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IscR-dependent gene expression links iron-sulphur cluster assembly to the control of O_2-regulated genes in *Escherichia coli*

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Summary

IscR is an iron-sulphur (Fe-S) cluster-containing transcription factor that represses transcription of the operon containing its own gene and the *iscSUA-hscBA-fdx* genes, whose products are involved in Fe-S cluster biogenesis. In this study, global transcriptional profiling of *Escherichia coli* IscR$^+$ and IscR$^-$ strains grown under aerobic and anaerobic conditions indicated that 40 genes in 20 predicted operons were regulated by IscR. DNase I footprinting and/or *in vitro* transcription reactions identified seven new promoters under direct IscR control. Among these were genes encoding known or proposed functions in Fe-S cluster biogenesis (*sufABCDSE*, *yadR* and *yhgI*) and Fe-S cluster-containing anaerobic respiratory enzymes (*hyaABCDEF*, *hybOABCDEFG* and *napFDAGHBC*). The finding that IscR repressed expression of the *hyaA*, *hybO* and *napF* promoters specifically under aerobic growth conditions suggests a new mechanism to explain their upregulation under anaerobic growth conditions. Phylogenetic footprinting of the DNase I protected regions of seven promoters implies that there are at least two different classes of IscR binding sites conserved among many bacteria. The findings presented here indicate a more general role of IscR in the regulation of Fe-S cluster biogenesis and that IscR contributes to the O_2 regulation of several promoters controlling the expression of anaerobic Fe-S proteins.

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Introduction

Iron-sulphur (Fe-S) clusters are cofactors for many proteins across all three branches of life. Fe-S proteins function in a number of cellular processes, including electron transfer, gene regulation, photosynthesis and nitrogen fixation, among others (reviewed in Johnson *et al*., 2005). Although Fe-S cluster formation into proteins can occur spontaneously *in vitro*, Fe-S clusters are synthesized *in vivo* by specialized Fe-S biogenesis proteins. While neither the mechanism nor the regulation of Fe-S biogenesis is well characterized, it has been established that, in *Escherichia coli*, IscR (iron-sulphur cluster regulator) represses the transcription of *iscRSUA-hscBA-fdx*, the operon encoding the housekeeping Fe-S biogenesis system (Isc) and the regulator IscR (Schwartz *et al*., 2001). Whether IscR regulates other functions involved in Fe-S biogenesis had not previously been addressed. The goal of this study was to investigate whether IscR regulates genes in addition to the *isc* operon.

The mechanism of Fe-S biogenesis is itself only partially understood (Johnson *et al*., 2005). In the housekeeping Isc system, IscS is a cysteine desulphurase that removes the sulphur from cysteine (Flint, 1996), generating an enzyme-bound persulphide, which is the sulphur donor for Fe-S cluster biogenesis (Smith *et al*., 2001; Urbina *et al*., 2001). IscU is postulated to be a scaffold upon which a transient Fe-S cluster is built and then transferred to an apo-protein (Agar *et al*., 2000; Hoff *et al*., 2000), while IscA has been proposed to serve either as an Fe-S scaffold (Krebs *et al*., 2001) or as the Fe donor for the assembly process (Ding and Clark, 2004). HscA and HscB are homologues of the protein folding chaperones DnaK and DnaJ, respectively, and have been shown to specifically interact with IscU (Hoff *et al*., 2002), perhaps to promote or stabilize a conformation suitable for assembly of an Fe-S cluster and transfer of the cluster to an apo-protein. The ferredoxin (Fdx) protein is likely involved in electron transfer, but the step at which it acts remains to be determined. In addition to the Isc system, a minor Fe-S cluster biogenesis pathway, Suf (sulfur mobilization), has been identified in *E. coli*. The Suf system is encoded by *sufABCDSE* and, like the Isc system, the Suf pathway is upregulated in response to oxidative stress and iron limitation (Patzer and Hantke,

1999; Zheng *et al*., 2001; Outten *et al*., 2004). In addition, there is some similarity among the proteins in the two pathways; the amino acid sequence of SufA is similar to IscA, and SufS exhibits cysteine desulphurase activity (Mihara *et al*., 1999), first described for IscS. A recent X-ray crystal structure of SufE (Goldsmith-Fischman *et al*., 2004), which is known to enhance the cysteine desulphurase activity of SufS (Loiseau *et al*., 2003; Outten *et al*., 2003), indicates that SufE and IscU have similar core structures even though they are not similar at the level of primary sequence. As found with HscBA (Vickery *et al*., 1997), SufBCD is an ATPase (Rangachari *et al*., 2002; Nachin *et al*., 2003), and this complex further increases the cysteine desulphurase activity of SufS in the presence of SufE (Nachin *et al*., 2003; Outten *et al*., 2003).

While the role of IscR in repressing the housekeeping Isc pathway is clearly established, how IscR activity is regulated to maintain an appropriate amount of Fe-S biogenesis is not well understood. Electron paramagnetic resonance data show that as-isolated IscR protein contains a [2Fe-2S] cluster that can be reversibly oxidized and reduced (Schwartz *et al*., 2001), a property characteristic of most Fe-S clusters. Furthermore, inactivation of the Isc Fe-S biogenesis pathway decreases repression of the *iscR* promoter (Schwartz *et al*., 2001), suggesting that the [2Fe-2S] cluster is required for IscR repressor function. Consistent with this observation, limitation of either iron (Outten *et al*., 2004) or sulphur (Gyaneshwar *et al*., 2005) also appears to derepress the *iscR* promoter. Confining the function of IscR to the Fe-S form provides an attractive feedback mechanism for Fe-S biogenesis to be sensitive to the Fe-S cluster status of cells; as previously proposed (Schwartz *et al*., 2001), when the Isc and Hsc proteins and the building blocks of Fe-S clusters (Fe^{2+}, cysteine) increase to the appropriate cellular concentrations, IscR itself will acquire an Fe-S cluster, thus enabling it to repress transcription of the *iscRSUA-hscBA-fdx* operon. This feedback mechanism may also explain the increase in transcription observed from the *iscR* promoter upon treating cells with the oxidant H_2O_2 (Zheng *et al*., 2001), as oxidant-induced damage of Fe-S clusters would be expected to increase the demand for Fe-S biogenesis and thus result in the upregulation of the *isc* operon. In agreement with this notion, the induction of the *isc* operon by H_2O_2 is dependent on IscR (Outten *et al*., 2004), and is independent of OxyR, unlike the induction of the *suf* operon (Zheng *et al*., 2001).

In this study, we investigated whether IscR regulates the expression of genes in addition to the *isc* operon. To identify additional members of the IscR regulon, we used global transcriptional profiling experiments on strains grown under aerobic or anaerobic growth conditions that contain or lack IscR. Additionally, we constructed promoter–*lacZ* fusions to identify the promoter regions responsible for the effects observed in the transcriptional profiling experiments and used *in vitro* transcription assays to test whether IscR directly affects the transcription of these promoters. Finally, binding sites for IscR were identified in seven promoters (*hyaA*, *iscR*, *napF*, *sufA*, *yadR*, *ydiU* and *yhgI*) via DNase I footprinting, and two IscR-binding motifs were constructed using both experimental and phylogenetic footprinting.

Results

Identification of IscR-regulated genes in E. coli *K-12*

Candidate IscR-regulated genes were identified by comparing the transcription profiles of strains PK6597 (an $IscR^-$ mutant containing a non-polar deletion of *iscR*) and its *E. coli* K-12 parent, MG1655. Of the transcripts that had an average signal intensity of 256 or above in the $iscR^+$ and/or $iscR^-$ strains, we identified 37 genes that showed greater than a twofold change in transcript intensity with a *P*-value (Student's *t*-test) of 0.1 or lower using cells grown under aerobic conditions (Fig. 1). (A signal intensity cut-off of 256 has been previously shown to provide sufficient separation of signal to noise; Kang *et al*., 2005.) Many of the 37 genes are in predicted (Bockhorst *et al*., 2003) or known operons, narrowing the number of candidate promoters regulated by IscR under aerobic conditions to 17. In addition, many of these operons contain genes that met some but not all of the above criteria, which, if included, would increase the total number of genes regulated by IscR under aerobic growth conditions to 62 (Fig. 1). To validate the cut-off values used in the transcriptional profiling experiments, we constructed chromosomal promoter–*lacZ* fusions to three genes with fold change values below 2 [*nikA* (–250 to –10 relative to the A of the ATG start codon), *fecA* (–200 to +40 relative to the transcription start site; Enz *et al*., 1995) and *mglB* (–200 to +40 relative to the transcription start site; Hogg *et al*., 1991), with fold change values of 1.4, 1.5 and 1.6 respectively]. None of these genes showed notable differences in β-galactosidase activity between the wild-type and Δ*iscR* strains (data not shown), indicating that our cut-off values were acceptable, assuming that the correct control regions of the promoters were assayed. The complete data set for the transcription profiling experiments is available in Table S1.

IscR regulates a diverse set of genes, including some encoding Fe-S proteins as well as genes encoding additional Fe-S biogenesis proteins

Thirty-one genes showed an increase in transcript levels in the strain lacking IscR compared with the wild-type

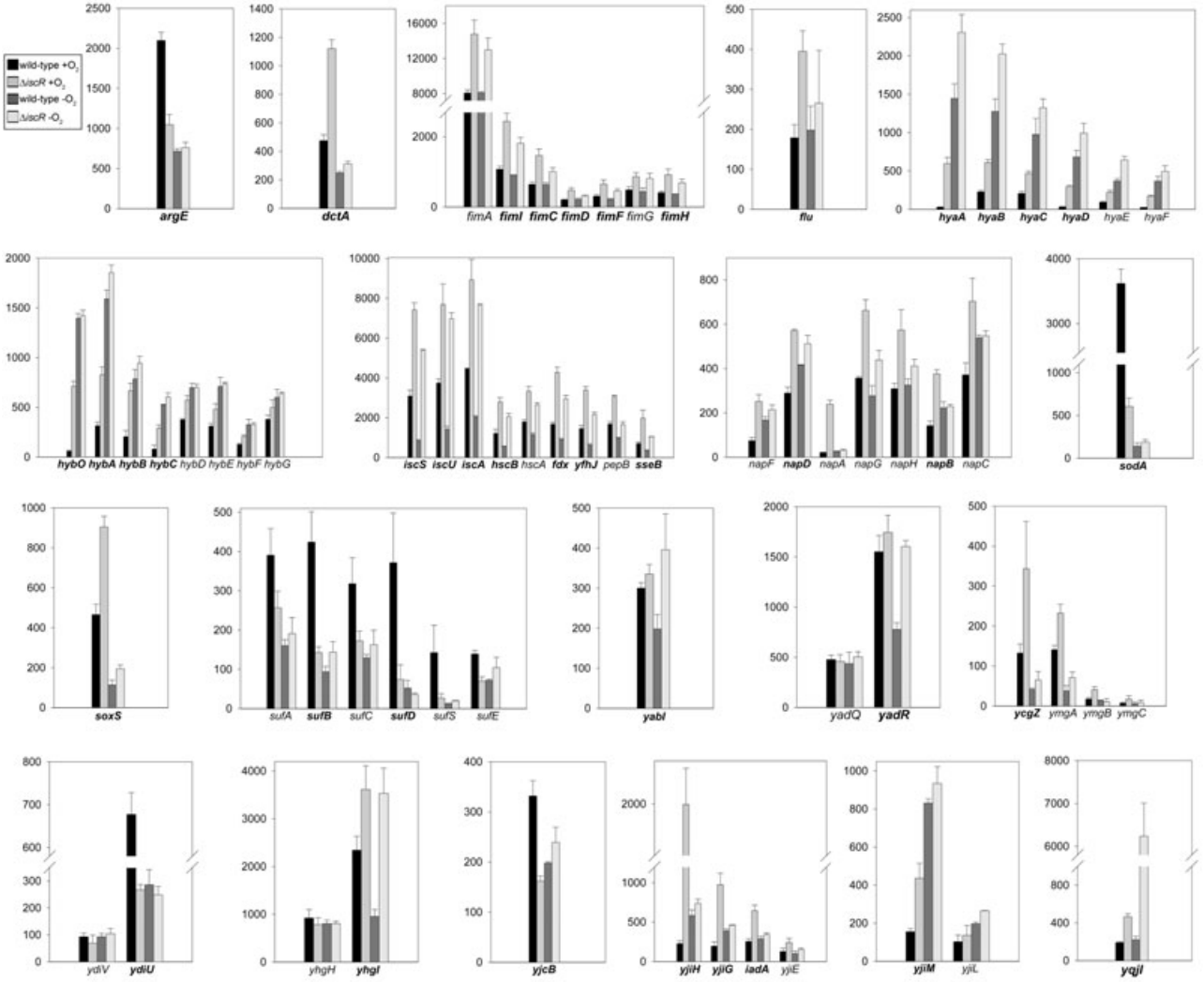

Fig. 1. Transcription profiling of IscR$^+$ and IscR$^-$ strains. Affymetrix GeneChip intensity values from wild-type (black bars) or Δ*iscR* (medium grey bars) strains grown aerobically and wild-type (dark grey bars) or Δ*iscR* (light grey bars) strains grown anaerobically. Genes are plotted as known or predicted operons (Bockhorst *et al*., 2003). Names in boldface are those that met the criteria of possessing an intensity value of 256 or greater, having a *P*-value of 0.1 or less, and having an IscR$^-$:IscR$^+$ ratio value of 2 or greater or 0.5 or less.

