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Differential control of *Bradyrhizobium japonicum* iron stimulon genes through variable affinity of the iron response regulator (Irr) for target gene promoters and selective loss of activator function

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Abstract

Bradyrhizobium japonicum Irr is a conditionally stable transcriptional activator and repressor that accumulates in cells under iron- limited, manganese-replete conditions, but degrades in a heme-dependent manner under high iron conditions, manganese limitation or upon exposure to H_2O_2. Here, we identified Irr-regulated genes that were relatively unresponsive to factors that promote Irr degradation. The promoters of those genes bound Irr with at least 200-fold greater affinity than promoters of the responsive genes, resulting in maintenance of promoter occupancy over a wide cellular Irr concentration range. For Irr-repressible genes, promoter occupancy correlated with transcriptional repression, resulting in differential levels of expression based on Irr affinity for target promoters. However, inactivation of positively controlled genes required neither promoter vacancy nor loss of DNA-binding activity by Irr. Thus, activation and repression functions of Irr may be uncoupled from each other under certain conditions. Abrogation of Irr activation function was heme-dependent, thus heme has two functionally separable roles in modulating Irr activity. The findings imply a greater complexity of control by Irr than can be achieved by conditional stability alone. We suggest that these regulatory mechanisms accommodate the differing needs for Irr regulon genes in response to the prevailing metabolic state of the cell.

INTRODUCTION

The ability of bacteria to sense nutrient availability and adapt accordingly contribute to their success in diverse environments. Iron is an essential nutrient required for many cellular processes. Bioavailability of iron is low in aerobic environments because it is mostly oxidized, and therefore insoluble. High affinity iron acquisition systems are expressed under iron limitation to scavenge the metal. Iron can also be toxic, as it catalyzes the generation of reactive oxygen species. Thus, metal homeostasis must be maintained.

Bradyrhizobium japonicum lives as a free-living soil organism or as the endosymbiont of soybean, where it fixes atmospheric nitrogen to ammonia to fulfill the nitrogen requirements of the host. *B. japonicum* belongs to the Alphaproteobacteria, a large taxonomic group that

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occupies diverse niches, including within eukaryotic cells in a symbiotic or pathogenic context. *B. japonicum* serves as a model system to understand metal metabolism and homeostasis in many Alphaproteobacterial species (Small *et al.*, 2009a). Soils are highly variable ecosystems, and symbiosis represents a niche with specific nutritional requirements. Thus, *B. japonicum* and other rhizobia must be able to accommodate changes in metal availability.

The iron response regulator (Irr) is the major transcriptional regulator of iron-responsive gene expression in *B. japonicum*, and the breadth of its role in other alpha-Proteobacteria varies. Some rhizobia employ RirA in addition to Irr (Todd *et al.*, 2002), and *Rhodobacter capsulatus* HbrL may control a larger iron-responsive regulon than Irr (Zappa and Bauer, 2013). *B. japonicum* Irr accumulates in iron-limited, manganese replete cells, and serves as both a positive and negative regulator of gene expression (Qi and O'Brian, 2002, Yang *et al.*, 2006b). Irr recognizes and binds to the iron control cis-acting element (ICE) within the promoters of target genes (Rudolph *et al.*, 2006, Yang *et al.*, 2006b, Todd *et al.*, 2006).

Irr senses iron through the status of heme by binding to ferrochelatase, a heme biosynthesis enzyme that uses iron as a substrate (Qi *et al.*, 1999). When the iron level is sufficient, heme binds directly to Irr leading to its degradation in *B. japonicum* and *Brucella abortus* (Anderson *et al.*, 2011, Martinez *et al.*, 2005), whereas the *Rhizobium leguminosarum* Irr level is not substantially altered by the iron status, but its binding activity is affected by heme, at least in vitro (Singleton *et al.*, 2010). Irr is an atypical member of the Fur family of metalloregulators in that it is active when the metal is limiting, it degrades or is inactivated in response to binding to its regulatory ligand, and it serves as a positive regulator of a large number of genes within its regulon.

Irr integrates the control of iron homeostasis and metabolism with other cellular phenomena such as the status of manganese and oxidative stress. In *B. japonicum*, manganese stabilizes Irr by interfering with heme binding, thereby increasing the heme concentration threshold that triggers Irr degradation (Puri *et al.*, 2010). As a result, manganese limitation creates an iron deficiency, possibly to counter the reduced anti-oxidant activity of manganese by attenuating iron acquisition. *B. japonicum* Irr degrades in response to H_2O_2 exposure, thereby linking peroxide stress to iron-responsive gene expression (Yang *et al.*, 2006a).

The Irr regulon is large and includes genes involved in a wide variety of cellular processes. Although they share a common iron-dependent regulatory mechanism, it is likely that additional levels of control are necessary in some cases to accommodate unique functions. In principle, this could be achieved by additional regulatory proteins or small RNAs that target a subset of the Irr regulon genes. Indeed, the heme biosynthesis genes *hemA* and *hemB* are negatively-regulated by Irr and activated by the FixL/FixJ/$FixK_2$ regulatory cascade in response to O_2 limitation (Page and Guerinot, 1995, Chauhan and O'Brian, 1997). Those genes are activated under low O_2, low iron conditions, suggesting that $FixK_2$-dependent activation overrides Irr-dependent repression. HmuP is a co-activator of the Irr-dependent heme utilization operon in *B. japonicum*, whereas utilization of other iron chelates do not require HmuP (Escamilla-Hernandez and O'Brian, 2012, Amarelle *et al.*, 2010). The regulatory rationale for this is unknown, but perhaps it is because heme can be both an iron

source and is also functional intact as the prosthetic group of heme enzymes. Irr also serves as an antirepressor of manganese-dependent repression of the *irr* gene by occluding binding of the Mur repressor to the *irr* promoter (Hohle and O'Brian, 2010).

In the current study, we identified two novel mechanisms of Irr-mediated control of gene expression that allow differential regulation of Irr operon genes without the apparent need for additional regulatory proteins.

RESULTS

In vivo occupancy of target promoters by Irr is strongly manganese-dependent for only some genes

Irr accumulates under iron limitation to positively or negatively regulate target genes. We found previously that manganese limitation results in substantial diminution of Irr levels in cells grown in low iron media (Puri *et al.*, 2010)(Fig. 1A). As a result, manganese affects promoter occupancy by Irr accordingly, and gene expression responds to manganese as well. In the current work, we examined six additional different genes within the Irr regulon for their responsiveness to manganese under iron limitation. *fhuE* and *bll7968* encode known and putative ferric siderophore receptors, respectively (Small and O'Brian, 2011, Small *et al.*, 2009b). These genes are positively controlled by Irr, thus promoter occupancy and expression are high under iron limitation, and low in an *irr* mutant (Small *et al.*, 2009b). The *bfr*, *blr7895*, *hemB* and *leuC* genes are repressed by Irr under iron limitation, and remain high independently of iron in an *irr* mutant (Hamza *et al.*, 1998, Sangwan *et al.*, 2008, Yang *et al.*, 2006b). The *bfr* (*bll6680*) gene encodes a putative bacterioferritin. The function of *blr7895* is not known, but its homolog in *Agrobacterium tumefaciens* contributes to resistance to hydrogen peroxide stress by an unknown mechanism (Ruangkiattikul *et al.*, 2012). *hemB* encodes 5-aminolevlinic acid dehydratase, an enzyme within the pathway that synthesizes heme, an iron containing prosthetic group (Chauhan and O'Brian, 1993). *leuC* is predicted to encode 3-isopropylmalate dehydratase large subunit, an iron-sulfur protein required for leucine biosynthesis. The rationale for choosing these genes is based on the fact that two of them (*bfr* and *blr7895*) did not respond to manganese as expected, and the others were useful for comparisons. This is described in detail below.

The effects of manganese on Irr occupancy of target gene promoters were examined using cross-linking and co-immunoprecipitation as previously described (Hohle and O'Brian, 2010) using anti-Irr antibodies. Co-precipitated DNA was examined by quantitative real time PCR (qPCR). Under low iron conditions, Irr occupancy of the *fhuE*, *bll7968*, *hemB* and *leuC* promoters were high in wild type cells when the medium was supplemented with manganese, and were low in cells grown without added manganese (Fig. 1B, C, F, G), although less so for the *leuC* promoter. These observations correlated with Irr levels in cells (Fig. 1A), and are consistent with that observed previously (Puri *et al.*, 2010).

Interestingly, diminution of cellular Irr levels by removal of manganese from low iron media did not result in a corresponding decrease in promoter occupancy of the *bfr* and *blr7895* genes (Fig. 1D, E). These findings show that not all genes within the Irr regulon are responsive to manganese, and Irr occupancy correlates with cellular Irr levels for only some

promoters. Moreover, substantial occupancy of the *bfr* and *blr7895* promoters by Irr persists even in cells grown under high iron, suggesting that they are less responsive to that metal as well.

