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Interchangeability and Distinct Properties of Bacterial Fe-S Cluster Assembly Systems: Functional Replacement of the *isc* and *suf* Operons in *Escherichia coli* with the *nifSU*-Like Operon from *Helicobacter pylori*

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The assembly of iron-sulfur (Fe-S) clusters, a key step in the post-translational maturation of Fe-S proteins, is mediated by a complex apparatus. In *E. coli*, this process involves two independent systems called ISC and SUF encoded by the *iscSUA-hscBA-fdx* gene cluster and *sufABCDSE* operon, respectively. Another system, termed NIF (*nifSU*), is required for the maturation of nitrogenase in nitrogen-fixing bacteria. We have developed a novel genetic system to gain further insight into these multi-component systems, and to determine how ISC, SUF and NIF might differ in their roles in Fe-S assembly. We have constructed an *E. coli* mutant lacking both the *isc* and *suf* operons, and this strain can only survive in the presence of a complementing plasmid. Using the plasmid replacement technique, we examined the *isc* and *suf* operons, and identified the genes essential for the function. Additionally, we have found that *nifSU*-like genes cloned from *Helicobacter pylori* are functionally exchangeable with the *isc* and *suf* operons. Thus, the NIF-like system participates in the maturation of a wide variety of Fe-S proteins. An increased ability of NIF to complement *isc* and *suf* loss was seen under anaerobic conditions. This may explain why the NIF system is only found in a limited number of bacterial species, and most other organisms prefer the ISC and/or SUF systems. While the differences between ISC and SUF were small with respect to the complementing activity, the SUF system appears to be more advantageous for bacterial growth in the presence of hydrogen peroxide.

Key words: biogenesis, Fe-S proteins, ISC, NIF, SUF.

Abbreviations: Cm, chloramphenicol; DIP, 2,2′-dipyridyl; FDH_H, formate dehydrogenase H; Fe-S, iron-sulfur; GltS, glutamate synthase; Gm, gentamycin; Km, kanamycin; PMS, phenazine methosulfate; ROS, reactive oxygen species; SDH, succinate dehydrogenase; Tc, tetracycline.

Iron-sulfur (Fe-S) clusters are cofactors in a number of cellular processes, including electron transfer, non-redox catalysis, and sensing for regulatory processes (*1–3*). The complex, multi-step process involved in the biosynthesis of Fe-S clusters is only now being clarified through the use of genetic and biochemical methods, and early work has identified three distinct systems termed NIF (nitrogen fixation), ISC (iron-sulfur cluster), and SUF (sulfur mobilization). In a pioneering report, Dean's group demonstrated the roles of the *nifS* and *nifU* gene products in the assembly of Fe-S clusters of the nitrogenase proteins in *Azotobacter vinelandii* (*4–6*). NifS is a pyridoxal phosphate-dependent cysteine desulfurase that initiates Fe-S cluster formation by eliminating elemental sulfur from cysteine and transferring it to NifU. NifU appears to serve as a scaffold for the assembly of transient Fe-S clusters prior to their delivery to apo–Fe-S protein targets.

In contrast to the NIF machinery that specifically deals with the maturation of nitrogenase, the ISC proteins are involved in the general biosynthesis pathway for numerous Fe-S proteins (*6*, *7*). Genetic experiments support a crucial role for the proteins encoded by the so-called *isc* operon (*iscSUA-hscBA-fdx* gene cluster) in Fe-S cluster assembly. Mutation of these genes in *Escherichia coli* decreases the activity of many Fe-S proteins, whereas overexpression of the operon leads to increased production of Fe-S proteins (*8–11*). More recently, a third system (SUF) encoded by the *sufABCDSE* operon has been identified which appears to represent a minor pathway for the assembly of Fe-S clusters (*12*). In an *E. coli* mutant from which the entire *isc* operon was deleted, the activity of Fe-S proteins was only 2–10% that in wild-type cells. The residual activity may arise from the contribution of the SUF system, since overexpression of the *suf* operon restores the growth phenotype and activity of Fe-S proteins in mutant cells lacking the ISC machinery. Disruption of the *suf* operon does not cause any major defects, whereas the loss of both the ISC and SUF systems leads to synthetic lethality.

Several similarities have been found among the NIF, ISC, and SUF systems (Fig. 1). First, IscS and SufS (formerly CsdB) are structurally similar to NifS, and all three function as a cysteine desulfurase (*7*, *13–16*). Second, IscU is homologous to the N-terminal domain of

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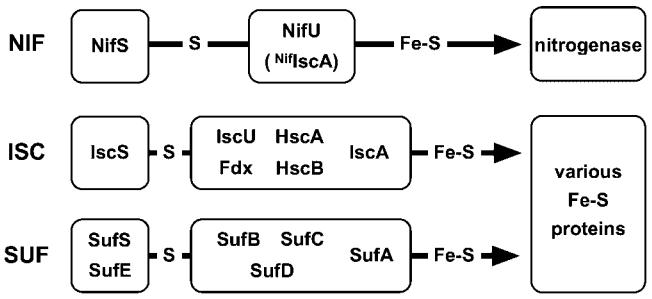


Fig. 1. **Comparison of the NIF, ISC and SUF systems involved in the biosynthesis of Fe-S clusters.** The three systems are composed of cysteine desulfurase (NifS/IscS/SufS) and other components that act in concert in the assembly of intermediate Fe-S clusters and the subsequent transfer of these clusters to target proteins. The NifS and NifU proteins are involved in the assembly of Fe-S clusters of nitrogenase Fe protein and MoFe protein in nitrogen-fixing bacterium *A. vinelandii*, but the role of ^{Nif}IscA has not been shown convincingly. The ISC and SUF systems in *E. coli* have redundant roles in separate, parallel pathways for the maturation of a wide number of Fe-S proteins.

