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A *suf* operon requirement for Fe–S cluster assembly during iron starvation in *Escherichia coli*

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Summary

The *suf* and *isc* operons of *Escherichia coli* have been implicated in Fe–S cluster assembly. However, it has been unclear why *E. coli* has two systems for Fe–S cluster biosynthesis. We have examined the regulatory characteristics and mutant phenotypes of both operons to discern if the two operons have redundant functions or if their cellular roles are divergent. Both operons are similarly induced by hydrogen peroxide and the iron chelator 2,2′-dipyridyl, although by different mechanisms. Regulation of the *isc* operon is mediated by IscR, whereas the *suf* operon requires OxyR and IHF for the response to oxidative stress and Fur for induction by iron starvation. Simultaneous deletion of *iscS* and most *suf* genes is synthetically lethal. However, although the *suf* and *isc* operons have overlapping functions, they act as distinct complexes because the SufS desulphurase alone cannot substitute for the IscS enzyme. In addition, *suf* deletion mutants are more sensitive to iron starvation than *isc* mutants, and the activity of the Fe–S enzyme gluconate dehydratase is diminished in the *suf* mutant during iron starvation. These findings are consistent with the model that the *isc* operon encodes the housekeeping Fe–S cluster assembly system in *E. coli*, whereas the *suf* operon is specifically adapted to synthesize Fe–S clusters when iron or sulphur metabolism is disrupted by iron starvation or oxidative stress.

Introduction

Fe–S clusters function as key cofactors in multiple cellular processes (reviewed in Beinert and Kiley, 1999; Frazzon and Dean, 2003). *In vitro* assembly of Fe–S clusters in proteins can occur spontaneously but *in vivo* Fe–S biogenesis requires specific accessory proteins (reviewed in Frazzon and Dean, 2003). Early studies of Fe–S cluster assembly in the complex nitrogenase enzyme of *Azotobacter vinelandii* identified two major components required for cluster building, NifS and NifU (Jacobson *et al*., 1989). NifS is part of a large family of pyridoxalphosphate dependent cysteine desulphurases that mobilize sulphur from L-cysteine (Zheng *et al*., 1993). NifU was found to be an Fe–S cluster scaffold upon which the nascent cluster can be constructed before its transfer to an apo-protein (Yuvaniyama *et al*., 2000). Homologues of these two core components can be found in organisms ranging from bacteria to humans.

The Gram-negative bacterium *Escherichia coli* contains three gene products that are homologous to NifS, namely IscS, SufS (also known as CsdB) and CsdA (also known as CSD or YgdJ). IscS and the NifU-like scaffold protein IscU are encoded by the *iscRSUA* operon and are required for assembly of a variety of Fe–S enzymes in *E. coli* (Flint, 1996; Schwartz *et al*., 2000; Urbina *et al*., 2001; Kato *et al*., 2002). The co-expressed IscA protein can form Fe–S clusters *in vitro* and may be an alternative scaffold or an iron handling protein (Krebs *et al*., 2001; Ollagnier-De-Choudens *et al*., 2001). Deletion mutants of *isc* possess a variety of growth defects as the result of the loss of Fe–S enzyme activity and the disruption of sulphur metabolism (Kambampati and Lauhon, 1999; Takahashi and Nakamura, 1999; Lacourciere *et al*., 2000; Schwartz *et al*., 2000; Tokumoto and Takahashi, 2001; Mihara *et al*., 2002).

The function of the *sufABCDSE* operon encoding SufS is less clear. SufA and SufS are homologous to IscA and IscS, respectively, but the *suf* operon does not contain a direct homologue to the Fe–S scaffold NifU. SufC is a soluble ATPase similar to those found associated with transporters (Rangachari *et al*., 2002; Nachin *et al*., 2003). The SufB and SufD proteins do not have assigned functions, although they do interact with SufC to form a complex (Rangachari *et al*., 2002; Nachin *et al*., 2003; Outten *et al*., 2003). SufE was recently shown to be a novel sulphur transfer protein that enhances SufS cysteine desulphurase activity by accepting sulfane sulphur (Loiseau *et al*., 2003; Ollagnier-De-Choudens *et al*., 2003; Outten *et al*., 2003). The SufBCD complex further stimulates SufE enhancement of SufS by an unknown

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mechanism (Outten *et al*., 2003). The *suf* operon was originally identified based on loss of the Fe–S cluster in the putative iron reductase FhuF in *sufD* or *sufS* deletion strains (Patzer and Hantke, 1999). FhuF is necessary for utilization of ferrioxamine B or rhodotorulic acid as sole iron sources (Muller *et al*., 1998), and the *sufS* and *sufD* mutants showed an inability to grow using these exclusive iron sources. Subsequently it was discovered that *suf* mutants of *Erwinia chrysanthemi* have similar limitations in their ability to utilize certain iron sources (Nachin *et al*., 2003). Recent work also has shown that, in certain media, *suf* mutants in *E. coli* are sensitive to phenazine methosulphate, a redox active reagent that can generate superoxide as well as oxidize protein thiols and intracellular iron, and that these defects are due to decreased activity of some Fe–S enzymes (Nachin *et al*., 2003).

The third NifS-like gene product, CsdA, can donate sulphur for molybdopterin synthesis *in vitro* but there have been no published phenotypes for *csdA* mutants (Leimkuhler and Rajagopalan, 2001). Interestingly, *csdA* is located on the chromosome adjacent to *ygdK*, which is homologous to SufE.

Recently it was discovered that the *iscRSUA* and *sufABCDSE* operons are induced during exposure to hydrogen peroxide (H_2O_2) in *E. coli* (Zheng *et al*., 2001). The only known regulator of *isc* is IscR, a transcriptional repressor co-expressed with the other *isc* genes. Repression of *isc* expression by IscR is relieved when a 2Fe–2S cluster present in IscR is damaged or fails to form, and a non-polar deletion in *iscR* causes a 17-fold increase in basal expression of the *isc* promoter (Schwartz *et al*., 2001). Induction of the *suf* promoter by H_2O_2 was found to be dependent on OxyR, the global regulator of the cellular response to H_2O_2 stress (Zheng *et al*., 2001). The expression of *sufS–* and *sufD–lacZ* fusions also were found to be regulated by Fur, a metalloregulatory protein that controls transcription of genes involved in iron metabolism (Patzer and Hantke, 1999). A subsequent DNA microarray study in *E. coli* confirmed that the *suf* operon is part of the Fur regulon in *E. coli* (McHugh *et al*., 2003). The *suf* system in *E. chrysanthemi* is also regulated by Fur in response to iron availability (Nachin *et al*., 2001).

