cat*	This study
PK8122	PK6364 Δ*iscSUA-hscBA-fdx*::*cat*	This study
PK8614	PK8122 + pGS0164	This study
PK8618	PK8614 Δ*sufABCDSE*::*kan*	This study
PK8615	PK8122 + pBAD/Myc-hisC	This study
PK7783	PK6364 Δ*iscS*	This study
PK6828	PK6364 Δ*iscU*	This study
PK7759	PK6364 Δ*iscA*	This study
PK6564	PK6364 Δ*sufABCDSE*::*kan*	This study
PK6826	PK6364 *fdx*::*kan*	This study
PK6848	PK6364 Δ*iscX*	This study
PK7540	PK6364 Δ*pepB*	This study
PK7751	PK6364 *sseB*::Tn5KAN2	This study
PK7854	PK6364 *iscR*-C92A *zfh-3601*::FRT	This study
PK7855	PK6364 *iscR*-C98A *zfh-3601*::FRT	This study
PK7856	PK6364 *iscR*-C104 *zfh-3601*::FRT	This study
PK7898	PK6364 *iscR*-C92A/C98A/C104A *zfh-3601*::FRT	This study
PK8616	PK6364 Δ*csdA*::*cat*	This study
PK8617	PK6364 Δ*ytfE*::*kan*	This study
PK7325	PK6364 + pACYC*lacI*^{Q}-CAM	This study
PK7329	PK6364 + pACYC*lacI*^{Q}-CAM + pDHB60	This study
PK7333	PK6364 + pACYC*lacI*^{Q}-CAM + pPK7332	This study
PK7328	PK6364 + pACYC*lacI*^{Q}-CAM + pPK7307	This study
PK7571	MG1655 P_{iscR}-*lacZ* (in *lac* region)	This study
PK8151	PK7571 Δ*lacY*	This study
PK9523	PK8151 *bla*-P_{tac}-*iscR*-FRT-*cat*-FRT-*araC*-P_{BAD}-*iscS* + pACYC*lacI*^{Q}	This study
PK8677	PK8151 Δ*iscR* + pACYC*lacI*^{Q}	This study
PK8521	MG1655 P_{iscR} (site A mutated)-*lacZ*	This study