NifU and contains three conserved cysteine residues that are essential for its function as a scaffold for intermediate Fe-S clusters (*17–20*). Additionally, SufA is closely related to IscA and also to the NIF counterpart (^{Nif}IscA), all of which were shown to acquire a labile Fe-S cluster and have been proposed to act as alternative scaffolds in Fe-S cluster biosynthesis (*21–23*). The ISC machinery contains at least three additional components, HscB (co-chaperone), HscA (Hsp70-type molecular chaperone), and Fdx ([2Fe-2S]ferredoxin), that appear to take part in a series of reactions that have yet to be fully characterized (*24–27*). The biochemical properties of the SUF-specific components are less well understood, but SufE interacts with SufS and stimulates its cysteine desulfurase activity. The SufB, SufC and SufD proteins associate in a stable complex, and SufC has been shown to possess ATPase activity in this context (*28–31*).

Components of the ISC machinery are conserved in several proteobacteria in the α-, β- and γ-subdivisions, and also in mitochondria of lower to higher eukaryotes (*32*, *33*). In contrast, the components of the SUF machinery are found in a wider range of Eubacteria as well as in Archaea, plants and parasites, in which SufB and SufC are ubiquitous (*12*, *34*). *E. coli* and its close relatives such as *Yersinia pestis*, *Shigella flexneri,* and several *Salmonella* species retain both the *isc* and *suf* operons, whereas other species possess only one of the two. Interestingly, the N_2-fixing bacterium *A. vinelandii* contains the *nif* and *isc* operons. In these cells, disruption of *nifUS* resulted in the reduction of nitrogenase activity, and cells without a functional *isc* operon were not viable (*6*, *7*). Therefore, it is generally thought that the NIF and ISC systems are functionally differentiated; the former executes specific functions in the assembly of Fe-S clusters of nitrogenase, while the latter has 'housekeeping' functions dealing with the maturation of a number of cellular Fe-S proteins. However, sequencing of the genomes of the ε-proteobacteria *Helicobacter pylori* and *Campylobacter jejuni*, neither of which fix nitrogen nor contain nitrogenase, posed an interesting question on the putative specific role of the NIF system, because the genomes of both bacteria encode the *nifSU*-like operon but do not possess the ISC and SUF components (*35*).

It has not yet been determined whether the *E. coli* ISC and SUF systems are completely redundant or if both have specific functions within the cell. Mutational analysis has demonstrated that the ISC system predominantly functions in the biosynthesis of Fe-S proteins, whereas the SUF system contributes only modestly (*12*). In contrast, expression analysis has demonstrated that the *suf* operon is controlled by OxyR and Fur, an H_2O_2-sensing regulator and an iron-dependent repressor, respectively, suggesting a stress response function for the SUF machinery under conditions of oxidative stress and iron limitation (*36*, *37*). Mutation of *sufC* has also been shown to decrease the activity of several Fe-S proteins, such as SoxR, 6-phosphogluconate dehydratase and fumarase A, during oxidative stress (*29*, *38*). Since damage to Fe-S clusters is a major consequence of oxidative stress, the expression of the *suf* operon appears to be regulated so as to satisfy the cellular need for Fe-S cluster formation. Regardless of the differential expression of the two operons, however, the central issue to be clarified is the difference between the ISC and SUF systems, if any, with respect to their biochemical properties such as substrate specificity and the ability to confer protection against reactive oxygen species (ROS).

Even though the components of the ISC and SUF systems have been isolated in pure forms, biochemical attempts to reconstitute the entire pathway have been unsuccessful. Such studies are complicated by the fact that Fe-S clusters spontaneously assemble *in vitro* into apoproteins from free Fe^{2+} and S^{2-} thereby masking the contributions of cellular factors to assembly. This further highlights the critical importance of genetic analysis. In this report we describe the construction of a mutant strain of *E. coli* from which the chromosomal *isc* and *suf* operons have both been deleted and replaced with a single operon carried by a complementing plasmid, with the aim of developing a simple genetic system for the *in vivo* examination of Fe-S cluster biosynthesis. The complementing plasmid is temperature-sensitive for replication and can be replaced by another plasmid carrying several genes, provided that the gene products are sufficient for the assembly of Fe-S clusters. This allows for the comparative study of the ISC and SUF systems expressed from the same promoter and in the absence of background activity encoded by the chromosome. Moreover, we report the application of our system to analysis of a NIF-like system from foreign species, which provided *in vivo* evidence for a "housekeeping" role responsible for the maturation of a wide variety of Fe-S proteins in *E. coli*. The properties of the SUF, ISC, and NIF systems will be discussed on the basis of their phylogenic background.

EXPERIMENTAL PROCEDURES

Strains and Cell Growth—The *E. coli* strains used in this study are listed in Table 1. Luria-Bertani (LB) medium was used as the standard medium. When required, kanamycin (Km), tetracycline (Tc), chloramphenicol (Cm), and gentamycin (Gm) were added at concentrations of 20, 10, 20, and 2 μg/ml, respectively. The minimal medium comprised M9 supplemented with glu-

Table 1. ***E. coli* strains used in this study.**

Strain	Properties	Source
MG1655	Wild-type	Laboratory strain
YT1014	MG1655 Δ(*iscRSUA-hscBA-fdx*-ORF3)::Km^r	*Ref. 9*
YT1023	MG1655 *iscS*::Km^r	*Ref. 9*
YT2512	MG1655 Δ (*sufABCDSE*)::Gm^r	*Ref. 12*
YT2281	MG1655 Δ (*iscSUA*)::Km^r; Δ (*sufABCDSE*)::Gm^r (pRKSUF106)	*Ref. 12*
UT109	MG1655 Δ (*iscUA-hscBA*)::Km^r; Δ (*sufABCDSE*)::Gm^r	This study