strain under aerobic conditions, indicating that these genes are candidates for repression by IscR. Within this group were the genes of the *isc* operon (*iscS*, *iscU*, *iscA*, *hscB* and *fdx*), which are known to be repressed by IscR (Schwartz *et al*., 2001). The *yfhJ-pepB-sseB* genes, located directly downstream of the IscR-repressed *iscSUA-hscBA-fdx* operon, also appeared to be repressed by IscR. In addition, transcript levels of *hyaABCDEF* and *hybOABCDEFG*, encoding hydrogenases-1 and -2, respectively, and *napFDAGHBC*, encoding the periplasmic nitrate reductase, were increased in the strain lacking IscR. These enzymes function in anaerobic respiration and all three utilize Fe-S clusters. Finally, IscR decreased the expression of *dctA*, encoding a C4-dicarboxylate transporter; *soxS*, encoding a transcription factor that activates genes in response to superoxide stress; *fimAICDFGH* and *flu*, two operons encoding surface structures; and several operons with genes of unknown function: *ycgZ*, the putative *yjiH-yjiG-iadA-yjiE* operon, *yjiM* and *yqjI*.

IscR also appears to increase the expression of a small subset of genes, including *sufABCDSE*; *argE*, involved in arginine biosynthesis; and *sodA*, the Mn-containing superoxide dismutase, as the transcript intensities decreased by at least twofold in this assay (Fig. 1). Finally, two genes of unknown function, *ydiU* and *yjcB*, also appeared to be activated by IscR. A subset of the candidate IscR-regulated genes were chosen for further study, and their regulation is addressed in the following sections.

IscR regulates the Suf pathway in addition to the Isc pathway

The transcriptional profiling data suggested that the alternate Fe-S cluster biogenesis system encoded by the *sufABCDSE* operon is also regulated by IscR; however, unlike the *isc* operon, the *suf* operon appears to be activated by IscR. To identify the region of the *sufA* promoter controlled by IscR, either nucleotides –393 to +90 (containing the previously described OxyR binding site located from –254 to –198; Zheng *et al*., 2001) or –200 to +40 relative to the transcription start site (lacking the OxyR binding site) was fused to *lacZ* on the chromosome, and β-galactosidase activity was assayed from cells grown under aerobic conditions. Expression of both of the P*sufA*–*lacZ* constructs increased about 2.5-fold when IscR is present, which was similar to the fold change values from the transcriptional profiling data (Table 1, Fig. 1). Thus, these data localized the IscR regulatory region to a site downstream of –200 and showed that the effect was independent of OxyR.

To show that the IscR effect was direct, *in vitro* transcription reactions were carried out with purified RNA polymerase and a plasmid containing the –200 to +40 *sufA* promoter region. The transcript initiating from the *sufA* promoter increased by four- to sixfold when IscR was added to the reaction, demonstrating that IscR directly activates transcription of the *sufA* promoter (Fig. 2A). Similar results were obtained with the –393 to +90 *sufA* promoter construct (data not shown). The increase in transcription was specific for the *sufA* promoter as no effect of IscR addition was observed for the control RNA-1 promoter. Using DNase I footprinting, an IscR binding site was identified upstream of the *sufA* promoter, spanning nucleotides –57 to –28 relative to the transcription start site (Figs 3A and 4A), with a weakly hypersensitive site at –57. This protected sequence is important for IscR function as deletion of nucleotides –56 to –51 from the *sufA* promoter region eliminated IscR-dependent transcription activation when assayed either by *in vitro* transcription reactions (data not shown) or by *in vivo* promoter *lacZ* fusions (Table 1). In addition, DNA binding was no longer detectable when bases –56 to –51 of *sufA* promoter were deleted (data not shown), indicating that these sequences are necessary for IscR binding.

Table 1. Identification of promoter regions regulated by IscR under aerobic and anaerobic conditions.

	Aerobic[a]			Anaerobic[a]		
Promoter	Wild type (Miller units)	Δ*iscR* (Miller units)	Fold change[b]	Wild type (Miller units)	Δ*iscR* (Miller units)	Fold change[b]
Anaerobically induced						
dmsA	12 ± 0.5	15 ± 0.5	1.3	400 ± 19	440 ± 23	1.1
fdnG[c]	8.0 ± 0.4	16 ± 0.4	2.0	430 ± 61	540 ± 34	1.2
frdA	7.0 ± 0.4	8.0 ± 0.3	1.1	35 ± 0.5	37 ± 1.0	1.1
hyaA	12 ± 1.0	480 ± 29	40	850 ± 63	890 ± 100	1.0
hybO	8.0 ± 0.2	21 ± 0.7	2.6	130 ± 2.0	130 ± 3.0	1.1
napF	10 ± 0.8	100 ± 9.0	10	140 ± 18	170 ± 22	1.3
narG[c]	15 ± 0.8	24 ± 1.0	1.6	490 ± 20	580 ± 30	1.2
Fe-S biogenesis						
iscR	1300 ± 61	7500 ± 250	5.6	280 ± 10	9300 ± 230	33
sufA[d]	120 ± 3.0	47 ± 3.0	0.4	83 ± 6.0	53 ± 5.0	0.6
sufA[e]	490 ± 39	200 ± 19	0.4	310 ± 19	200 ± 5.0	0.7
$sufA_{\Delta}$[f]	36 ± 2.0	34 ± 3.0	0.9	49 ± 1.0	38 ± 1.0	0.8
yadR	1700 ± 77	1900 ± 170	1.1	1250 ± 28	2800 ± 77	2.2
yhgI	380 ± 2.6	620 ± 26	1.6	240 ± 5.3	870 ± 25	3.6
Oxidative stress						
sodA	1400 ± 48	310 ± 11	0.2	560 ± 32	120 ± 7.0	0.2
soxS	81 ± 1.0	160 ± 2.0	2.0	38 ± 1.0	43 ± 3.0	1.1
Unknown function						
ydiU	280 ± 19	65 ± 6.7	0.2	200 ± 14	70 ± 3.2	0.4
yjiH	4.0 ± 0.4	41 ± 1.0	10	29 ± 4.0	67 ± 5.0	2.3
yqjI	61 ± 2.0	89 ± 5.0	1.5	63 ± 6.0	1900 ± 120	31

a. Expression levels of chromosomal promoter–*lacZ* fusions as measured via β-galactosidase assays on wild-type and Δ*iscR* strains grown under aerobic and anaerobic conditions in MOPS minimal medium with 0.2% glucose. The activity, expressed in Miller units, represents the average activity of three independently isolated strains performed in triplicate. Strains with no promoter fused to *lacZ* grown under aerobic and anaerobic conditions gave 4 and 3 Miller units respectively.
b. Represents the Miller units from the Δ*iscR* strain divided by those of the wild-type strain.
c. Cultures also contained a final concentration of 40 mM KNO_3.
d. Promoter spans –200 to +40 relative to the transcription start site of *sufA*.
e. Promoter spans –393 to +90 relative to the transcription start site of *sufA*.
f. Promoter spans –200 to +40 relative to the transcription start site of *sufA*; has bases –56 to –51 deleted.

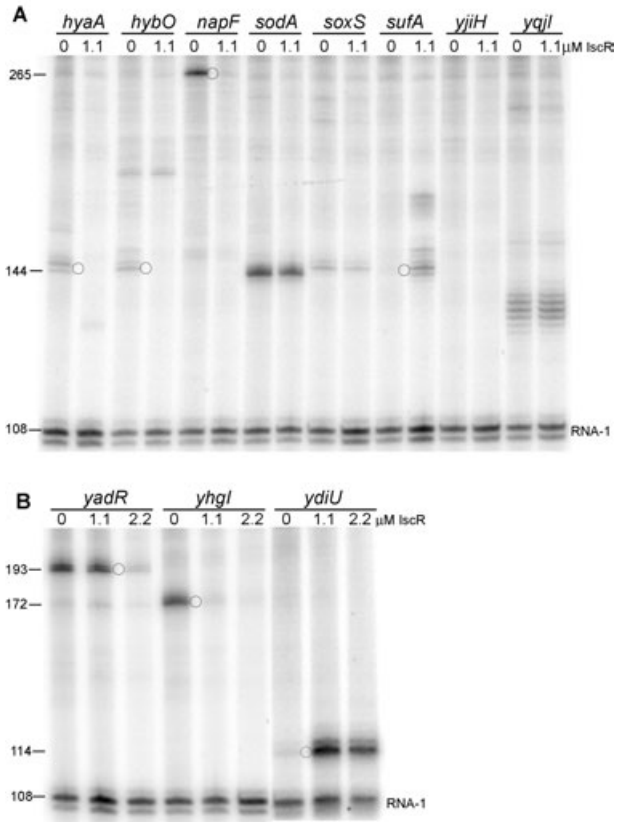

Fig. 2. IscR directly regulates a number of promoters, as shown by *in vitro* transcription assays. Reactions contained plasmids (2 nM) containing the promoter of interest (A: *hyaA*, *hybO*, *napF*, *sodA*, *soxS*, *sufA*, *yjiH* and *yqjI*, and B: *yadR*, *yhgI* and *ydiU*), $E\sigma^{70}$ RNA polymerase, and where indicated, 1.1 or 2.2 μM IscR protein. Open circles indicate the transcripts regulated by IscR, numbers indicate the size of the transcripts in nucleotides and transcripts from the control RNA-1 promoter are indicated.

under anaerobic conditions (Choe and Reznikoff, 1993; Brondsted and Atlung, 1994; Darwin and Stewart, 1995; Richard *et al*., 1999). To isolate the regions of the *hyaA*, *hybO* and *napF* promoters that are subject to IscR regulation, bases –200 to +40 relative to the previously mapped transcription start sites (Richard *et al*., 1999) were fused to *lacZ* on the chromosome, and β-galactosidase activity was measured from cells grown aerobically (Table 1). In agreement with the transcriptional profiling data, IscR decreased expression of the *hyaA*, *hybO* and *napF* promoters. However, although the β-galactosidase assays indicated that *hybO* is repressed by IscR, the magnitude of the regulation for the *hybO* promoter was lower than that observed when measuring transcript abundance via microarrays. We eliminated the possibility that this difference arose from differences in methods of culture aeration as the same amount of β-galactosidase in cells was obtained when cells were aerated by shaking versus aerated by sparging with a gas mixture of 25% O_2, 70% N_2 and 5% CO_2 (data not shown).