The fact that the *isc* and *suf* operons both contain NifS homologues and both have phenotypes that connect them to Fe–S cluster assembly raises the question of why *E. coli* has two loci for Fe–S cluster assembly. The two operons may have distinct roles, such as is the case with the *nif* and *isc* operons of *A. vinelandii*, where the *nif* operon is specialized for the construction of nitrogenase whereas the *isc* operon assembles clusters for other Fe–S enzymes (Jacobson *et al*., 1989; Zheng *et al*., 1998). The *isc* and *suf* operons do contain different accessory genes that are co-expressed with the NifS homologue, indicating some likelihood of differential function. Alternatively, the two operons may be regulated differently to provide similar functions under distinct conditions in the cell. To gain more insight into these possibilities, we examined the regulatory characteristics and the mutant phenotypes of the *isc* and *suf* operons. Based on reported iron phenotypes for *suf* mutant strains and on the results presented here, we propose that the *suf* operon in *E. coli* is an Fe–S cluster assembly pathway specifically adapted for constructing Fe–S clusters under iron limiting conditions.

Results

Strong suf *and* isc *induction by* H_2O_2 *and 2,2′-dipyridyl*

To assess whether the *suf* or *isc* operons are differentially induced by H_2O_2, we carried out primer extension assays to measure *suf* and *isc* transcript levels in cells exposed to a range of H_2O_2 concentrations. Both operons were similarly responsive to H_2O_2, with the highest induction occurring at 300 μM H_2O_2 (Fig. 1A). We also examined the *suf* and *isc* mRNA levels in cells treated with various amounts of the intracellular iron (II) chelator 2,2′-dipyridyl. The basal levels of the *suf* transcript appear to be lower than the basal levels of the *isc* transcript, but the two operons showed similar fold induction by 2,2′-dipyridyl-mediated iron starvation (Fig. 1B). These results show that the induction of the *suf* and *isc* operons in response to H_2O_2 and 2,2′-dipyridyl is similar.

iscR *is required for* isc *induction in response to* H_2O_2 *and 2,2′-dipyridyl*

To ascertain if the induction of the *isc* operon in response

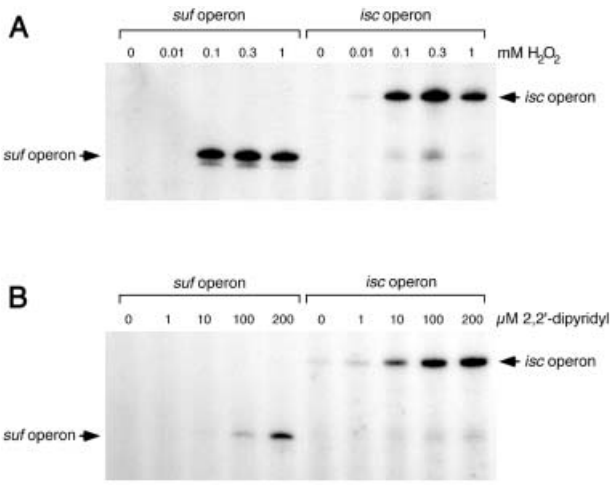

Fig. 1. Regulation of *suf* and *isc* in response to H_2O_2 and 2,2-dipyridyl.
A. Primer extension analysis of *suf* and *isc* transcription in cells exposed to varying concentrations of H_2O_2 for 5 min.
B. Primer extension analysis of *suf* and *isc* transcription in cells exposed to varying concentrations of 2,2′-dipyridyl for 60 min.

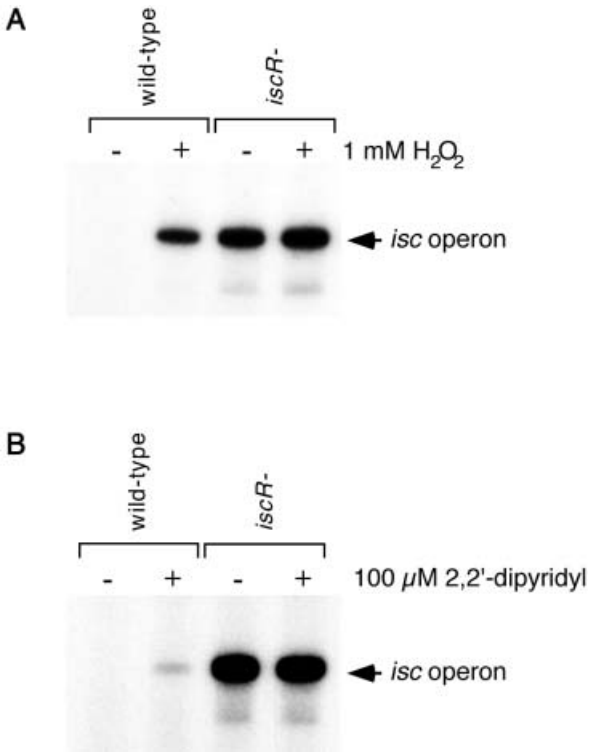

Fig. 2. Regulation of *isc* in response to H_2O_2 and 2,2-dipyridyl in an *iscR* mutant. Autoradiograph exposure times were optimized for each panel to visualize the *isc* transcript in the wild-type background, and therefore do not allow direct comparison between panels A and B.
A. Primer extension analysis of *isc* transcription in wild-type and *iscR* mutant cells exposed to 1 mM H_2O_2 for 5 min.
B. Primer extension analysis of *isc* transcription in wild-type and *iscR* mutant cells exposed to 100 μM 2,2′-dipyridyl for 60 min.

to oxidative stress and iron starvation is mediated through the IscR repressor, we examined *isc* induction in response to H_2O_2 and 2,2′-dipyridyl in a mutant strain with a non-polar deletion of *iscR*. An *iscR* deletion strain shows constitutive expression of the *isc* operon and *isc* expression is not further induced in response to H_2O_2 or 2,2′-dipyridyl (Fig. 2A and B). This result indicates that the IscR repressor accounts for the transcriptional induction of the *isc* promoter in response to oxidative stress and iron starvation.

Both OxyR and IHF are required for suf *induction in response to* H_2O_2

To further characterize the *suf* transcriptional response to H_2O_2 and 2,2′-dipyridyl, we defined the regulatory features of the *suf* promoter. It was previously shown that OxyR regulates *suf* in response to H_2O_2 and that OxyR binds to the *suf* promoter (Zheng *et al*., 2001). Interestingly, the OxyR binding site is located at −197 to −244, much further from the −35 promoter element than is typically seen with OxyR regulated genes (Fig. 3A). Examination of the *suf* promoter revealed a putative binding site for integration host factor (IHF) located at −139 to −151 (Fig. 3A). IHF, a DNA-bending protein, plays a key role in the regulation of a number of stress response genes and is thought to be important for the cellular adaptations that occur upon entry into stationary phase (Nash and Robertson, 1981; Stenzel *et al*., 1987; Altuvia *et al*., 1994; Ditto *et al*., 1994).