Table 2. **Oligonucleotides used in this study.**

Oligonucleotide	Sequence[a]	Restriction site
HpNifF	5′-CGGTCTAGAGGATTAACCATGTTACAACGAATTTATTTAG-3′	*Xba*I
HpNifR	5′-GCCGGAGCTCAAATCGGTAACACCCTGAT-3′	*Sac*I
Hp1492F	5′-GAGCTCAGGTTTTAAAAAATGATAGAATTTAGCG-3′	*Sac*I
Hp1492R	5′-CTCGAGCCTTAAAGCTTATCAAACTCCT-3′	*Xho*I
HpNifSR	5′-GCCGGAGCTCTTAATAAGAGCTTGAAATATTTCTC-3′	*Sac*I
HpNifUF	5′-CGGTCTAGAGGAATCAAAATGGCAAAACATGA-3′	*Xba*I
*Pac*I linker	5′-TTAATTAATTAA-3′	

[a]The underlined bases comprise restriction sites.

cose (0.4%), lactose (0.4%), or sodium succinate (20 mM). For growth on anaerobic respiration, the medium was supplemented with glycerol (0.4%) and sodium fumarate (40 mM). When indicated, the growth medium was supplemented with nicotinic acid (1 μM), thiamine (1 μg/ml), and vitamin-free casein hydrolysate (0.1%). IPTG was used at a concentration of 0.2 mM to induce expression of the *lac* promoter.

Operon Deletion—For deletion of the *iscUA-hscBA* genes in the *isc* operon, a 1.2-kb DNA fragment containing the 5′ upstream region of *iscU* (i.e. *iscS*), the Km^r cassette (*9*), and a 1.8-kb *Hpa*I–*Eco*RI fragment containing Δ*hscA* (3′ half of *hscA*)-*fdx*-ORF3 were ligated into the *Not*I site of pKO3 (*39*). The resulting plasmid was designated as pKO3-ΔUABA, in which the correct insertion of *iscS*-Km^r-Δ*hscA-fdx*-ORF3 was confirmed by digestion with several restriction enzymes. For double disruption of the chromosomal *isc* and *suf* operons, pKO3-ΔUABA was introduced into *E. coli* YT2512 (Δ*sufABCDSE*::Gm^r) cells (*12*) in the presence of a complementing plasmid, pRKSUF106, carrying the *sufABCDSE* operon, and cells were selected for chromosomal integration and excision as described previously for pKO3-based mutagenesis (*39*). The genotype of the resulting strain, UT109 (Δ*iscUA-hscBA*::Km^r; Δ*sufABCDSE*::Gm^r) harboring pRKSUF106, was confirmed by PCR using several sets of primers described elsewhere (*12*, *24*).

Construction of Complementation Plasmids—The coding regions of the *nifS*-like gene (Hp0220, here called *nifS*), *nifU*-like gene (Hp0221, here called *nifU*), and *nfu* (Hp1492) and the preceding ribosome binding sites were amplified by PCR using *H. pylori* (ATCC 700392) genomic DNA as a template and the primers listed in Table 2; the initiation codon of *nifS* was altered from TTG to ATG. The PCR products were first cloned into the PCR2.1-TOPO vector (Invitrogen) by the TA cloning method and then the sequences were verified. The 1.2-kb *Xba*I–*Sac*I fragment containing *nifS*, the 1.0-kb *Xba*I–*Sac*I fragment containing *nifU*, the 0.3-kb *Sac*I–*Xho*I fragment containing *nfu*, and the 2.2-kb *Xba*I-*Sac*I fragment containing *nifSU* were excised and ligated into the respective restriction sites of the pRKNMC vector (*10*) in the same direction as the *lac* promoter. For construction of pKO3-SUF, the *Nhe*I site of pRKSUF106 (*12*) was first converted to a *Bam*HI site by linker ligation. The 5.6-kb *Bam*HI fragment carrying the *sufABCDSE* operon was subsequently excised and ligated into the *Bam*HI site of pKO3. For construction of pKO3-NIF, the 2.2-kb *Sal*I/*Xho*I fragment containing *nifSU* was excised from pRKHpSU and then ligated into the *Sal*I site of pKO3. The pKO3-SUF and pKO3-NIF plasmids were first introduced into YT1014 (Δ*iscRSUA-hscBA-fdx*-ORF3::Km^r) cells (*9*), and complementation was verified at a permissive temperature (30°C).

Plasmid Replacement—Starting with strain UT109 (Δ*iscUA-hscBA*::Km^r; Δ*sufABCDSE*::Gm^r), Tc^r plasmid pRKSUF106 carrying *sufABCDSE* was replaced by a temperature-sensitive, Cm^r plasmid, pKO3-SUF or pKO3-NIF, carrying the *suf* or *nifSU* operon, respectively. Transformants were grown on Cm plates at the permissive temperature (30°C). The cells were diluted and plated again on Cm plates, and then screened for Tc-sensitivity to detect the loss of pRKSUF106. Next, UT109 cells harboring the plasmid pKO3-SUF or pKO3-NIF were transformed with Tc^r plasmids carrying the intact or modified *isc*, *suf,* or *nif*-like operon. Transformants were selected on Tc plates at a non-permissive temperature (43°C) of the pKO3-replicon. The cells were diluted and grown again on Tc plates at 43°C, and then screened for Cm-sensitivity at 30°C to detect the loss of pKO3-derivatives. In our experiments, 20–100% of Tc^r colonies were Cm^s, and the frequency was dependent on the complementation allowed by the newly introduced plasmids. No Cm^s cells were obtained in the control experiments using the plasmids lacking the related genes.

Growth Assays—*E. coli* cells were grown overnight at 37°C in LB medium. A 200–500-fold dilution was made in 5 ml LB medium (the starting OD_{660} was routinely 0.01), and the cultures were then incubated in L-shaped culture tubes at 37°C with vigorous seesaw shaking at 70 rpm.