As the *hya*, *hyb* and *nap* operons are known to be upregulated under anaerobic conditions, we also examined whether IscR had any effect under anaerobic growth conditions (Table 1). Expression of *hyaA*, *hybO* and *napF* did not appear to be repressed by IscR under anaerobic conditions as the amount of β-galactosidase activity was similar in strains containing or lacking IscR, suggesting that IscR might be an aerobic-specific repressor. However, the possibility that IscR is non-functional in the absence of O_2 was excluded as IscR-dependent repression of the *iscR* promoter was detectable anaerobically and, in fact, was enhanced under anaerobic growth conditions (Fig. 1, Table 1).

Transcription of a subset of anaerobically induced genes is repressed by IscR under aerobic conditions

Unexpectedly, the transcriptional profiling data indicated that three operons, *hyaABCDEF*, *hybOABCDEFG* and *napFDAGHBC*, encoding respiratory enzymes that are known to contain Fe-S clusters and are active only under anaerobic conditions, were derepressed under aerobic conditions in the absence of IscR. Hydrogenase-1 and -2 are encoded by the *hyaABCDEF* and *hybOABCDEFG* operons, respectively, and are membrane-bound proteins that enable cells to use H_2 as an energy source, coupling its oxidation to the donation of electrons to anaerobic electron acceptors. The *napFDAGHBC* operon encodes a periplasmic nitrate reductase, which catalyses the reduction of nitrate to nitrite under anaerobic conditions.

The finding that IscR represses the expression of the *hya*, *hyb* and *nap* operons under aerobic conditions was surprising as previous work indicated that the regulation of these genes by O_2 resulted from specific activation

IscR does not repress expression of all anaerobic Fe-S respiratory enzymes

We considered the possibility that transcriptional repression of the *hyaA*, *hybO* and *napF* promoters by IscR under aerobic conditions may be a general mechanism to prevent inappropriate Fe-S cluster insertion into anaerobic Fe-S respiratory enzymes. As the promoters of other anaerobically induced Fe-S enzymes (*frd*, *fdnG*, *narG*, *dmsA*, driving the expression of operons encoding fumarate reductase, formate dehydrogenase, nitrate reductase and DMSO reductase respectively) are known to be poorly expressed under aerobic conditions and were below the limits of detection in the microarray experiments, we tested whether any of these other anaerobic respiratory enzymes were also controlled by IscR using the more sensitive β-galactosidase assay. Strains containing promoter–*lacZ* fusions for the *frd*, *fdnG*, *narG* and *dmsA* promoters and isogenic Δ*iscR* strains were grown aerobically and anaerobically (with 40 mM potassium

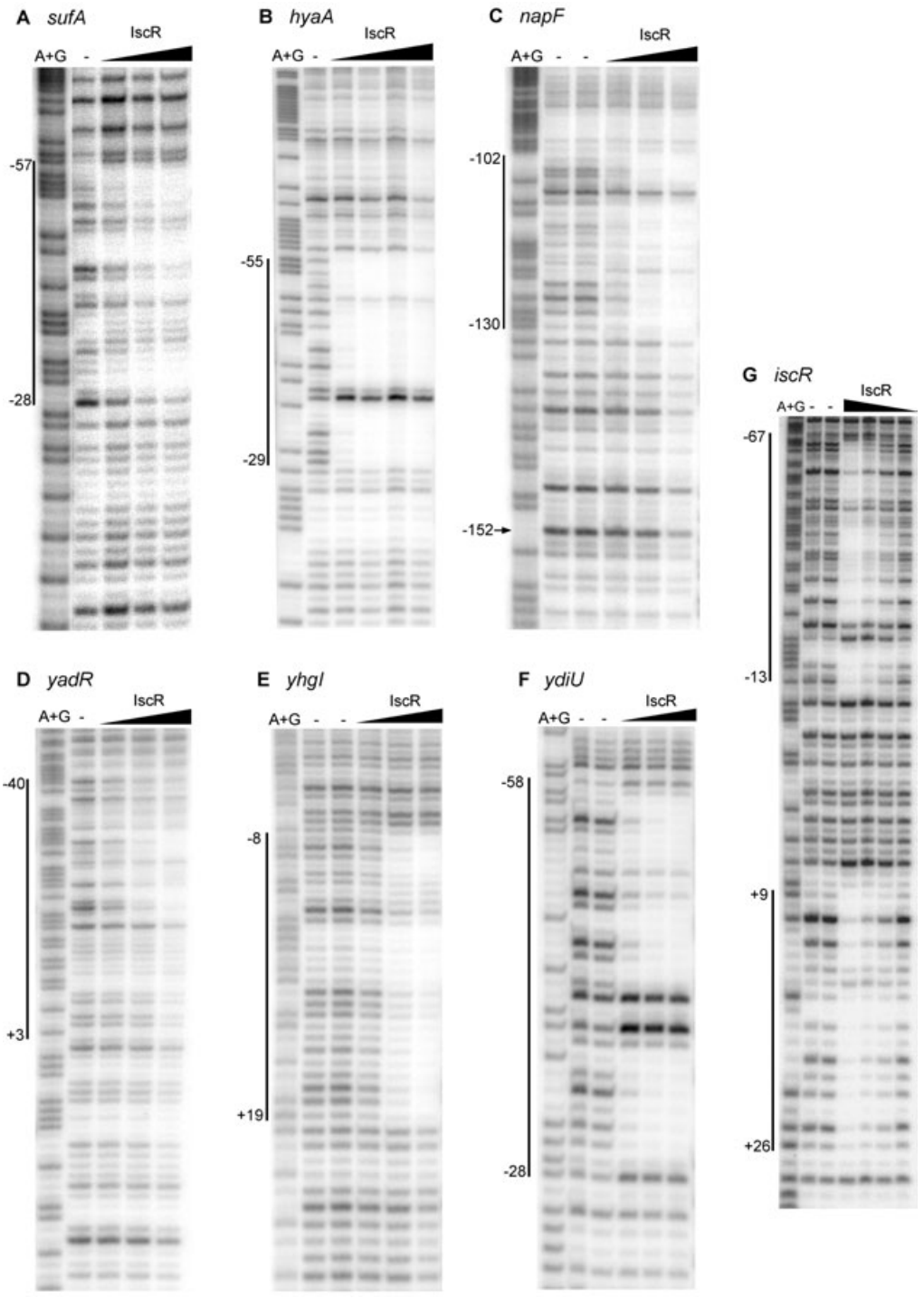

Fig. 3. Identification of IscR binding sites via DNase I footprinting. The regions protected by IscR are indicated with vertical lines and numbers indicate the distance relative to the transcription start site. Samples were electrophoresed with Maxam–Gilbert ladders (A + G) made using the same DNA. IscR protein concentrations are given from left to right in terms of nM total protein.
A. Top strand of the *sufA* promoter. IscR: 569, 853 and 1137 nM.
B. Top strand of the *hyaA* promoter. IscR: 125, 250, 500 and 1000 nM.
C. Bottom strand of the *napF* promoter. IscR: 250, 500 and 1000 nM.
D. Top strand of the *yadR* promoter. IscR: 250, 500 and 1000 nM.
E. Top strand of the *yhgI* promoter. IscR: 250, 500 and 1000 nM.
F. Top strand of the *ydiU* promoter. IscR: 250, 500 and 1000 nM.
G. Top strand of the *iscR* promoter. IscR: 1509, 1132, 753 and 377 nM.

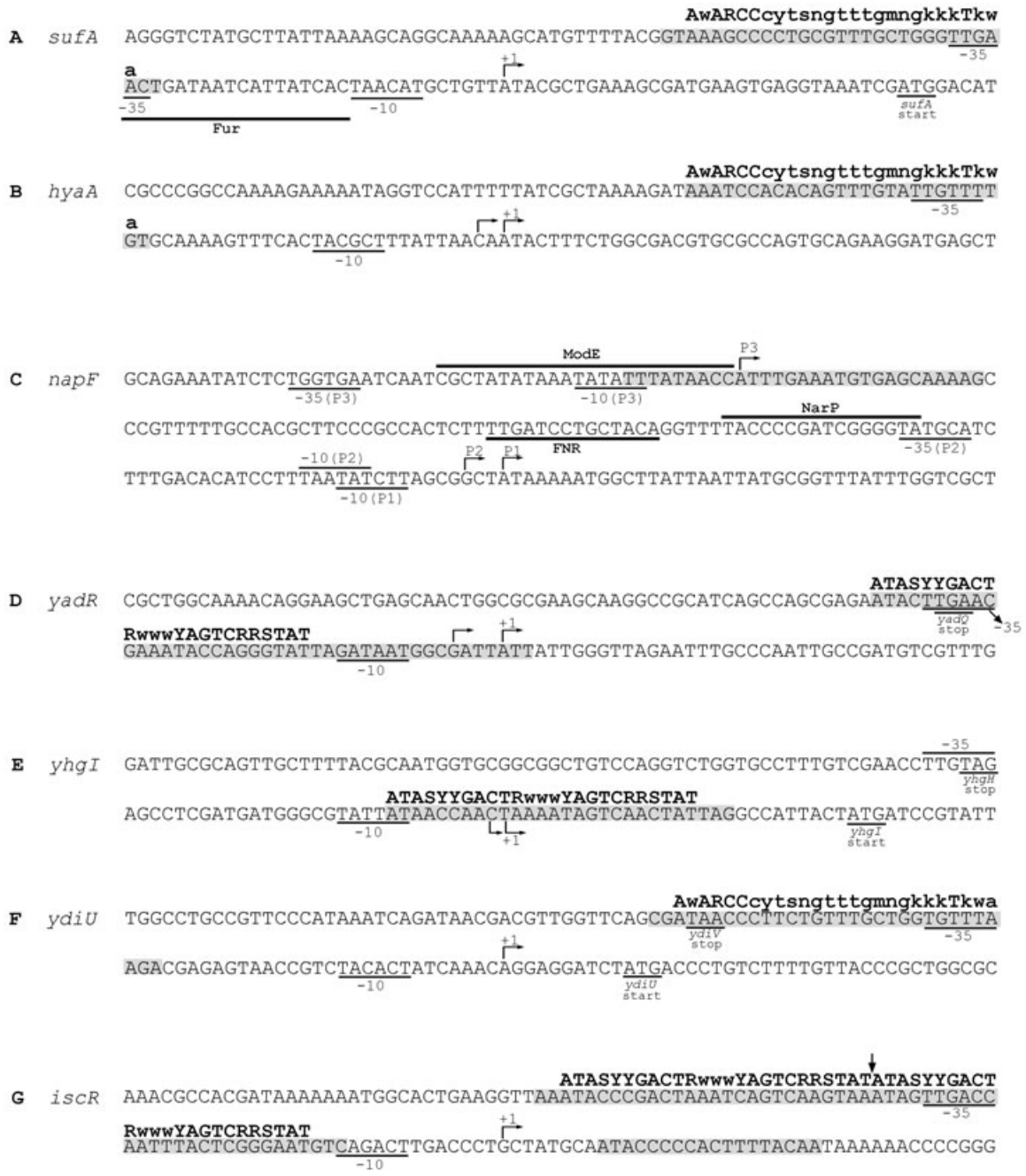

Fig. 4. Promoter regions under direct IscR control. Nucleotides protected by IscR in the DNase I footprinting experiments (Fig. 3) are shaded, IscR-binding motifs (see Fig. 6) appear in bold, arrows indicate transcription initiation sites, and start and stop codons, where applicable, are indicated by underlines as are putative −10 and −35 hexamers.

A. Nucleotides −100 to +40 relative to the transcription start site (Zheng *et al*., 2001) of the *sufA* promoter with the Fur binding site (Outten *et al*., 2004) underlined. (The IHF and OxyR binding sites lie outside this region and are not depicted).