To test whether IHF is necessary for OxyR-dependent induction of the *suf* operon, we examined *suf* expression in strains lacking either *himA* or *himD*, which encode the subunits of IHF. Deletion of either *himA* or *himD* abolished the H_2O_2 induction of *suf* by OxyR (Fig. 3B). This suggests that the DNA bending activity of IHF may be required to bring the atypically distant OxyR site closer to RNA polymerase bound at the −10 and −35 promoter elements. There was still some residual activation of *suf* in response to H_2O_2 even in the *oxyR*, *himA* and *himD* deletion strains. Because Fe-Fur may be damaged during oxidative stress, the residual transcriptional activation of *suf* seen in the mutant strains (Fig. 3B) could be the result of loss of Fur repression of *suf* upon H_2O_2 exposure. Alternatively, the residual induction could be mediated by an as yet unidentified regulator (Lee *et al*., 2003).

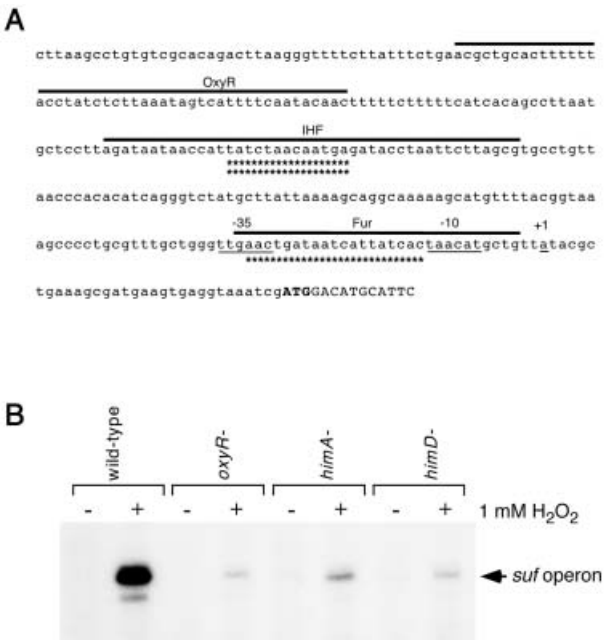

Fig. 3. Regulation of the *suf* promoter.
A. The +1, −10 and −35 elements of the *suf* promoter are underlined, the start codon for *sufA* is shown in bold capital letters, the putative Fur site is indicated with asterisks under the sequence, and the putative IHF site is indicated with double asterisks under the sequence. The sequences protected by Fur, IHF and OxyR in DNase I footprinting assays are denoted with solid bars.
B. Primer extension analysis of *suf* transcription in response to 1 mM H_2O_2 in *oxyR*, *himA*, and *himD* mutant strains.

Independent binding of IHF, OxyR and Fur to the suf *promoter*

Although genetic evidence indicates that both Fur and IHF regulate *suf*, it was not known where these two regulatory factors bind to the *suf* promoter. To localize the IHF and Fur binding sites we carried out DNase I footprinting experiments. These assays showed that IHF binding protects from –121 to –164 of the *suf* promoter, centred at the predicted binding site between the OxyR site and the promoter (Fig. 3A and Fig. 4). At 100 nM and higher concentrations of IHF, some protection was observed at a second lower affinity site from +7 to –14, which partially overlaps the –10 promoter element (Fig. 4A and data not shown). We observed Fur binding to the predicted Fur box centred between the –10 and –35 promoter elements resulting in DNase I protection of the region from –3 to –32 (Fig. 3A and Fig. 4). No Fur binding was observed elsewhere in the *suf* promoter even at Fur concentrations of up to 1 μM (Fig. 4, data not shown). Oxidized OxyR bound at a single site located at –197 to –244, as has been previously reported (Zheng *et al*., 2001).

To determine if binding of one regulatory protein to the *suf* promoter might interfere with subsequent binding of the other proteins to their mapped sites, we mixed different combinations of IHF, oxidized OxyR, and Fur with the *suf* promoter fragment before DNase I treatment (Fig. 4B). Prior binding of IHF to the *suf* promoter did not affect the OxyR or Fur footprint. Addition of OxyR before Fur also did not effect the footprints for either protein. Finally, mix-