Turbidity was monitored every 10 minutes with a Bio-Photorecorder TN1506 (ADVANTEC) at an optical density of 660 nm. Anaerobic growth was monitored in airtight tubes containing degassed LB medium supplemented with glycerol and fumarate. All analysis was performed at least in triplicate with independent cultures of each strain. The wild-type (MG1655) cells grew with a doubling time of 20.2 ± 0.7 min in aerobic LB medium at 37°C, and 22.5 ± 0.9 min in anaerobic LB/glycerol/fumarate medium. When indicated, 2,2′-dipyridyl (DIP), hydrogen peroxide or phenazine methosulfate (PMS) was added during the exponential growth phase (OD_{660} ~0.3), and the growth was followed by measuring OD_{660}. Nutritional requirements were assessed by monitoring the growth on solid M9 medium supplemented with glucose, lactose, or succinate as a carbon source. Anaerobic growth was examined on M9 solid plates supplemented with glucose, lactose, or glycerol/fumarate in a GasPak anaerobic jar (BBL Microbiology Systems).

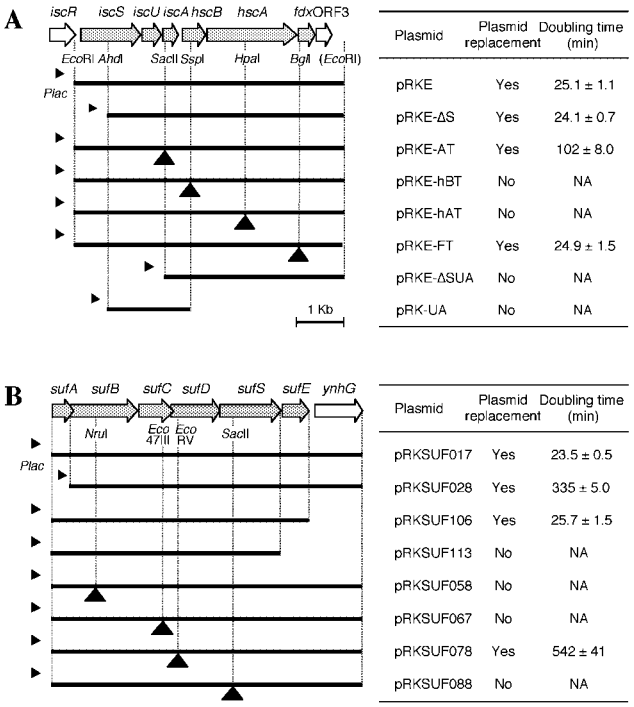
Enzyme Assays—Cells were grown aerobically in LB medium to the early stationary phase. Cells from a 3-ml sample were pelleted and washed with cold 50 mM potassium phosphate buffer, pH 7.5. The cells were suspended in the same buffer, and then sonicated and clarified as described previously (*9*). Succinate dehydrogenase (SDH), glutamate synthase (GltS), and malate dehydrogenase assays were conducted as described (*9*). Protein was determined by means of Coomassie Blue dye-binding assaying (Nacalai Tesque, Inc., Japan). One unit of enzyme activity was defined as 1 μmol product/min. The formate dehydrogenase (FDH_H) activity was assessed by the benzyl viologen agar overlay method (*40*). All analysis was performed in triplicate with three independent cultures of each strain.

RESULTS

Operon Deletions and Plasmid Replacement—An *E. coli* strain was constructed in which the chromosomal *isc* and *suf* operons were both replaced by antibiotic resistance cassettes. In an earlier study, we constructed mutant YT2512 (Δ*sufABCDSE*::Gm^r), in which the entire *suf* operon was precisely deleted by substituting the coding regions and the preceding ribosome binding sequence with a Gm^r cassette (*12*). With YT2512 as a host, in the presence of a complementing plasmid, pRKSUF106, carrying *sufABCDSE*, it was possible to substitute chromosomal *iscUA-hscBA* with a Km^r cassette by homologous recombination, resulting in strain UT109 (Δ*iscUA-hscBA*::Km^r; Δ*sufABCDSE*::Gm^r). Among the eight genes in the *isc* operon (*iscRSUA-hscBA-fdx*-ORF3), we decided to leave chromosomal *iscRS* and *fdx*-ORF3 intact due to their potential roles in metabolic processes other than Fe-S cluster assembly. IscS is involved in thiamine biosynthesis and tRNA modification via its cysteine desulfurase activity, and in selenocysteine metabolism (*15*, *40*, *41*). Fdx is a substrate of CoA-acetylating pyruvate:ferredoxin (flavodoxin) oxidoreductase (*42*). IscR may function as a regulator of mRNA degradation (T. Yonesaki, personal communication) in addition to its regulatory role as a repressor of the *isc* operon (*43*). When we compared the growth of UT109 and YT2281 (Δ*iscSUA*::Km^r; Δ*sufABCDSE*::Gm^r), both harboring the same complementing plasmid, pRKSUF106, the former grew as well as the wild-type (MG1655) cells with a doubling time of 25 min in LB medium, while the latter showed retarded growth with a doubling time of 44 min, which suggests the important role of IscS. Thus, we chose UT109 for further investigation.

The viability of UT109 was dependent on the presence of the complementing plasmid, and this plasmid can be replaced by any other plasmid provided it carries the necessary genes for the assembly of Fe-S clusters and a different antibiotic resistance marker. To further facilitate the plasmid replacement steps, we constructed pKO3-SUF that carries *sufABCDSE*, a Cm^r marker, the temperature-sensitive pSC101 replicon, and the *sacB* gene. After transformation of UT109/pRKSUF106 with pKO3-SUF, Cm^r transformants were screened for Tc^s at the permissive temperature (30°C). The resultant colonies harboring only the pKO3-SUF plasmid were tested for Km^r, Gm^r and temperature sensitivity at a non-permissive temperature (43°C) to confirm the chromosomal genotype and dependency on pKO3-SUF. The growth of UT109/pKO3-SUF cells was severely impaired at 43°C, although tiny colonies appeared on LB plates after 3–5 d. All colonies were Cm^r and may have retained pKO3-SUF in a reduced copy number. Homologous recombination likely did not occur, since pKO3-SUF contained no chromosomal sequences of UT109. The pKO3-SUF plasmid could then be replaced by another plasmid by a more direct procedure: transformation at 43°C followed by screening for Cm^s colonies at 30°C.