B. Nucleotides −100 to +40 relative to the A transcription start site (Richard *et al*., 1999) of the *hyaA* promoter.

C. Nucleotides −170 to +40 relative to the A (P1) transcription start site (Stewart *et al*., 2003) of the *napF* promoter. NarP (Darwin and Stewart, 1995), FNR (Darwin *et al*., 1998) and ModE (McNicholas and Gunsalus, 2002) binding sites are over- or underlined. The P3 transcription start site was determined by primer extension assays (Fig. 5A).

D. Nucleotides −100 to +40 relative to the A transcription start site of the *yadR* promoter, which was determined by primer extension assays (Fig. 5B).

E. Nucleotides −100 to +40 relative to the T transcription start site of the *yhgI* promoter, which was determined by primer extension assays (Fig. 5C).

F. Nucleotides −100 to +40 relative to the transcription start site of the *ydiU* promoter, which was determined by primer extension assays (Fig. 5D).

G. Nucleotides −100 to +40 of the *iscR* promoter (Schwartz *et al*., 2001). The vertical arrow denotes the boundary of the tandem Isc-R-binding motifs.

nitrate for the *fdnG* and *narG* promoter–*lacZ* strains), and β-galactosidase activity was assayed (Table 1). Little to no change in expression between the wild-type and Δ*iscR* strains was detected for any of these promoters under either aerobic or anaerobic growth conditions. Thus, IscR does not regulate the expression of all anaerobic Fe-S containing respiratory enzymes.

IscR directly represses hyaA, hybO *and* napF *transcription*

To test whether the effect of IscR at the *hyaA*, *hybO* and *napF* promoters was direct, *in vitro* transcription assays were performed (Fig. 2A). Addition of IscR led to a decrease in transcript levels from the previously characterized *hyaA* and *hybO* transcription start sites, indicating that IscR directly repressed the known *hyaA* and *hybO* promoters. In contrast, for the *napF* promoter, we found that IscR repressed expression of a novel transcript initiating upstream of the previously identified transcription initiation sites at +1 (P1) and –3 (P2) (Choe and Reznikoff, 1993; Stewart *et al*., 2003) in the *napF* promoter region. The new start site (P3) was identified by primer extension assays of *in vitro* generated RNA, which showed the initiation site mapped at an A located 121 nucleotides upstream of the previously identified P1 transcriptional start site (Fig. 5A). Transcripts originating from P1 and P2 were present at such low levels in the absence of additional factors, as has been previously reported (Darwin *et al*., 1998), that it could not be determined whether IscR repressed these transcripts. Thus, two possible mechanisms have emerged to explain the O_2 and IscR regulation of the *hyaA*, *hybO* and *napF* promoters: in the case of the *hyaA* and *hybO* promoters, IscR represses expression of the previously known O_2-regulated promoters, but in the case of *napF*, a previously unidentified promoter is subject to regulation by IscR.

To identify the location of the IscR binding sites, DNase I footprinting was performed on the *hyaA* and *napF* promoters. For the *hyaA* promoter, IscR protected a region from –55 to –29 relative to the transcription start site with a hypersensitive site at –36, indicating a bend or structural change in the DNA (Figs 3B and 4B). IscR may act by preventing RNA polymerase from binding to the –35 hexamer and/or by blocking the binding site of other known activators such as ArcA and AppY (Brondsted and Atlung, 1994; Richard *et al*., 1999). At the *napF* promoter, IscR bound a sequence from –130 to –102 relative to the previously identified P1 transcription start site (–19 to +19 relative to the novel P3 *napF* transcription initiation site) with a hypersensitive site at –122, and at higher protein concentrations, partial protection from –152 to –102 was observed (Figs 3C and 4C). In this case, it seems likely that IscR may act by blocking RNA polymerase from binding to the promoter elements of the novel start site or from clearing the promoter region.

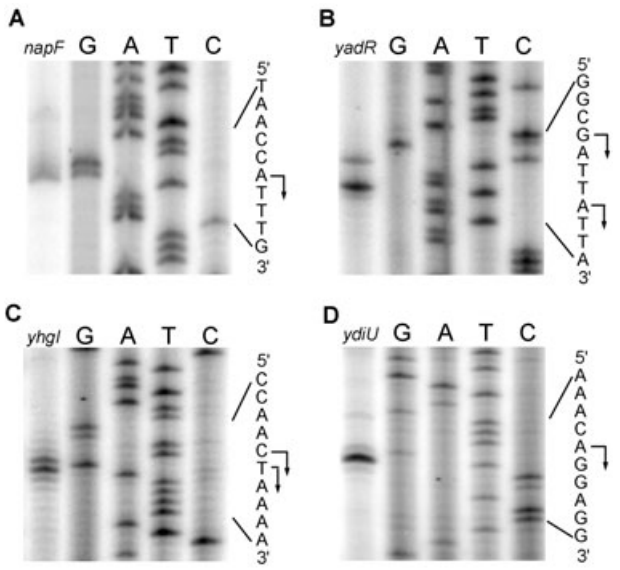

Fig. 5. Identification of transcription start sites using primer extension of *in vitro* transcribed RNA. Samples were electrophoresed with sequence markers generated using the fmol Cycle Sequencing System (Promega). Primer extension of the transcription start sites was performed for the (A) *napF*, (B) *yadR*, (C) *yhgI* and (D) *ydiU* regions.

Connection of other O_2-regulated genes to the IscR regulon

In addition to the *hya*, *hyb* and *nap* operons, a recent global gene expression study (Kang *et al*., 2005) indicates that the expression of other candidate promoters identified in this study (*flu*, *sufABCDSE*, *sodA*, *yjiH* and *fimICDFGH*) is also regulated by O_2. To determine whether IscR had an effect on expression of these genes under anaerobic growth conditions, global transcriptional profiling experiments were carried out on strains grown anaerobically (Fig. 1). The only genes that appeared to be regulated by IscR under both aerobic and anaerobic conditions were *iscSUA-hscBA-fdx-yfhJ-sseB*, *fimIF* and *yqjI*. In contrast, some genes (*argE*, *dctA*, *flu*, *ydiU*, *yjiH*, *yjiM*) were regulated like the *hya*, *hyb* and *nap* operons in that they showed no significant IscR-dependent changes in expression under anaerobic conditions. In addition, we found that under anaerobic conditions, expression of some operons (*sodA*, *soxS*, *sufABCDSE*, *ycgZ*, *yjcB*) was decreased relative to aerobic conditions, but regulation by IscR could not be determined as expression levels of these operons in both strains were below the limit of detection. However, three new genes of unknown function (*yabI*, *yadR* and *yhgI*) were identified as candidates for repression by IscR specifically under anaerobic conditions. YabI is a predicted inner membrane protein, while both YadR and YhgI are in the HesB/YadR/YfhF protein family (Dombrecht *et al*.,

2002) of which IscA (formerly known as YfhF) is also a member. Complementation studies have also implicated YhgI (also called GntY) and YhgH (GntX), encoded directly upstream of *yhgI*, in gluconate uptake (Porco *et al*., 1998); however, a direct role for YhgI alone in this process was not tested.

IscR directly represses yadR *and* yhgI*, predicted Fe-S biogenesis proteins*

Promoter–*lacZ* fusions were constructed for *yadR* and *yhgI*, and assays of β-galactosidase activity (Table 1) indicated that IscR decreased expression of these genes only under anaerobic growth conditions as was found with the transcriptional profiling experiments. *In vitro* transcription assays showed that IscR directly repressed transcription from the same promoter regions of *yadR* and *yhgI* that were fused to *lacZ* (Fig. 2B). Primer extension assays of *in vitro* transcribed RNA (Fig. 5B and C) demonstrated that transcription of *yadR* initiates at an A (major band) and G (minor band) located 49 and 53 bp upstream of the *yadR* start codon, respectively, and transcription of *yhgI* initiates at a C and a T located 29 and 28 bp away, respectively, from the *yhgI* start codon. As these transcripts from each promoter are very similar in size, we were unable to distinguish any differences in IscR repression on the major and minor initiation sites in the *in vitro* transcription assays. DNase I footprinting localized the IscR binding sites to –40 to +3 nucleotides relative to the A transcription start site of the *yadR* promoter (Figs 3D and 4D) and to –8 to +19 nucleotides relative to the T transcription start site of the *yhgI* promoter (Figs 3E and 4E). Given the location of the IscR binding sites in the *yadR* and *yhgI* promoters, IscR likely interferes with binding of RNA polymerase.

IscR decreased expression of soxS*, encoding a general regulator of oxidative stress, but increased expression of a superoxide dismutase*

The levels of transcription of two genes involved in the response to oxidative stress also were affected by IscR. Expression of *soxS*, encoding a transcription factor that upregulates a number of genes in response to superoxide stress, was measured via β-galactosidase assays in wild-type and Δ*iscR* strains (Table 1). IscR decreased expression of *soxS* twofold under aerobic conditions but had no effect under anaerobic conditions, which paralleled the results of the transcriptional profiling experiments (Fig. 1). However, the physiological relevance of the increase in *soxS* expression in strains lacking IscR is questionable as no corresponding increase in SoxS target gene expression was observed in the strain lacking IscR. In fact, one SoxS target, *sodA*, encoding the Mn-containing superoxide dismutase, showed a decrease in expression. Additionally, *in vitro* transcription assays showed no effect of IscR addition on *soxS* transcription even when IscR was added to 2.2 μM (Fig. 2A, data not shown), suggesting that IscR may not directly regulate the *soxS* promoter. As SoxR, the transcription factor that activates *soxS* under superoxide stress, is an Fe-S protein, this small change in *soxS* expression may reflect an indirect effect on SoxR function. A similar analysis of the *sodA* promoter indicated that IscR increased expression from a *sodA* promoter–*lacZ* fusion *in vivo*, but like the *soxS* promoter, the *sodA* promoter exhibited no change in *in vitro* transcript levels even upon addition of IscR to 2.2 μM (Fig. 2A, data not shown), indicating that IscR regulation may be indirect or that an additional *sodA* transcription factor (Compan and Touati, 1993) is necessary to observe an effect of IscR *in vitro*.

IscR directly activates ydiU*, a gene of unknown function*

We examined the effect of IscR on *ydiU* expression as, like the *sufA* promoter, the transcription profiling data suggested that IscR activates this gene. Using a promoter–*lacZ* fusion, we found that IscR increased expression of *ydiU* under both aerobic and anaerobic conditions (Table 1). *In vitro* transcription assays showed that IscR directly activates transcription of *ydiU* (Fig. 2B), and primer extension assays identified the transcription start site 10 nucleotides upstream of the ATG start codon (Fig. 5D). DNase I footprinting indicates that IscR binds a region located from –58 to –28 relative to the transcription start site (Figs 3F and 4F), a region with similar spacing to that of the IscR binding site in the *sufA* promoter. Hypersensitive sites located at –58, –37 and –28 also indicate a structural change in the DNA upon binding of IscR.

Other promoters repressed by IscR in vivo

We also examined whether the promoters for *yqjI* and the putative *yjiH-yjiG-iadA-yjiE* operons were directly repressed by IscR. In both cases, the promoter region fused to *lacZ* showed IscR-dependent repression when assayed *in vivo* (Table 1). However, when the same promoters were tested by *in vitro* transcription assays, no effect of IscR was observed even at IscR protein concentrations of 2.2 μM (Fig. 2A, data not shown); a transcript from *yjiH* was not even detected in this assay despite the fact that the control RNA-1 transcript was present.