The affinity of Irr for target promoters varies greatly in vitro

The simplest explanation for the high occupancy of the *bfr* or *blr7895* promoter by Irr when the cellular Irr level is low is that Irr has a higher affinity for those promoters than for the *fhuE*, *bll7968*, *hemB* or *leuC* promoters. This variability could be due to an accessory protein that affects affinity, or Irr may simply have differing affinities for the promoters examined. We measured the affinity of Irr for ^{32}P-labeled 45 bp double stranded DNA probes in vitro that correspond to each of the six promoter regions by electrophoretic mobility shift analysis (EMSA). Each DNA probe contained the 21 bp ICE motif within each promoter flanked by an additional 12 bp on each side (Fig. S1). Binding curves were generated by titrating the reactions with varying amounts of Irr, and the fraction bound was determined by the mobility of the bands (Fig. 2, Fig. S2). Dissociation binding constants (K_d) values derived from then are listed in Table 1. The K_d values for the *bfr* and *blr7895* promoters were 3.3 nM and 1.5 nM, respectively. The K_d values for the *fhuE* and *bll7968*, *hemB* and *leuC* promoters were estimated to be greater than 440 nM, 320 nM, 1000 nM and 1000 nM, respectively. These latter 4 values are only approximations because binding did not saturate at 1000 nM Irr, which was the highest concentration that could be achieved in the reactions. Nevertheless, the data show a very large range of Irr binding affinities for target promoters. The high affinities of the *bfr* and *blr7895* promoters for Irr can explain high occupancy even when Irr levels are low, hence less responsive to manganese. Similarly, weak binding of Irr to the other promoters is consistent with low promoter occupancy under low manganese conditions where the cellular Irr level is low, resulting in responsiveness to manganese.

The iron control element (ICE) motif is sufficient to confer binding affinity of Irr to target promoters in vitro

The ICE motif is a 21 bp imperfect inverted repeat consensus sequence necessary for Irr binding (Rudolph *et al.*, 2006, Yang *et al.*, 2006b). We wanted to determine whether differences in the ICE motif sequence within each promoter was sufficient to explain the wide range of Irr binding affinities. The ICE motifs within the promoters of *fhuE* and *bfr* differ from each other by 6 bp. Therefore, substitutions were made so that the weak binding *fhuE* promoter contained the ICE motif of the strong binding *bfr* promoter (P_{fhuE}/ICE_{bfr}) and vice versa (P_{bfr}/ICE_{fhuE}) (Fig. S1). These oligonucleotides were analyzed by EMSA to determine the dissociation binding constants (Fig. 2; Fig. S2, Table 1). The wild type *bfr* promoter had a K_d value of 3.3 nM, but the K_d for the P_{bfr}/ICE_{fhuE} promoter was >730 nM. Correspondingly, the K_d values of the wild type *fhuE* promoter and the P_{fhuE}/ICE_{bfr} promoter were >550 nM and 16 nM, respectively.

Binding experiments were also carried out between the respective high and low affinity promoters of *blr7895* and *bll7968*, which contain ICE motifs that differ from each other by 6 bp (Fig. S1; Table 1). Insertion of the *blr7895* ICE into *bll7968* promoter sequence (P_{7968}/ICE_{7895}) increased the Irr binding affinity from >320 nM to 4.6 nM. In addition,

insertion of the *bll7968* ICE motif into the *blr7895* promoter (P_{7895}/ICE_{7968}) significantly reduced binding, from 1.5 nM to >340 nM.

Collectively, the in vitro binding experiments show that the ICE motif is a major determinant in promoter affinity for Irr.

Responsiveness of Irr occupancy to hydrogen peroxide in vivo depends on promoter affinity for Irr

The data presented thus far suggest that the status of promoter occupancy by Irr in response to manganese is dependent on the affinity of Irr to the target gene promoter. As a result, diminution of the cellular Irr level has less effect on the occupancy of high affinity promoters compared with those of low affinity for Irr. This idea predicts that factors other than manganese that affect cellular Irr levels will also differentially influence promoter occupancy. We found previously that Irr degrades in response to short term exposure to exogenous H_2O_2 in cells grown with 2 μM $FeCl_3$, an iron concentration that is not sufficient to fully degrade Irr (Yang *et al.*, 2006a). The Irr level in cells was followed over 90 min after H2O2 exposure by western blotting (Fig. 3A). Irr decreased with time after H_2O_2 addition, similar to what was reported previously (Yang *et al.*, 2006a). Corresponding decreases in promoter occupancy of the *fhuE*, *bll7968*, *hemB* and *leuC* genes were observed (Fig. 3B, C, F, G). By contrast, occupancy of the high Irr affinity promoters of the *bfr* and *blr7895* genes remained relatively high upon exposure to H_2O_2 even though Irr decreased over that time period (Fig. 3D,E). These observations support the conclusion that environmental factors that affect the cellular Irr level will result in differential occupancy of target genes based on promoter affinity for Irr, and that these responses are not specific to manganese.

Expression of negatively-regulated genes in response to environmental factors correlates with Irr occupancy of their promoters

We sought to address whether environmental factors that affect Irr levels regulate gene expression in a manner consistent with occupancy of their promoters. Gene expression was monitored by measuring mRNA abundance by quantitative real time PCR (qPCR). The negatively regulated genes *bfr* and *blr7895* showed low mRNA abundance under iron limitation that was essentially independent of the manganese status (Figs. 1J, K), which is consistent with high occupancy of their promoters under low or high manganese exposure (Fig. 1D, E). Expression of the *leuC* and *hemB* genes were more responsive than *bfr* or *blr7895* to manganese in iron-limited cells, showing partial derepression when manganese is limiting (Fig. 1L, M). This derepression correlated with decreased Irr occupancy of the *leuC* and *hemB* promoters (Fig. 1F, G).

Transcript abundance was also measured in cells grown in 2 μM iron that were exposed to 2 mM H_2O_2 over a 90 minute time course (Fig. 3H–M). The *bfr* and *blr7895* genes remained repressed over that time compared to the 20 μM iron control as discerned by the low mRNA abundance (Fig. 3J, K). This is consistent with the maintenance of high Irr occupancy over that period (Fig. 3D, E). By contrast, *leuC* and *hemB* mRNA increased with time upon H_2O_2

exposure (Fig. 3L, M) concomitant with decreasing promoter occupancy (Fig. 3F, G), which in turn correlates with low affinity of Irr for those promoters.

To further address the idea that promoter affinity for Irr strongly influences gene expression in response to environmental factors that affect Irr levels, we examined the consequences of replacing the ICE motif in the *bfr* promoter with a low affinity one. As noted above, the ICE motifs within the *bfr* and *fhuE* promoters differ by 6 bp, and changes in those residues were sufficient to drastically alter the affinity of the respective promoters to Irr in vitro (Table 1). We constructed a mutant strain in which the *bfr* gene contains the ICE motif of the *fhuE* promoter (*bfr*/ICE$_{fhuE}$), and the 6 bp substitutions are the only changes in the genome.

Cells of the wild type and *bfr*/ICE$_{fhuE}$ mutant were grown in manganese replete or depleted media under iron limitation. In the presence of manganese, where Irr levels are high (Fig. 4A), promoter occupancy of the wild type and mutant were similar (Fig. 4B), and the genes were likewise similarly repressed in each strain (Fig. 4C). Under low manganese conditions, occupancy of the wild type promoter was essentially the same as in manganese-replete cells, but the *bfr*/ICE$_{fhuE}$ promoter occupancy by Irr was lower (Fig. 4B). Correspondingly, *bfr* expression from the mutant promoter was derepressed resulting in expression even higher than that found under high iron conditions of the wild type (Fig. 4C). These data show that introduction of a low affinity Irr binding site into the *bfr* gene promoter is sufficient to confer strong manganese responsiveness to expression from that promoter. Even under iron replete conditions where Irr levels are extremely low, there was a large difference in promoter occupancy and expression between the wild type and the mutant (Figs. 4B, C). This shows that *bfr* is not fully derepressed in wild type cells even when grown in 20 μM Fe due to having a promoter with very high affinity for Irr.

The effects of H_2O_2 exposure on occupancy of, and expression from, the wild type and *bfr*/ICE$_{fhuE}$ promoters were examined (Fig. 5A–C). Cells grown in media supplemented with 2 μM $FeCl_3$ were exposed to 2 mM H_2O_2 over 90 minutes (Fig. 5B, C), similar to that described in Fig. 3. Occupancy of the *bfr*/ICE$_{fhuE}$ promoter was more dependent on H_2O_2, decreasing throughout the time course of the experiment, as did Irr levels (Fig. 5A, B). Also, *bfr* mRNA levels expressed from the wild type promoter remained very low through 90 minutes, but expression from the *bfr*/ICE$_{fhuE}$ promoter was strongly responsive to H_2O_2 (Fig. 5C), showing a 10-fold increase in *bfr* transcripts relative to the wild type after 90 minutes exposure (Fig. 5C). Thus, introduction of a low affinity ICE motif into the *bfr* promoter conferred H_2O_2 responsiveness on that gene.

Finally, we examined the effects of iron on substituting the high affinity ICE motif in the *bfr* promoter for a low affinity one. Irr regulon genes with low or high affinity promoters respond to iron when comparing cells in iron deplete media compared with >10 μM supplementation. We chose a narrow range from 2 to 4 μM of iron where Irr levels vary but remain detectable (Fig. 6A) to better gauge sensitivity. The wild type *bfr* gene was mostly unresponsive to iron in this range both in terms of promoter occupancy (Fig. 6B) and mRNA abundance level that remained repressed (Fig. 6C). In the mutant, however, occupancy of the *bfr*/ICE$_{fhuE}$ promoter was iron responsive (Fig. 6B), and expression was strongly derepressed (Fig. 6C).

Collectively, the evidence shows that, for genes that are negatively regulated by Irr, expression correlates strongly with promoter occupancy, which in turn is strongly influenced by the affinity of Irr to target promoters.