Replacement with Plasmids Carrying the isc or suf Operon—The *isc* and *suf* operons, and their variants shown in Fig. 2 were cloned into low copy number vector pRKNMC that carries the IncP1 replicon and a Tc^r marker. The genes were expressed under the control of the *lac* promoter, but we have previously observed that basal expression in the absence of IPTG was sufficient for complementation (*9*, *12*). When plasmids pRKE, pRKE-ΔS, and pRKE-FT were introduced into UT109/pKO3-SUF cells, the transformants showed normal growth even at 43°C. Most of the cells were Tc^r Cm^s, indicating that pKO3-SUF had segregated; this was confirmed by genomic PCR and plasmid preparations (data not shown). These cells grew as well as the wild-type (MG1655) cells with a doubling time of about 25 min in LB medium at 37°C (Fig. 2A). Proper growth in the absence of plasmid-borne *iscS* (pRKE-ΔS) and *fdx* (pRKE-FT) may confirm that their chromosomal copies are fully functional. When control plasmid pRKNMC (no insert) was introduced into UT109/pKO3-SUF, transformants were detected only after 3–5 d at 43°C. None of the cells were Cm^s, suggesting that they were unable to segregate pKO3-SUF. Similar results were obtained with cells transformed with plasmid pRKE-hBT, pRKE-hAT, pRKE-ΔSUA, or pRK-UA. However, transformation with pRKE-AT gave slightly faster growing cells compared to those transformed with pRKNMC. In this experiment about 20% of the Tc^r transformants were Cm^s. The UT109/pRKE-AT cells exhibited retarded growth with a doubling time of about 100 min in LB medium. Taken together, these results indicate an essential role for *iscU*, *hscB*, and *hscA* in the absence of a functional *suf* operon. A nonessential, auxiliary role for IscA is compatible with



Plasmid	Plasmid replacement	Doubling time (min)
pRKE	Yes	25.1 ± 1.1
pRKE-ΔS	Yes	24.1 ± 0.7
pRKE-AT	Yes	102 ± 8.0
pRKE-hBT	No	NA
pRKE-hAT	No	NA
pRKE-FT	Yes	24.9 ± 1.5
pRKE-ΔSUA	No	NA
pRK-UA	No	NA

Plasmid	Plasmid replacement	Doubling time (min)
pRKSUF017	Yes	23.5 ± 0.5
pRKSUF028	Yes	335 ± 5.0
pRKSUF106	Yes	25.7 ± 1.5
pRKSUF113	No	NA
pRKSUF058	No	NA
pRKSUF067	No	NA
pRKSUF078	Yes	542 ± 41
pRKSUF088	No	NA

Plasmid	Plasmid replacement	Doubling time (min)
pRKHpSUN	Yes	62.1 ± 0.2
pRKHpSU	Yes	63.3 ± 2.9
pRKHpUN	No	NA
pRKHpSN	No	NA
pRKHpS	No	NA
pRKHpU	No	NA
pRKHpN	No	NA

Fig. 2. **Complementation analysis by the plasmid replacement technique.** A: *E. coli isc* operon; B: *E. coli suf* operon; and C: *H. pylori nif* operon (synthetic operon with *nfu*). The DNA fragments (thick lines) used for the plasmid replacement are shown below the restriction maps. They were inserted into a Tc^r, low–copy-number vector and expressed under the control of the *lac* promoter indicated by an arrowhead. Filled triangles denote the insertion of a *Pac*I linker (TTAATTAATTAA) that contains stop codons in all reading frames. After introduction into UT109/pKO3-SUF (A and C) or UT109/pKO3-NIF (B) cells, plasmid replacement was examined as Cm-sensitivity for at least fifty Tc^r transformants, and further confirmed by genomic PCR and restriction analysis of the plasmid preparations. The doubling time was measured by monitoring the growth in LB medium at 37°C. NA, not applicable.

our previous data obtained in overexpression experiments and on mutational analysis carried out in the suf^+ backgound (*9*, *11*).

Similar experiments were carried out using plasmids carrying the *sufABCDSE* operon (Fig. 2B). To avoid the risk of homologous recombination between pKO3-SUF and the newly introduced pRKSUF derivatives, UT109/pKO3-NIF carrying the *nifSU*-like operon cloned from *H. pylori* (see below) was used in the following experiments. Transformation of UT109/pKO3-NIF with pRKSUF017 or pRKSUF106 carrying the intact *suf* operon resulted in plasmid replacement and allowed the cells to grow almost as fast as the wild-type cells. Four *suf* genes, *i.e.* *sufB*, *sufC*, *sufS*, and *sufE*, were necessary for plasmid replacement, since inactivation of any one of these genes (plasmids pRKSUF058, pRKSUF067, pRKSUF088, and pRKSUF113, respectively) abolished the segregation of pKO3-NIF. In contrast, when plasmid pRKSUF028 or pRKSUF078 was introduced into UT109/pKO3-NIF, Tc^r Cm^s cells were obtained, and pKO3-NIF was completely replaced by these plasmids. However, deletion of *sufA* (pRKSUF028) or disruption of *sufD* (pRKSUF078) caused a severe growth defect; the former grew in LB medium with a doubling time of about 5.5 h and the latter with 9 h. These results suggest a nonessential but crucial role for SufA and SufD in the SUF system. Interestingly, SufA and IscA are paralogous proteins exhibiting 47% identity, and both have been shown to acquire a labile Fe-S cluster, a possible intermediate yet to be transferred to Fe-S protein targets (*22*, *23*).

Expression of a Foreign NIF-Like System in UT109—One objective in constructing the UT109 strain was to examine foreign genes or operons in a background with no chromosomally encoded activity. As a first step, we chose the *nifSU*-like operon encoded in the *H. pylori* chromosome. Although the bacterium does not fix nitrogen nor possess nitrogenase, the genes have been referred to as "*nif* (nitrogen fixation)" because of the high similarity to NifS and NifU in diazotrophic bacteria in terms of their amino acid sequences and biochemical properties (*35*). *H. pylori* also encodes another gene, Hp1492 (here called *nfu*), in a separate locus on the chromosome that exhibits similarity with the C-terminal domain of NifU. Nfu-like proteins found in *Saccharomyces cerevisiae, Synechocystis* PCC6803, and *Arabidopsis thaliana* have been proposed to be involved in Fe-S metabolism (*44–46*).

