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Genetic Analysis of the *isc* Operon in *Escherichia coli* Involved in the Biogenesis of Cellular Iron-Sulfur Proteins[1]

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The iron-sulfur (Fe-S) cluster, the nonheme-iron cofactor essential for the activity of many proteins, is incorporated into target proteins with the aid of complex machinery. In bacteria, several proteins encoded by the *iscRSUA-hscBA-fdx*-ORF3 cluster (*isc* operon) have been proposed to execute crucial tasks in the assembly of Fe-S clusters. To elucidate the *in vivo* function, we have undertaken a systematic mutational analysis of the genes in the *Escherichia coli isc* operon. In all functional tests, *i.e.* growth rate, nutritional requirements and activities of Fe-S enzymes, the inactivation of the *iscS* gene elicited the most drastic alteration. Strains with mutations in the *iscU, hscB, hscA,* and *fdx* genes also exhibited conspicuous phenotypical consequences almost identical to one another. The effect of the inactivation of *iscA* was small but appreciable on Fe-S enzymes. In contrast, mutants with inactivated *iscR* or ORF3 showed virtually no differences from wild-type cells. The requirement of *iscSUA-hscBA-fdx* for the assembly of Fe-S clusters was further confirmed by complementation experiments using a mutant strain in which the entire *isc* operon was deleted. Our findings support the conclusion that IscS, *via* cysteine desulfurase activity, provides the sulfur that is subsequently incorporated into Fe-S clusters by assembler machinery comprising of the *iscUA-hscBA-fdx* gene products. The results presented here indicate crucial roles for IscU, HscB, HscA, and Fdx as central components of the assembler machinery and also provide evidence for interactions among them.

Key words: cysteine desulfurase, *Escherichia coli*, gene disruption, iron-sulfur cluster, IscS.

Proteins containing iron-sulfur clusters (Fe-S) are ubiquitous in biological systems; they perform crucial roles in electron transfer, and have catalytic and regulatory functions (for recent reviews *1–4*). Structural data are available for a number of Fe-S proteins containing a variety of Fe-S clusters (*5*), but details of their biosynthesis in proteins are not understood. Only recently, have the components and mechanisms involved in the assembly of Fe-S clusters been approached by genetic and biochemical methods. In a pioneering report, Dean's group demonstrated the involvement of the NifS and NifU proteins in the generation of Fe-S clusters of nitrogenase in *Azotobacter vinelandii* (*6*). NifS is a pyridoxal phosphate–dependent cysteine desulfurase that initiates Fe-S cluster formation by producing elemental sulfur from cysteine (*7, 8*). The functions of NifU in binding iron and transient Fe-S clusters have recently been demonstrated (*9, 10*).

nifS-related genes are distributed in all known genome sequences including non-nitrogen-fixing organisms. *Escherichia coli* contains three copies of *nifS*-like genes, one of which (*iscS*) is located in the *iscRSUA-hscBA-fdx*-ORF3 cluster. The IscU protein encoded adjacent to *iscS* also shows homology to the N-terminal third of diazotrophic NifU. The gene cluster is conserved in many eubacteria and designated as the *isc* (iron-sulfur cluster) operon since it appears likely that the genes are cotranscribed and encode proteins with functions coupled with IscS in the formation of Fe-S clusters (*11*). Consistent with this proposal, we have recently demonstrated that the overexpression of the entire *isc* operon increases the cellular ability to assembly of Fe-S clusters, which leads to the overproduction of recombinant ferredoxins in *E. coli* (*12*). In addition, the *iscS, iscA, hscB, hscA,* and *fdx* genes have been shown to be crucial for the assembly of [2Fe-2S], [3Fe-4S], and [4Fe-4S] clusters in a variety of ferredoxin polypeptides (*13*). Thus, in contrast to the limited function of NifS and NifU in nitrogenase maturation, the proteins encoded in the *isc* operon probably serve a general function in the biosynthesis of a number of Fe-S proteins.

In the past three years, genes related to *iscS, iscU, iscA, hscB, hscA,* and *fdx* have been identified in yeast *Saccharomyces cerevisiae*. Biochemical and genetic studies have shown that the eukaryotic homologs are localized in mitochondria and involved in a complex machinery that cata-

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Abbreviations: Fe-S, iron-sulfur; Km^r, kanamycin-resistant; GltS, glutamate synthase; OD, optical density; ORF, open reading frame; SDH, succinate dehydrogenase.

lyzes the formation of Fe-S clusters (for recent reviews *4*, *14*, *15*). Most of the mitochondrial homologs are indispensable for the viability of yeast cells. Studies on the diazotrophic bacterium *A. vinelandii* have also demonstrated that the *iscS*, *hscA*, and *fdx* genes are essential for cell survival (*11*, *16*). In contrast, dispensable roles have been shown for the following bacterial genes: *hscA* and *fdx* in *Pseudomonas aeruginosa*, *iscR* and *iscA* in *Salmonella enterica*, and *hscA* and *iscS* in *E. coli* (*17–22*). In *E. coli*, the mutation in *hscA* coding for an Hsp70-type molecular chaperone was first described in 1994 as a suppressor of gene-regulation defects associated with mutations in the *hns* gene (*19*). Subsequent characterization demonstrated that HscA is unable to replace DnaK, a major Hsp70 chaperone in *E. coli*, in the chaperone-assisted folding and refolding of various proteins (*20*). However, the lack of HscA did not result in observable growth defects, and the exact role of HscA remained unclear. In contrast with *hscA*, mutations in the *iscS* gene cause severe growth defects in *E. coli* cells. Recent studies on the *iscS*-inactivated strain have revealed that IscS, in addition to its role in generating Fe-S clusters, is involved in the biosynthesis of the thiazole moiety of thiamine and 4-thiouridine in tRNA via a sulfur-transfer reaction to the ThiI protein (*21*, *22*). Until now, no genetic study has been reported on the bacterial *iscU* or *hscB* genes.