Inactivation of a positively regulated gene can occur without Irr vacancy of its promoter

The *fhuE* and *bll7986* genes have promoters with low affinity for Irr which renders occupancy sensitive to the manganese status under low iron conditions (Fig. 1B,C). We found that expression of *fhuE* and *bll7986* as measured by mRNA abundance was high in cells grown in low iron, high manganese media, but not in cells grown in low manganese or high iron media (Fig. 1H, I). Thus, expression of these genes was manganese-responsive.

Expression of the *fhuE* and *bll7986* genes were also followed in response to H_2O_2 in cells grown with 2 μM iron as described above for the promoter occupancy experiments. Transcript levels of both genes dropped sharply by 30 minutes after H_2O_2 exposure (Fig. 3H, I). Thus, factors that promote Irr degradation lead to inactivation of positively-controlled genes.

The sensitivity of *fhuE* and *bll7968* expression to the status of H_2O_2 and manganese appears to have a causal relationship with promoter occupancy, and the findings for the Irr-repressible genes predict that a positively regulated gene expressed from a promoter with high affinity for Irr would be less sensitive to those environmental factors. However, experiments described below show that this is not the case.

Based on microarray data (Yang *et al.*, 2006b), binding studies (Small *et al.*, 2009b) and bioinformatics predictions (Rodionov *et al.*, 2006), we identified 8 genes or operons that are positively controlled by Irr and also have a known or predicted ICE motif. Both published (Small *et al.*, 2009b) and unpublished studies failed to find ICE motifs of these genes with high affinity for Irr as estimated by those that are at least half-bound by 250 nM Irr in EMSA analysis. Nevertheless, we constructed a mutant strain containing an *fhuE* gene derivative with a high affinity promoter. To do this, we made a 6 bp change within the ICE motif of *fhuE* promoter so that it is identical to the ICE motif from the *bfr* promoter (*fhuE*/ICE$_{bfr}$). These are the only changes in the genome of the *fhuE*/ICE$_{bfr}$ mutant strain.

Occupancy of the *fhuE*/ICE$_{bfr}$ promoter was very high in iron limited cells compared with the wild type promoter grown in either high or low manganese media (Fig. 4D), and thus high Irr affinity resulted in greater occupancy. However, *fhuE* transcript levels in the wild type and mutant strains were similar in low iron, high manganese cells despite the large difference in Irr occupancy of their promoters (Fig. 4E). Moreover, removal of manganese from the low iron medium resulted in nearly undetectable *fhuE* transcripts even though Irr occupancy of the mutant promoter remained very high. Finally, occupancy of the mutant promoter by Irr in iron replete cells was only modestly lower than that found in the wild type promoter in iron-limited cells. Thus, expression of *fhuE* in the mutant strain was manganese-responsive even though occupancy of its promoter by Irr remained and high was relatively unresponsive to the metal.

We then compared the effects of H_2O_2 exposure on wild type and *fhuE*/ICE$_{bfr}$ cells grown in media supplemented with 2 μM $FeCl_3$ (Fig. 5A, D, E). Whereas Irr occupancy of the wild type *fhuE* promoter decreased over time upon H_2O_2 exposure, occupancy of the *fhuE*/ICE$_{bfr}$ promoter remained high throughout the time course. Nevertheless, transcript levels in the mutant strain declined rapidly in response to H_2O_2 exposure as was also observed in the wild type.

Responses to iron were also examined in the narrow range of 2 to 4 μM as described above (Fig. 6A, D, E). Again, introduction of a high affinity Irr binding site into the *fhuE* promoter yielded very high Irr occupancy that changed little from 2 to 4 μM iron (Fig. 6D). Despite the high Irr occupancy, *fhuE* transcripts decreased in the mutant strain with increasing iron concentrations (Fig. 6E).

Collectively, the findings show that environmental factors that decrease the cellular Irr level can deactivate expression of target genes by a mechanism that does not require promoter vacancy. This differs from Irr-repressible genes, where repression correlates with promoter occupancy.

Exposure of iron-limited cells to hydrogen peroxide inactivates expression of Irr target genes, but does not affect cellular Irr levels, promoter occupancy or repression

Whereas Irr degrades in response to H_2O_2 in cells grown in 2 μM iron, very little degradation occurs upon short term exposure of iron-limited cells to H_2O_2 (Yang *et al.*, 2006a), which was reproduced here (Fig. 7A). In vitro, H_2O_2 can oxidize Irr in the presence of heme (Yang *et al.*, 2006a), and thus we wanted to address whether H_2O_2 exposure affected Irr occupancy or expression of target genes in vivo.

Promoter occupancy of genes positively or negatively regulated by Irr remained high throughout the 90 minute exposure to H_2O_2 (Figs. 7B–G), consistent with the maintenance of a high level of cellular Irr over that time period (Fig. 7A). Correspondingly, the negatively-regulated genes *blr7895*, *bfr*, *leuC* and *hemB* remained repressed under those conditions compared with a high iron control (Fig. 7J–M), showing that Irr remains functional as a repressor. However, transcript levels of the positively-controlled genes *fhuE* and *bll7968* decreased by 30 minutes after H_2O_2 exposure and remained low even though occupancy by Irr of those promoters remained high. Thus, loss of activation function of Irr can occur without loss of DNA-binding activity or repressor activity in wild type cells.

Loss of Irr-dependent activation of target genes is heme-dependent

To determine whether the effects of H_2O_2 on Irr activity in iron-limited cells depended on heme, we measured promoter occupancy and expression of Irr-responsive genes in a heme-deficient strain in response to H_2O_2 exposure. Irr is stable in a heme-deficient strain under conditions where it degrades in the wild type (Puri *et al.*, 2010, Qi and O'Brian, 2002, Yang *et al.*, 2006a), and thus Irr levels remained high in iron-replete control cells of the mutant (Fig. 8A). As in the wild type, cellular Irr levels remained high throughout the 90 exposure of H_2O_2 in the heme-deficient strain (Fig. 8A). Consistent with this, promoter occupancy by Irr remained high in the six genes examined (Fig. 8B–G), and expression of the negatively

controlled genes *blr7895*, *bfr*, *leuC* and *hemB* remained repressed (Fig. 8J–M). However, unlike the wild type, expression of *fhuE* and *bll7968* did not diminish in response to H_2O_2 exposure (Fig. 8H, I). These findings show that H_2O_2-dependent loss of Irr activation function requires heme by a mechanism that is distinct from heme-dependent degradation of Irr.

DISCUSSION

In previous work, we showed that *B. japonicum* Irr is a conditionally stable protein that accumulates in cells under iron limitation and when manganese is sufficient, but degrades in a heme-dependent manner under high iron conditions, manganese limitation or upon exposure to H_2O_2. Conditions that degrade Irr result in the inactivation of positively controlled genes and derepression of negatively regulated genes. In the present study, we identified two additional levels of control, one which relies on differential affinity of Irr for target promoters, and another which abrogates the activation function of Irr without loss of DNA-binding activity or repressor function. Heme is required for the loss of activation function by a mechanism that does not require degradation. We suggest that these additional levels of control allow differential regulation among negatively regulated genes, and the uncoupling of activation from repression under certain conditions. Thus, the Irr regulon shows a greater complexity of control than can be achieved by conditional stability alone.

The K_d values of Irr for target promoters spanned from low nanomolar to the micromolar range. Irr levels in cells were estimated by quantifying protein levels in cells by Western blots using pure protein as a standard (data not shown), and also estimating the cell volume to be 0.6–1 μm^3 (Kanbe *et al.*, 2007), yielding a cellular Irr concentration of 0.7 to 1.1 μM under iron limitation. The comparison between K_d values and Irr concentration is imperfect because supercoiled DNA is the target for Irr in vivo, purified Irr may not be as robust as the in vivo protein, and cell volume estimates include the periplasm, but Irr is cytosolic. Nevertheless, the estimations imply that the cellular Irr concentration is sufficiently high to occupy promoters with low affinity for it, as was confirmed by the in vivo analyses described here. A broad range of binding affinities of the Fur family proteins Zur from *Streptomyces coelicolor* (Shin *et al.*, 2011) and Fur from *Neisseria gonorrhoeae* (Yu and Genco, 2012) for target promoters has been observed, but the concentration of those proteins do not vary greatly in response to their respective regulatory metal. Instead, the metal primarily controls DNA-binding activity.

Irr occupancy of target promoters correlated well with its affinity for the promoter, and for the ICE motif in particular. We suggest that, for negatively regulated genes, the broad range of affinities of Irr for target promoters allows different levels of repression at a given Irr concentration to accommodate the physiological function of each gene product and the cellular status (Fig. 9). The large subunit 3-isopropylmalate dehydrogenase encoded by *leuC* is an iron-sulfur protein, and the *hemB* product 5-aminolevulinic acid dehydrogenase is involved in the synthesis of heme, an iron containing prosthetic group. Repression of these genes under low iron conditions ensures that the synthesis of the proteins does not exceed iron availability. However, *B. japonicum* cells grow aerobically under that condition, hence some expression of housekeeping genes such as *hemB* and *leuC* must be maintained. Thus, a

weaker affinity promoter may allow some level of expression (derepression) under a low or moderate iron availability where Irr levels are high. By contrast, bacterioferritin and perhaps Blr7895 are likely to be important in managing stress associated with iron-dependent chemistry, hence strong repression is appropriate under low or moderate iron levels, which can be achieved by having those genes controlled by promoters with a high affinity Irr-binding site. Similarly, manganese limitation creates an iron deficiency (Puri *et al.*, 2010), and repression of *bfr* and *blr7895* is maintained even though Irr levels are diminished under that condition.