Strain	Relevant Genotype or Phenotype	Source
PK8528	MG1655 P_{iscR} (site B mutated)-*lacZ*	This study
PK8582	MG1655 P_{iscR} (sites A & B mutated)-*lacZ*	This study
PK8551	MG1655 P_{iscR} (site C mutated)-*lacZ*	This study
PK8801	MG1655 P_{iscR} (sites A & C mutated)-*lacZ*	This study
PK8808	MG1655 P_{iscR} (sites B & C mutated)-*lacZ*	This study
PK8820	MG1655 P_{iscR} (sites A, B, & C mutated)-*lacZ*	This study
PK4854	MG1655 Δ*iscR*	(Schwartz *et al*., 2001)
PK7572	PK4854 P_{iscR}-*lacZ*	This study
PK8523	PK4854 P_{iscR} (site A mutated)-*lacZ*	This study
PK8530	PK4854 P_{iscR} (site B mutated)-*lacZ*	This study
PK8584	PK4854 P_{iscR} (sites A & B mutated)-*lacZ*	This study
PK8553	PK4854 P_{iscR} (site C mutated)-*lacZ*	This study
PK8803	PK4854 P_{iscR} (sites A & C mutated)-*lacZ*	This study
PK8810	PK4854 P_{iscR} (sites B & C mutated)-*lacZ*	This study
PK8822	PK4854 P_{iscR} (sites A, B, & C mutated)-*lacZ*	This study
PK7599	BL21 *himA*: :*tet* + pPK6161	(Nesbit *et al*., 2009)
PK7881	BL21 *himA*: :*tet*Δ*iscR*: :*kan* + pPK7862	(Nesbit *et al*., 2009)
DY329	W3110 Δ*lacU169nadA*: :*Tn10gal490 λcI857* Δ(*cro-bioA*)	(Yu *et al*., 2000)
CSH115	F-, *ara-600*, Δ*(gpt-lac)5* λ^-, *mutS104*: :*mini-Tn10 relA1?, spoT1?, thi-1*	The Coli Genetic Stock Center (Yale)
BW25993	$lacI^q hsdR514 \Delta araBAD_{AH33} \Delta rhaBAD_{LD78}$	(Datsenko and Wanner, 2000)
Plasmid		
pPK6161	*iscR* cloned into pET-11a	(Schwartz *et al*., 2001)
pPK7862	*iscR*-C92A/C98A/C104A cloned into pET-11a	(Nesbit *et al*., 2009)
pPK823	*fnr* cloned into pET-11a	(Lazazzera *et al*., 1993)
pPK1868	*fnr*-L28H cloned into pET-11a	(Bates *et al*., 2000)
pDHB60	Ap^R, P_{tac} (pBR322-derived)	(Boyd *et al*., 2000)
pPK7332	XbaI-HindIII *fnr* fragment from pPK823 cloned into XbaI and HindIII sites of pDHB60	This study
pPK7307	XbaI-HindIII *fnr*-L28H fragment from pPK1868 cloned into XbaI and HindIII sites of pDHB60	This study
pKD13	FRT-*kan*-FRT	(Datsenko and Wanner, 2000)
pKD32	Same as pKD13 but with FRT-*cat*-FRT	B. L. Wanner
pCP20	Ap^R	(Datsenko and Wanner, 2000)
pKD46	Phage λ*gam-bet-exo* genes under P_{araB} control	B. L. Wanner
pBR322	Ap^R	(Bolivar *et al*., 1977)
pPK7035	pBR322 with *kan* from pHP45Ω and BamHI-NdeI fragment from pRS1553	(Kang *et al*., 2005)
pPK8508	pPK7035 with P_{iscR} (−428 to +144 bp relative to the IscR start codon) cloned into BamHI and XhoI sites	This study
pBAD/Myc-hisC	Ap^R	Invitrogen

Strain	Relevant Genotype or Phenotype	Source
pGS0164	*sufABCDSE* cloned into pBAD/Myc-hisC	(Outten *et al*., 2003)
pPK9125	FRT-*cat*-FRT from pKD32 cloned into SphI site of pBAD/Myc-hisC	This study
pACYC184	Tet^R, Cm^R cloning vector	(Chang and Cohen, 1978)
pPK5960	pACYC184 with StuI-EcoRV 985 bp fragment containing *iscR* and part of *iscS*	(Schwartz *et al*., 2001)
pPK9112	*iscRS* fragment from pPK5960 cloned into EcoRV site of pBR322	This study
pPK9127	FRT-*cat*-FRT-*araC*-P_{BAD} fragment from pPK9125 cloned into EcoRI site of pPK9112	This study
pRZ7411	HindIII-BamHI fragment of *fnr* in pACYC184	(Lazazzera *et al*., 1993)
pPK9003	pRZ7411 based vector with *bla* from pBR322 at AflII site and *Ptac* from pPK7332 at AflII and NdeI sites	This study
pACYC*lacI*^{Q}	*lacI*^{Q} in pACYC184, Tet^R	(Boyd *et al*., 1987)
pACYC*lacI*^{Q} CAM	*lacI*^{Q} in pACYC184, Cm^R	(Derman *et al*., 1993)
pPK6511	P*iscR* cloned into pUC19-*spf*'	(Schwartz *et al*., 2001)
pPK8515	pPK6511 site A mutated	This study
pPK6806	pPK6511 site B mutated	This study
pPK8547	pPK6511 site C mutated	This study

Table 2

In vivo levels of IscR protein measured via quantitative Western blotting.

	IscR monomers, μM		IscR monomers/cell	
Chromosome	**Aerobic**	**Anaerobic**	**Aerobic**	**Anaerobic**
wild-type IscR	17.1 ± 1.2	1.8 ± 0.1	10,300 ± 750	1060 ± 80
IscR-C92A/C98A/C104A	29.9 ± 2.0	13.1 ± 1.2	18,000 ± 1200	7900 ± 750