Few data are available for determining the comparative roles of the various components encoded in the bacterial *isc* operon or homologous proteins in yeast. Several bacterial mutations appear to have polar effects on the downstream genes in the multicistronic *isc* operon, which makes it difficult to evaluate the function of the mutated gene. In this study we have undertaken a systematic directed mutational analysis of the eight ORFs, including those as yet poorly characterized. We have found new mutant strains that exhibit previously undescribed defects in the synthesis of Fe-S proteins. This study also provides a comparative characterization of the mutant strains, which leads to the identification of central components of the Fe-S cluster biosynthesis apparatus.

MATERIALS AND METHODS

Strains and Cell Growth—Escherichia coli strain MG1655 served as the wild type. Rich media were LB or Terrific broth. Minimal media were generally M9 supplemented with glucose (20 mM), glycerol (40 mM), or sodium succinate (20 mM). When indicated, the growth medium was supplemented with nicotinic acid (1 μM), thiamine (1 μg/ml), ampicillin (50 μg/ml), chloramphenicol (20 μg/ml), tetracyclin (5 μg/ml), and kanamycin (20 μg/ml). Methionine, isoleucine, and valine were used at a concentration of 40 μg/ml.

Construction of pKO3-Based Plasmids for Gene-Disruption—Recombinant DNA techniques were carried out according to the established procedures (*23*). A 6.8-kb fragment containing the *isc* operon was excised from the pRKISC plasmid (*12*) by *Nco*I–*Nhe*I digestion and subcloned into the *Kpn*I–*Xba*I sites of the pHSG396 vector (TaKaRa) after appropriate end-repair reactions with T4 DNA polymerase. The resulting plasmid (pHSG-ISC) was digested with *Kpn*I, *Ahd*I, *Sac*II, *Ssp*I, *Hpa*I, and *Nsi*I to inactivate *iscR*, *iscS*, *iscA*, *hscB*, *hscA*, and ORF3, respectively, the ends were made blunt, and a blunt-ended Km^r cartridge from Tn5 was inserted by ligation. For the deletion of *iscSUA* and *iscRSUA-hscBA-fdx*-ORF3, pHSG-ISC was digested with *Ahd*I–*Sac*II and *Kpn*I–*Nsi*I, respectively, and the coding regions were replaced with the Km^r cartridge by blunt-end ligation. The correct orientation of the Km^r gene was confirmed by digestion with several restriction enzymes. Fragments containing the Km^r-marked insertion/deletion alleles of the *isc* operon were excised from the plasmids and cloned into pKO3 (*24*). For the deletion of *iscU*, the 1.2-kb fragment corresponding to the 5′ upstream region (*iscU*-5′) and the 1.0-kb fragment in the 3′ downstream region (*iscU*-3′) were amplified by PCR. The PCR products were cloned into the PCR2.1-TOPO vector (Invitrogen) by the TA cloning method, and the correct sequences were confirmed by the dideoxynucleotide chain-termination method using an Applied Biosystems 373A sequencer. The *iscU*-5′ fragment was excised by *Bam*HI–*Spe*I digestion and the *iscU*-3′ fragment by *Xho*I–*Bam*HI digestion. These fragments, together with the *Xba*I–*Sal*I fragment containing the Km^r gene, were cloned into the *Bam*HI site of pKO3 using the compatible cohesive ends. The resulting plasmid prepared from Km^r colonies had an *iscU*-5′/Km^r/*iscU*-3′ insertion in the correct orientation, as confirmed by digestion with several restriction enzymes. Similarly, a pKO3-based plasmid was constructed for the deletion of *fdx* using the 1.9-kb and 0.5-kb PCR fragments corresponding to the 5′ upstream and the 3′ downstream regions, respectively. The sequences of the PCR primers used in this work are listed in Table I.

Isolation of Mutant Strains—Following the transformation of *E. coli* strain MG1655 with the pKO3-derived plasmids, the gene-disrupted strains were isolated essentially as described by Church and co-workers (*24*). First, transformants containing the integrated plasmid were selected at a nonpermissive temperature (43°C) of the temperature-sensitive replicon (*repA*) in pKO3. Subsequently, cells that had lost the plasmid were selected by plating on 5% sucrose, taking advantage of the *sacB* gene in pKO3. Sucrose-resistant, Km^r, and chloramphenicol-sensitive colonies arose at a frequency of 10^{-3} to 10^{-5}. Genomic DNA was prepared from the mutant strains and analyzed by Southern hybridization as described (*25*).

Construction of Complementation Plasmids—Several DNA fragments in the *isc* gene cluster were subcloned into the pRKNSE vector (*12*), a low copy-number plasmid that contains an IncP1-type replicon and tetracyclin resistance marker, in the same direction as the *lac* promoter. The pISCΔ1 plasmid containing the entire *isc* operon was previously constructed by subcloning the 6.8-kb *Nco*I–*Eco*RI fragment from Kohara clone #430 (*13*). The pRKE plasmid was generated by ligating the 5.4-kb *Eco*RI fragment containing the *iscSUA-hscBA-fdx*-ORF3 cluster into the *Eco*RI

TABLE I. **PCR primers used in this work.** The underlined bases indicate restriction sites.