For genes positively regulated by Irr, we showed that abrogation of activation can occur by a process that is distinct from Irr degradation. As a result, diminution of gene expression can occur even when promoter occupancy by Irr remains high (Fig. 9). Experiments with the *fhuE*/ICE$_{bfr}$ mutant show that the low level of Irr remaining in cells after exposure to iron, low manganese conditions or H_2O_2 plus 2 μM Fe is not functional as an activator. Similarly, *fhuE* and *bll7986* transcripts declined in iron-limited wild type cells in response to H_2O_2 even though cellular Irr levels and promoter occupancy remained high. Retention of promoter occupancy under these conditions shows that loss of activation function of Irr does not require loss of DNA binding activity.

The retention of DNA binding activity of Irr by treatments that modify activation function or stability may explain why Irr was able to repress gene expression under those conditions. It is likely that DNA binding is sufficient to occlude the promoter from RNA polymerase, and therefore repression by Irr is not affected by modifications that do not change promoter occupancy. This is consistent with previous work showing that Irr binding to a target promoter is sufficient to repress transcription in vitro (Sangwan *et al.*, 2008). We suggest that loss of repression function by Irr occurs primarily through its diminution in cells via degradation resulting in promoter vacancy. The extent of this vacancy is dictated by the affinity of the promoter for the regulator (Fig. 9). Irr-dependent activation also requires promoter occupancy, hence Irr stability, but loss of activity need not be accompanied by promoter vacancy. As a result, inactivation is not sensitive to affinity of Irr to the target promoter. It is plausible that the activated genes have promoters with low affinity for Irr because there is no evolutionary selection for a high affinity binding site.

The loss of activation function of Irr without degradation uncouples activation from repression in a manner that control by degradation alone cannot, and this may be advantageous under certain conditions. For example, cells exposed to H_2O_2 under low iron conditions where Irr functions may respond by down regulating genes involved in iron transport (e.g. *fhuE*, *bll7968*) as a means to mitigate the formation of reaction oxygen species through the Fenton reaction. Under these conditions, a concomitant derepression of genes that encode iron-containing proteins would not be appropriate because there is no increase in the iron level. More generally, this control may be appropriate for any cellular state that does not call for simultaneous inactivation and derepression of the respective Irr regulon genes.

The decrease in *fhuE* or *bll7968* in iron-limited wild type cells in response to H_2O_2 was not observed in the heme-defective mutant strain, showing that heme is involved in Irr-

dependent gene expression that is distinct from its role in degradation. Specifically, heme is required for abrogation of the activation function of Irr. The data suggest that Irr has DNA-binding and activation domains that can be functionally distinguished based on the ability of the latter to be specifically altered by heme (Fig. 9). This alteration may interfere with Irr binding to RNA polymerase or to another regulatory protein. Heme is known to catalyze Irr oxidation in vitro, and evidence indicates that this is important for degradation in vivo (Yang *et al.*, 2005, Yang *et al.*, 2006a). Oxidation may also play a role in inactivation, but if so degradation must require at least one additional event since heme-dependent degradation and inactivation are functionally separable.

Heme binds to Irr to inactivate it, but this does not abrogate DNA-binding in vivo. This conclusion differs from that reported for *Rhizobium leguminosarum* Irr, where heme inhibits DNA binding in vitro (Singleton *et al.*, 2010). It is also at variance with findings for essentially all other characterized Fur family proteins, where in those cases the protein-binding regulatory ligand greatly diminishes DNA binding activity (Bsat *et al.*, 1998, Gaballa and Helmann, 1998, Patzer and Hantke, 1998, Ahn *et al.*, 2006, Hohle and O'Brian, 2009, Platero *et al.*, 2007).

The auxiliary regulator HmuP was identified in Irr-dependent control of the heme transport cluster of *B. japonicum* (Escamilla-Hernandez and O'Brian, 2012), and control of the *irr* gene by Irr proceeds by anti-repression of the Mur protein (Hohle and O'Brian, 2010). Although we cannot rule out additional regulatory factors that mediate control of Irr regulon genes, the observations described here do not mandate one, and can be explained in terms of differential promoter affinity and abrogation of its activation function while keeping repressor activity intact.

MATERIALS AND METHODS

Strains and media

Bradyrhizobium japonicum USDA I110 is the parent strain used in this study. Strain ΔhemAH is a double mutant defective in *hemA* and *hemH* genes, encoding heme biosynthesis enzymes (Qi and O'Brian, 2002). *B. japonicum* strains were routinely grown at 29°C in glycerol-salts-yeast (GSY) medium as previously described (Frustaci *et al.*, 1991). Strain ΔhemAH was grown in media supplemented with 50 μg/ml kanamycin, 50 μg/ml streptomycin, 100 μg/ml spectinomycin, with 1 μM hemin hydrochloride to fulfill its heme auxotrophy. For experiments requiring low heme conditions, the medium was supplemented with 0.15 μM heme, which allows growth comparable to the wild type but retains other heme-defective phenotypes (Qi and O'Brian, 2002).

For low iron or low manganese conditions, modified GSY was used, which contains 0.5 g per liter of yeast extract instead of 1 g per liter, with no exogenous iron or manganese added. The actual iron and manganese concentrations in the medium were 0.3 and 0.4 μM, respectively, as determined with a PerkinElmer model 1100B atomic absorption spectrophotometer. Media for growth under high iron or high manganese conditions are supplemented with 20 μM $FeCl_3$ or 50 μM $MnCl_2$, respectively.

Hydrogen peroxide treatments

Cells were grown to mid log phase in modified GSY medium supplemented with no iron or 2 μM $FeCl_3$ and with 50 μM $MnCl_2$. Cultures were then treated with 2 mM hydrogen peroxide (Sigma, MO) with continuous shaking at 29°C. Aliquots were collected at specified time points for analysis.

Construction of the ICE mutant strains

The promoter regions of *bfr* and *fhuE* were modified so that the ICE motif sequence within one gene promoter was substituted for the other. To do this, DNA fragments containing the 21bp ICE motif flanked on either side by 700bp DNA were amplified by PCR using genomic DNA as template, and the products were ligated into pBluescript SK+. The 6 bp substitutions in each construct were made by site-directed mutagenesis (Stratagene, La Jolla, CA). The mutated constructs were introduced into pLO1 (Lenz *et al.*, 1994), mobilized into strain USDA I110, and recombinants were selected as described previously (Escamilla-Hernandez and O'Brian, 2012). Mutants were confirmed by DNA sequencing.

Immunoblotting

Cultures were harvested, washed and re-suspended in phosphate-buffered saline (10 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl [pH 7.4]). Total protein concentrations were determined by BCA protein assay (Pierce, Rockford, IL). Fifteen μg of total cell protein were boiled in loading buffer and resolved by electrophoresed through 15% SDS-polyacrylamide gels. Immunoblotting was carried out with Irr or GroEL (Stressgen, Vancouver, Canada) polyclonal antibodies and HRP-conjugated goat anti-rabbit IgG (Southern Biotech, Birmingham, AL) with detection using the Immobion system (Millipore, Billerica, MA).

Overexpression and purification of Irr

The *irr* gene was amplified by PCR and cloned into pETM-11 vector containing an N-terminal 6xHis tag. The vector with insert was transformed into chemical competent BL21 (DE3) *E. coli* cells. Cells were inoculated from an overnight culture grown in Luria-Bertani media containing 20 μg/ml kanamycin and 25 μg/ml chloramphenicol into 1 l of fresh 2×YT medium (Sambrook *et al.*, 1989) containing the same antibiotics and 50 μM $MnCl_2$. Overexpression was induced in cells at the midlog phase by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 37 °C for 4 h with shaking. Cells were pelleted by centrifugation, washed in lysis buffer (5 mM Tris-HCl, pH 8.0). The pellet was further washed in lysis buffer containing 4% Thesit (Sigma, MO) followed by a wash in lysis buffer with 2M urea, before finally solubilizing in lysis buffer with 8M urea. Cells were disrupted by passage through a French pressure cell at 1,200 psi and clarified by centrifugation at 13,000 × *g* for 10 min. Two milliliters of Ni-NTA slurry (Qiagen) was added to the cleared lysate and rocked for 60 min at room temperature (22 °C).

The Ni-NTA slurry-protein mixture was poured into a column and washed with ten column volumes of lysis wash buffer (5 mM Tris-HCl, 8 M Urea, and 30 mM imidazole, pH 8.0). His-Irr was eluted using lysis elution buffer (5 mM Tris-HCl, 8 M Urea, and 250 mM

Imidazole, pH 8.0). The purified protein was run through an FPLC buffer exchange column so that the final buffer consisted of 10 mM Tris-HCl, 100 μM $MnCl_2$, pH 8.0.

EMSA

Electrophoretic mobility shift assays (EMSA) were used to analyze the binding of purified Irr to various DNA probes *in vitro* as described previously (Friedman and O'Brian, 2004). The negative-control DNA corresponds to the sequence found in the multiple-cloning site of pBluescript SK+. The test DNA probes were 45 bp in length and contained 21-bp ICE (underlined in the sequences below) flanked either by the original genomic flanks or the genomic flanks of a different ICE motif. The sequences of the probes are shown in Fig. S1. EMSA reactions were analyzed as autoradiograms of 5% non-denaturing polyacrylamide gels. Autoradiograms were scanned using GS-700 densitometer (Bio-Rad), and signal intensities were determined and quantified by Quantity One software (Bio-Rad). To determine the dissociation binding constant (K_d), binding reactions containing 0.1 nM ^{32}P-labelled DNA were titrated with various concentrations of Irr. Bound and unbound DNAs were quantified by comparing relative signal intensities and analyzed using GraphPad Prism software (GraphPad software Inc., San Diego, CA)

Analysis of RNA by quantitative real-time PCR

Steady state transcript levels of selected genes were determined by qPCR with SsoAdvanced SYBR Green supermix (Bio-Rad) using iCycler thermal cycler (Bio-Rad). RNA was isolated from *B. japonicum* cells using a hot phenol method as described previously (Yang *et al.*, 2006a). cDNA was synthesized from 1 μg total RNA using iScript cDNA synthesis kit (Bio-Rad). qPCRs were carried out as previously described (Hohle and O'Brian, 2009). Data were normalized to *gapA* and are expressed as average of triplicates, with standard deviation represented by the error bars.

