

The cysteine desulfurase, IscS, has a major role in *in vivo* Fe-S cluster formation in *Escherichia coli*

Christopher J. Schwartz*, Ouliana Djaman[†], James A. Imlay[†], and Patricia J. Kiley**

*Department of Biomolecular Chemistry, University of Wisconsin, Madison, WI 53706; and [†]Department of Microbiology, University of Illinois, Urbana, IL 61801

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The cysteine desulfurase, IscS, provides sulfur for Fe-S cluster synthesis *in vitro*, but a role for IscS in *in vivo* Fe-S cluster formation has yet to be established. To study the *in vivo* function of IscS in *Escherichia coli*, a strain lacking IscS was constructed and characterized. Using this *iscS* deletion strain, we have observed decreased specific activities for proteins containing [4Fe-4S] clusters from soluble (aconitase B, 6-phosphogluconate dehydratase, glutamate synthase, fumarase A, and FNR) and membrane-bound proteins (NADH dehydrogenase I and succinate dehydrogenase). A specific role for IscS in *in vivo* Fe-S cluster assembly was demonstrated by showing that an Fe-S cluster independent mutant of FNR is unaffected by the lack of IscS. These data support the conclusion that, via its cysteine desulfurase activity, IscS provides the sulfur that subsequently becomes incorporated during *in vivo* Fe-S cluster synthesis. We also have characterized a growth phenotype associated with the loss of IscS. Under aerobic conditions the deletion of IscS caused an auxotrophy for thiamine and nicotinic acid, whereas under anaerobic conditions, only nicotinic acid was required. The lack of IscS also had a general effect on the growth of *E. coli* because the *iscS* deletion strain grew at half the rate of wild type in many types of media even when the auxotrophies were satisfied.

NifS | FNR | oxidative stress repair | sulfur metabolism

Iron-sulfur proteins are ubiquitous in nature and participate in many vital cellular functions such as DNA repair, transcriptional regulation, nucleotide and amino acid biosynthesis, and energy metabolism. Although many studies have focused on the activities of Fe-S proteins, little is known about how Fe-S clusters are assembled and inserted into these proteins *in vivo*. Under reducing conditions *in vitro*, such clusters are slowly assembled into proteins when only Fe²⁺ and S²⁻ are provided (1). However, studies of the N₂-fixation (*nif*) gene cluster from *Azotobacter vinelandii* suggest that cells contain activities that increase the efficiency of Fe-S cluster assembly (2). In particular, the characterization of NifS led to the discovery that this protein is a pyridoxal 5'-phosphate-containing cysteine desulfurase that transfers the sulfur from cysteine to an active site cysteinyl residue of NifS, forming an enzyme-bound persulfide (3). Upon reduction and addition of an iron source, the S⁰ can be released and efficiently incorporated into the cluster of the Fe-S protein of nitrogenase (4).

Proteins similar to NifS have been discovered in non-N₂-fixing bacteria (5), eukaryotes and archaea (BLAST, National Center for Biotechnology Information), indicating that such cysteine desulfurases might serve a general role in Fe-S cluster formation. In support of this notion, a homolog of NifS, called Nfs1, was identified in the yeast *Saccharomyces cerevisiae*, where it appears to play a role in mitochondrial Fe-S cluster assembly (6–8). In addition, cysteine desulfurases homologous to NifS have been purified from *A. vinelandii* and *Escherichia coli* (9, 10). These proteins have been named IscS for iron sulfur cluster to distinguish them from their counterparts encoded within the N₂-fixation gene clusters of N₂-fixing bacteria (5). Although IscS from *E. coli* and *A. vinelandii* have been shown to facilitate Fe-S

cluster assembly *in vitro*, their role in *in vivo* Fe-S cluster formation has not yet been established. In addition, Flint *et al.* (11) also showed by using *E. coli* cell extracts that other proteins can function to donate sulfur for Fe-S cluster assembly, leaving open the question as to which protein is the physiologically relevant sulfur donor. Thus, the goal of this study was to investigate the role of IscS in donating sulfur for *in vivo* Fe-S cluster assembly.

Fe-S cluster assembly is important for the *de novo* synthesis of a broad spectrum of proteins and also the repair of certain Fe-S proteins that are damaged during oxidative stress. A family of dehydratases, including the major fumarase and aconitase isozymes, use [4Fe-4S]²⁺ clusters as Lewis acids during catalysis. These solvent-exposed clusters can be rapidly oxidized by superoxide, and the oxidized clusters are unstable, losing one and perhaps more iron atoms (12). The inactivation of these enzymes is responsible for many of the characteristic deficiencies of superoxide-stressed cells. Work in several labs has shown that these clusters are reassembled *in vivo*, using iron drawn from ferritin and a presumptive electron donor that has not been identified (13, 14). In fact, the fully demetallated form of one such enzyme, dihydroxyacid dehydratase, was used as a substrate during the purification of the *E. coli* IscS protein (10). However, it is not clear whether cluster disintegration *in vivo* progresses to the point where sulfur atoms are lost, so that the role of IscS in cluster repair is unclear.