Identification of DNA binding sites for IscR

To determine whether there is a common DNA sequence required for IscR binding, DNase I footprinting assays

were also performed at the *iscR* promoter (Fig. 3G). The addition of IscR revealed two regions of protection from DNase I, extending from bases –67 to –13 and bases +9 to +26 relative to the start site of transcription (Fig. 4G); reactions performed on both strands of promoter DNA gave similar results (Fig. 3G, data not shown). A DNase I hypersensitive band was observed at –21 in the *iscR* promoter, indicative of a DNA bend or other structural change dependent on IscR binding. The upstream protected region encompassed 55 bp, which is larger than the sites protected in the *hyaA*, *napF*, *sufA*, *yadR*, *ydiU* and *yhgI* promoters. In addition, the presence of two well-defined regions separated by roughly 20 bp suggests that multiple oligomers of IscR may bind to the *iscR* promoter. While two related inverted repeats could be identified in the upstream region (from –67 to –13), no similarity between the sequence of the downstream and upstream region was observed. Despite this difference in DNA sequences, the occupancy of the upstream (–67 to –13) sites was the same as the downstream (+9 to +26) site upon titration of IscR, indicating either that these sites bind IscR equally well or that binding is cooperative. Furthermore, alignment of the sequences that were protected by IscR in the additional six promoter regions did not reveal a highly conserved inverted repeat sequence typical of sites recognized by helix–turn–helix DNA-binding domains, a motif also predicted to be in IscR. Thus, we used a computational approach guided by the DNA footprinting results to identify sequences recognized by IscR.

A phylogenetic footprinting approach (Florea *et al*., 2003) was used to define the level of conservation of IscR-protected regions in related bacteria and to define a conserved IscR-binding motif. For this purpose, the sets of multiple sequence alignments of orthologous upstream regions were generated by CLUSTALX (Chenna *et al*., 2003). The IscR-protected regions showed generally higher conservation than surrounding regions and contain runs of exact matches (Fig. S1). An iterative signal detection procedure implemented in the program SIGNALX (Gelfand *et al*., 2000) was used to identify a common IscR DNA-binding motif. The training set included both IscR-protected regions in promoters of seven genes (*hyaA*, *iscR*, *napF*, *sufA*, *yadR*, *ydiU* and *yhgI*) from *E. coli* and additional orthologous upstream regions from various bacteria that were found to be conserved by phylogenetic footprinting. All analysed species of Enterobacteria possess an IscR orthologue with the same $CysX_{5-7}CysX_{5-9}Cys$ motif that is present in *E. coli* IscR. The highly conserved sequence motif (Type 1) with consensus ATA-SYYGACTRwwwYAGTCRRSTAT, and inverted repeat symmetry was identified for the *iscR*, *yadR* and *yhgI* promoter regions (Fig. 6A). A string of two such palindromic sites were located in the *iscR* promoter within the upstream IscR-protected region (from –65 to –41 and –40 to –16) and both of them were highly conserved in 10 other species of Enterobacteria (Fig. S1). The downstream protected site in the *iscR* promoter (from +9 to +26) does not contain this motif and is not well conserved in other bacteria (Fig. S1). On the other hand, like the upstream sites in the *iscR* promoter, the IscR sites in the *yadR* and *yhgI* promoter regions also showed a high conservation across Enterobacteria. Interestingly, all of these genes are repressed by IscR and are known or implicated in Fe-S biogenesis.

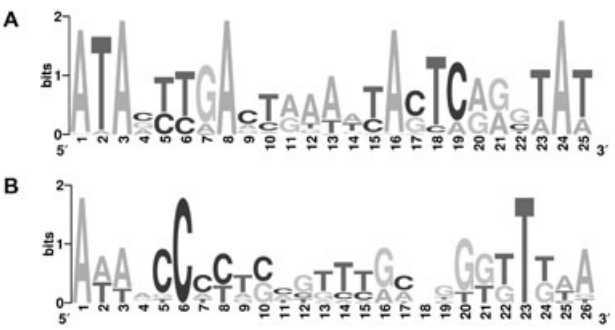

Fig. 6. Sequence logos for the IscR binding sites identified for (A) the *iscR*, *yhgI* and *yadR* genes (Type 1); and for (B) the *sufA*, *ydiU* and *hyaA* genes (Type 2) from *E. coli* and related Enterobacteria generated using the SIGNALX program and WebLogo package. R and Y are puRine and pYrimidine, respectively; S is G or C; W is A or T; K is T or G; and M is A or C.

The remaining promoters, *hyaA*, *napF*, *sufA* and *ydiU*, did not appear to contain the same IscR consensus site. Using the signal detection procedure and a set of these *E. coli* regions together with orthologous regions from Enterobacteria, another conserved sequence (Type 2) with consensus AWARCCCYTSnGTTTGMnGKKKTKWA was found to be present in the promoter regions of *hyaA*, *sufA* and *ydiU*, but not in the *napF* promoter (Fig. 6B). This motif is highly conserved in promoter regions of the IscR-activated *sufA* and *ydiU* genes and the IscR-repressed *hyaA* gene (Fig. S1). Interestingly, the distances between the IscR motifs and transcription start sites of *sufA* and *ydiU* are almost the same, 29 and 30 nucleotides respectively.

To examine the conservation of the IscR regulon in other species, a weight matrix and sequence logo for the aforementioned IscR signals from Enterobacteria were constructed by the SIGNALX program and the WebLogo package (Crooks *et al*., 2004) respectively. Each genome encoding an IscR orthologue with a $CysX_{5-7}CysX_{5-9}Cys$ motif was scanned with the constructed profile using the GenomeExplorer software (Mironov *et al*., 2000), and genes with candidate regulatory sites in the upstream regions were selected. The threshold for the site search was defined as the lowest score observed in the training set. As a result, high scoring IscR Type 1 motifs were found upstream of *iscRSUA-hscBA-fdx*, *yhgI* and *yadR*

genes in various γ- and β-proteobacteria and in *Rickettsia* species (Table S2). Among additional putative targets of IscR identified in some genomes, the *cysE* gene for serine acetyltransferase is preceded by a high scoring IscR motif in *E. coli* and closely related Enterobacteria, as well as in *Vibrio* species and some β-proteobacteria. CysE catalyses the rate-limiting step in biosynthesis of cysteine that is needed for Fe-S cluster formation (Zheng *et al*., 1998). No other IscR sites above the threshold were found in the *E. coli* genome. A weight matrix search of the *E. coli* genome using the Type 2 motif yielded three additional sites (Table S3), but the relevance of these is unknown as they are not conserved in other Enterobacteria.

Discussion

A broader role for IscR in regulating Fe-S biogenesis

Our finding that IscR, in addition to negatively regulating the Isc Fe-S biogenesis pathway, also positively regulates an alternative Fe-S biogenesis pathway, Suf, suggests that IscR has a more general role in regulation of this key metabolic process. The Suf pathway has been suggested to function under conditions of oxidative and iron stress (Lee *et al*., 2003; 2004; Outten *et al*., 2004) via OxyR and Fur, respectively, but the finding that IscR also controls the Suf pathway suggests that this alternate pathway is involved in Fe-S biogenesis under normal aerobic growth conditions. It is surprising, however, that *suf* is activated by IscR, unlike the *isc* operon, which is repressed. In addition, the IscR binding site in the *sufA* promoter corresponds to the oxidant-responsive element III (ORE-III) from −56 to −35 identified in Lee *et al*. (2004). While the specific factor required for *sufA* promoter activation within ORE-III was not previously identified, substitution of the sequence within this region abolished the mobility shift of *sufA* promoter DNA when incubated with cell-free extracts (Lee *et al*., 2004). Given that we found that IscR binds to a site with an almost identical location as ORE-III and that nucleotides −56 to −51 were necessary for IscR binding, it is likely that IscR is the third oxidant-responsive regulator in the *sufA* promoter. Thus, under conditions of oxidative stress, IscR would be involved in the upregulation of both the *isc* and *suf* operons (Zheng *et al*., 2001), although whether this occurs by the IscR feedback mechanism is unknown.

In addition, we found that the expression of two genes, *yadR* and *yhgI*, encoding proteins related to IscA are also repressed by IscR, similar to the *isc* operon. Remarkably, the IscR DNA binding sites upstream of *yadR* and *yhgI* showed the greatest similarity to the footprinted sites upstream of *iscR* and also showed the most extensive conservation in a phylogenetic analysis. This conserved mode of regulation of *yadR*, *yhgI* and the *isc* operon and the similarity to IscA suggests a common function in Fe-S biogenesis and supports the notion that YadR and YhgI may function as alternate scaffolds or bind Fe, as has been proposed for IscA (Krebs *et al*., 2001; Ding and Clark, 2004).

Are yfhJ-pepB-sseB *involved in Fe-S biogenesis?*

These three genes are located directly downstream of *iscRSUA-hscBA-fdx* and are regulated in an identical fashion to the upstream *isc* operon. In fact, the small spacing (11 nt) between the termination codon of *fdx* and the initiation codon of *yfhJ* is most consistent with *yfhJ-pepB-sseB* being transcribed as part of the same transcriptional unit as *iscRSUA-hscBA-fdx* or from the *hscB* (Lelivelt and Kawula, 1995) or *hscA* (Seaton and Vickery, 1994) promoters, which may indicate a function in Fe-S biogenesis. While no function has been ascribed to YfhJ, it has been shown to bind IscS (Tokumoto *et al*., 2002), and the three dimensional structure of YfhJ revealed a negatively charged surface (Shimomura *et al*., 2005), which was proposed to bind iron. PepB is an aminopeptidase that was shown to interact with HscA via tandem affinity purification in *E. coli* (Butland *et al*., 2005); however, it is not obvious what function a peptidase would have in Fe-S biogenesis. The *sseB* gene was first identified in a screen for mutants that enhance serine sensitivity (Hama *et al*., 1994), perhaps indicating a connection to cysteine biosynthesis and thus sulphur metabolism. Thus, given their genomic location, regulation by IscR, and ability to specifically bind Isc and Hsc proteins, the *yfhJ-pepB-sseB* gene products may also contribute to Fe-S cluster biogenesis.

Expression of many Fe-S biogenesis functions is downregulated under anaerobic conditions

We were surprised to find that expression of *iscRSUA-hscBA-fdx-yfhJ-pepB-sseB*, *yadR*, *yhgI* and *sufABCDSE* was decreased under anaerobic conditions as many of the respiratory enzymes that are synthesized under anaerobic conditions contain Fe-S clusters. This observation suggests that there may be a decreased requirement for Fe-S biogenesis under anaerobic growth conditions; O_2 and reactive oxygen species are known to damage or destroy Fe-S clusters, perhaps explaining why more Fe-S biogenesis machinery would be produced under aerobic conditions. Nevertheless, expression of *csdA-ygdK*, encoding another IscS/SufS homologue, CsdA, and a second SufE homologue, YgdK, was not regulated by either IscR or O_2 in our experiments; YgdK enhances the cysteine desulphurase activity of CsdA in a manner analogous to that of SufE and SufS (Liu *et al*., 2005; Loiseau *et al*., 2005), although no *in vivo* function has been established for these proteins.

IscR contributes to the O_2 regulation of several genes

For several genes (*dctA*, *flu*, *hyaABCDEF*, *hybOABCDEFG*, *napFDAGHBC*, *soxS*, *ycgZ*, *yjiM*), repression by IscR was observed only under aerobic growth conditions even though IscR can function anaerobically. The *in vitro* transcription and footprinting data suggest at least two plausible mechanisms to explain how IscR can contribute to the O_2 regulation of these genes without requiring a change in IscR activity. Previous studies of the *hyaA*, *hybO* and *napF* promoters suggested that the selective expression of these genes under anaerobic conditions resulted from activation by a global anaerobic regulator (Choe and Reznikoff, 1993; Brondsted and Atlung, 1994; Darwin and Stewart, 1995; Richard *et al*., 1999). To incorporate a role for IscR, we would suggest that for the *napF* promoter, anaerobic activation of the downstream promoter(s) via FNR, a *bona fide* anaerobic global regulator, and NarP, a nitrate-dependent activator (Darwin *et al*., 1998; Stewart *et al*., 2003), masks detection of IscR-dependent changes in expression from the upstream promoter (Fig. 4C). Thus, the effect of IscR on the upstream promoter is only observed under aerobic conditions, when the downstream promoter is not functional. We also note that the IscR binding site overlaps with that of ModE, which increases *napF* transcription in the presence of molybdate (McNicholas and Gunsalus, 2002), although it has not been established which start site ModE regulates.