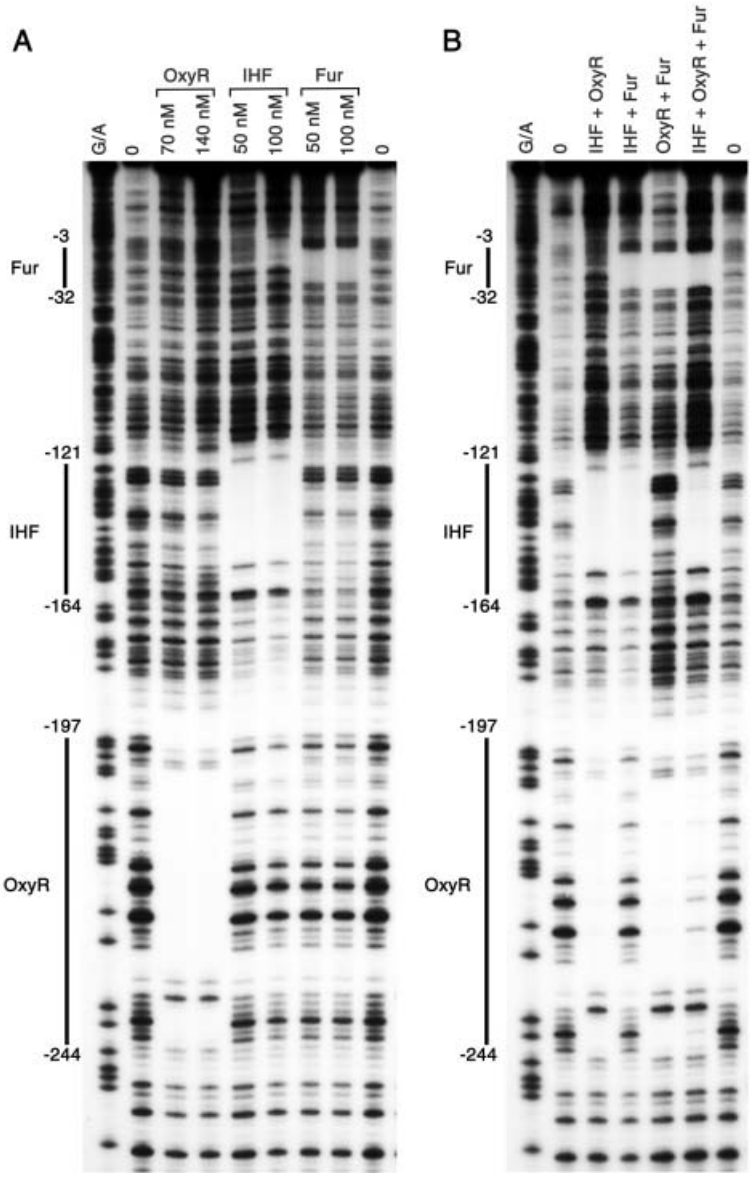

Fig. 4. DNase I footprinting assays of the *suf* promoter. The samples were run in parallel with Maxam–Gilbert G/A sequencing ladders. The regions protected by each protein are shown as a solid bar to the left of the footprint. Numbering of the protected regions are relative to +1 of the *suf* promoter.
A. Binding of various amounts of oxidized OxyR, IHF and Fur to the top strand of the *suf* promoter followed by DNase I treatment.
B. Binding of stepwise mixtures of the three proteins at 50 nM levels followed by DNase I treatment. The order of addition is indicated by the label order.

ing of IHF, OxyR and Fur did not significantly block or alter the footprints of any of the three proteins. These results indicate that each regulatory protein binds at a distinct site within the *suf* promoter independent of the other proteins.

Most suf *gene products are needed to complement an* iscS *deletion*

The *isc* operon of *E. coli* is important for Fe–S cluster assembly in a variety of enzymes, and the *iscS* deletion mutant shows a range of growth defects including a slow growth phenotype and several auxotrophies (Takahashi and Nakamura, 1999; Schwartz *et al*., 2000). In contrast, no obvious phenotypes for a *sufABCDSE* deletion mutant under normal growth in rich or minimal media have been reported. However, a recent study has shown that a plasmid expressing *sufABCSE* was required to construct an *iscRSUA* mutant in a *sufABCDSE* deletion background, indicating that deletion of the two operons is synthetically lethal (Takahashi and Tokumoto, 2002).

To test if individual *suf* gene products can substitute for deletion of *iscS*, we measured the co-transduction of an *iscS::kan*^{R} mutation and a nearby Tn10 insertion into various *suf* mutant backgrounds. The results revealed that all *suf* mutations constructed, except *sufA*, were synthetically lethal with the *iscS* mutation (Table 1). The synthetic lethality assays suggest that the cysteine desulphurase SufS alone cannot directly substitute for IscS but requires the other *suf* accessory proteins. In addition, the synthetic lethality observed upon deletion of the *sufE* or *sufBCD* genes with *iscS* indicated that SufE and SufBCD are absolutely essential for proper *suf* function. Thus the *suf* gene products are likely to act as a co-dependent complex rather than as separate enzymes. This interpretation is consistent with a recent study that shows that SufB, SufC, and SufD interact as a biochemical complex and that one function for SufE and the SufBCD complex is to enhance the unusually low cysteine desulphurase activity of SufS (Outten *et al*., 2003). The synthetic lethality phenotype was complemented if the *suf* operon was provided *in trans* (Table 1).

Table 1. Synthetic lethality of *iscS* and most *suf* deletion mutants.

Strain	Co-transduction frequency (*iscS*::*kan*^{R} and *zff*208-Tn10)
Wild type	81%
sufABCDSE	0
sufA	79
sufBCD	0
sufB	0
sufD	0
sufSE	0
sufE	0
sufABCDSE/pBAD*sufABCDSE*	71

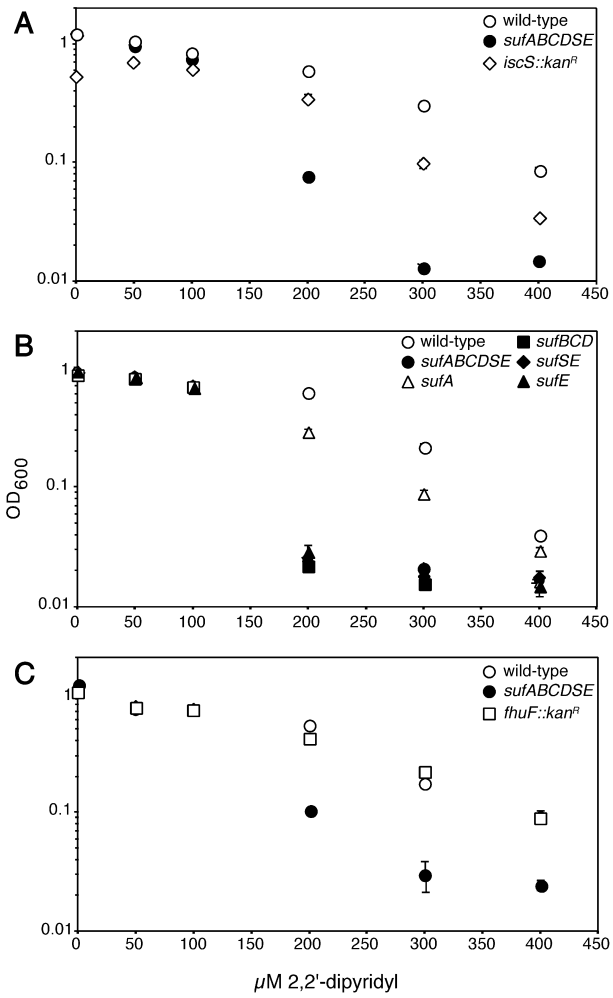

Fig. 5. Iron starvation sensitivity of *suf* mutants. Stationary phase cultures of wild-type, *iscS*::*kan*^{R} , and *sufABCDSE* mutant strains (A); wild-type and various *suf* mutant strains (B); and wild-type, *fhuF*::*kan*^{R}, and *sufABCDSE* mutant strains (C) were diluted into M9 gluconate minimal media containing increasing levels of 2,2′-dipyridyl. Final OD_{600} measurements were taken after 14 h of growth at 37°C in 2,2′-dipyridyl containing media. Error bars are ± one standard deviation for experiments carried out in triplicate.