An attempt to delete *iscS* from *A. vinelandii* was unsuccessful, apparently because the gene is essential (5). In addition, studies in yeast have demonstrated that the IscS homologue (Nfs1) is also essential in this organism (6). In this study, we report the successful disruption of *iscS* from *E. coli*, allowing us to analyze the function of IscS *in vivo*. We have found that the lack of IscS leads to an overall decrease in the activity of a number of Fe-S proteins and results in a general growth defect.

Materials and Methods

Growth Experiments. Cultures were grown aerobically overnight at 37°C in Luria broth (15), pelleted, washed, resuspended in glucose minimal medium (15), and used as an inoculum at a 1:1,000 dilution. Growth was monitored by measuring the OD₆₀₀. When indicated, the growth medium was supplemented with yeast extract (5 mg/ml), thiamine (2 µg/ml), nicotinic acid (12.5 µg/ml), chloramphenicol (20 µg/ml), ampicillin (50 µg/ml), tetracycline (10 µg/ml), and kanamycin (Kn, 40 µg/ml).

Abbreviations: Kn, kanamycin; FNR, fumarate nitrate reduction; WT, wild type; SDH, succinate dehydrogenase; DH, dehydrogenase.

[‡]To whom reprint requests should be addressed at: 1300 University Avenue, Department of Biomolecular Chemistry, University of Wisconsin Medical School, Madison, WI 53706. E-mail: pj.kiley@facstaff.wisc.edu.

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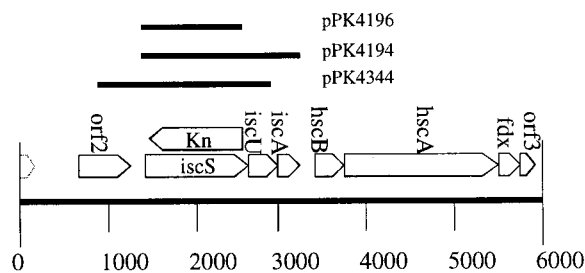


Fig. 1. Genomic organization of the *isc* region. Plasmids used in this study are indicated. The genotype of the *iscS* mutant is indicated with the *iscS* coding region having been replaced by the Kn^R gene.

Plasmid Construction. Plasmids pPK4071 and pPK4083 were made by digesting λ 430 (16) with *Nru*I and cloning this 5-kb fragment containing *iscRSUAhscB* (17) into the *Sma*I site of pUC19 (18) and pAlter-1 (Promega), respectively. Plasmids pPK4196 and pPK4194 were generated by cloning PCR products containing only the ORFs for *iscS* and *iscSUA* into pET11a (19), respectively (Fig. 1). Plasmid pPK4344 was generated by cloning into pGem2 (Promega), a PCR product containing sequence beginning within the *iscR* ORF and ending within the *iscA* ORF (Fig. 1). For DNA amplification by PCR from chromosomal or plasmid DNA, either *Pfu* DNA polymerase (Stratagene) or *Taq* DNA polymerase (Promega) was used. The sequences of oligonucleotides (Operon Technologies, Alameda, CA) used in this work are available on request.

Strain Construction. Codons 7 and 399 of *iscS* in pPK4083 were altered by site-directed mutagenesis (Promega Altered Sites), creating *Xho*I restriction endonuclease sites and generating plasmid pPK4119. The *iscS* coding region was deleted by digestion of pPK4119 with *Xho*I, the ends made blunt, and a blunt-ended Kn^R cartridge from Tn903 (20) was inserted by ligation to generate pPK4155 (Fig. 1). A DNA fragment containing approximately 1 kb of flanking sequence on either side of the Kn^R gene was PCR-amplified from pPK4155 and then cloned into pK03 (chloramphenicol, *sacB*, pSC101ori), which is temperature-sensitive for replication (21). The resulting plasmid, pPK4258, was transformed into PK4304, a strain containing a chromosomal Tn10 insertion in *phe* (located approximately 2 min from *iscS*) and F'142 that covers both the *isc* and *phe* regions. Strain PK4304 was made by mating CAG18469 (*phe*::Tn10) with KL709 (F'142) (22). Strains containing pPK4258 integrated into the chromosome of PK4304 were selected at the nonpermissive temperature (42°C) with a frequency of 10^{-3} – 10^{-4} . Cells containing the integrated plasmid were plated on 5% sucrose to select for those that had resolved and subsequently lost the plasmid. Sucrose- Kn^R -resistant colonies arose at a frequency of 10^{-3} , which was the source of PK4326. P1 phage were generated

from PK4326, and the Δ *iscS*:: Kn^R marker was transduced into RZ4500, PK3292, or PK3293 (Table 1) by selecting on Kn^R , generating strains PK4331, PK4357, and PK4359, respectively.