In contrast, for the *hyaA* and *hybO* promoters, IscR repressed the previously identified O_2-regulated transcription start sites; under anaerobic growth conditions, AppY and the ArcA/B two-component regulatory system have been implicated in the activation of transcription of the *hya* operon (Brondsted and Atlung, 1994; Richard *et al*., 1999), whereas FNR appears to contribute to activation of the *hybO* promoter (Richard *et al*., 1999). As IscR protected a region of the *hyaA* promoter that overlaps the −35 promoter element and site(s) where an activator may bind, one mechanism to explain the lack of IscR repression of the *hyaA* and *hybO* promoters under anaerobic conditions would be the occlusion of IscR binding by anaerobic regulators (ArcA, AppY or FNR) and/or RNA polymerase. Such a model implies that IscR cannot compete effectively for binding when RNA polymerase and the anaerobic activators are present. Additional studies will be required to determine whether either of these mechanisms can explain the role of IscR in the control of other O_2-regulated genes identified in this study.

One question that remains is why only a subset of anaerobically induced Fe-S proteins are regulated in this manner. Perhaps transcriptional repression of the *hyaA*, *hybO* and *napF* promoters by IscR under aerobic conditions prevents inappropriate Fe-S biogenesis of the Fe-S subunits under aerobic conditions, which might lead to their destruction and increase oxidative stress. Such a model implies that transcription of these promoters in the absence of activators is greater than other anaerobically induced genes and thus they require additional mechanisms to limit their synthesis under aerobic conditions. The relative strength of the *hyaA*, *napF* and *hybO* promoters observed in this study supports this notion.

IscR appears to bind to two different classes of DNA sites

The use of bioinformatic methods in addition to DNase I footprinting of IscR binding sites was critical in identifying conserved sequences for binding of IscR as it appears that IscR can recognize two different DNA sequences. The first motif, ATASYYGACTRwwwYAGTCRRSTAT, built using conserved regions in the *iscR*, *yadR* and *yhgI* promoters, appears to be important for repression of promoters driving the expression of Fe-S biogenesis proteins. The second motif, AWARCCCYTSnGTTTGMnGKKKTKWA, built from conserved regions in the *hyaA*, *sufA* and *ydiU* promoters, was more degenerate. This degeneracy could reflect the differences in binding affinity of the various sites; for example, the DNase I footprinting shows better protection of the *hyaA* promoter than the *sufA* promoter. The *iscR* control region was the only gene found to contain two of the conserved Type 1 motifs and both are found in the same position in related Enterobacteria. In contrast, the third protected site (+9 to +26) is not related to either motif and is also not highly conserved in other Enterobacteria. None of the footprinted sites contained the marbox element previously noted (Schwartz *et al*., 2001; Martin *et al*., 2002) in the −82 to −63 region of the *iscR* promoter, indicating that these sequences are not relevant to IscR function. Additional studies are necessary to determine the mechanism by which IscR recognizes different DNA binding sites and which nucleotides in the two identified motifs are important for recognition by IscR.

In summary, this study extends the role of IscR in the regulation of Fe-S cluster biogenesis in *E. coli* to include the *suf* operon as well as the genes encoding the putative Fe-S cluster assembly proteins, YadR and YhgI, and suggests a similar function of IscR in other related bacteria. Surprisingly, IscR activates the Suf pathway yet represses the other Fe-S biogenesis genes. The analysis of IscR binding sites suggests two modes of DNA binding, one of which is specific for repression of the Fe-S biogenesis proteins. As the Fe-S form of IscR has only been shown to be necessary for repression of the *isc* operon (Schwartz *et al*., 2001), it is possible that IscR lacking an Fe-S cluster may also bind DNA. Whether this indicates that the Fe-S form of IscR is specific for sites that function in repression of Fe-S biogenesis functions and that IscR lacking an

Fe-S cluster recognizes the second class of binding sites awaits additional studies.

Experimental procedures

DNA microarray experiments and data analysis

MG1655 from the laboratory of F.R.B. was used to create PK6597, a Δ*iscR* strain, in the manner described in Schwartz *et al*. (2001). This source of MG1655 and the isogenic Δ*iscR* strain PK6597 (Table 2) were grown in MOPS minimal medium (Neidhardt *et al*., 1974) with 0.1% glucose at 37°C to saturation overnight and subcultured 1:250 into fresh media. Cells were grown aerobically by sparging the culture with a gas mixture of 25% O_2, 70% N_2, 5% CO_2 or anaerobically with a mixture of 95% N_2 and 5% CO_2 as previously described (Sutton and Kiley, 2003). Cultures were grown to an OD_{600} of 0.2 (for aerobic cultures, about nine generations) or 0.1 (for anaerobic cultures, about five generations), and aliquots were mixed with two volumes of RNAprotect Bacteria Reagent (Qiagen) according to the manufacturer's recommendations. RNA was isolated from three independent cultures for each strain and condition (with the exception of MG1655 grown anaerobically, which was performed twice), and RNA purification, cDNA synthesis and biotin labelling were performed as previously described (Kang *et al*., 2005). Biotin-labelled cDNA was then hybridized to *E. coli* Antisense GeneChips (Affymetrix), incubated and scanned according to the manufacturer's protocol.

Gene expression levels were calculated using the Microarray Suite 5.0 software (Affymetrix), which generates a signal value (after subtracting the background and adjusting for noise) for each gene. Those values are then imported into a relational database and averaged for each gene across replicates. The correlation coefficient of intensity values between any two replicates was greater than 0.9. Data obtained from MG1655 grown aerobically in the sparging apparatus were also compared with experiments on MG1655 aerated at 225 r.p.m. in a shaking water bath, and no significant differences were observed (Y. Kang and J.L. Giel, unpubl. data).

*Construction of promoter–*lacZ *fusions and β-galactosidase assays*

DNA fragments containing the control regions of candidate IscR-regulated genes (see Table 2) were generated by PCR amplification of chromosomal DNA and cloned into plasmid pPK7035. The –200 to +40 bp relative to previously identified transcription start sites were cloned for *hyaA* (Richard *et al*., 1999), *hybO* (Richard *et al*., 1999), *napF* (Choe and Reznikoff, 1993), *sodA* (Compan and Touati, 1993), *soxS* (Hidalgo and Demple, 1994) and *sufA* (Zheng *et al*., 2001). Bases –485 to +40 and –310 to –3 relative to the A of the start codons were cloned for *yjiH* and *yqjI* respectively. For *yadR* and *yhgI*, bases –300 to +40 relative to the start codon were cloned, and 360 bp upstream of the A of the ATG start codon were cloned for *ydiU*. Another *sufA* promoter region, from –393 to +90, was also cloned, and the Δ(–56 to –51)*sufA* promoter construct was created by QuikChange (Stratagene) deletion of bases –56 to –51 in the –200 to +40 *sufA* promoter plasmid. The *lacI*-Kn-promoter–*lacZ* fragment was amplified from the resulting plasmid with primers 5′-GGCACGACAGG TTTCCCGACTGGAAAGCGGGCAGTGAGCCGGATCAATT CCCCTGCTC-3′ and 5′-CGTTTCACCCTGCCATAAAG-3′ and recombined into the chromosome in the *lacZ* region of BW25993/pKD46 as previously described (Kang *et al*., 2005), resulting in a Kn-promoter–*lacZ* fusion in the *lac* region of the chromosome, replacing DNA starting 1 bp after the *lacI* stop codon and ending 17 bp upstream of the *lacZ* start ATG. Strains containing no promoter cloned into the pPK7035 template were also constructed as controls. Kn-promoter–*lacZ* fusions were moved into MG1655 and PK4854 strains via P1 transduction and selected for kanamycin resistance. Resulting strains (Table 2) were grown to an OD_{600} of 0.2 in MOPS minimal medium containing 0.2% glucose either by shaking at 250 r.p.m. or by incubating cultures anaerobically in filled screw-capped tubes and were subsequently assayed for β-galactosidase activity (Miller, 1972). When indicated, potassium nitrate was added to a final concentration of 40 mM.

Protein purification

IscR was purified anaerobically in a Coy anaerobic chamber (10% CO_2, 10% H_2 and 80% N_2) from *E. coli* BL21 containing pPK6161 as described previously (Schwartz *et al*., 2001) except that 10 mM HEPES, pH 7.4 replaced Tris-HCl in all solutions and a second chromatography step was added. Briefly, cells were resuspended in buffer R (10 mM Hepes, pH 7.4, 10% glycerol, 100 mM KCl, 1 mM DTT) containing 1.7 mM dithionite and 0.1 mM phenylmethylsulphonyl fluoride. Buffer R was also utilized for the heparin-ion exchange chromatography step, eluting with a linear gradient of KCl as described previously. Fractions from the heparin column containing IscR were pooled and loaded onto a HiPrep 16/60 S-100 high-resolution gel filtration column (Amersham Pharmacia) equilibrated in buffer R (containing an additional 100 mM KCl) at a flow rate of 0.5 ml min^{-1}. Fractions containing IscR were pooled and diluted twofold in buffer R lacking KCl before concentration over a gravity-flow BioRex-70 column. IscR was eluted from the BioRex-70 column in buffer R containing an additional 0.9 M KCl. The iron and sulphide content and protein concentration were determined as described previously (Schwartz *et al*., 2001). IscR utilized in these experiments was 26–66% occupied with Fe-S cluster.

In vitro *transcription*

Candidate IscR-regulated promoter regions (see Table 2) were amplified by colony PCR using MG1655 as a template and primers containing XhoI and HindIII sites, digested with XhoI and HindIII, and cloned into pPK7179, a pUC19-*spf′* derivative containing a XhoI site (Kang *et al*., 2005). The plasmid DNA was purified with the QIAfilter Maxi kit (Qiagen). The effect of IscR on σ^{70}-dependent promoter activity from the candidate control regions was determined as previously described (Schwartz *et al*., 2001); 2 nM supercoiled plasmid DNA was incubated with IscR, [α-^{32}P]-UTP and NTPs for 30 min at 37°C. Eσ^{70} RNA polymerase (50 nM) (Epicentre)

Table 2. Strains and plasmids used in this work.