Quantitative *in vivo* cross-linking and immunoprecipitation

Cells were grown to mid-log phase. Twenty-five-milliliter aliquots were spun down and the pellets were saved at −80°C until further use. For cross-linking, pellets were re-suspended in phosphate-buffered saline (10 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) and formaldehyde was added to 1% (vol/vol) and the samples were gently rocked at room temperature for 10 min. To quench the cross-linking, glycine was added to a final concentration of 10 mg/ml, and the cells were shaken gently at 4°C for 30 min. Cells were collected by centrifugation, and the pellets were resuspended and washed twice with phosphate-buffered saline. Washed cells were re-suspended in 2 ml lysis buffer (100 mM Tris pH 8.0, 300 mM NaCl, and 10 mM EDTA) and lysed with a French pressure cell as described previously. The cell extracts were aliquoted and frozen at −80°C until further use. Protein-A agarose beads, pre-blocked with salmon sperm DNA and bovine serum albumin (Millipore, Billerica, MA), were washed with lysis buffer and resuspended to a slurry of 33%. The lysates were pre-cleared by adding 20 μl of bead slurry to 200 μl cell lysate and mixed slowly at room temperature for 1 h with a rotation mixer, followed by centrifugation for 1 min at 1,000 × g. One microliter of the lysate was diluted with 9 μl of the dilution buffer and the mixture was saved at −80 °C, to serve as the input-control (1% of input

DNA). One hundred microliters of the remaining supernatant was diluted with 900 μl of dilution buffer. Serum containing anti-Irr poly- clonal antibodies was added to a dilution of 0.08 μl for every 15 μg of total protein and the tube was left rotating slowly at 4°C overnight with a rotation mixer. Fifty microliters of washed bead slurry was added to the samples and mixed by rotation at room temperature for 1 h. The protein-DNA complex was washed and eluted from the beads at room temperature as per the manufacturer's instructions. The input-control samples were also diluted with the elution buffer to the same final volume as the samples. The DNA from both the samples and the input controls was uncrosslinked from the protein by adding NaCl to 0.2 M to the eluted samples, and the samples were incubated at 65°C overnight. DNA was purified using the Qiagen PCR purification kit and eluted in 50 μl of elution buffer. Immunoprecipitated DNA of selected promoter regions was quantified using qPCR as described above, using 0.4 μl as template. The data were normalized to input DNA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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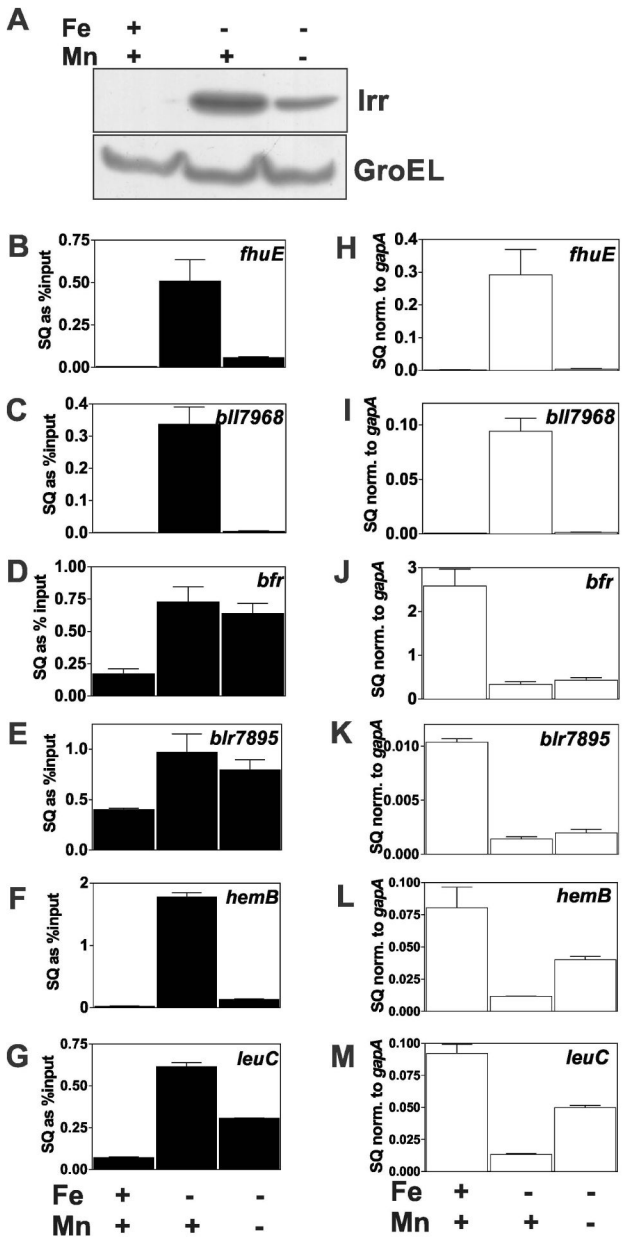

Fig. 1.

Effects of manganese on promoter occupancy and expression of Irr-regulated genes. Cells were grown in media supplemented with 20 μM $FeCl_3$ (Fe) or 50 μM $MnCl_2$ (Mn) (+) or not supplemented with metal (−). The actual Fe and Mn concentrations in the unsupplemented media were 0.3 μM and 0.4 μM, respectively. (A) Steady state levels of Irr were detected by immunoblotting using anti-Irr antibodies. GroEL was used as a control for an unregulated protein, and was detected using anti-GroEL antibodies. Fifteen micrograms of protein was loaded per lane. (B–G) In vivo promoter occupancy of the respective genes in cells grown under different metal conditions was measured by crosslinking and immunoprecipitation using anti-Irr antibodies as described in *Materials and Methods*. The precipitated DNA was analyzed by qPCR using primers delimiting the promoter regions of the respective genes.

The data are expressed as the relative starting quantities (SQ) of immunoprecipitated DNA normalized to the input DNA and are presented as the average of three replicates ± the standard deviation. (H–M) Steady-state transcript levels of the respective genes obtained from cells grown under different metal conditions were analyzed by qPCR. The data are expressed as relative starting quantities (SQ) of respective mRNAs normalized to the housekeeping gene *gapA*, and presented as average of three replicates ± the standard deviation.

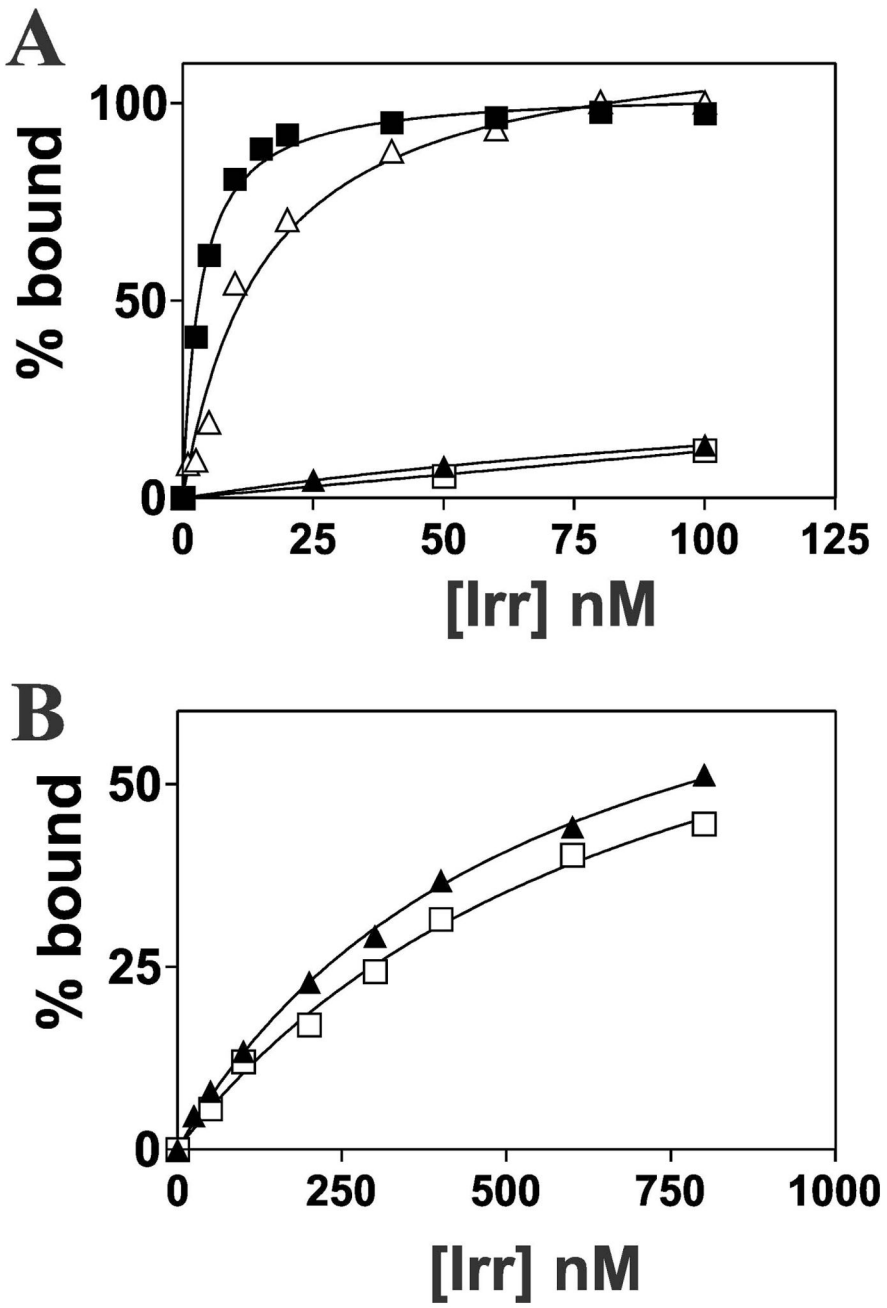

Fig. 2.