iscU-5′F	5′-GGATCCAAATTACCGATTTATCTCGACTAC
iscU-5′R	5′-GTCGACTATTAATGATGAGCCCATTCGAT
iscU-3′F	5′-CCGCTCGAGTTGAGGTTTGGTTATGTC
iscU-3′R	5′-CGGGATCCGCCAGACGACGCTG
fdx-5′F	5′-GGATCCGCCTTATTACAAATTAGTGAACC
fdx-5′R	5′-GTCGACTATTAAACCTCGTCCACGGAATG
fdx-3′F	5′-CGGCTCGAGAGGTTAGTATGGGACTTAAG
fdx-3′R	5′-CGGGATCCCCGTTCAAATGCAGGG

site of pRKNSE. The correct orientation was confirmed with several restriction enzymes. Similarly, 5.4-kb *Eco*RI fragments were excised from the pISC-ST, pISC-AT, pISC-hBT, pISC-hAT, pISC-FT plasmids (*13*), and subcloned into pRKNSE to generate the pRKE-ST, pRKE-AT, pRKE-hBT, pRKE-hAT, and pRKE-FT plasmids, respectively. These plasmids had an insertion of an oligonucleotide linker (TTAATTAATTAA, the underlined bases indicate stop codons) at appropriate restriction sites, which inactivates the genes by translational termination. The 4.7-kb *Ahd*I–*Eco*RI fragment (the *Ahd*I site was end-repaired) containing truncated *iscS* and intact *iscUA-hscBA-fdx*-ORF3 was inserted into pRKNSE, which had been cut with *Kpn*I, blunt-ended, and digested with *Eco*RI to generate pRKE-ΔS. Similarly, the pRKE-Δ3 and pRKS plasmids were generated by subcloning the 5.0-kb *Eco*RI–*Nsi*I and the 1.4-kb *Eco*RI-*Drd*I fragments, respectively, into the *Eco*RI-*Xho*I site of the pRKNSE vector after appropriate end-repair reactions with T4 DNA polymerase.

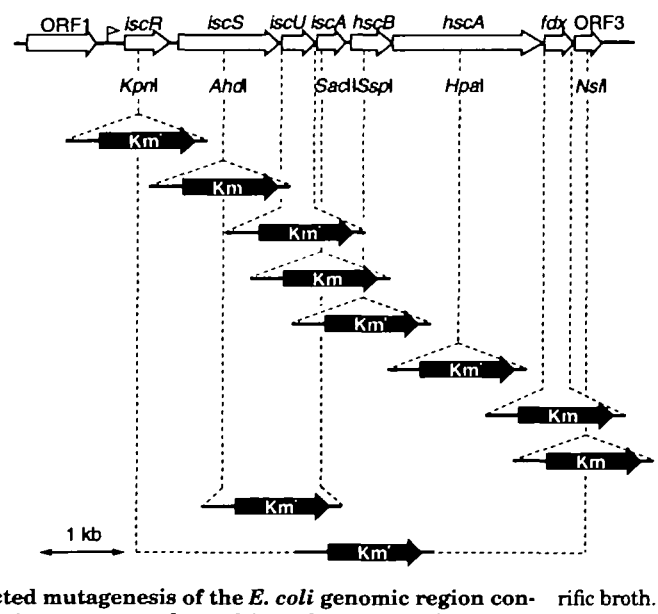
Measurement of Growth Rates—Growth rates of wild-type cells and mutant strains were measured as follows. A single colony of the strain was grown in Terrific broth to saturation. A 500-fold dilution was then made into 5 ml Terrific broth, and cell density (OD_{660}) was monitored every 15 minutes automatically with a Bio-Photorecorder TN-1506 (ADVANTEC). For monitoring the lag phase of growth upon switching from rich to minimal media, cells grown in Terrific broth were harvested, washed with minimal medium, and then diluted into medium containing additives as indicated. Growth was followed by measuring the turbidity (OD_{660}) with a colorimetor (mini photo 518, TAITEC).

Enzyme Assays—Cells were grown in Terrific broth and harvested in early stationary phase. The cells were pelleted, washed and resuspended in cold 50 mM potassium phosphate buffer, pH 7.4, and then disrupted by sonication in the presence of 0.1-mm glass beads. The cell extract was clarified by centrifugation at 15,000 ×*g* for 15 min and subjected to enzyme assays. SDH activity was measured by recording the succinate-dependent absorbance change of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2,4-tetrazolium bromide (MTT) at 570 nm in the presence of phenazine methosulfate (*26, 27*). GltS and malate dehydrogenase assays were conducted essentially as described previously (*28–31*). Protein was determined by the method of Bradford (*32*). One unit of activity was defined as 1 μmol product/min.

RESULTS

Mutagenesis of the isc Operon—To facilitate the investigation of the *in vivo* functions of the *isc* operon, a series of mutant strains was constructed. As shown in Fig. 1, the *iscR*$^-$, *iscS*$^-$, *iscA*$^-$, *hscB*$^-$, *hscA*$^-$, and ORF3$^-$ strains had the Km^r gene inserted at the *Kpn*I site in *iscR*, the *Ahd*I site in *iscS*, the *Sac*II site in *iscA*, the *Ssp*I site in *hscB*, the *Hpa*I site in *hscA*, and the *Nsi*I site in ORF3, respectively. The Δ*iscU* and Δ*fdx* strains were generated by replacing the coding region with the Km^r gene. In addition, the Δ*isc* strain was created by replacing the 5.4-kb *Kpn*I–*Nsi*I fragment with the Km^r gene, which led to the inactivation of all eight ORFs. In the Δ*iscSUA* strain, the 1.1-kb *Ahd*I–*Sac*II fragment in the *iscSUA*-coding region was replaced with the Km^r gene. To reduce the possibility of polarity, we used the Km^r gene derived from Tn5, which contains no known transcriptional termination site. In addition, the Km^r gene was introduced into the chromosome in the same direction of transcription as the gene it was disrupting.