Fe-S Protein Assays. Isocitrate and potassium cyanide were from Aldrich; all other reagents were from Sigma. Cultures for assays of enzymatic activities were grown to 0.4–0.7 OD in media that would ensure consistent, substantial synthesis of the relevant enzymes. In all cases except for that of fumarase, the media were chosen to avoid a dependence on Fe-S enzymes for central metabolism. NADH DH1 (Ndh1) and succinate dehydrogenase (SDH) were assayed by using inverted membrane vesicles prepared from cells grown in minimal A salts (15) containing 1% casein hydrolysate, 0.5% yeast extract, and 0.5% NaCl (pH 7), and NADH DH2 (Ndh2) was assayed in vesicles derived from cells grown in LB medium containing 0.2% glucose. For aconitase assays cells were grown in LB medium; for 6-phosphogluconate dehydratase assays, in minimal A salts containing 0.2% gluconate and 0.2% casein hydrolysate; for glutamate synthase, in minimal A salts containing 0.2% glucose; and for fumarase and isocitrate dehydrogenase, in minimal A salts containing 40 mM fumarate.

To stabilize Ndh1 for assay, vesicles were prepared by sonication in 50 mM MES, pH 6. Ndh1 activity was measured by the oxidation of deaminoNADH (23), which was prepared by the enzymatic reduction of deaminoNAD⁺ using the protocol for NADH preparation (24). Ndh2 was assayed by the oxidation of NADH after Ndh1 had been inactivated by the overnight incubation of the vesicles in 50 mM KPi, pH 7.8, on ice. Succinate oxidase was measured by monitoring O₂ consumption with an OROBOROS oxygraph (Paar, Gratz, Austria) during incubation of inverted vesicles with 0.1 M succinate in 50 mM KPi, pH 7.8, at 37°C; succinate/quinone oxidoreductase activity was determined by plumbagin-dependent cytochrome *c* reduction (25).

Because anaerobiosis is essential for maintenance of the full activity of aconitase (S. M. Varghese and J.A.I., unpublished results), cell extracts were prepared by sonication in anaerobic 50 mM Tris-HCl, pH 7.8, at 1% the culture volume, in the anaerobic chamber. Aliquots were immediately diluted into 100 mM isocitrate in the Tris buffer and sealed inside air-tight cuvettes, and activity was quantitated by the production of cis-aconitase (26). Because some aconitase activity is from the stable isozyme aconitase A, this latter activity was determined by the inactivation of aconitase B by treatment with 1 mM EDTA for 1 h before assay (S. M. Varghese and J.A.I., unpublished results) and subtracted to indicate the aconitase B activity.

Total fumarase activity was assayed by the conversion of 50 mM malate to fumarate. To distinguish the oxidatively labile fumarase A isozyme from fumarase C, fumarase A was inactivated by exposure of the extract to xanthine oxidase and xanthine for 15 min at room temperature before reassay (27). The residual activity was caused by fumarase C. Isocitrate DH was assayed by the production of NADPH (28). To test the responsiveness of the SoxRS (superoxide) regulon to superoxide stress, the cells were cultured in the presence or absence of 10 μ M paraquat for more than three generations before harvesting for fumarase C determinations.

The activity of 6-phosphogluconate dehydratase was assayed by the two-step procedure for the determination of pyruvate (29). Glutamate synthase activity was determined by the oxidation of NADPH (30, 31). Protein was measured by the Coomassie dye-binding assay (Pierce).

The activity of FNR (for fumarate nitrate reduction) in whole cells was determined in strains containing *lacZ* under the control of an FNR-activated promoter, *PdmsA* (DMSO reductase). Cells were grown aerobically or anaerobically to an OD₆₀₀ of 0.1–0.2 or 0.3–0.7, respectively, in glucose minimal medium supple-

Table 1. *E. coli* strains

Strain	Genotype	Reference
CAG18469	MG1655 <i>phe</i> ::Tn10	(56)
CAG18481	MG1655 <i>zff-208</i> ::Tn10	(56)
RZ4500	MG1655 <i>lacZ</i> Δ 145	(57)
PK3292	RZ4500, <i>fnr</i> ⁺ , <i>dmsA'</i> - <i>lacZ</i>	This lab
PK3293	RZ4500, <i>fnr</i> ⁻ , <i>dmsA'</i> - <i>lacZ</i>	This lab
PK4304	CAG18469, F'142	This study
PK4326	CAG18469, Δ <i>iscS</i> :: Kn^R , F'142	This study
PK4331	RZ4500 Δ <i>iscS</i> :: Kn^R	This study
PK4357	PK3292 Δ <i>iscS</i> :: Kn^R	This study
PK4359	PK3293 Δ <i>iscS</i> :: Kn^R	This study