suf *mutants are more sensitive to iron starvation than* isc *mutants*

As both *suf* and *isc* were induced by iron starvation, we compared the ability of *suf* and *isc* deletion mutants to grow under iron starvation conditions. Stationary phase cultures were diluted directly into media containing the iron chelator 2,2′-dipyridyl and allowed to grow for 14 h. Although initial growth rates were similar for the wild-type and *sufABCDSE* mutant strains, with time, at 2,2′-dipyridyl concentrations of 200 μM or greater, the *sufABCDSE* mutant strain showed a marked decrease in growth compared to the wild-type strain (Fig. 5A and data not shown). The *sufABCDSE* mutant also was more sensitive to iron starvation than an *iscS* mutant despite the pleiotropic

growth defects observed for the *iscS* mutant strain (Fig. 5A). The iron starvation phenotype of the *sufABCDSE* mutant was observed in a variety of media compositions but was most pronounced in minimal media with gluconate as the carbon source. The iron starvation phenotype could be complemented by the *suf* operon expressed from a plasmid or by the addition of iron to the media (data not shown). All *suf* mutants tested showed the same iron starvation phenotype with the exception of *sufA*, paralleling the results we observed with the synthetic lethality phenotype (Fig. 5B). When exponential phase cultures of the *sufABCDSE* deletion strain were exposed to 2,2′-dipyridyl, they also showed sensitivity to the iron chelator (data not shown). However, the growth defect exhibited by the exponential phase cells was less severe than the defect observed for the stationary phase cultures.

To determine if overexpression of the *isc* operon might compensate for the *sufABCDSE* deletion under iron starvation conditions, we compared the sensitivity of a nonpolar *iscR* and *sufABCDSE* double mutant strain with that of wild-type and *sufABCDSE* single mutant strains when stationary phase cultures were diluted directly into gluconate minimal media containing 2,2′-dipyridyl. The constitutive overexpression of *iscSUA* that occurs in an *iscR* deletion strain (Fig. 2 and Schwartz *et al*., 2001) was not sufficient to rescue the *sufABCDSE* phenotype as the *iscR sufABCDSE* double mutant exhibited the same sensitivity to 2,2′-dipyridyl as the *sufABCDSE* single mutant (data not shown).

One phenotype reported for *sufD* or *sufS* mutant strains is the loss of the FhuF Fe–S centre (Patzer and Hantke, 1999). FhuF, a putative iron reductase, is needed for the utilization of ferrioxamine B or rhodotorulic acid as iron sources (Muller *et al*., 1998). The exact range of FhuF substrates has not been determined, and it is possible that the FhuF ferric reductase may play a role in releasing iron from other siderophore complexes. To test whether the iron starvation phenotype of the *suf* mutants could be attributed to loss of FhuF activity, we deleted *fhuF* and compared the iron starvation phenotype of the *fhuF*::*kan*^{R} strain with that of the *sufABCDSE* deletion strain. The iron starvation phenotype of the *suf* mutant was more severe than that of the *fhuF* mutant, which grew as well as the wild-type strain under these conditions (Fig. 5C). Thus the iron starvation sensitivity of a *suf* deletion strain was not solely caused by loss of FhuF activity, but was the result of a more general growth defect when iron is limiting.

Activity of the Fe–S enzyme 6-phosphogluconate dehydratase is reduced during iron starvation in a suf *mutant*

Although iron starvation sensitivity also was observed in glucose and succinate minimal media (data not shown), the effects of 2,2′-dipyridyl were most pronounced in gluconate minimal media. Similarly, phenazine methosulphate sensitivity was most pronounced for *suf* mutants grown with gluconate as the carbon source (Nachin *et al*., 2003). The enhanced phenotypes seen in gluconate media may be the result of the labile nature of the 4Fe–4S cluster in 6-phosphogluconate dehydratase (Gardner and Fridovich, 1991). Loss of phosphogluconate dehydratase activity would block the Entner–Doudoroff pathway and restrict growth with gluconate as the sole carbon source.

To test this possibility, we measured the specific activity of phosphogluconate dehydratase in wild-type and *sufABCDSE* deletion strains diluted from stationary phase directly into gluconate minimal media containing 2,2′-dipyridyl (Fig. 6A). Phosphogluconate dehydratase activity decreased more rapidly in response to increasing amounts of 2,2′-dipyridyl in the *suf* mutant strain compared to the wild-type strain. The activity of a more stable Fe–S enzyme, glutamate synthase, decreased in response to iron starvation but the decrease was the same in the wild-type strain and in the *sufABCDSE* mutant (Fig. 6A). Decreased gluconate dehydratase activity in the *sufABCDSE* mutant was not simply the result of decreased levels of gluconate dehydratase protein since Western blot analysis showed similar protein levels in both wild-type and *sufABCDSE* mutant strains at 2,2′-dipyridyl levels below 250 μM (Fig. 6B).

To determine if the *suf* defect in phosphogluconate dehydratase was due to decreased *de novo* synthesis of Fe–S clusters or to lack of Fe–S cluster repair, we grew wild-type and *sufABCDSE* deletion strains in glucose minimal media to ensure that transcription of the gluconate utilization pathway was fully repressed (Zwaig *et al*., 1973; Isturiz *et al*., 1986). Cells in exponential phase then were harvested and shifted into gluconate media containing 2,2′-dipyridyl so that only the activity of phosphogluconate dehydratase newly synthesized under iron starvation conditions would be measured. Phosphogluconate dehydratase activity increased more slowly and to a lesser degree in the *sufABCDSE* mutant compared to the wild-type strain at 100 μM 2,2′-dipyridyl, suggesting that *de novo* synthesis of the 4Fe-4S cluster in phosphogluconate dehydratase is impaired in the *sufABCDSE* mutant strain under iron starvation conditions (Fig. 6C).