Southern analyses were carried out to verify the geno-



Strain	Doubling time (min)	Growth phenotype			
		Nad	Thi	Met	Ilv
MG1655 (wild type)	20 ± 1.5	+	+	+	+
iscR$^-$	19 ± 1.2	+	+	+	+
iscS$^-$	76 ± 6.8	-	-	-	-
Δ*iscU*	54 ± 1.5	+	+	+	+
iscA$^-$	23 ± 2.3	+	+	+	+
hscB$^-$	54 ± 1.5	+	+	+	+
hscA$^-$	56 ± 1.7	+	+	+	+
Δ*fdx*	57 ± 0.6	+	+	+	+
ORF3$^-$	18 ± 0.6	+	+	+	+
Δ*iscSUA*	78 ± 5.5	-	-	-	-
Δ*isc*	147 ± 3.8	-	-	-	-

Fig. 1. **Directed mutagenesis of the *E. coli* genomic region containing the *isc* operon and resulting phenotypes.** Restriction sites used for deletion or insertion of interposons are illustrated in the physical map of the *iscRSUA-hscBA-fdx*-ORF3 locus in *E. coli*. Note that ORF2 has been redesignated *iscR* (*18*). The arrowhead indicates the potential −35/−10 promoter. The Km^r gene is indicated by a filled arrow. Doubling time was measured by monitoring the growth in Terrific broth. Nutritional auxotrophies of nicotinic acid and thiamine for growth on solid minimal media are represented by Nad "−" and Thi "−", respectively. The effect of amino acids were examined by monitoring the lag phase upon switching from rich to minimal media. The methionine requirement is represented by Met "−" and that of isoleucine and valine by Ilv "−".

type of the mutant strains. Chromosomal DNA isolated from parent cells (MG1655) and mutant strains was digested with *Bgl*II or *Pvu*II and hybridized with a probe containing the authentic *isc* operon. All hybridization signals observed were the results expected after the replacement of the wild-type allele with Km^r-marked insertion/deletion alleles by double crossover recombination. The genotype was further confirmed by hybridization with a probe containing the Km^r gene (data not shown).

Growth Characteristics of Mutant Strains—The mutant strains, all viable in rich media, showed considerable differences in their growth rates. As shown in Fig. 1, the *iscR*$^-$, *iscA*$^-$, and ORF3$^-$ strains grew as well as the wild-type parent cells (MG1655) with a doubling time of about 20 min in Terrific broth at 37°C. The Δ*iscU*, *hscB*$^-$, *hscA*$^-$, and Δ*fdx* strains exhibited retarded growth with a doubling time of about 55 min. The doubling time of the *iscS*$^-$ and Δ*iscSUA* strains was about 77 min, 4-fold greater than wild-type cells. The Δ*isc* strain showed still more retarded growth with a doubling time of 147 min. The slow growth phenotype of the mutant strains was also observed in solid media; for instance, the Δ*isc* cells took 2–3 days to form small colonies on LB plates.

The three strains lacking IscS (*iscS*$^-$, Δ*iscSUA*, and Δ*isc*) exhibited negligible growth on solid minimal media without the addition of either nicotinic acid or thiamine (Fig. 1). The nicotinic acid and thiamine auxotrophies, consistent with earlier observation for the *iscS*-inactivated strains (*21*, *22*), were appreciable on a variety of carbon sources including glucose, glycerol and succinate. The biosynthetic pathway of nicotinic acid, a precursor of NAD(P)$^+$, utilizes a single Fe-S enzyme quinolinate synthase that condenses α-iminoaspartate and dihydroxyacetone phosphate to give quinolinic acid (*33*). The nicotinic acid auxotrophy of the three mutant strains was satisfied by the addition of quinolinic acid to the growth medium (data not shown), indicating a deficiency in the quinolinate synthase activity. With respect to the thiamine auxotrophy in the strains lacking IscS, the ThiH protein, an enzyme involved in the thiamine biosynthetic pathway that may contain an Fe-S cluster and require IscS for its activity is a proposed target (*18*, *22*). In addition, thiamine itself contains sulfur, and IscS could function as the sulfur donor *via* a ThiI intermediate (*21*).

The addition of casamino acid markedly promoted the growth of the three strains lacking IscS on minimal media containing nicotinic acid and thiamine (data not shown). Lauhon and Kambampati have identified two amino acids, isoleucine and valine, that accelerate the growth of the *iscS*-inactivated strain. Without these amino acids, growth in minimal medium is preceded by a considerable lag period (*21*). We have examined various combinations of amino acids and found methionine to be another requirement. As shown in Fig. 2, the lag time for the growth of the *iscS*$^-$ strain was substantially reduced in the presence of either methionine or isoleucine and valine, and further reduced in the presence of all three amino acids. Similar effects of these three amino acids were observed on the growth of the Δ*iscSUA* and Δ*isc* strains, whereas no significant effects were detectable on the growth of other mutant strains or wild-type cells (data not shown). The biosynthesis of isoleucine and valine involves a common enzyme, [4Fe-4S] dihydroxy-acid dehydratase (*34*), and, therefore, a defect in this enzyme is likely to be a cause of the isoleucine

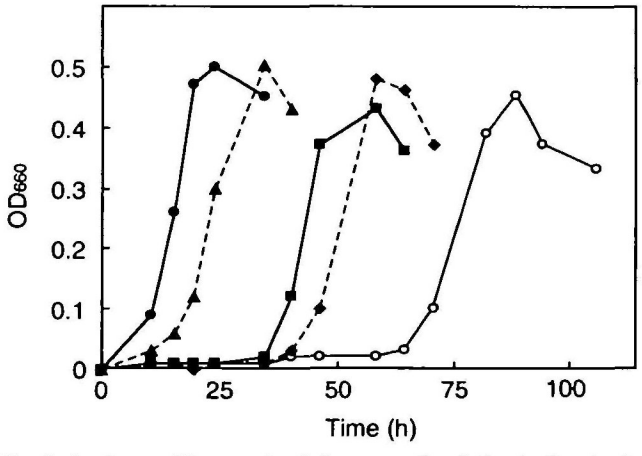


Fig. 2. **Amino acids required for growth of the *iscS*$^-$ strain.** *iscS*$^-$ cells pre-cultivated in Terrific broth were washed and diluted with M9/succinate medium containing nicotinic acid and thiamine. The following amino acids were added to the medium: ●, all amino acids; ▲, methionine, isoleucine, and valine; ■, methionine; ◆, isoleucine and valine; ○, no amino acids. Cell density was estimated from the optical density (OD) at 660 nm. Isoleucine alone had no observable effect (not shown). Valine inhibits the growth of *E. coli* K-12 in the absence of isoleucine.