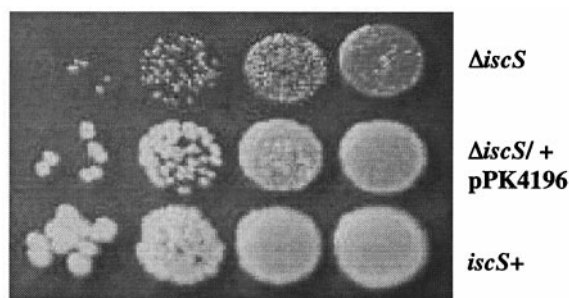


Fig. 2. The *iscS* mutant has a small colony phenotype. Colony phenotype of the *iscS* mutant (PK4331; *Top*) after 48 h of growth on LB plates at 37°C compared with the isogenic *IscS*⁺ strain (RZ4500; *Bottom*). Each spot represents a 10-fold serial dilution. The *iscS* mutant complemented with *iscS* in trans on plasmid pPK4196 (*Middle*).

mented with yeast extract (5 g/liter). β-galactosidase activity was measured as described (15).

Results

Generation of a *iscS* Null Strain. To address the function of *IscS* in *E. coli*, a gene disruption was created by replacing the *iscS* coding region with a gene encoding *Kn*^R. The genotype of the *ΔiscS::Kn*^R strain was verified by PCR using gene-specific primers (data not shown). Genetic mapping using P1 generalized transduction and a Tn10 (*zff-208*, 57.4 min) tightly linked to *iscS* (57.3 min) also showed that the *Kn*^R marker was at the expected location in the *E. coli* chromosome.

***IscS* Is Not Essential in *E. coli*.** The *iscS* mutant (PK4331) had a small colony phenotype on LB plates (Fig. 2), with colony formation taking twice as long for the mutant as for the *iscS*⁺ wild-type (WT) parent cells (RZ4500). P1 transduction demonstrated that the small colony phenotype was always associated with the *Kn*^R marker (data not shown), and when the WT *iscS* locus was cotransduced with a nearby Tn10 marker (*zff-208*) into the *Kn*^R mutant, all of the *Kn*-sensitive (*iscS*⁺) transductants grew as well as WT. In addition, the phenotype was corrected by the addition of *iscS* in trans on plasmid pPK4196 (Fig. 2). Because the *iscS* mutant took 2 days to form colonies, and because similar attempts to create a *iscS* null strain in *A. vinelandii* were unsuccessful (5), we wanted to address the possibility that the *iscS* mutant strain might have acquired a secondary mutation for viability. This appears not to be the case, because upon selection of the *ΔiscS::Kn*^R marker or an unlinked *phe::Tn10* marker transduced independently from the same strain similar numbers of transductants were obtained. If a suppressor mutation was required for viability, then fewer *ΔiscS::Kn*^R transductants would be expected because of the additional event that would be needed for growth. Therefore, in contrast to the situation in *A. vinelandii* and yeast (5–7), *iscS* is not essential for viability in *E. coli*, allowing us to analyze the function of *IscS* *in vivo*.

The *iscS* Mutant Has a Slow Growth Phenotype in Rich Media and Is Auxotrophic for Thiamine and Nicotinic Acid Under Aerobic Growth Conditions. In addition to producing small colonies on LB solid medium, the *iscS* mutant also exhibited a slow growth phenotype in LB liquid medium with a doubling time of 62 min for the *iscS* mutant compared with 31 min for WT (Fig. 3A). The *iscS* mutant did not grow at all in glucose minimal medium (Fig. 3B), even when casamino acids were present (data not shown). However, the addition of yeast extract to glucose minimal medium restored growth to the rate observed in LB medium (data not shown). Subsequent experiments determined that the addition of thiamine and nicotinic acid (Fig. 3B) to glucose minimal medium

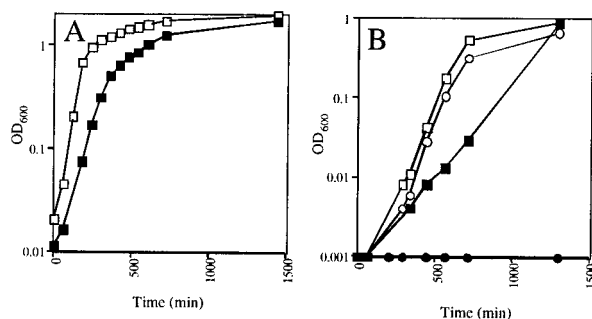


Fig. 3. Growth phenotype of the *iscS* mutant. (A) Growth curves of the *iscS* mutant (PK4331; ■) and the isogenic *IscS*⁺ parent strain (RZ4500; □) grown aerobically in LB medium. (B) Growth curves of the *iscS* mutant (●) and the parent strain (○) grown aerobically in glucose minimal medium, and the *iscS* mutant (■) and the parent strain (□) grown in glucose minimal medium supplemented with thiamine and nicotinic acid.

was sufficient for growth of the *iscS* mutant under aerobic conditions, with doubling times of 67 min for WT and 122 min for the *iscS* mutant. However, under anaerobic growth conditions the growth requirement for thiamine was eliminated (data not shown). To verify that the growth phenotypes resulted from the *iscS* deletion, we added *iscS* in trans (pPK4196), which relieved the thiamine and nicotinic acid auxotrophies and restored the growth rate to that of WT (data not shown). The *iscS* mutant also grew more slowly than WT in fumarate minimal medium, with doubling times of 230 min and 110 min, respectively. Therefore, under all conditions where the *iscS* mutant grew, the growth rate was consistently half the rate of WT growth, suggesting that this effect on growth rate is not related to nutritional requirements.