Analysis of Irr-binding to the promoters of Irr-regulated genes. (A) EMSA was carried out using 0.1 nM ^{32}P-labelled promoter DNA of *bfr* (closed squares), *fhuE* (closed triangles), P_{fhuE}/ICE_{bfr} (open triangles) and P_{bfr}/ICE_{fhuE} (open squares) titrated with various concentrations of Irr. Bound and unbound DNA was resolved on a 5% non-denaturing polyacrylamide gel and visualized by autoradiography. Autoradiographs were scanned, and bands were quantified to determine bound and unbound DNA. (B) Binding curves of *fhuE* (closed triangles) and P_{bfr}/ICE_{fhuE} (open squares) at higher Irr concentrations.

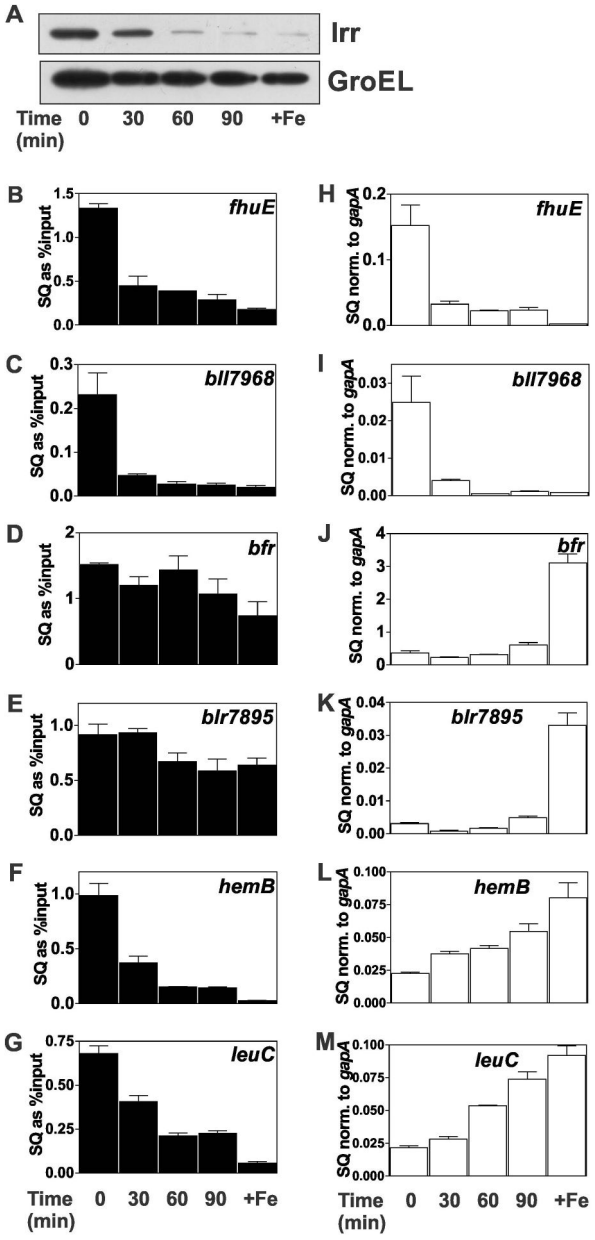

Fig. 3.

Effects of H_2O_2 on promoter occupancy and expression of Irr-regulated genes. Cultures were grown to mid-log phase in media supplemented with 2 μM $FeCl_3$ and 50 μM $MnCl_2$. At time 0, 2 mM H_2O_2 was added to the cultures and were continually aerated at 29°C. Cells were grown in 20 μM (+Fe) without H_2O_2 treatment as a control. Aliquots were harvested at indicated time points for analysis. (A) Steady-state levels of Irr and GroEL were detected by immunoblotting as described in Fig. 1. (B–G) Promoter occupancy of respective genes by Irr in cells treated with H_2O_2 were carried out as described in the Fig. 1 legend. The data are expressed as the relative starting quantities (SQ) of immunoprecipitated DNA normalized to the input DNA and are presented as average of three replicates ± the standard deviation. (H–M) Steady-state transcript levels of the respective genes obtained from cells treated with

H_2O_2 were analyzed by qPCR as described in the Fig. 1 legend. The data are expressed as relative starting quantities (SQ) of respective mRNAs normalized to the housekeeping gene *gapA*, and presented as average of three replicates ± the standard deviation.

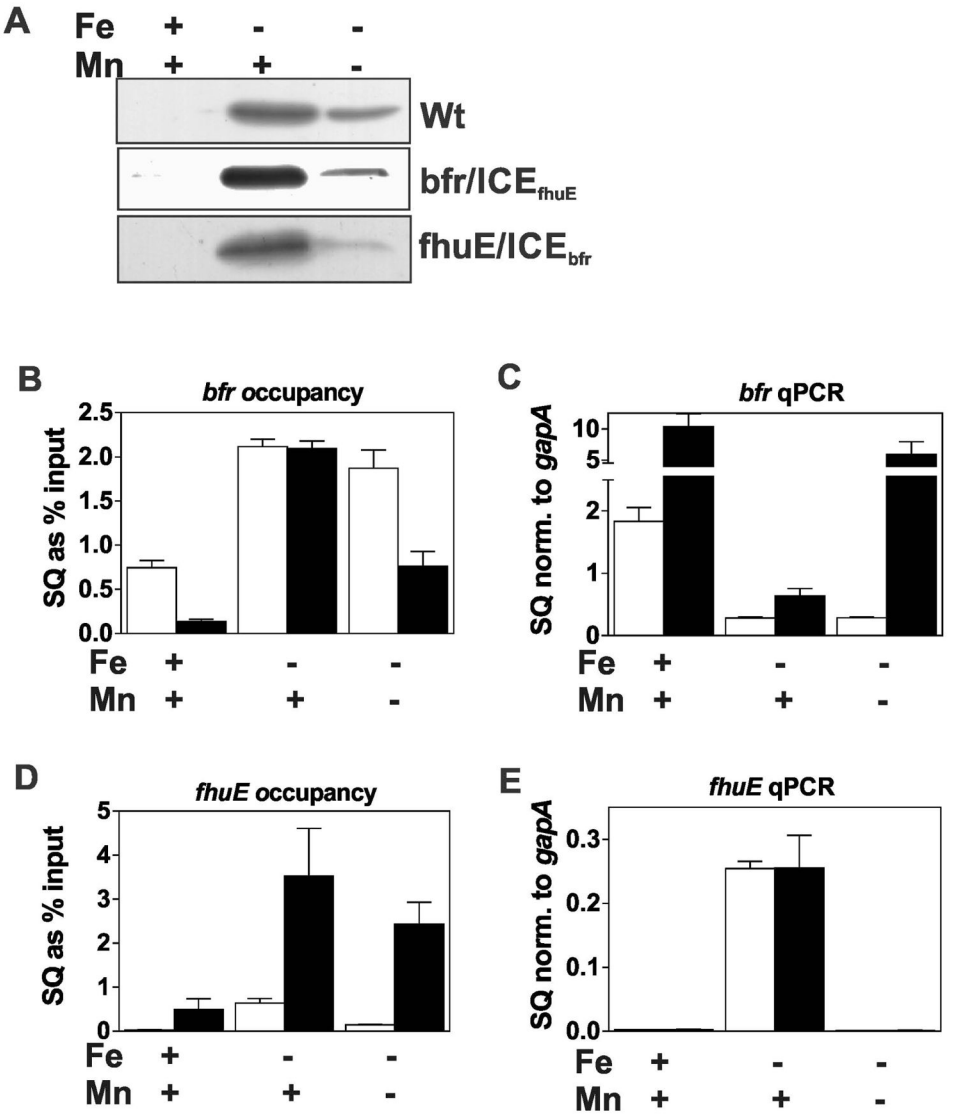

Fig. 4.