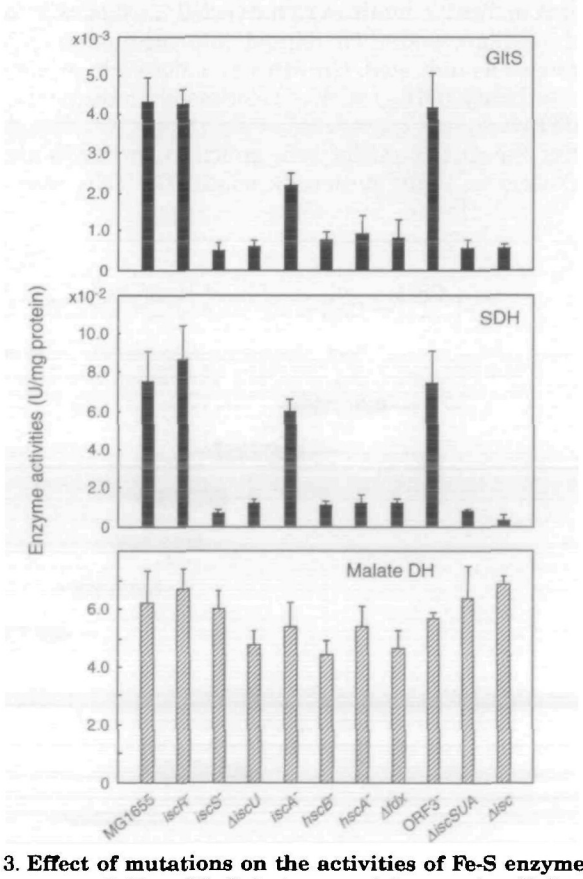


Fig. 3. **Effect of mutations on the activities of Fe-S enzymes.** The enzyme activities of Fe-S cluster–containing proteins, GltS and SDH, and of malate dehydrogenase (DH) were measured in crude extracts prepared from the indicated strains, and represented by specific activities (units/mg protein). Experiments were carried out using three independent cultures, and the values are the mean ± SD of the measurements.

and valine auxotrophies (*21*). At present, we cannot unequivocally assign the Fe-S enzyme in the biosynthetic pathway of methionine, although one candidate is sulfite reductase, a [4Fe-4S] protein that catalyzes the reduction of sulfite to sulfide. This is because the methionine requirement can be partially satisfied by the addition of cysteine (data not shown).

Activity of Fe-S Proteins in Mutants—To assess the *in vivo* role of the genes in the assembly of the Fe-S cluster, the mutant strains were employed to examine the effects of gene-disruption on the activity of Fe-S proteins. As shown in Fig. 3, a marked decrease in the activities of Fe-S proteins, glutamate synthase (GltS) and succinate dehydrogenase (SDH) was observed in mutant strains lacking IscS, IscU, HscB, HscA, or Fdx. Their specific activities were reduced 5- to 10-fold as compared to wild-type cells. Hardly any change was detectable in the activity of a control protein (malate dehydrogenase), which does not contain an Fe-S cluster. The results are consistent with the earlier characterization of the *iscS*-inactivated strain, in which the GltS and SDH activities were several fold smaller than in controls (*22*). The *iscA*$^-$ strain showed slight but appreciable decreases in these activities. In contrast, the activities in the *iscR*$^-$ and ORF3$^-$ strains were normal. Accordingly, disruption of the *iscR* gene or ORF3 had no observable effect. Taken together, these data indicate a requirement of IscS, IscU, IscA, HscB, HscA, and Fdx for the activity of cellular Fe-S proteins, and suggest functions in the generation of these proteins.

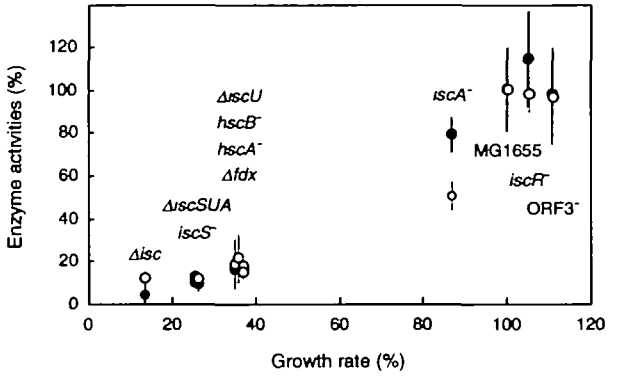
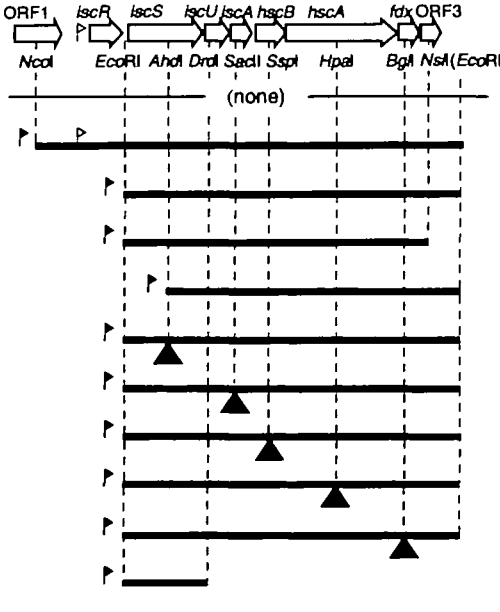


Fig. 4. **Activities of Fe-S enzymes in mutant strains in comparison with their growth rate.** The values obtained for MG1655 (wild-type) cells are represented as 100%. ○, GltS activity; ●, SHD activity.