***IscS* Is Required for Full Activity of Many Fe-S Proteins.** The cysteine desulfurase *IscS* previously has been shown to participate in *in vitro* Fe-S cluster assembly. Therefore, the activity of a number of Fe-S proteins was measured in the *iscS* mutant to assess the role of *IscS* *in vivo*. The lack of *IscS* resulted in a 2- to 50-fold decrease in the specific activities of a number of proteins containing [4Fe-4S] clusters (Table 2 and Fig. 4). *IscS* appeared to be important for the activity of both soluble and membrane-bound Fe-S enzymes, because proteins from both classes had reduced activities in the *iscS* mutant. Enzymes that did not contain Fe-S clusters, such as isocitrate DH (Table 2) and the respiratory *Ndh2* (0.99 unit/mg in the mutant compared with 0.94 unit/mg in the parent), were unaffected by the mutation.

Table 2. Effect of the *iscS* mutation upon enzyme activities

	+	-	-/S	-/P _S SU
NADH DH I	100 (0.47)	1.4	6.3	28
SDH (succinate oxidase)	100 (0.033)	17	22	73
(quinone reduction)	100 (0.047)	12	23	70
Glutamate synthase	100 (0.42)	34	57	51
Aconitase B	100 (0.021)	52	60	90
6-phosphogluconate dehydratase	100 (0.098)	11	43	52
Fumarase A	100 (1.3)	49	59	ND
Isocitrate DH	100 (1.04)	107	113	103

*Averages are shown from 2–3 assays with <5% variation and are normalized to those of the parent *iscS*⁺ strain. The values in parentheses are the specific activities (units/mg) of the *iscS*⁺ strain. 1 unit = 1 μmol/min of product, except for succinate oxidase, where it represents 1 μmol per min O₂ consumed. ND, not determined. +, the *iscS*⁺ strain, -, the *iscS*⁻ strain, -/S, the *iscS*⁻ strain containing plasmid pPK4196, and -/P_SSU, the *iscS*⁻ strain containing plasmid pPK4344.

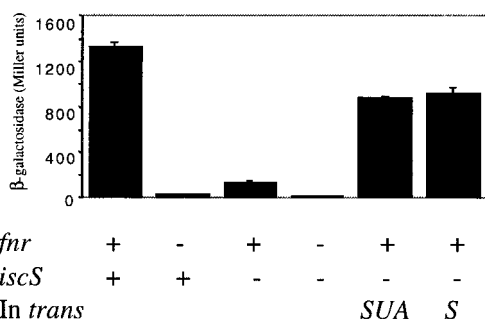


Fig. 4. The *iscS* mutant has reduced FNR activity that can be complemented by the addition of *iscS* in trans. FNR activity was measured by assaying the amount of β -galactosidase (Miller units) produced in strains grown under anaerobic conditions containing the FNR-dependent *dmsA'::lacZ* promoter fusion. The genotypes are indicated below the figure. Complemented strains contained either pPK4196 (*iscS*) or pPK4194 (*iscSUA*) and as with complemented strains in Figs. 2 and 3, expression of the *isc* gene(s) depended on fortuitous promoter elements from the vector (pET11a). The strains are all derivatives of either PK3292 (FNR⁺) or PK3293 (FNR⁻; Table 1).

Ndh1 activity was decreased to a greater extent (50-fold) than aconitase B activity (2-fold) even when both were assayed from the same cell culture, demonstrating that the distinct growth conditions necessary to assay certain enzyme activities (e.g., fumarase) did not cause the variable effect the *iscS* mutation had on different enzymes.