Discussion

Our comparison of the regulation and mutant phenotypes of the *isc* and *suf* operons suggests overlapping as well as divergent roles for the two systems. Both operons encode Fe–S cluster assembly proteins and are induced by oxidative stress and iron starvation. The induction of

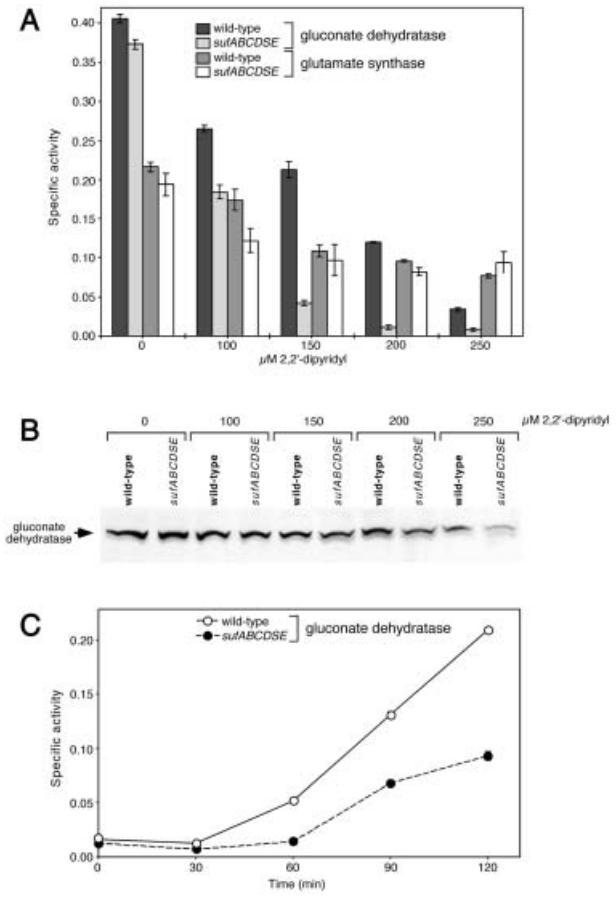

Fig. 6. Decreased 6-phosphogluconate dehydratase activity in response to iron starvation.
A. Stationary phase cultures of wild-type and *sufABCDSE* mutant strains were diluted to OD_{600} = 0.25 directly in gluconate minimal media with various amounts of 2,2′-dipyridyl for 2 h at 37°C. Specific activities of phosphogluconate dehydratase and glutamate synthase were measured in cell extracts after exposure. Error bars are ± one standard deviation for triplicate reactions carried out from each extract. Specific activity is defined as μmol of product formed per min per mg of total protein.
B. Levels of 6-phosphogluconate dehydratase protein in 20 μg aliquots from each of the cell extracts used in Fig. 6A were compared via immunoblot with antibodies specific for the enzyme.
C. Wild-type and *sufABCDSE* mutant strains were grown in glucose minimal media at 37°C to mid-exponential phase, harvested, and washed with minimal salts. Cells were then suspended in gluconate minimal media containing 100 μM 2,2′-dipyridyl. Specific activity of phosphogluconate dehydratase was measured in cell extracts at various time-points after suspension.

isc by oxidative stress and iron starvation likely reflects a failure in the formation of the Fe–S cluster in the IscR repressor, a regulatory system designed to sense perturbations in the overall Fe–S cluster status of the cell (Schwartz *et al*., 2001). In contrast, *suf* is regulated by OxyR and Fur, which are optimized to sense hydrogen peroxide stress and iron starvation, respectively. Thus whereas *isc* induction reflects disruption of overall Fe–S cluster status and the inability of IscS, IscU and IscA to maintain the cluster of IscR, *suf* induction is a specific response to oxidative stress and iron starvation.

Though both operons are induced in response to oxidative stress and iron starvation, a deletion of the *suf* operon renders the cell more sensitive to iron starvation than a deletion of the *isc* operon. This result shows that the *isc*-encoded proteins cannot function effectively to synthesize Fe–S clusters when iron is limiting. Thus the physiological roles of the *suf* and *isc* operons diverge under iron starvation conditions, explaining the requirement for two separate Fe–S cluster assembly operons in *E. coli*.

Our finding that the *E. coli suf* mutants show a greater sensitivity to iron starvation than *isc* mutants is consistent with the reported sensitivity of *Erwinia chrysanthemi suf* mutants to iron chelators (Nachin *et al*., 2003). Those results were interpreted to indicate the loss of a *suf*-dependent reductase in *Erwinia*, however, we have determined that the *suf* iron starvation sensitivity in *E. coli* is not solely caused by loss of the FhuF reductase. Indeed, we found that a decrease in phosphogluconate dehydratase activity is at least partially responsible for the *suf* growth defects under iron starvation conditions. We suggest that the *suf* operon plays an essential role in Fe–S assembly during iron starvation and FhuF is one of multiple Fe–S target enzymes disrupted in a *suf* mutant under iron limiting conditions. Based on assays of gene reporter constructs and the iron-activated antibiotic streptonigrin, *suf* mutants in *E. chrysanthemi* were reported to have elevated intracellular iron levels (Nachin *et al*., 2001). However, as both of these methods assay changes in intracellular iron availability rather than changes in overall iron accumulation, the results may be difficult to interpret. The reduced pathogenesis of *E. chrysanthemi suf* mutants (Nachin *et al*., 2001) and the induction of *sufA* in *Shigella* upon entry into host cells (Runyen-Janecky and Payne, 2002) are both consistent with a *suf* operon role in adaptation to the iron starvation faced by pathogens during infection.

The close interplay between iron homeostasis and oxidative stress complicates the interpretation of defects observed in *suf* mutants exposed to oxidative stress, such as H_2O_2 or phenazine methosulphate. One possibility is that the *suf*-encoded complex functions to repair damaged Fe–S clusters. However, in our assay measuring new synthesis of phosphogluconate dehydratase activity after a shift from glucose to gluconate under iron starvation conditions, the *suf* deletion strain showed decreased induction, consistent with disruption of *de novo* synthesis of the 4Fe-4S cluster in the enzyme. The *suf* operon also does not appear to be directly involved in Fe–S cluster repair under oxidative stress conditions (O. Djaman and J. A. Imlay, unpublished results). Another possibility is that the *suf* operon is required to synthesize Fe–S clusters in a

subset of enzymes distinct from those that require the *isc* operon. This second hypothesis is weakened by the synthetic lethality observed in the *isc* and *suf* double mutants, which implies at least some overlap in the Fe–S synthesis capacity of the two systems.

We favour a third hypothesis that the iron available for Fe–S cluster assembly is limiting during oxidative stress and that the *suf* operon has a critical function under such conditions. Cellular iron pools are thought to consist of two major components; iron bound tightly by iron proteins, including Fe–S enzymes, haeme proteins, and iron storage proteins, and a more labile pool of iron that is available for the synthesis of iron enzymes or control of iron responsive genes (Woodmansee and Imlay, 2002). The distribution of iron between these two components can be altered upon oxidative stress. The global iron regulator Fur is induced by OxyR in response to H_2O_2 (Zheng *et al*., 1999), which may lead to the repression of iron uptake genes. The pool of available iron may be further depleted in *E. coli* as it is sequestered into tightly binding iron proteins such as Dps. Dps (*D*NA-binding *p*rotein from *s*tarved cells) is strongly induced by hydrogen peroxide and during stationary phase and binds iron to form a ferritin-like microcrystalline core with 255 ± 42 atoms per dodecamer (Altuvia *et al*., 1994; Ilari *et al*., 2002). Restricting iron uptake and sequestering intracellular iron during H_2O_2 stress protects the cell from the Fenton reaction whereby Fe^{2+} reacts with H_2O_2 to form hydroxyl radicals (Imlay *et al*., 1988). However, the shift of total cellular iron into storage proteins could reduce the amount of iron available for synthesis of essential iron proteins even as some of these proteins, including Fe–S enzymes, are damaged by H_2O_2 stress. In this way, H_2O_2 stress may lead to *de facto* iron starvation and disrupt synthesis of iron enzymes required for basic metabolic functions.