In most mutant strains, the residual activity of Fe-S proteins correlates with their growth rate (Fig. 4). In particular, cells lacking IscU, HscB, HscA, and Fdx exhibited almost identical activities as well as growth rates. The *iscS*$^-$ cells showed slightly lower activities and growth rate than the *ΔiscU*, *hscB*$^-$, *hscA*$^-$, and *Δfdx* cells. The values for the *ΔiscSUA* strain were similar to those for *iscS*$^-$, indicating that mutations in *iscS*, *iscU*, and *iscA* are nonadditive. The *Δisc* strain grew at the slowest rate, although corresponding decreases in the activities of Fe-S proteins were not clearly observed.

Complementation of the Δisc Strain—Transformation of the *Δisc* strain with a plasmid expressing the entire *isc* operon (pISCΔ1) resulted in the complementation of all phenotypic effects (Fig. 5). As shown in Fig. 6, the activities of GltS and SDH were also restored to the wild-type level (Fig. 3) by complementation. In contrast, no restoration was observed in cells with the control vector pRKNSE. The slightly lengthened doubling time of the *Δisc*(pRKNSE) cells compared to the *Δisc* cells might be caused by the additional task of maintaining the plasmid and its tetracycline-resistance activity. These results indicate that the phenotypes observed in the *Δisc* strain are clearly associated with the deletion of the *isc* operon. The generation of secondary mutations or polar effects on downstream genes appear not to be involved. Full complementation was also observed with the pRKE plasmid in which the *iscR* gene and putative promoter sequence in the upstream region are deleted from pISCΔ1 (Figs. 5 and 6). Thus, the dispensable role of IscR was demonstrated in both *iscR*$^-$ and *Δisc*



Plasmid	Doubling time (min)	Growth phenotype			
		Nad	Thi	Met	Ilv
pRKNSE	169 ± 4.6	-	-	-	-
pISCΔ1	24 ± 1.5	+	+	+	+
pRKE	25 ± 1.2	+	+	+	+
pRKE-Δ3	24 ± 1.0	+	+	+	+
pRKE-ΔS	166 ± 1.7	-	-	-	-
pRKE-ST	166 ± 3.5	-	-	-	-
pRKE-AT	67 ± 2.1	+	+	+	+
pRKE-hBT	127 ± 0.6	+	±	+	+
pRKE-hAT	125 ± 0.6	+	±	+	+
pRKE-FT	128 ± 2.5	+	±	+	+
pRK-S	128 ± 1.7	+	±	+	+

Fig. 5. **Complementation analysis of the *Δisc* strain.** The gene fragments used in the complementation experiments are illustrated by the physical map of the *isc* operon. The *Eco*RI site in parentheses was derived from the polylinker sequence of the EMBL4 vector in Kohara clone #430. The fragments were cloned into the polylinker site of pRKNSE in the same direction as the *lac* promoter indicated by a filled arrowhead. An oligonucleotide linker (TTAATTAATTAA, the underlined bases indicate stop codons) inserted into the restriction sites is denoted by filled triangles. The doubling time and nutritional requirements were examined as described in the legend to Fig. 1. Thi "±" indicates retarded growth in the absence of thiamine.

(pRKE) cells. The growth rate of the Δ*isc* (pRKE) cells was almost equal to the parent strain and Δ*isc* (pISCΔ1) cells in both the presence and absence of IPTG, indicating that leaky expression from the uninduced *lac* promoter is sufficient for complementation (not shown).

Genes in the *iscSUA-hscBA-fdx*-ORF3 cluster in pRKE were further modified either by truncating or by introducing translational stop codons into the coding regions. As shown in Figs. 5 and 6, transformation of the Δ*isc* strain with the pRKE-Δ3 plasmids resulted in full complementation, indicating that the ORF3 product is not essential for the function. In contrast, no complementation was observed with plasmids pRKE-ΔS or pRKE-ST, which contain intact *iscUA-hscBA-fdx*-ORF3 but not *iscS*. The results confirm the crucial role of the IscS protein and further indicate that IscS is absolutely required for the function of IscU, IscA, HscB, HscA, and Fdx in the biosynthesis of Fe-S clusters. This conclusion is supported by additional experiments showing that plasmids containing *iscUA* (*Ahd*I–*Ssp*I fragment), *hscBA* (*Sac*II–*Bgl*I fragment), *fdx* (PCR product), or ORF3 (*Bgl*I–*Eco*RI fragment) do not alter the growth of Δ*isc* cells (data not shown).

Similar Phenotypical Consequences Caused by the Inactivation of hscB, hscA, and fdx—The plasmids pRKE-AT, pRKE-hBT, pRKE-hAT, and pRKE-FT were found to confer partial complementation on the Δ*isc* strain. Among them, the pRKE-AT plasmid relieved all nutritional requirements of the Δ*isc* strain and considerably restored the growth rate and activities of Fe-S proteins (Figs. 5 and 6). The effect of *iscA*-inactivation was small in comparison with *hscB*-, *hscA*-, and *fdx*-inactivation, consistent with the observations of the gene-disrupted mutants described above.