To demonstrate a direct role for IscS in *in vivo* Fe-S cluster formation, the anaerobic global regulator, FNR, was analyzed to take advantage of FNR mutants that have altered Fe-S cluster requirements. FNR is normally active only under anaerobic conditions because of the O₂ lability of its [4Fe-4S] cluster. FNR activity was monitored by assaying β -galactosidase activity from strains containing an FNR-dependent *dmsA'::lacZ* reporter fusion construct. As was the case with other Fe-S proteins analyzed, FNR activity was significantly decreased in the *iscS* mutant (PK4357) to 10% of the activity observed in the *iscS*⁺ strain (PK3292) grown under anaerobic conditions (Fig. 4). This decrease in FNR activity cannot be explained by a decrease in FNR protein levels because Western blot analysis showed that FNR protein levels were equivalent in both the WT and the *iscS* mutant strains (data not shown). To demonstrate that the decrease in FNR activity was directly caused by a defect in Fe-S cluster synthesis, we measured the activity of a FNR mutant (D154A), which unlike WT FNR, has measurable activity under aerobic conditions when the protein is devoid of a Fe-S cluster (32, 33). The aerobic activity of FNR-D154A was not altered by the deletion of *iscS* (Fig. 5 Upper), consistent with the fact that FNR-D154A activity is independent of a Fe-S cluster under aerobic growth conditions. A decrease in the activity of FNR-D154A is observed in the *IscS*⁻ strain under anaerobic conditions as is found with WT FNR (Fig. 5 Lower), consistent with previous studies showing that the majority of FNR-D154A activity under anaerobic conditions depends on the [4Fe-4S] cluster (33, 34). Thus these results show that the loss of IscS only reduces the activity of those forms of FNR that depend on an Fe-S cluster for activity.

The IscS Defects Resulted from a Block in *de Novo* Fe-S Cluster Synthesis. Two lines of evidence suggest that the decrease in Fe-S cluster protein activity in the *iscS* deletion strain was caused by a decrease in *de novo* cluster synthesis rather than by an inability to repair oxidized clusters. First, using another well-characterized FNR mutant, L28H, we show that the decreased activity observed in the *iscS* mutant is independent of O₂. The [4Fe-4S] cluster in FNR-L28H has been shown to be less O₂

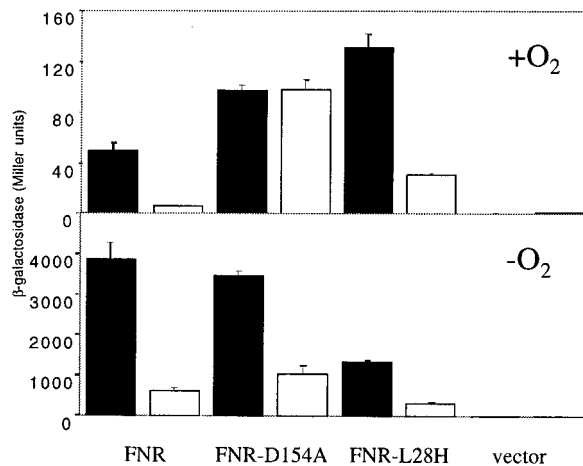


Fig. 5. The effect of the *iscS* mutation on the activity of FNR mutants that exhibit altered Fe-S cluster requirements. β -galactosidase activity produced from the *dmsA'::lacZ* promoter fusion was assayed from strains (*IscS*⁺; PK3293, filled columns; *IscS*⁻; PK4359, open columns) grown under aerobic conditions (Upper) and anaerobic conditions (Lower). WT FNR, FNR-D154A, and FNR-L28H were present on the multicopy plasmid pACYC184 (58).

labile than the WT protein, resulting in increased activity of this protein under aerobic conditions (35). Nevertheless, when cells are grown aerobically the [4Fe-4S] cluster of FNR-L28H is likely to be more susceptible to oxidative damage than when cells are grown anaerobically. Thus, if IscS were functioning in the repair of oxidatively damaged clusters, then the aerobic activity of FNR-L28H should show a greater dependence on IscS. However, the *iscS* mutant shows the same 4-fold decrease in activity under both aerobic and anaerobic growth conditions (Fig. 5), suggesting that IscS does not preferentially function in a cluster repair pathway; rather, IscS appears to function primarily in *de novo* cluster synthesis.

Additional evidence supporting the role of IscS in *de novo* cluster synthesis is that neither 6-phosphogluconate dehydratase nor aconitase B could be reconstituted by treatment of the *iscS* mutant extracts with DTT and Fe²⁺ (data not shown). In contrast, this protocol restored the activities of these enzymes when they were oxidatively damaged *in vitro* or in WT cells (36, 37) (data not shown). It is likely that cluster oxidation to the [4Fe-4S]³⁺ state triggers demetallation to an inactive [3Fe-4S]¹⁺ form that can be converted back to the active form without a sulfur source. Evidently the proteins in the *iscS* mutant accumulate incomplete clusters that are deficient in sulfur atoms, which differ from protein clusters after oxidation by superoxide, suggesting that the Fe-S protein defects observed in the *iscS* deletion strain reflect deficiencies in *de novo* Fe-S cluster assembly.