Effects of ICE motif substitutions on promoter occupancy by Irr and gene expression in response to manganese. Cultures of wild type and the ICE mutant strains, *bfr*/ICE$_{fhuE}$ and *fhuE*/ICE$_{bfr}$, were grown in media supplemented with 20 μM $FeCl_3$ (Fe) or 50 μM $MnCl_2$ (Mn) (+) or not supplemented with metal (−). The actual Fe and Mn concentrations in the unsupplemented media were 0.3 μM and 0.4 μM, respectively. (A) Steady state levels of Irr in wild-type and the ICE mutants were detected by immunoblotting as described in Fig. 1. (B) Promoter occupancy of *bfr* (open bars) and P$_{bfr}$/ICE$_{fhuE}$ gene (closed bars). (C) Steady state transcript levels of *bfr* (open bars) and P$_{bfr}$/ICE$_{fhuE}$ gene (closed bars). (D) Promoter occupancy of *fhuE* (open bars) and P$_{fhuE}$/ICE$_{bfr}$ gene (closed bars). (E) Steady state transcript levels of the *fhuE* (open bars) and P$_{fhuE}$/ICE$_{bfr}$ genes (closed bars).

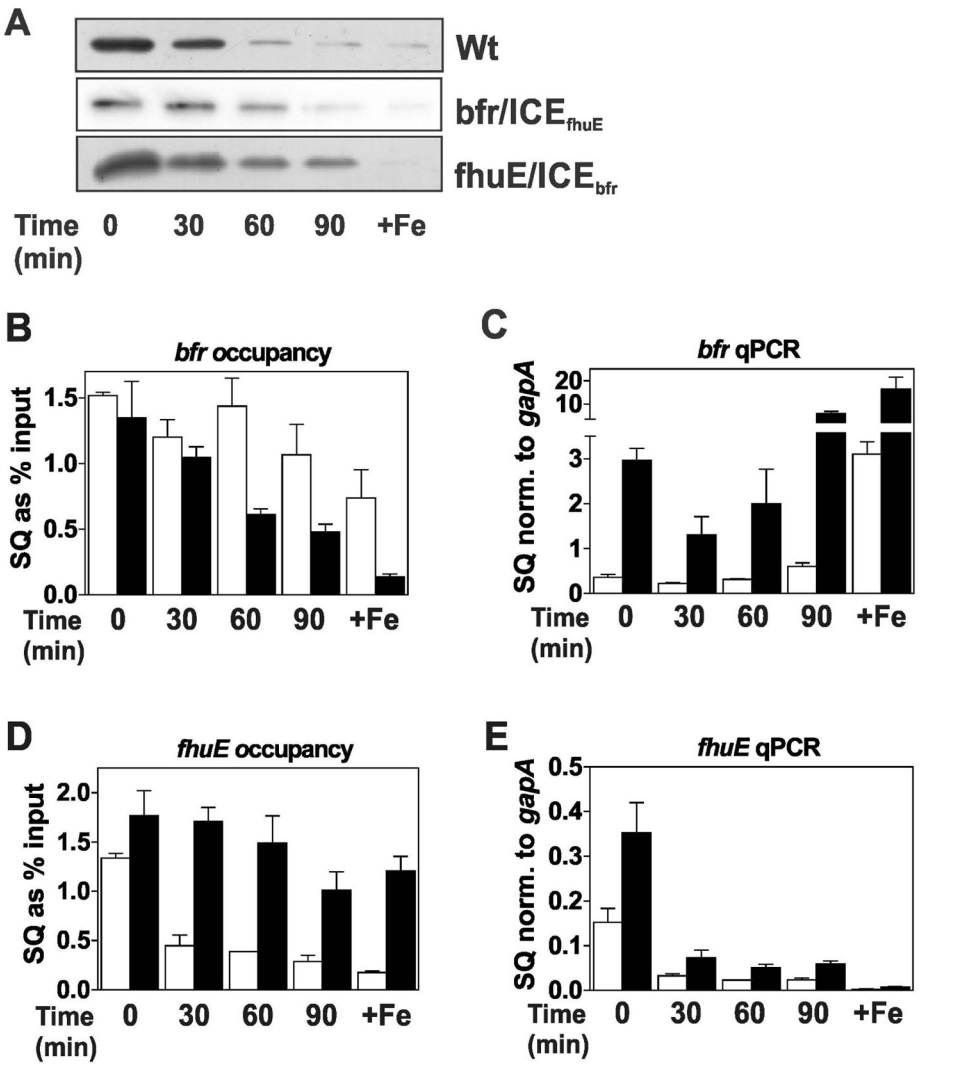

Fig. 5.

Effects of ICE motif substitutions on promoter occupancy by Irr and gene expression in response to H_2O_2. Cultures of wild type and the ICE mutant strains, *bfr*/ICE$_{fhuE}$ and *fhuE*/ICE$_{bfr}$, were grown to mid-log phase in media supplemented with 2 μM $FeCl_3$ and 50 μM $MnCl_2$. At time 0, 2 mM H_2O_2 was added to the cultures and were continually aerated at 29°C. Cells were grown in 20 μM (+Fe) without H_2O_2 treatment as a control. (A) Steady-state levels of Irr in wild-type and the ICE mutants were detected by immunoblotting as described in Fig. 1. (B) Promoter occupancy of *bfr* (open bars) and P$_{bfr}$/ICE$_{fhuE}$ gene (closed bars). (C) Steady state transcript levels of *bfr* (open bars) and P$_{bfr}$/ICE$_{fhuE}$ gene (closed bars). (D) Promoter occupancy of *fhuE* (open bars) and P$_{fhuE}$/ICE$_{bfr}$ gene (closed bars). (E) Steady state transcript levels of the *fhuE* (open bars) and P$_{fhuE}$/ICE$_{bfr}$ genes (closed bars).

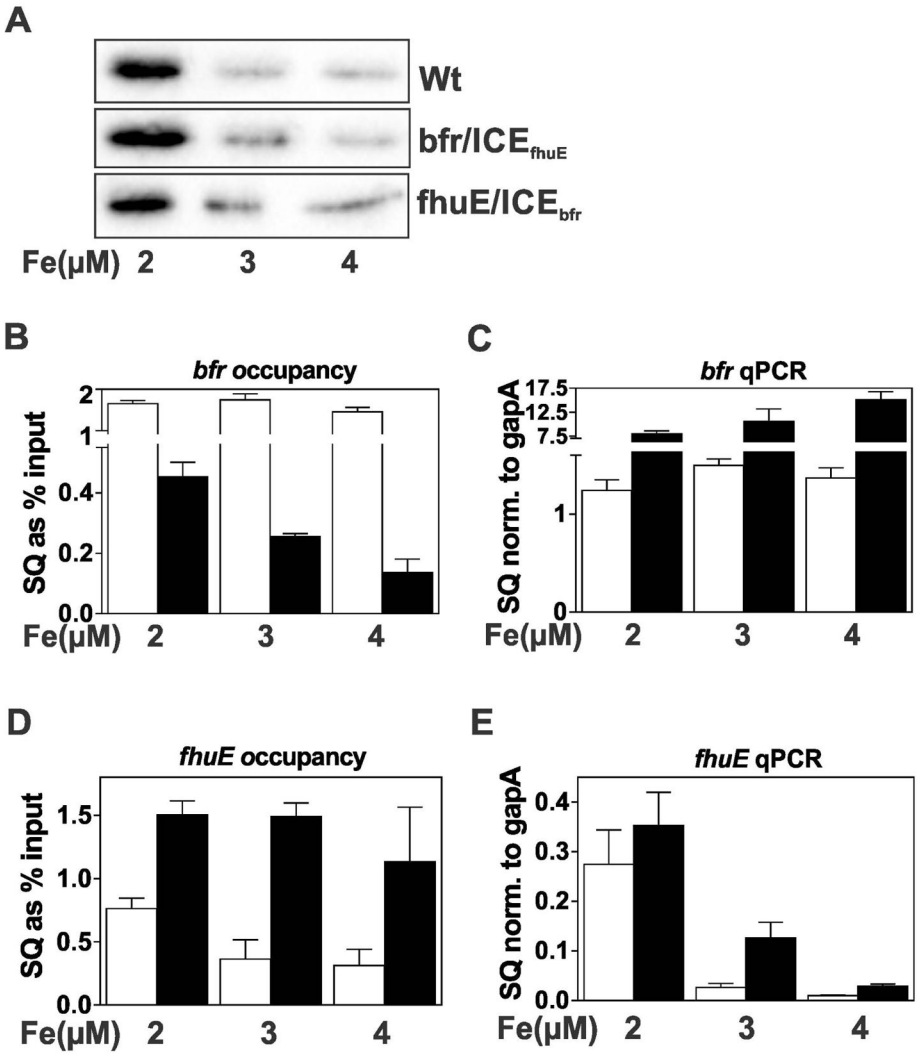

Fig. 6.

Effects of ICE motif substitutions on promoter occupancy by Irr and gene expression in response to iron. Cultures of wild type and the ICE mutant strains, *bfr*/ICE$_{fhuE}$ and *fhuE*/ICE$_{bfr}$, were grown in media supplemented with 2 μM, 3 μM or 4 μM $FeCl_3$ and with 50 μM $MnCl_2$. (A) Steady-state levels of Irr in wild type and the ICE mutants were detected by immunoblotting as described in Fig. 1. (B) Promoter occupancy of *bfr* (open bars) and P$_{bfr}$/ICE$_{fhuE}$ gene (closed bars). (C) Steady state transcript levels of *bfr* (open bars) and P$_{bfr}$/ICE$_{fhuE}$ gene (closed bars). (D) Promoter occupancy of *fhuE* (open bars) and P$_{fhuE}$/ICE$_{bfr}$ gene (closed bars). (E) Steady state transcript levels of the *fhuE* (open bars) and P$_{fhuE}$/ICE$_{bfr}$ genes (closed bars).