The coding regions and the preceding ribosome binding sequences of *H. pylori nifS*, *nifU*, and *nfu* were amplified by PCR and then cloned in tandem into the pRKNMC vector (Fig. 2C). These plasmids were introduced into UT109/pKO3-SUF cells, and then Tc^r Cms cells were screened as described above. Two plasmids, pRKHpSUN and pRKHpSU, allowed plasmid replacement and thus sufficed for the complementation of UT109. The resulting cells, UT109/pRKHpSUN and UT109/pRKHpSU, grew slowly in LB medium with a doubling time of about 1 h.

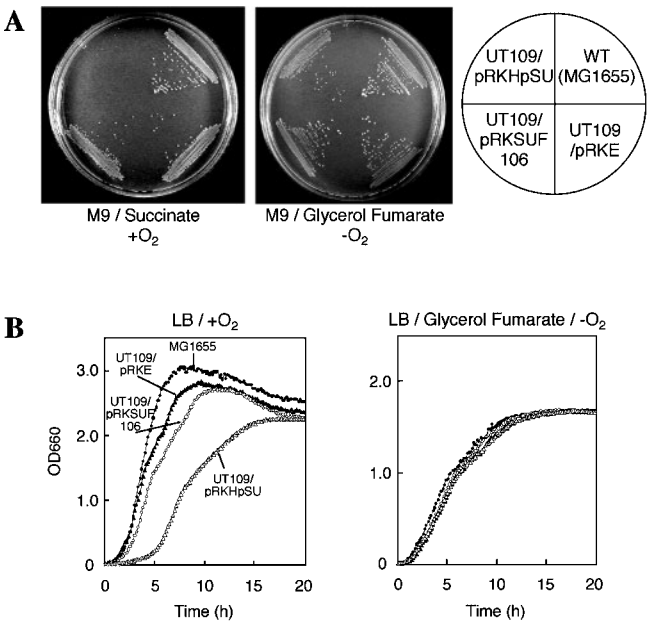


Fig. 3. **Phenotypic characterization of the *E. coli* UT109 strain complemented by the ISC, SUF, and NIF systems.** A: wild-type (MG1655) and UT109 cells harboring plasmid pRKE, pRKSUF106 or pRKHpSU were aerobically grown on agar plates containing minimal (M9) medium supplemented with succinate (left), or anaerobically on M9 medium supplemented with glycerol and fumarate (right). B: cells were aerobically grown in liquid LB medium (left), or anaerobically in LB medium supplemented with glycerol and fumarate (right). Filled circles, MG1655 (wild type); filled triangles, UT109/pRKE; open circles, UT109/pRKSUF106; and open triangles, UT109/pRKHpSU.

The addition of IPTG did not improve the doubling time (70 min), suggesting that the low level of expression from the uninduced *lac* promoter was not the cause of their retarded growth. All strains lacking either the *nifS* or *nifU* gene were not able to complement UT109. We concluded from these results that the *H. pylori nifSU* operon is exchangeable for the *E. coli isc* and *suf* operons, and sufficient, at least in part, for the assembly of Fe-S clusters. The requirement of the Nfu protein was negligible for this heterologous complementation since plasmids pRKHpSUN and pRKHpSU did not show any observable differences in any of the functional tests described below (data not shown).

Phenotypic Analysis—Strains UT109/pRKE, UT109/pRKSUF106 and UT109/pRKHpSU live on the ISC, SUF and NIF systems, respectively, that are expressed from the same promoter in an isogenic background. Thus, *E. coli* requires one of the three systems for the maturation of cellular Fe-S proteins, although the three systems are quite different from one another not only in the amino acid sequences but also in the number of components. To address the question of whether the three systems might differ in their functional roles, their phenotypic characteristics were compared.

The UT109/pRKHpSU cells grew slowly in LB medium as described above and formed small colonies on solid plates. This strain exhibited negligible growth on minimal media unless nicotinic acid, thiamine and casamino acid were added (Fig. 3A). This auxotrophic behavior was also observed for an *isc* operon deletion mutant (YT1014; Δ*iscRSUA-hscBA-fdx*-ORF3::Km^r) and is indicative of a low degree of Fe-S cluster assembly, since the biosynthesis pathways for NAD$^+$, thiamine, isoleucine and valine require Fe-S enzymes (*9*). The auxotrophy was not affected by the carbon source (glucose, lactose, or succinate), suggesting that neither the induction of the *lac* promoter with lactose nor catabolite repression by glucose is the major cause of the observed phenotype. In contrast, when grown under anaerobic conditions, the UT109/pRKHpSU cells grew as well as the wild-type, UT109/pRKE, and UT109/pRKSUF106 cells, with a doubling time of about 25 min in LB medium containing glycerol and fumarate (Fig. 3B). The UT109/pRKHpSU cells were also able to grow on anaerobic minimal media containing glucose, lactose, or glycerol/fumarate even in the absence of nicotinic acid, thiamine, and casamino acids. Thus, UT109/pRKHpSU showed an almost normal ability to grow under anaerobic conditions requiring either fermentation or anaerobic respiration. In marked contrast, the growth defects of YT1014 cells were not abrogated by growth under anaerobic conditions (data not shown).