Complementation of the *iscS* Mutant. In addition to pPK4196 (*iscS*⁺) complementing the growth phenotype of the *iscS* mutant, the level of FNR activity restored by the presence of the same plasmid was 80% of the levels observed in the *iscS*⁺ parent (Fig. 4). Although this represents a significant restoration in FNR activity, we were surprised that the levels were not the same as that observed in the *iscS*⁺ strains. Because the *iscS* mutant strain contained a Kn^R gene in place of *iscS*, we considered the possibility that the Kn^R insertion also might be affecting expression of the downstream genes *iscUA*. *iscUA* (Fig. 1) show similarity to two other *A. vinelandii* *nif* genes, *nifU* and *orf6*, that also have been implicated in Fe-S cluster assembly (5). However, the amount of FNR activity observed in the strain containing *iscSUA* in trans (pPK4194) was no different from that observed

in the strain containing just *iscS* in trans (Fig. 4), suggesting that a decrease in the amount of the *iscUA* gene products is not contributing to the mutant phenotype. In contrast to the results obtained with FNR, the activity of several of the other Fe-S proteins was only minimally restored in strains containing either pPK4196 (*iscS*; Table 2) or pPK4194 (*iscSUA*; data not shown). Because all of the strains in Table 1 lack T7 RNA polymerase, expression of the *isc* genes from these plasmids depends on fortuitous vector promoter sequences, and thus IscS levels are likely to be low. Therefore, we introduced a plasmid containing the native *iscS* promoter along with *iscSU* (pPK4344). Although strains containing pPK4344 did not show any increase in FNR activity (data not shown), this plasmid led to increased activity for many of the Fe-S proteins (Table 2). Taken together, these data suggest that the degree of complementation may be related to the level of *iscS* expression. If this is indeed the case, then the decreases in Fe-S protein activities observed in the $\Delta iscS::Kn^R$ strain are most easily explained by the lack of *iscS*.

Discussion

The Role of IscS and Other Possible Cysteine Desulfurases in *in Vivo* Fe-S Cluster Formation. Previous studies of IscS have indicated that the cysteine desulfurase activity of this enzyme is used for *in vitro* Fe-S cluster formation (10). In this study, we observed that all examined Fe-S proteins had decreased levels of activity in the strain lacking IscS, indicating that IscS is also a major donor of sulfur atoms for the construction of Fe-S clusters *in vivo*. The fact that all Fe-S enzymes exhibited some activity demonstrates that a modest amount of cluster assembly persists in the *iscS* mutant. The efficiency of the residual cluster building pathway(s) varied among the enzymes that were tested, being particularly inadequate for Ndh1, SDH, and 6-phosphogluconate dehydratase. Although Ndh1 and SDH share the property of requiring multiple clusters for activity and these clusters are buried inside the holoenzyme, neither of these properties holds true for 6-phosphogluconate dehydratase, indicating no clear trends with this data set.

The *E. coli* genome contains three ORFs that encode proteins homologous to *A. vinelandii* NifS. IscS, the focus of this study, is 40% identical to NifS, and this homology exists through the entire protein. The two remaining NifS homologs, CSD and CsdB, represent a separate class in that they are both 24% identical to NifS with the highest homology in the N-terminal half of the proteins. Although both CSD and CsdB have cysteine desulfurase activity, selenocysteine is the preferred substrate (38, 39). In addition to CSD and CsdB, several other enzymes from *E. coli* were able to desulfurylate cysteine *in vitro*, stimulating Fe-S cluster assembly in apoenzymes (11). However, the enzymes that were shown to carry out this activity have other, well-established roles in cysteine metabolism, so their cluster assembly activity *in vitro* could be fortuitous or they could represent minor reactions for these enzymes. Nevertheless, these activities may account for the residual cluster assembly that occurs in the *iscS* mutant.

IscS Appears to be Involved in *de Novo* Fe-S Cluster Synthesis. Assembly of Fe-S clusters must occur in *E. coli* in at least two circumstances: during *de novo* cluster synthesis on the nascent apoenzyme and during the repair of oxidatively damaged clusters. Several lines of evidence indicate that the properties of the IscS mutant reported here resulted from inadequate *de novo* assembly rather than failed repair. The magnitude of the decrease in FNR-L28H activity was similar in cells grown both aerobically and anaerobically, whereas a defect in cluster repair would be expected to have a greater impact in air. The defects in FNR function and nicotinic acid biosynthesis persisted during anaerobiosis, when oxidative damage could not occur. Further, several proteins with oxidatively stable clusters (SDH, Ndh1, and

glutamate synthase) were affected as much by the *iscS* mutation as were proteins with unstable clusters (aconitase B, fumarase A, 6-phosphogluconate dehydratase, and FNR). Finally, oxidative inactivation of even the labile dehydratase Fe-S clusters has been estimated to occur fairly slowly in routinely cultured aerobic cells ($t_{1/2} = 40$ min) (27). Because this rate is relatively low, the activity deficits that we measured cannot be ascribed solely to a defect in cluster repair. These results all indicate that the defects in Fe-S cluster assembly that were observed in this study occurred because IscS is necessary for efficient *de novo* cluster assembly in most or all Fe-S proteins. Whether IscS is additionally involved in some cluster repair processes remains to be determined.