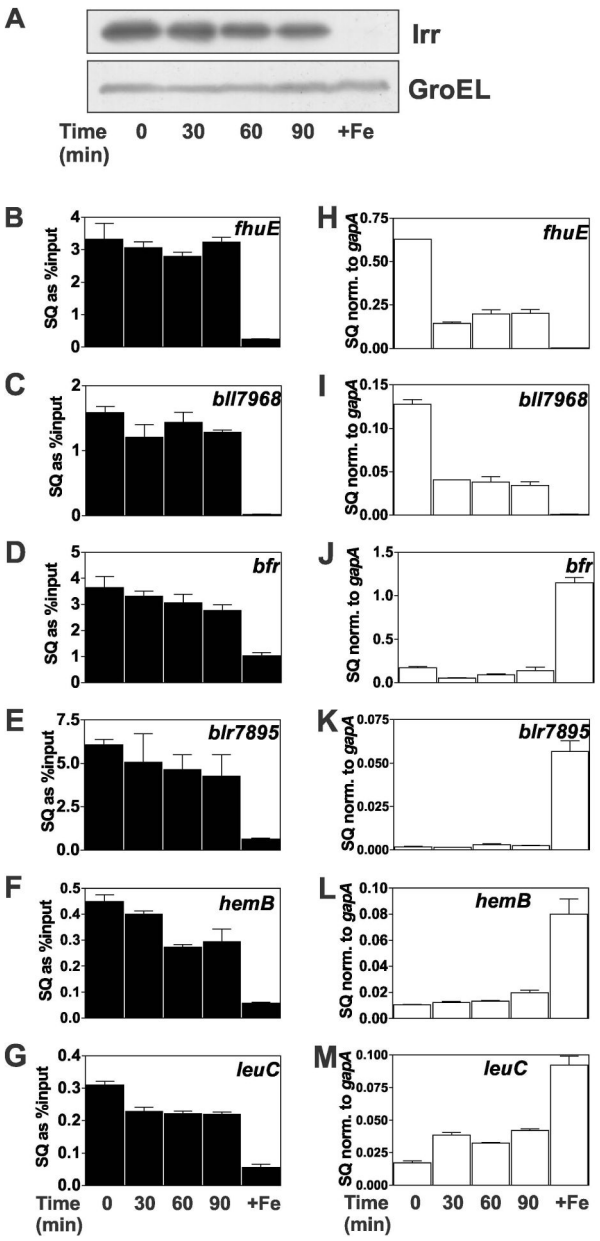

Fig. 7.

Effect of H_2O_2 on promoter occupancy by Irr and expression of Irr-regulated genes in wild type cells grown in iron-limited media. Cultures were grown to mid-log phase in media not supplemented with iron. The actual iron concentration was 0.3 μM. At time 0, 2 mM H_2O_2 was added to the cultures and were continually aerated at 29°C and harvested at the times shown. Cells were grown in 20 μM (+Fe) as a control. (A) Steady-state levels of Irr and GroEL were detected by immunoblotting as described in Fig. 1. (B–G) Promoter occupancy of respective genes by Irr in cells treated with H_2O_2 were carried out as described in the text. The data are expressed as the relative starting quantities (SQ) of immunoprecipitated DNA normalized to the input DNA and are presented as average of three replicates ± the standard deviation. (H–M) Steady-state transcript levels of the respective genes obtained from cells

treated with H_2O_2 were analyzed by qPCR. The data are expressed as relative starting quantities (SQ) of respective mRNAs normalized to the housekeeping gene *gapA*, and presented as average of three replicates ± the standard deviation.

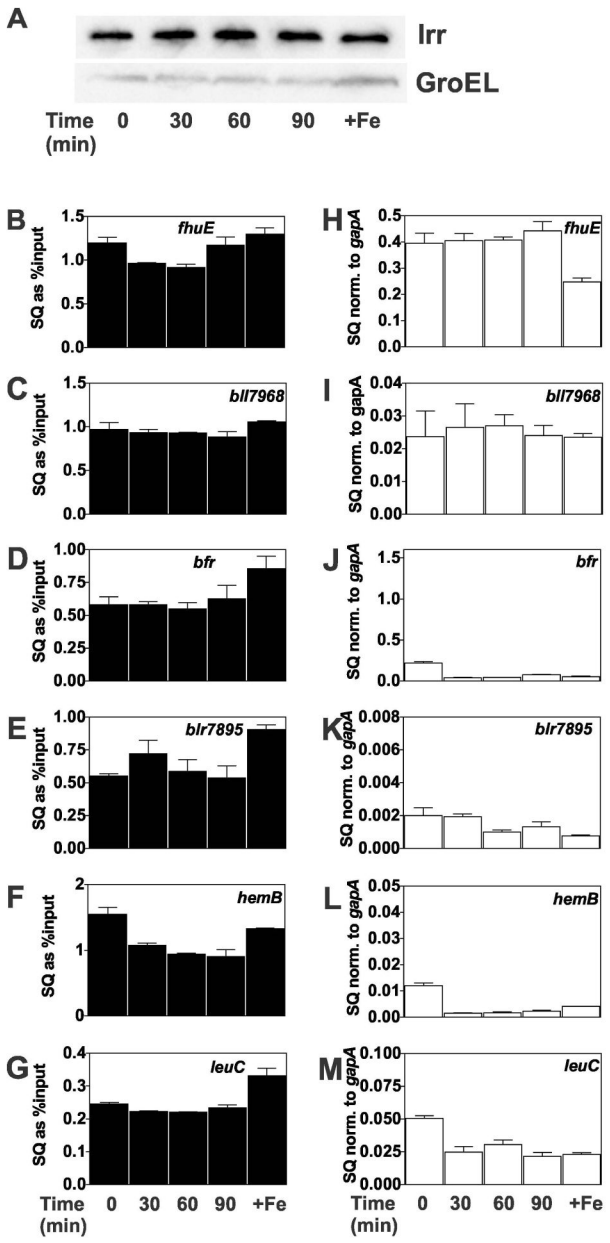

Fig. 8.

Effect of H_2O_2 on promoter occupancy by Irr and expression of Irr-regulated genes in heme-deficient strain ΔhemAH grown in iron-limited media. Cultures were grown to mid-log phase in media not supplemented with iron and supplemented with 150 nM hemin to satisfy heme auxotrophy. The actual iron concentration was 0.3 μM. At time 0, 2 mM H_2O_2 was added to the cultures and were continually aerated at 29°C, and harvested at the times shown. (A) Steady-state levels of Irr and GroEL were detected by immunoblotting as described in Fig. 1. (B–G) Promoter occupancy of respective genes by Irr in cells treated with H_2O_2 were carried out as described in the Fig. 1 legend. The data are expressed as the relative starting quantities (SQ) of immunoprecipitated DNA normalized to the input DNA and are presented as average of three replicates ± the standard deviation. (H–M) Steady state

transcript levels of the respective genes obtained from cells treated with H_2O_2 were analyzed by qPCR. The data are expressed as relative starting quantities (SQ) of respective mRNAs normalized to the housekeeping gene *gapA*, and presented as average of three replicates ± the standard deviation.

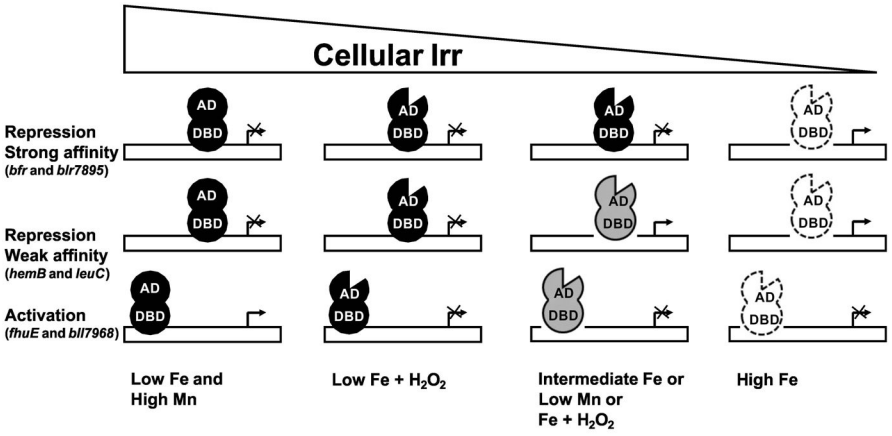

Fig. 9.

Representation of the effects of environmental factors on cellular Irr levels, promoter occupancy by Irr and gene expression. AD and DBD are activation domains and DNA-binding domains of Irr, respectively. The notch in the AD depicts heme-dependent loss of activation function of Irr. The lighter shading of Irr denotes decreasing occupancy on the Irr promoter.

TABLE 1

Dissociation binding constants (K_d values) of Irr for ICE motif sequences[a]

ICE motif	K_d (nM)
bfr	3.3 ± 0.3
blr7895	1.5 ± 0.3
fhuE	>440[b]
bll7968	>320[b]
P_{bfr}/ICE_{fhuE}	>730[b]
P_{fhuE}/ICE_{bfr}	16.3 ± 3.1
P_{7895}/ICE_{7968}	>340
P_{7968}/ICE_{7895}	4.6 ± 0.7
leuC	>1000[b]
hemB	>1000[b]
control[c]	NB[d]

[a] K_d values were determined by measuring bound and unbound ^{32}P-labeled DNA fragments 39 bp in length in EMSA analysis as described in the text.

[b] Values are approximate because binding did not saturate at 1000 nM Irr, which was the highest concentration that could be achieved in the binding reactions.

[c] The control is a 39 bp DNA sequence corresponding to the multiple cloning site of pBluescript SK+.

[d] No binding was detected.