Strain/plasmid	Relevant genotype	Sources
Bacterial strains		
MG1655	λ^- F$^-$*rph*-1	Laboratory stock
PK6597[a]	MG1655 Δ*iscR*	This study
PK4854[a]	MG1655 Δ*iscR*	Schwartz *et al*. (2001)
BW25993	*lacI*^{q}*hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	Datsenko and Wanner (2000)
PK7573	MG1655 P*hyaA–lacZ*	This study
PK6886	PK4854 P*hyaA–lacZ*	This study
PK7574	MG1655 P*hybO–lacZ*	This study
PK6888	PK4854 P*hybO–lacZ*	This study
PK7538	MG1655 P*napF–lacZ*	This study
PK7539	PK4854 P*napF–lacZ*	This study
PK7564	MG1655 P*sodA–lacZ*	This study
PK7565	PK4854 P*sodA–lacZ*	This study
PK7503	MG1655 P*soxS–lacZ*	This study
PK7504	PK4854 P*soxS–lacZ*	This study
PK6879	MG1655 (–200 to +40) P*sufA–lacZ*	This study
PK6880	PK4854 (–200 to +40) P*sufA–lacZ*	This study
PK7722	MG1655 (–393 to +90) P*sufA–lacZ*	This study
PK7723	PK4854 (–393 to +90) P*sufA–lacZ*	This study
PK7720	MG1655 [–200 to +40, Δ(-56 to –51)] P*sufA–lacZ*	This study
PK7721	PK4854 [–200 to +40, Δ(-56 to –51)] P*sufA–lacZ*	This study
PK8034	MG1655 P*yadR–lacZ*	This study
PK8035	PK4854 P*yadR–lacZ*	This study
PK8004	MG1655 P*ydiU–lacZ*	This study
PK8005	PK4854 P*ydiU–lacZ*	This study
PK8039	MG1655 P*yhgI–lacZ*	This study
PK8040	PK4854 P*yhgI–lacZ*	This study
PK7596	MG1655 P*yjiH–lacZ*	This study
PK7597	PK4854 P*yjiH–lacZ*	This study
PK7743	MG1655 P*yqjI–lacZ*	This study
PK7744	PK4854 P*yqjI–lacZ*	This study
PK7519	MG1655 promoterless–*lacZ*	This study
PK7520	PK4854 promoterless–*lacZ*	This study
RZ4500	MG1655 *lacZ*Δ145	Choe and Reznikoff (1991)
PK1815	RZ4500 *fdnG–lacZ*	This laboratory
PK7728	PK1815 Δ*iscR*	This study
PK3292	RZ4500 λPC25 (*dmsA–lacZ*)	This laboratory
PK7731	PK3292 Δ*iscR*	This study
RZ7350	RZ4500 *narG*234::MudI1734	Kiley and Reznikoff (1991)
PK7727	RZ7350 Δ*iscR*	This study
RZ7382	RZ4500 *frd*::MudI1734	This laboratory
PK7729	RZ7382 Δ*iscR*	This study
Plasmids		
pKD46	Phage λ *gam-bet-exo* genes under P*araB* control	B.L. Wanner
pPK7035	Kn^R gene from pHP45Ω and BamHI–NdeI fragment from pRS1553 into pBR322	Kang *et al*. (2005)
pPK7179	pUC19-*spf*′ with XhoI site replacing SalI site	Kang *et al*. (2005)
pPK6511	P*iscR* cloned into pUC19-*spf*′	Schwartz *et al*. (2001)
pPK6831	As pPK6511, but P*iscR* in opposite orientation	This study
pPK6842	P*hyaA* cloned into pPK7179	This study
pPK6843	P*hybO* cloned into pPK7179	This study
pPK6844	P*napF* cloned into pPK7179	This study
pPK6869	P*sodA* cloned into pPK7179	This study
pPK6868	P*soxS* cloned into pPK7179	This study
pPK6845	P*sufA* cloned into pPK7179	This study
pPK8018	P*yadR* cloned into pPK7179	This study
pPK7794	P*ydiU* cloned into pPK7179	This study
pPK8019	P*yhgI* cloned into pPK7179	This study
pPK7589	P*yjiH* cloned into pPK7179	This study
pPK6870	P*yqjI* cloned into pPK7179	This study

a. Strain PK6597 is a derivative of MG1655 from the laboratory of F.R.B. and was used for the transcription profiling experiments; strain PK4854 is a derivative of the P.J.K. laboratory stock of MG1655 and was used in constructing strains for the β-galactosidase assays. No differences between these strains have been observed.

was added and the reaction was terminated after 5 min. Assays were performed under aerobic conditions with anaerobically purified IscR that was diluted immediately before the start of the incubation of IscR with DNA.

DNase I footprinting

DNA fragments containing the *iscR* promoter region were isolated from pPK6831 or pPK6511 (Table 2) after digestion

with XbaI and HindIII. Klenow fragment (NEB) was used to 3′ radiolabel the HindIII end of the fragment with [α-^{32}P]-dATP (Amersham). For all other promoter regions (cloned into pPK7179-based plasmids), the DNA was digested with HindIII and BamHI and then either the BamHI end was 3′ labelled with [α-^{32}P]-dGTP or the HindIII end was 3′ labelled with [α-^{32}P]-dATP. Labelled DNA fragments were isolated from a non-denaturing 5% acrylamide gel and were subsequently purified with elutip-d columns (Schleicher and Schuell). As with the *in vitro* transcriptions, assays were performed under aerobic conditions with anaerobically purified IscR that was diluted immediately before the start of the incubation of IscR with DNA. Labelled DNA (20 000–100 000 cpm) was incubated with IscR for 30 min at 37°C in 40 mM Tris (pH 7.9), 70 mM KCl, 100 μg ml^{-1} BSA and 1 mM DTT followed by the addition of 2 μg ml^{-1} DNase I (Worthington) for 30 s. The DNase I reaction was terminated by the addition of sodium acetate and EDTA to final concentrations of 300 mM and 20 mM respectively. The reaction mix was ethanol precipitated, resuspended in loading dye, heated for 30–60 s at 90°C, and loaded onto a 7 M urea, 8% polyacrylamide gel in 0.5× TBE buffer. A+G sequencing ladders were generated as previously described (Maxam and Gilbert, 1980). The reaction products were visualized by phosphorimaging and ImageQuant software.

Primer extension assays

RNA was synthesized using the same protocol as the *in vitro* transcription assays, with the exception that the UTP was unlabelled and the reactions were allowed to proceed for 15 min. This *in vitro* synthesized RNA was phenol extracted, ethanol precipitated and hybridized at 65° to ^{32}P-labelled primers (for *napF*, primer 5′-GGATGTGTCAAAGATGCATAC CCCG-3′; for *yadR*, primer 5′-TAGGATCCCGTCGGTAAAC TCCAGCGGCAG-3′; for *yhgI*, primer 5′-TTGGATCCTGGCA AAGTGCGCTTGTGCAGC-3′; for *ydiU*, primer 5′-CGTGAA GTAAAAGGTCTGAAAGATAGAACATCTTACCTCTGTGGG-3′). Primer extension with ThermoScript reverse transcriptase (Invitrogen) was carried out according to the manufacturer's instructions. Sequencing reactions using the same primer from the primer extension assays were performed using the fmol DNA Cycle Sequencing System (Promega).

Sequence data

Complete genomes and unfinished annotated genomic sequences of proteobacteria were downloaded from GenBank (Benson *et al*., 2005). Unpublished genomes of *Citrobacter rodentum* and *Proteus mirabilis* were downloaded from the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk), and genomic sequences of *Erwinia chrysanthemi* and *Klebsiella pneumoniae* were obtained from the websites of the Institute for Genomic Research (http://www.tigr.org) and Washington University (http://genomeold.wustl.edu) respectively.

Identification of IscR-binding motifs

The training set for motif identification was composed of the IscR-protected regions and the corresponding orthologous regions defined by phylogenetic footprinting. The latter approach uses aligning regions upstream of orthologous genes from different species in order to find conserved motifs that may represent common regulatory elements (McCue *et al*., 2001). Multiple sequence alignments were generated by CLUSTALX (Chenna *et al*., 2003). An iterative signal detection procedure implemented in the program SIGNALX (Gelfand *et al*., 2000) was used for identification of a common DNA motif and profile construction. Each IscR-encoding genome of proteobacteria was scanned with the constructed profile using the GenomeExplorer software (Mironov *et al*., 2000) and additional genes with candidate IscR motifs in the upstream regions were selected (Table S2). Positional nucleotide weights in the recognition profile and *Z* scores of candidate sites were calculated as the sum of the respective positional nucleotide weights as described in Mironov *et al*. (1999). The threshold for the site search was defined as the lowest score observed in the training set.

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References

Agar, J.N., Krebs, C., Frazzon, J., Huynh, B.H., Dean, D.R., and Johnson, M.K. (2000) IscU as a scaffold for iron-sulfur cluster biosynthesis: sequential assembly of [2Fe-2S] and [4Fe-4S] clusters in IscU. *Biochemistry* **39:** 7856–7862.

Benson, D.A., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., and Wheeler, D.L. (2005) GenBank. *Nucleic Acids Res* **33:** 1.

Bockhorst, J., Qiu, Y., Glasner, J., Liu, M., Blattner, F., and Craven, M. (2003) Predicting bacterial transcription units using sequence and expression data. *Bioinformatics* **19 (Suppl.):** i34–43.

Brondsted, L., and Atlung, T. (1994) Anaerobic regulation of the hydrogenase 1 (*hya*) operon of *Escherichia coli*. *J Bacteriol* **176:** 5423–5428.

Butland, G., Peregrin-Alvarez, J.M., Li, J., Yang, W., Yang, X., Canadien, V., *et al.* (2005) Interaction network containing conserved and essential protein complexes in *Escherichia coli*. *Nature* **433:** 531–537.

Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T.J., Higgins, D.G., and Thompson, J.D. (2003) Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res* **31:** 3497–3500.

Choe, M., and Reznikoff, W.S. (1991) Anaerobically expressed *Escherichia coli* genes identified by operon fusion techniques. *J Bacteriol* **173:** 6139–6146.

Choe, M., and Reznikoff, W.S. (1993) Identification of the regulatory sequence of anaerobically expressed locus *aeg-46.5*. *J Bacteriol* **175:** 1165–1172.

Compan, I., and Touati, D. (1993) Interaction of six global transcription regulators in expression of manganese superoxide dismutase in *Escherichia coli* K-12. *J Bacteriol* **175:** 1687–1696.

Crooks, G.E., Hon, G., Chandonia, J.M., and Brenner, S.E. (2004) WebLogo: a sequence logo generator. *Genome Res* **14:** 1188–1190.

Darwin, A.J., and Stewart, V. (1995) Nitrate and nitrite regulation of the Fnr-dependent *aeg-46.5* promoter of *Escherichia coli* K-12 is mediated by competition between homologous response regulators (NarL and NarP) for a common DNA-binding site. *J Mol Biol* **251:** 15–29.

Darwin, A.J., Ziegelhoffer, E.C., Kiley, P.J., and Stewart, V. (1998) Fnr, NarP, and NarL regulation of *Escherichia coli* K-12 *napF* (periplasmic nitrate reductase) operon transcription *in vitro*. *J Bacteriol* **180:** 4192–4198.

Datsenko, K.A., and Wanner, B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* **97:** 6640–6645.

Ding, H., and Clark, R.J. (2004) Characterization of iron binding in IscA, an ancient iron-sulphur cluster assembly protein. *Biochem J* **379:** 433–440.

Dombrecht, B., Tesfay, M.Z., Verreth, C., Heusdens, C., Napoles, M.C., Vanderleyden, J., and Michiels, J. (2002) The *Rhizobium etli* gene *iscN* is highly expressed in bacteroids and required for nitrogen fixation. *Mol Genet Genomics* **267:** 820–828.

Enz, S., Braun, V., and Crosa, J.H. (1995) Transcription of the region encoding the ferric dicitrate-transport system in *Escherichia coli*: similarity between promoters for *fecA* and for extracytoplasmic function sigma factors. *Gene* **163:** 13–18.

Flint, D.H. (1996) *Escherichia coli* contains a protein that is homologous in function and N-terminal sequence to the protein encoded by the *nifS* gene of *Azotobacter vinelandii* and that can participate in the synthesis of the Fe-S cluster of dihydroxy-acid dehydratase. *J Biol Chem* **271:** 16068–16074.

Florea, L., McClelland, M., Riemer, C., Schwartz, S., and Miller, W. (2003) EnteriX 2003: visualization tools for genome alignments of Enterobacteriaceae. *Nucleic Acids Res* **31:** 3527–3532.

Gelfand, M.S., Koonin, E.V., and Mironov, A.A. (2000) Prediction of transcription regulatory sites in Archaea by a comparative genomic approach. *Nucleic Acids Res* **28:** 695–705.

Goldsmith-Fischman, S., Kuzin, A., Edstrom, W.C., Benach, J., Shastry, R., Xiao, R., *et al.* (2004) The SufE sulfur-acceptor protein contains a conserved core structure that mediates interdomain interactions in a variety of redox protein complexes. *J Mol Biol* **344:** 549–565.

Gyaneshwar, P., Paliy, O., McAuliffe, J., Popham, D.L., Jordan, M.I., and Kustu, S. (2005) Sulfur and nitrogen limitation in *Escherichia coli* K-12: specific homeostatic responses. *J Bacteriol* **187:** 1074–1090.

Hama, H., Kayahara, T., Ogawa, W., Tsuda, M., and Tsuchiya, T. (1994) Enhancement of serine-sensitivity by a gene encoding rhodanese-like protein in *Escherichia coli*. *J Biochem (Tokyo)* **115:** 1135–1140.