The Δ*isc* cells harboring the pRKE-hBT, pRKE-hAT, and pRKE-FT plasmids exhibited similar growth characteristics (Fig. 5). They grew slowly in rich media with a doubling time of about 126 min. The auxotrophies for nicotinic acid, methionine, isoleucine, and valine were relieved by the plasmids, whereas the requirement for thiamine was not completely eliminated. As shown in Fig. 6, the activities of Fe-S proteins were increased by a factor of 3 to 4 compared with control cells harboring the pRKNSE plasmid, but were still about 30% of those in cells with pRKE. Subsequent experiments indicated that the *iscS* gene is sufficient for partial complementation. Introduction of the pRK-S plasmid into Δ*isc* cells resulted in phenotypical consequences indistinguishable from those observed with plasmids pRKE-hBT, pRKE-hAT, and pRKE-FT (Figs. 5 and 6). It has been demonstrated that IscS is involved in several cellular functions *via* its cysteine desulfurase activity (*21*, *35*, *36*). This versatility of IscS appears to cause the partial restoration of nutritional requirements and activities of Fe-S proteins. In addition, the results suggest a tightly coupled function of the HscB, HscA, and Fdx proteins, in which depletion of one particular component causes the loss of function of the remaining proteins.

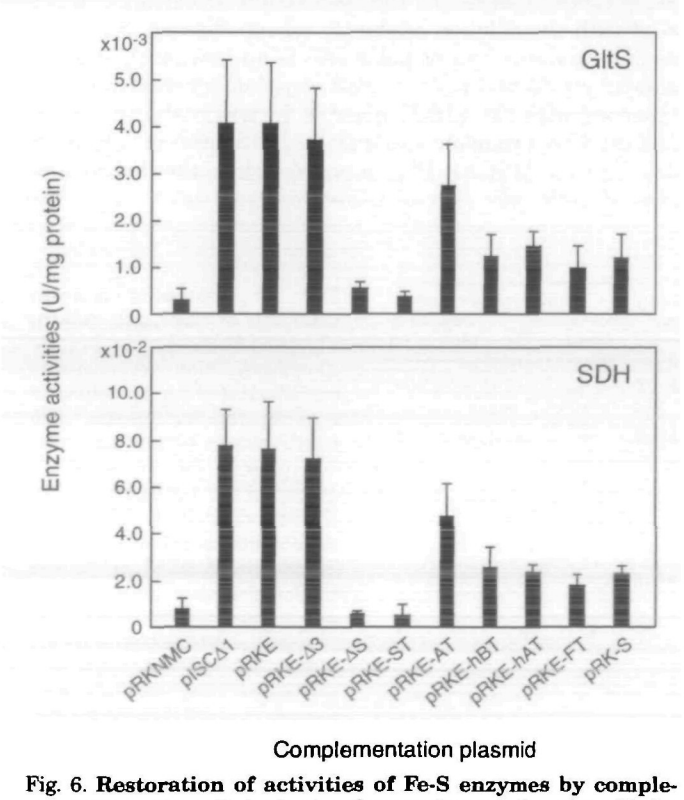


Fig. 6. **Restoration of activities of Fe-S enzymes by complementation.** Δ*isc* cells harboring the complementation plasmids indicated in the figure were grown in Terrific broth and harvested in early stationary phase. The enzyme activities of GltS and SDH were determined as described in the legend to Fig. 3.

DISCUSSION

In this study, we have obtained a series of gene disruption mutants for the *isc* operon and proved that as many as six genes are required for the biosynthesis of Fe-S proteins. Today, over 50 different types of Fe-S proteins have been identified in *E. coli*. We observed that the examined Fe-S proteins, GltS and SDH, show decreased activity in strains lacking IscS, IscU, IscA, HscB, HscA, or Fdx. Similarly, a recent characterization of the *iscS*-inactivated strain has shown defects in the following Fe-S proteins: NADH dehydrogenase I, aconitase B, 6-phosphogluconate dehydrogenase, fumarase A, and FNR (fumarate nitrate reduction protein) in addition to GltS and SDH (*22*). The nutritional requirements for nicotinic acid, methionine, isoleucine and valine observed in the IscS-lacking strains are most likely explained by a Fe-S cluster protein defect. Further, the overexpression of the entire *isc* operon has been shown to increase the cellular ability to assemble Fe-S clusters (*12*, *13*). Hence, several lines of evidence support the conclusion that proteins encoded in the *isc* operon serve predominant functions in the biosynthesis of cellular Fe-S proteins.

In all functional parameters examined in this study, *i.e.* growth rate, nutritional requirements and activities of Fe-S enzymes, mutations in *iscS* were found to cause the most drastic alterations. Our data also indicate that there are no additive effects of combining *iscS*, *iscU*, and *iscA* mutations. The crucial role of IscS is clearly demonstrated by the complementation analysis, in which providing *iscSUA-hscBA-fdx* to the Δ*isc* strain in *trans* restored all phenotypical effects, whereas *iscUA-hscBA-fdx* did not. Apparently, the product of IscS (cysteine desulfurase) is prerequisite to the function of IscU, IscA, HscB, HscA, and Fdx in the biosynthesis of Fe-S clusters. In addition to its role in generating Fe-S clusters, recent studies have revealed that IscS provides sulfur to the ThiI protein for the biosynthesis of the thiazole moiety of thiamine and the formation of 4-thiouri-