Activity of Fe-S Proteins—To assess the ability to assemble Fe-S clusters, we determined the activity of endogenous Fe-S proteins glutamate synthase (GltS) and succinate dehydrogenase (SDH) in cell extracts grown aerobically in LB medium. As shown in Fig. 4, the UT109/pRKHpSU cells exhibited decreased GltS activity (about 40%) and SDH activity (about 25%) while the UT109/pRKE and UT109/pRKSUF106 cells exhibited enzymatic activities similar to the wild-type level. Virtually no changes were detectable in the activity of a control enzyme (malate dehydrogenase) that does not contain an Fe-S cluster. These results are consistent with the

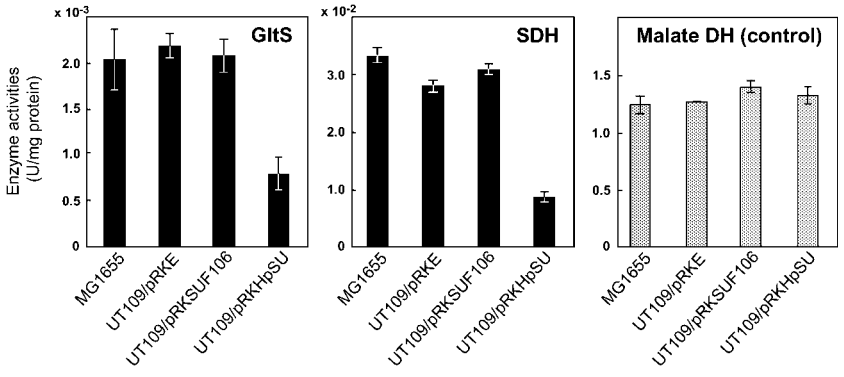


Fig. 4. **Functional but insufficient complementation by the *H. pylori* NIF system under aerobic conditions.** *E. coli* strains were grown aerobically at 37°C in LB medium and harvested at the early stationary phase. The activities of Fe-S enzymes (GltS and SDH), and a non-Fe-S enzyme, malate dehydrogenase, were measured in crude extracts of each strain and presented as specific activity (units/mg protein). Experiments were carried out with three independent cultures, and error bars represent standard deviations.

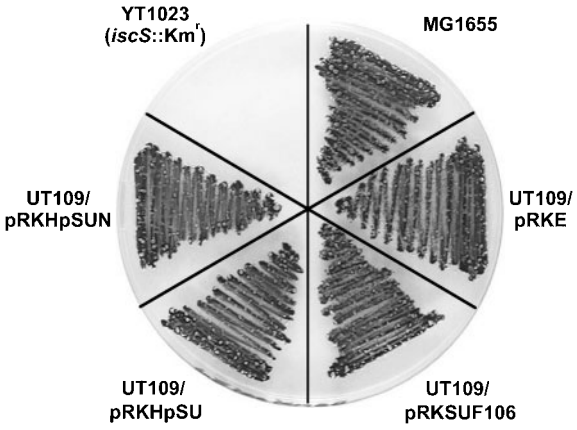


Fig. 5. **Comparison of the anaerobic FDH_H activity.** *E. coli* MG1655 (wild-type), YT1023 (*iscS*::Km^r), UT109/pRKE, UT109/pRKSUF106, UT109/pRKHpSU, and UT109/pRKHpSUN cells were grown anaerobically on an LB agar plate containing 0.4% lactose. After growth at 37°C for 24 h, each plate was overlaid with a dye solution containing 0.75 % agar, 1 mg/ml of benzyl viologen, 0.25 μM sodium formate, and 25 mM KH_2PO_4. The color of the cells gradually turned purple with the formate-dependent reduction of benzyl viologen.

growth characteristics observed under aerobic conditions described above, and together indicate partial complementation by the NIF system expressed from pRKHpSU. Complementation by the ISC (pRKE) or SUF (pRKSUF106) system appears to satisfy the cellular demand for the Fe-S clusters.

Due to difficulty in disrupting cells under anaerobic conditions, we took advantage of the agar overlay assay to assess the activity of formate dehydrogenase H (FDH_H), an anaerobic Fe-S protein containing an oxygen-labile [4Fe-4S] cluster, selenocysteine, and molybdopterin guanine dinucleotide as cofactors for its catalytic activity. Enzymatic activity is negligible in mutant strain YT1023 (*iscS*::Km^r), due to defects in both the assembly of Fe-S clusters and selenocysteine metabolism (*40*). As shown in Fig. 5, all the cells except for YT1023 reduced benzyl viologen. We carried out triplicate experiments carefully monitoring the color developing process, and observed that FDH_H activity in the UT109/pRKE, UT109/pRKSUF106 and UT109/pRKHpSU cells was comparable and almost equal to the wild-type level. These results suggest that the ISC, SUF, and NIF systems expressed from the respective plasmids are able to execute anaerobic Fe-S cluster biosynthesis sufficiently for the maturation of active FDH_H.

Sensitivity to Reactive Oxygen Species (ROS)—The NIF system expressed in the UT109 cells showed unique properties; the operon was fully functional under anaerobic conditions but only partially under oxidative conditions. These observations led us to further examine these cells under oxidative stress conditions. We used phenazine methosulfate (PMS), an artificial electron carrier that produces ROS ($O_2^{-\bullet}$) inside the cells, at a concentration that does not significantly affect the growth of wild-type cells (Fig. 6A). UT109 cells expressing either the ISC or SUF system exhibited slower growth after the addition of 15 μM PMS, but they were able to grow continuously at this slower rate. In contrast, growth of the UT109/pRKHpSU cells ceased after the addition of 15 μM PMS and never resumed. It should be noted that the ISC, SUF and NIF systems expressed from the respective plasmids did not show toxic effects nor accumulate toxic byproducts that magnify the deleterious effects of oxidants,