Expression of non-iron alternative enzymes, such as fumarase C, during oxidative stress may circumvent the *de facto* iron starvation but not all iron-utilizing enzymes have non-iron alternatives. Therefore, the cell must have mechanisms to channel iron to essential iron proteins during oxidative stress, and Fe–S cluster assembly may require specialized enzymes, such as the *suf*-encoded proteins, to interface with iron trafficking pathways utilized under these conditions. It has been shown that recovery of Fe–S enzyme activity following challenge with peroxynitrite is reduced in mutants that lack the iron storage proteins ferritin (FtnA) or bacterioferritin (Bfn) (Keyer and Imlay, 1997).

Although we have discussed potential roles for the *suf* gene products in iron acquisition, it is possible that the limiting component for Fe–S cluster assembly under stress conditions is sulphur. Thus the key role of the *suf* operon during iron starvation or oxidative stress may actually be to provide a shielded pathway for donating sulfane sulphur to Fe–S clusters. Sulphur availability is likely to be limited as the result of direct oxidation of sulfane sulphur by reactive oxygen species or loss of sulfane sulphur from aborted clusters when iron is limited. Recent work has shown that the transfer of sulfane sulphur from SufS to SufE is more resistant to reductant than transfer of sulphur from IscS to IscU (Urbina *et al*., 2001; Outten *et al*., 2003), indicating that the sulphur transfer pathway is shielded in the *suf*-encoded complex.

In summary, we propose that the *suf* operon can compensate for the loss of iron or sulphur availability for Fe–S cluster biosynthesis that occurs during both iron starvation and H_2O_2 stress and is adapted to build Fe–S clusters under these conditions. The *suf* pathway could acquire iron through direct competition with other cellular chelators for the remaining labile iron pool or through specific interactions between Suf proteins and iron storage or trafficking proteins. Alternatively or in addition, the carefully shielded sulphur transfer pathway between SufS and SufE may be necessary to protect sulfane sulphur from reactive oxygen species and from loss if iron is unavailable. The next challenge is to elucidate the biochemical mechanisms by which *suf* gene products build Fe–S clusters during iron starvation or oxidative stress.

Experimental procedures

Media

Lennox Broth (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl per litre) was used for experiments with rich media. M9 minimal media with 0.2% carbon source was used for iron starvation sensitivity assays. Initial selection for gene disruption mutants was carried out on TYE plates (10 g of yeast extract, 5 g of tryptone, 8 g of NaCl per litre) with either chloramphenicol (30 μg ml^{-1}) or kanamycin (25 μg ml^{-1}). Other antibiotics used were ampicillin (100 μg ml^{-1}) and tetracycline (6 μg ml^{-1}).

Strain construction

Mutagenesis was performed using the *E. coli* λ Red targeted mutagenesis system (Datsenko and Wanner, 2000; Yu *et al*., 2000). Briefly, PCR fragments were amplified from either pKD3 or pKD4 plasmid templates encoding chloramphenicol and kanamycin resistance cassettes respectively (Datsenko and Wanner, 2000). The primers used for targeted gene disruptions are listed in Table S1. The 5′ end of each primer contained 30–40 nucleotides of sequence specific for the targeted gene(s) and the 3′ end of the primer contained 20 nucleotides of sequence specific for the template plasmid. Where necessary, primer sequence was designed to fuse optimized ribosomal binding site and start codon in frame with a downstream gene to construct non-polar mutations. Following transformation of PCR products into DY330, kanamycin- or chloramphenicol-resistant colonies were selected. Mutations were confirmed using colony PCR with

Table 2. Strains used in this study.

Strain	Relevant genotype	Reference
MG1655	wild-type	Laboratory strain
GSO77	*oxyR*::*kan*^{R}	Zheng *et al*. (2001)
GSO82	*fur*::*kan*^{R}	Mukhopadhyay *et al*. (2004)
GSO92	*sufABCDSE*	This work
GSO93	*sufA*	This work
GSO94	*sufB*	This work
GSO95	*sufBCD*	This work
GSO96	*sufD*	This work
GSO97	*sufSE*	This work
GSO98	*sufE*	This work
GSO99	*fhuF*::*kan*^{R}	This work
GSO100	*iscR*	This work
GSO101	*iscS*::*kan*^{R}	This work
GSO102	*iscS*::*kan*^{R} *zff*208-Tn10	This work
GSO103	*himA*::*cat*^{R}	This work
GSO104	*himD*::*cat*^{R}	This work
DY330	λ*cI857* Δ*(cro-bioA)*	Yu *et al*. (2000)

primers flanking the mutation. Each mutation was then transduced via P1 phage into MG1655. In some cases antibiotic resistance cassettes were later removed by FLP via plasmid pCP20 as described previously (Datsenko and Wanner, 2000). Removal of antibiotic resistance cassettes was confirmed by colony PCR with flanking primers. Previously described mutations in *himA* (Granston and Nash, 1993), *himD* (Flamm and Weisberg, 1985), and *iscS* (Schwartz *et al*., 2000) were P1 transduced into the MG1655 background. All strains used in this study are listed in Table 2.

RNA isolation and primer extension assays

Total RNA was isolated from cell cultures during early exponential growth (OD_{600} = 0.2–0.3) using either Trizol according to the product protocol (Invitrogen) or acid phenol as previously described (Kawano *et al*., 2002). Cells in early exponential growth were treated for 5 min with varying amounts of H_2O_2 or with varying amounts of 2,2′-dipyridyl for 1 h.

Primer extension was conducted on total RNA as described previously (Zheng *et al*., 2001). For quantitative measurements, gels were exposed to a phosphoimager screen and analysed using a Typhoon 9200 (Amersham Pharmacia) and ImageQuant 5.2 software (Molecular Dynamics). The primers used for primer extension are listed in Table S1.