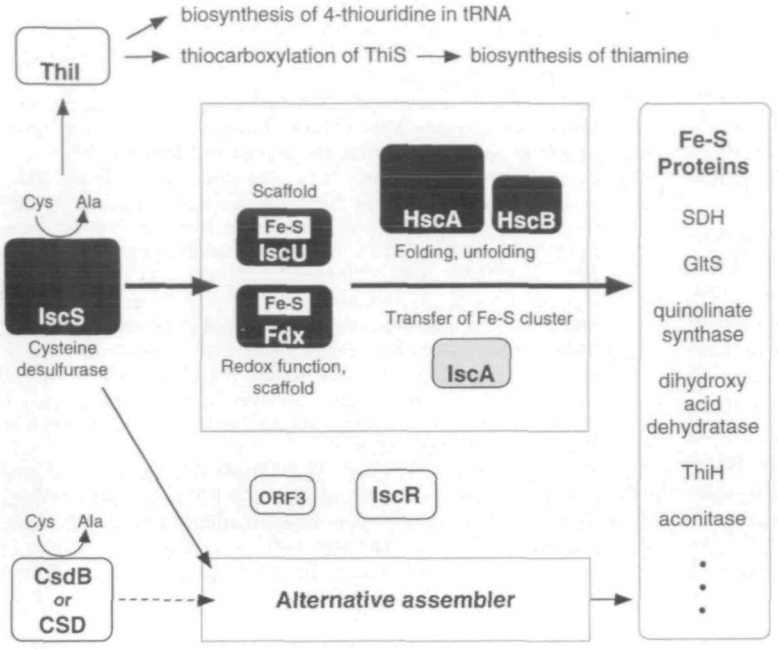


Fig. 7. **Working model for the biogenesis of Fe-S proteins in *E. coli*.** Arrows indicate possible interactions for transferring sulfur or Fe-S clusters. Filled boxes represent components with crucial functions in the assembly of Fe-S clusters. See text for details.

dine in tRNA (*21*, *35*, *36*). The thiamine requirement of the IscS-lacking strains seems more complex because another enzyme, ThiH in the biosynthetic pathway of thiazole, has been proposed to contain an Fe-S cluster (*18*). We, therefore, speculate that mutations in *iscS* affect thiamine synthesis in at least two steps, but the precise effect remains to be defined.

According to a current working model (Fig. 7), the assembly process of Fe-S clusters is depicted as follows: The cysteine desulfurase IscS catalyzes the removal of sulfur from cysteine to form alanine and enzyme-bound S^0 (*37*). Upon reduction to S^{2-}, presumably by the [2Fe-2S]-containing ferredoxin Fdx, the sulfur is transferred to a dimer of the IscU protein where the intermediate [2Fe-2S] or [4Fe-4S] cluster is constructed (*38*). The cluster is then released and transferred to apoproteins with the possible assistance of the IscA protein (*13*). Two heat shock cognate proteins, HscB and HscA, might be responsible for a chaperone function during Fe-S cluster assembly (*39*, *40*). So far, the IscS and IscU proteins have been extensively characterized, although the roles of remaining components remain unclear. With respect to *iscU, hscB, hscA,* and *fdx*, the present study demonstrates almost identical phenotypical consequences of mutations in these genes. Further, our complementation analysis indicates their tightly coupled function, in which the depletion of one component among the HscB, HscA, and Fdx proteins causes the loss of function of the remaining proteins. Presumably, these proteins work in concert either in a linear way or together as a complex to assemble the intermediate Fe-S cluster and transfer it to apoproteins. The overall function of this assembler machinery depends strictly on the sulfur supplied by the IscS protein as described above. To our knowledge, the results are the first genetic evidence for the interaction of the components encoded in the *isc* operon.

The inactivation of *iscA* has comparatively mild phenotypical consequences. For instance, *iscA*$^-$ cells have GltS and SDH activities that are only slightly decreased. It appears from these findings that the IscA protein does not contribute to a crucial step in contrast to IscS, IscU, HscB, HscA, and Fdx. Rather, IscA appears to improve the efficiency of Fe-S cluster assembly. An auxiliary role for IscA is compatible with previous data obtained in overexpression experiments (*13*). A weaker contribution to the biogenesis of the Fe-S cluster has also been reported for eukaryotic homologs of IscA, *Saccharomyces cerevisiae* Isa1p and Isa2p (*41–43*).

With respect to *iscR* and ORF3, the mutant strains exhibited virtually no difference from wild-type cells. At present, their physiological significance is not clear. Genes homologous to *iscR* and ORF3 are conserved in several bacterial species but not found in the genome sequences of eukaryotes (*13*). However, it is noteworthy that there is a significant difference between the Δ*isc* cells and *iscS*$^-$ or Δ*iscSUA* strains with regard to their doubling times. Slower growth rates are also remarkable in Δ*isc* cells complemented partially with plasmids lacking *iscR* and several other genes. We therefore speculate that *iscR* might affect the growth of *E. coli* on the mutant background of other genes in the *isc* gene cluster, although the activities of the Fe-S enzymes appear to be IscR-independent. Further studies should be directed elucidating the genetic interactions of *iscR* to clarify its functional role.

In contrast to the situation in *A. vinelandii* and yeast, the genes in the *isc* operon are not essential for the viability of *E. coli* cells. The fact that mutant strains retain some Fe-S enzyme activities may represent a redundant system for Fe-S cluster formation. *E. coli* contain two paralogous proteins of IscS and several other enzymes responsible for cysteine desulfurase activity (*44–47*), some of which might be involved in the sulfur-transfer reaction to the alternative assembler machinery that contributes to a modest amount of Fe-S cluster formation. Probably, the alternative assembler also utilizes sulfur supplied by IscS, because our complementation study demonstrates an important function of IscS even in the absence of IscU, IscA, HscB, HscA, and

Fdx. A possible candidate responsible for the components of the alternative machinery is the *sufABCDSE* operon that contains paralogous genes of *iscS* (*sufS*) and *iscA* (*sufA*). At present, the physiological function of this operon is unclear, but seems to be related to the stability of FhuF, a [2Fe-2S] cluster-containing protein (*48*). Several mutants obtained in this study should provide a means by which identification of the alternative machinery can be addressed.

To date, it has become clear that yeast mitochondria contain proteins homologous to bacterial IscS IscU, IscA, HscB, HscA, and Fdx for the biosynthesis of Fe-S proteins (*14, 15*). Similar components are encoded in the genomes of other eukaryotes including human and plants. The conserved proteins should function as central components in the machinery for the biogenesis of Fe-S clusters in both *E. coli* and yeast. The findings presented here provide strong evidence for interactions among the central components. A more detailed biochemical analysis will be required to clarify the molecular events leading to Fe-S cluster assembly.

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