Hidalgo, E., and Demple, B. (1994) An iron-sulfur center essential for transcriptional activation by the redox-sensing SoxR protein. *EMBO J* **13:** 138–146.

Hoff, K.G., Silberg, J.J., and Vickery, L.E. (2000) Interaction of the iron-sulfur cluster assembly protein IscU with the Hsc66/Hsc20 molecular chaperone system of *Escherichia coli*. *Proc Natl Acad Sci USA* **97:** 7790–7795.

Hoff, K.G., Ta, D.T., Tapley, T.L., Silberg, J.J., and Vickery, L.E. (2002) Hsc66 substrate specificity is directed toward a discrete region of the iron-sulfur cluster template protein IscU. *J Biol Chem* **277:** 27353–27359.

Hogg, R.W., Voelker, C., and Von Carlowitz, I. (1991) Nucleotide sequence and analysis of the *mgl* operon of *Escherichia coli* K12. *Mol Gen Genet* **229:** 453–459.

Johnson, D.C., Dean, D.R., Smith, A.D., and Johnson, M.K. (2005) Structure, function, and formation of biological Fe-S clusters. *Annu Rev Biochem* **74:** 247–281.

Kang, Y., Weber, K.D., Qiu, Y., Kiley, P.J., and Blattner, F.R. (2005) Genome-wide expression analysis indicates that FNR of *Escherichia coli* K-12 regulates a large number of genes of unknown function. *J Bacteriol* **187:** 1135–1160.

Kiley, P.J., and Reznikoff, W.S. (1991) Fnr mutants that activate gene expression in the presence of oxygen. *J Bacteriol* **173:** 16–22.

Krebs, C., Agar, J.N., Smith, A.D., Frazzon, J., Dean, D.R., Huynh, B.H., and Johnson, M.K. (2001) IscA, an alternate scaffold for Fe-S cluster biosynthesis. *Biochemistry* **40:** 14069–14080.

Lee, J.H., Yeo, W.S., and Roe, J.H. (2003) Regulation of the *sufABCDSE* operon by Fur. *J Microbiol* **41:** 109–114.

Lee, J.H., Yeo, W.S., and Roe, J.H. (2004) Induction of the *sufA* operon encoding Fe-S assembly proteins by superoxide generators and hydrogen peroxide: involvement of OxyR, IHF and an unidentified oxidant-responsive factor. *Mol Microbiol* **51:** 1745–1755.

Lelivelt, M.J., and Kawula, T.H. (1995) Hsc66, an Hsp70 homolog in *Escherichia coli*, is induced by cold shock but not by heat shock. *J Bacteriol* **177:** 4900–4907.

Liu, G., Li, Z., Chiang, Y., Acton, T., Montelione, G.T., Murray, D., and Szyperski, T. (2005) High-quality homology models derived from NMR and X-ray structures of *E. coli* proteins YgdK and SufE suggest that all members of the YgdK/Suf E protein family are enhancers of cysteine desulfurases. *Protein Sci* **14:** 1597–1608.

Loiseau, L., Ollagnier-de-Choudens, S., Nachin, L., Fontecave, M., and Barras, F. (2003) Biogenesis of Fe-S cluster by the bacterial Suf system: SufS and SufE form a new type of cysteine desulfurase. *J Biol Chem* **278:** 38352–38359.

Loiseau, L., Ollagnier-de-Choudens, S., Lascoux, D., Forest, E., Fontecave, M., and Barras, F. (2005) Analysis of the heteromeric CsdA-CsdE cysteine desulfurase, assisting

Fe-S cluster biogenesis in *Escherichia coli*. *J Biol Chem* **280:** 26760–26769.

McCue, L., Thompson, W., Carmack, C., Ryan, M.P., Liu, J.S., Derbyshire, V., and Lawrence, C.E. (2001) Phylogenetic footprinting of transcription factor binding sites in proteobacterial genomes. *Nucleic Acids Res* **29:** 774–782.

McNicholas, P.M., and Gunsalus, R.P. (2002) The molybdate-responsive *Escherichia coli* ModE transcriptional regulator coordinates periplasmic nitrate reductase (*napFDAGHBC*) operon expression with nitrate and molybdate availability. *J Bacteriol* **184:** 3253–3259.

Martin, R.G., Gillette, W.K., Martin, N.I., and Rosner, J.L. (2002) Complex formation between activator and RNA polymerase as the basis for transcriptional activation by MarA and SoxS in *Escherichia coli*. *Mol Microbiol* **43:** 355–370.

Maxam, A.M., and Gilbert, W. (1980) Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol* **65:** 499–560.

Mihara, H., Maeda, M., Fujii, T., Kurihara, T., Hata, Y., and Esaki, N. (1999) A *nifS*-like gene, *csdB*, encodes an *Escherichia coli* counterpart of mammalian selenocysteine lyase. Gene cloning, purification, characterization and preliminary X-ray crystallographic studies. *J Biol Chem* **274:** 14768–14772.

Miller, J.H. (1972) *Experiments in Molecular Genetics.* Plainview, NY: Cold Spring Harbor Laboratory Press.

Mironov, A.A., Koonin, E.V., Roytberg, M.A., and Gelfand, M.S. (1999) Computer analysis of transcription regulatory patterns in completely sequenced bacterial genomes. *Nucleic Acids Res* **27:** 2981–2989.

Mironov, A.A., Vinokurova, N.P., and Gel'fand, M.S. (2000) GenomeExplorer: software for analysis of complete bacterial genomes. *Mol Biol* **34:** 222–231.

Nachin, L., Loiseau, L., Expert, D., and Barras, F. (2003) SufC: an unorthodox cytoplasmic ABC/ATPase required for [Fe-S] biogenesis under oxidative stress. *EMBO J* **22:** 427–437.

Neidhardt, F.C., Bloch, P.L., and Smith, D.F. (1974) Culture medium for Enterobacteria. *J Bacteriol* **119:** 736–747.

Outten, F.W., Wood, M.J., Munoz, F.M., and Storz, G. (2003) The SufE protein and the SufBCD complex enhance SufS cysteine desulfurase activity as part of a sulfur transfer pathway for Fe-S cluster assembly in *Escherichia coli*. *J Biol Chem* **278:** 45713–45719.

Outten, F.W., Djaman, O., and Storz, G. (2004) A *suf* operon requirement for Fe-S cluster assembly during iron starvation in *Escherichia coli*. *Mol Microbiol* **52:** 861–872.

Patzer, S.I., and Hantke, K. (1999) SufS is a NifS-like protein, and SufD is necessary for stability of the [2Fe-2S] FhuF protein in *Escherichia coli*. *J Bacteriol* **181:** 3307–3309.

Porco, A., Alonso, G., and Isturiz, T. (1998) The gluconate high affinity transport of GntI in *Escherichia coli* involves a multicomponent complex system. *J Basic Microbiol* **38:** 395–404.

Rangachari, K., Davis, C.T., Eccleston, J.F., Hirst, E.M., Saldanha, J.W., Strath, M., and Wilson, R.J. (2002) SufC hydrolyzes ATP and interacts with SufB from *Thermotoga maritima*. *FEBS Lett* **514:** 225–228.

Richard, D.J., Sawers, G., Sargent, F., McWalter, L., and Boxer, D.H. (1999) Transcriptional regulation in response to oxygen and nitrate of the operons encoding the [NiFe] hydrogenases 1 and 2 of *Escherichia coli*. *Microbiology* **145:** 2903–2912.

Schwartz, C.J., Giel, J.L., Patschkowski, T., Luther, C., Ruzicka, F.J., Beinert, H., and Kiley, P.J. (2001) IscR, an Fe-S cluster-containing transcription factor, represses expression of *Escherichia coli* genes encoding Fe-S cluster assembly proteins. *Proc Natl Acad Sci USA* **98:** 14895–14900.

Seaton, B.L., and Vickery, L.E. (1994) A gene encoding a DnaK/hsp70 homolog in *Escherichia coli*. *Proc Natl Acad Sci USA* **91:** 2066–2070.

Shimomura, Y., Takahashi, Y., Kakuta, Y., and Fukuyama, K. (2005) Crystal structure of *Escherichia coli* YfhJ protein, a member of the ISC machinery involved in assembly of iron-sulfur clusters. *Proteins* **60:** 566–569.

Smith, A.D., Agar, J.N., Johnson, K.A., Frazzon, J., Amster, I.J., Dean, D.R., and Johnson, M.K. (2001) Sulfur transfer from IscS to IscU: the first step in iron-sulfur cluster biosynthesis. *J Am Chem Soc* **123:** 11103–11104.

Stewart, V., Bledsoe, P.J., and Williams, S.B. (2003) Dual overlapping promoters control *napF* (periplasmic nitrate reductase) operon expression in *Escherichia coli* K-12. *J Bacteriol* **185:** 5862–5870.

Sutton, V.R., and Kiley, P.J. (2003) Techniques for studying the oxygen-sensitive transcription factor FNR from *Escherichia coli*. *Methods Enzymol* **370:** 300–312.

Tokumoto, U., Nomura, S., Minami, Y., Mihara, H., Kato, S., Kurihara, T., *et al.* (2002) Network of protein–protein interactions among iron-sulfur cluster assembly proteins in *Escherichia coli*. *J Biochem (Tokyo)* **131:** 713–719.

Urbina, H.D., Silberg, J.J., Hoff, K.G., and Vickery, L.E. (2001) Transfer of sulfur from IscS to IscU during Fe/S cluster assembly. *J Biol Chem* **276:** 44521–44526.

Vickery, L.E., Silberg, J.J., and Ta, D.T. (1997) Hsc66 and Hsc20, a new heat shock cognate molecular chaperone system from *Escherichia coli*. *Protein Sci* **6:** 1047–1056.

Zheng, L., Cash, V.L., Flint, D.H., and Dean, D.R. (1998) Assembly of iron-sulfur clusters. Identification of an *iscSUA-hscBA-fdx* gene cluster from *Azotobacter vinelandii*. *J Biol Chem* **273:** 13264–13272.

Zheng, M., Wang, X., Templeton, L.J., Smulski, D.R., LaRossa, R.A., and Storz, G. (2001) DNA microarray-mediated transcriptional profiling of the *Escherichia coli* response to hydrogen peroxide. *J Bacteriol* **183:** 4562–4570.

Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Multiple sequence alignments of upstream regions of the IscR-regulated genes from *E. coli* and their orthologues in other Enterobacteria. Genome abbreviations: EC, *Escherichia coli*; CR, *Citrobacter rodentium*; ST, *Salmonella typhi*; KP, *Klebsiella pneumoniae*; SM, *Serratia marcescens*; YE, *Yersinia enterocolitica*; YP, *Yersinia pestis*; PL, *Photorhabdus luminescens*; PM, *Proteus mirabilis*; EO, *Erwinia carotovora*; ER, *Erwinia chrysanthemi*; SF, *Shigella flexneri*. IscR-protected regions are underlined. Translation start codons

and putative ribosome binding sites are in red and green respectively. Experimentally determined transcription start sites and associated promoter elements are in blue. Nucleotides matching the derived IscR consensus sites are highlighted in green. Asterisks indicate base pairs that are 100% conserved among these bacteria.

Table S1. Normalized Affymetrix GeneChip intensities for RNA from wild-type (Ctrl) and *ΔiscR* (Expt) strains grown under both aerobic and anaerobic conditions and *P*-values calculated using these intensity values. Numbers in the top row indicate the number of the experimental replicate.

Table S2. High scoring promoters containing the Type 1 *IscR* motif constructed from the *iscR*, *yhgI* and *yadR* promoter regions from *E. coli* and other Enterobacteria encoding IscR with the $CysX_{5\text{-}7}CysX_{5\text{-}9}Cys$ motif.

Table S3. High scoring promoters containing the Type 2 IscR motif constructed from the *hyaA*, *sufA*, and *ydiU* promoter regions from *E. coli* and other Enterobacteria.

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