DNase I footprinting assays

To generate a footprinting probe for the top strand of the *suf* promoter, the 435 bp *Bam*HI–*Eco*RV fragment of pGSO133 was labelled with ^{32}P at the *Bam*HI site. DNase I footprinting of oxidized OxyR or IHF binding to the *suf* promoter was carried out in 25 mM Tris-Cl, pH 7.9, 6 mM $MgCl_2$, 10% glycerol, 0.5 mM EDTA, 0.05% Nonidet P40 (Fluka), and 50 mM KCl. Footprinting with Fur alone and Fur together with IHF was carried out in Fur buffer (10 mM Bis-Tris-HCl, pH 7.5, 5 μg ml^{-1} sonicated salmon sperm DNA, 5% glycerol, 1 mM $MgCl_2$, 40 mM KCl, 100 μg ml^{-1} BSA, and 100 μM $MnCl_2$). Footprinting with OxyR and combinations of Fur and IHF was carried out in 25 mM Tris-Cl, pH 7.9, 6 mM $MgCl_2$, 10% glycerol, 0.05% Nonidet P40 (Fluka), and 50 mM KCl. Proteins were allowed to incubate with DNA probe for 15 min at 25°C before DNase I treatment. For footprints containing various mixtures of the three proteins, proteins and DNA probe were allowed to incubate 15 min at 25°C before addition of next protein. Samples were run in parallel with Maxam–Gilbert G/A sequencing ladders. Oxidized OxyR was purified as described previously. IHF protein was kindly provided by H. A. Nash. Fur protein was kindly provided by C. E. Outten and T. V. O'Halloran.

Growth assays

Sensitivity to iron starvation was measured as follows: overnight stationary phase cultures grown in M9 minimal media containing 0.2% gluconate were adjusted to the same starting OD_{600} and diluted 1:100 in 2 ml of M9 minimal media containing 0.2% gluconate and varying amounts of 2,2′-dipyridyl. Media used for assays with the *iscS*::*kan*^{R} strain also contained 12.5 μg ml^{-1} nicotinic acid. Cells were grown with shaking at 37°C for 14 h and OD_{600} was measured throughout growth. To assay the effects of added $FeCl_3$, equalized stationary phase cultures were diluted 1:100 into M9/gluconate media with 200 μM 2,2′-dipyridyl and varying amounts of $FeCl_3$. All assays were conducted in triplicate.

Synthetic lethality was assessed by measuring co-transduction frequency of the *iscS::kan*^{R} mutation with a nearby Tn10 insertion (*zff*208-Tn10) into various *suf* mutant backgrounds. Briefly, *iscS::kan*^{R} – *zff*208-Tn10 was transduced via P1 phage into MG1655 and various *suf* mutants followed by initial selection on tetracycline at 30°C. Tet^R colonies were then tested for kanamycin resistance at 30°C. Co-transduction frequency was calculated by dividing the number of Kan^R colonies by the total number of Tet^R colonies. A minimum of 50 colonies was screened for each measurement. The plasmid pGSO164, containing the *sufABCDSE* operon under the control of the pBAD promoter, was used for complementation studies (Outten *et al*., 2003). Basal expression from the pBAD promoter was sufficient to complement the *suf* mutant strains without addition of arabinose.

6-Phosphogluconate dehydratase activity assays and immunoblots

To assay activity in stationary phase cultures, overnight cultures of MG1655 and *sufABCDSE* (OD_{600} = 1.3) grown in M9 minimal media containing 0.2% gluconate were diluted to OD_{600} = 0.25 in M9 minimal media containing 12.5 μg ml^{-1} nicotinic acid and 0.2% gluconate with various amounts of 2,2′-dipyridyl. After 2 h at 37°C with shaking, cells were harvested by centrifugation at 8000 *g* for 10 min, washed once in 10 ml of M9 salts, and frozen in liquid nitrogen. Cell pellets were stored at −70°C until use. For time-course of gluconate dehydratase induction, cells were grown in M9 minimal media containing 12.5 μg ml^{-1} nicotinic acid and 0.2% glucose at 37°C to an OD_{600} = 0.4–0.5. Cells were collected by centrifugation, washed once with M9 salts, and resuspended in M9 minimal media containing 12.5 μg ml^{-1} nicotinic acid and 0.2% gluconate. Samples were taken at the indicated time points, harvested, washed, and frozen as described above.

Cell pellets were thawed on ice in an anaerobic chamber (Coy), resuspended in 800 μl of 50 mM Tris-Cl, pH 7.65, and lysed by sonication. Cell lysate (240 μl) was mixed with 30 μl of 100 mM $MgCl_2$. Reactions were initiated by addition of 30 μl of 80 mM 6-phosphogluconate and quenched by addition of 500 μl of boiling Tris buffer followed by boiling for 2–3 min. Pyruvate formed from 6-phosphogluconate and 2-dehydro-3-deoxyphosphogluconate aldolase was determined by mixing 50 μl of reaction with 930 μl of 50 mM Tris-Cl, pH 7.65, 10 μl 20 mM NADH, and 10 μl of 5% L-lactic dehydrogenase. The decrease in absorbance at 340 nm was measured and a millimolar extinction coefficient of 6.22 was used for NADH to calculate the amount of pyruvate present (amount of NADH consumed = amount of pyruvate present in original 5 min reaction). Total protein present in the cell extracts was measured. Specific activity is defined as μmol of product formed per min per mg of total protein.

Twenty microgram aliquots of total protein from each of the cell extracts prepared above were separated on a 4–20% Tris-glycine gel and transferred to a nitrocellulose membrane. The membrane was probed with a 1:5000 dilution of polyclonal antibodies prepared from rabbit specific for 6-phosphogluconate dehydratase that were kindly provided by L. O. Ingram. 6-Phosphogluconate protein levels were visualized using a 1:10 000 dilution of an HRP-conjugated secondary antibody specific for rabbit and the ECL Western Blotting Detection Reagents Kit (Amersham Biosciences).

Glutamate synthase activity assays

Twenty microlitre aliquots from the cell extracts prepared above were mixed with 830 μl 100 mM Tris-Cl, pH 7.8, 50 μl of 0.1 M α-ketoglutarate, 50 μl of 0.2 M L-glutamine, and 50 μl of 4 mM NADPH. The decrease in absorbance at 340 nm was measured over time and a millimolar extinction coefficient of 6.22 was used for NADPH to calculate the amount of L-glutamate formed from α-ketoglutarate and L-glutamine. Total protein present in the cell extracts was measured. Specific activity is defined as μmol of product formed per min per mg of total protein.

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Supplementary material

The following material is available from
http://www.blackwellpublishing.com/products/journals.suppmat/mmi/mmi4025/mmi3025sm.htm

Table S1. Sequences of oligonucleotides used in this study.

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