The Role of IscS in [2Fe-2S] Cluster Synthesis. All of the enzymes in Table 2 contain clusters of the [4Fe-4S] type, precluding any conclusion regarding the role of IscS in building [2Fe-2S] clusters. However, we found that treatment of the *iscS* mutant with paraquat led to a 10-fold induction of fumarase C, exactly as in the WT parent (data not shown). This result suggests that a substantial amount of SoxR protein, which mediates this response, contains its required [2Fe-2S] cluster (40). However, other work has shown that neither [4Fe-4S] nor [2Fe-2S] ferredoxins can be amply overproduced in *E. coli* unless the *isc* and *hsc* gene clusters are overexpressed as well (41). Additional work will be necessary to address the specificity of IscS in formation of [2Fe-2S] clusters and in the conversion of a [2Fe-2S] to a [4Fe-4S] cluster that occurs in some Fe-S proteins (42).

The Conserved Genomic Organization of the *isc* Genes and the *hsc* Genes Implies a Functional Relationship. The grouping of *iscU* and *iscA* immediately downstream of *iscS* suggests that these two gene products are also likely to be involved in Fe-S cluster assembly, and this has recently been demonstrated *in vitro* for IscU from *A. Vinelandii* (43). Downstream from the *isc* genes are *dnaJ* and *dnaK* molecular chaperone homologs (*hscBA*, respectively) and a ferredoxin (*fdx*) that have been implicated in Fe-S cluster formation (5), and furthermore, this genomic organization is conserved in other prokaryotes (17, 44) (Fig. 1). Although *hscBAfdx* are located immediately downstream of *iscSUA*, a transcription start site was identified upstream of *hscB* (45), indicating that these genes are transcribed independently of *iscSUA* in *E. coli*. Thus it seems unlikely that the *iscS* gene disruption is affecting levels of the *hscBAfdx* gene products. Nevertheless, the *hsc* gene products appear to be playing a role in Fe-S cluster assembly, because FNR activity is reduced 4-fold in a *hscA* strain (data not shown), and both the *isc* genes and the downstream *hsc* genes are needed for efficient overproduction of ferredoxins (41, 46). In addition, *S. cerevisiae* strains deficient in *iscU* homologs (*isu1* and *nfu1*) or a *hscA* homolog (*ssq1*) exhibit a similar phenotype of decreased activity of Fe-S proteins (7, 47), indicating that these genes are functionally related in the process of Fe-S cluster assembly.

The Growth Phenotype of the *iscS* Mutant May Have Both Fe-S Cluster Protein-Dependent and -Independent Components. The observation that the *iscS* mutant grew at a slower rate than WT was not unexpected because a number of metabolic enzymes contain Fe-S clusters. However, one cannot conclude at this time whether the slower growth rate is caused only by Fe-S protein defects or is related to other cellular functions requiring IscS as a sulfur donor, such as formation of thioridine in tRNA (48).

In contrast, the requirement for nicotinic acid in the *iscS* mutant is most likely explained by a Fe-S cluster protein defect. In *Bacillus subtilis*, mutations in a *nifS*-like gene or the putative Fe-S protein NadA (quinolinate synthase A) in the NAD biosynthetic pathway lead to an auxotrophy for nicotinic acid, a precursor for NAD (49, 50). To explain the thiamine require-

ment is more complex because the thiazole ring itself contains sulfur and IscS could function as the sulfur donor. In support of this, ThiI, a protein required for both thiamine and tRNA modification (51, 52) recently has been shown to function in the transfer of sulfur from IscS to form thiouridine in tRNA (48). In addition, an enzyme in the thiamine biosynthetic pathway (ThiH) has been proposed to contain an Fe-S cluster (53), and the activity of this enzyme may be insufficient in the *iscS* mutant. Consistent with an Fe-S protein defect, oxidative stress, a condition known to damage Fe-S clusters, also induces a growth requirement for thiamine and nicotinic acid (54). Interestingly, similar growth requirements have been observed in a *Salmonella typhimurium* strain with an insertion into *iscR*, the gene upstream of *iscS* (59). It remains an open question as to why the thiamine requirement was alleviated under anaerobic conditions. If thiamine production is reduced in the IscS⁻ strain, a simple explanation is that the level of thiamine needed to support anaerobic growth is less than that needed under aerobic conditions. This would not be surprising because expression of two major thiamine requiring enzymes, pyruvate dehydrogenase and α -ketoglutarate dehydrogenase, is repressed under anaerobic conditions (55).

The recent plethora of information about the *isc* genes and Fe-S cluster formation has led to a greater understanding of this essential cellular process. However, many questions remain, such as what other roles the IscS protein might perform *in vivo*, the source of the residual cluster assembly activity in the *iscS* mutant, whether [2Fe-2S] clusters are formed by the same assembly pathway as the [4Fe-4S] clusters, how the chaperone-like proteins are involved, and how these genes are regulated and this process is coordinated. Mutants in *E. coli* should provide a means by which these issues can be addressed at the molecular level.

Note Added in Proof. Lauhon *et al.* (60) have recently made a similar observation that *E. coli* strains lacking Isc S require nicotinic acid and thiamin.

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