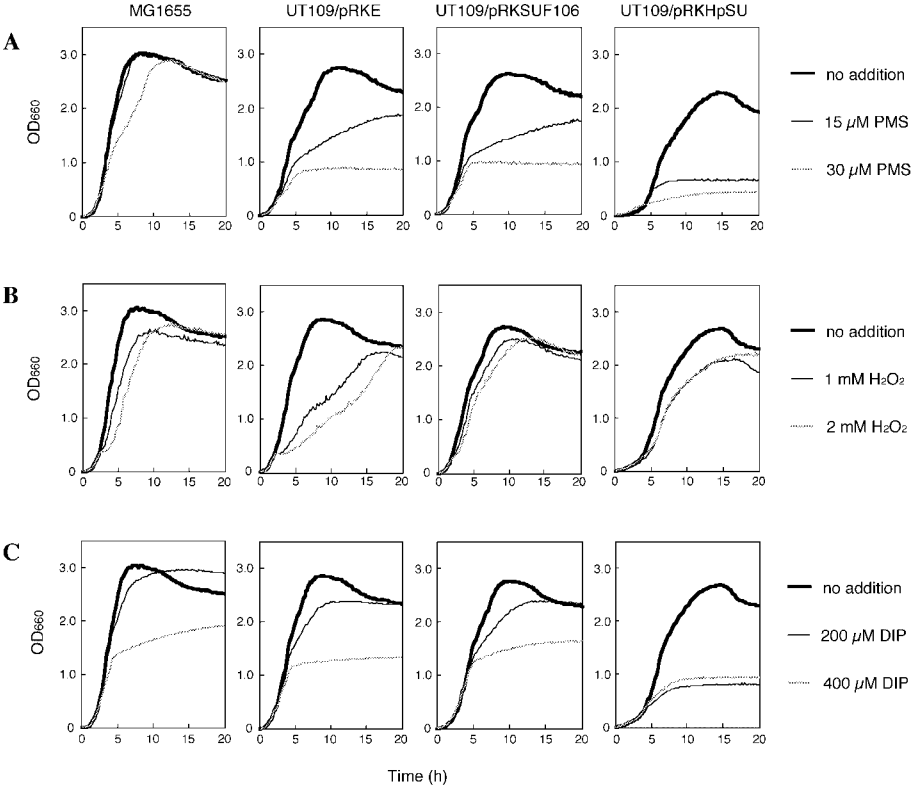
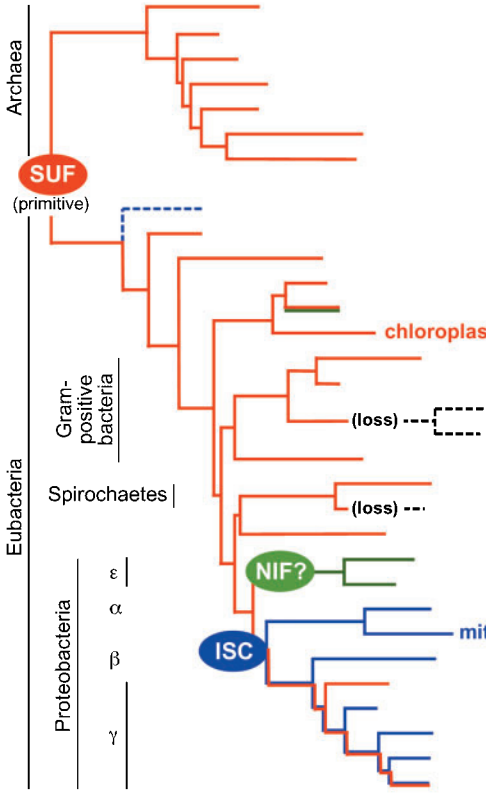


Fig. 6. **Growth defects caused by oxidative stress and iron limitation.** *E. coli* strains were aerobically grown in liquid LB medium. PMS (A), H_2O_2 (B), or DIP (C) was added to the cultures at the exponential phase (OD_{660} ~0.3). Growth was monitored at OD_{660}.



Organism	Related genes	Fe-S proteins
Aeropyrum pernix	/ C B D / S / S /	+
Pyrococcus horikoshii	/ C B / C B / S /	+
Methanococcus jannashii	/ C B /	+
Methanobacterium thermoautotrophicum	/ C B / S /	+
Archaeoglobus fulgidus	/ C B / S U / S U /	+
Thermoplasma acidophilum	/ C B B /	+
Halobacterium sp.	/ C B B / S U /	+
Aquifex aeolicus	/ A / S / S / U / F /	+
Thermotoga maritima	/ C B D S U / C B / S /	+
Deinococcus radiodurans	/ A / A / C B x x x x B / E S / S /	+
Synechocystis sp. 6803	/ A / A / B C D S / E / S / S / S /	+
Anabaena sp. 7120	/ A / A / A / B C D S / E / S / S / S / S / S nU /	+
Lactococcus lactis	/ C B S x U B / S / S /	+
Bacillus subtilis	/ A / C B S U B / S / S / S / S /	+
Ureaplasma urealyticum	/ S U /	ND
Mycoplasma pneumoniae	/ S U /	ND
Mycobacterium tuberculosis	/ A / B D C S U / E / S / S / S /	+
Treponema pallidum	/ C B D S U / S /	+
Borrelia burgdorferi	/ S U /	ND
Chlamydia trachomatis	/ B C D S / E / S /	+
Campylobacter jejuni	/ S nU / S /	+
Helicobacter pylori	/ S nU / S /	+
Rickettsia prowazekii	/ A / S S U A / hB hA F /	+
Neisseria meningitidis	/ S U A x hB / hA x x F /	+
Xylella fastidiosa	/ A / A / B C D S / E /	+
Pseudomonas aeruginosa	/ S A / S E / S / S / S / S U A hB hA F /	+
Haemophilus influenzae	/ S E / S U A hB hA F /	+
Vibrio cholerae	/ A / E / S / S / S U A hB hA F /	+
Escherichia coli	/ A / A B C D S E / S E / S U A hB hA F /	+

Fig. 7. **Distribution of the *suf*-, *nif*-, and *isc*-related genes among the completely sequenced microbial genomes.** Genes involved in all three systems are shown in black letters (S, *sufS*/*nifS*/*iscS*; and A, *sufA*/^{nif}*iscA*/*iscA*). The *suf*-specific genes (B, *sufB*; C, *sufC*; D, *sufD*; and E, *sufE*) are shown in red. The *nif*-specific genes (nU, *nifU*) are shown in green and the *isc*-specific ones (U, *iscU*; hB, *hscB*; hA, *hscA*; and F, *fdx*) in blue. Slanting lines indicate the boundaries of the gene clusters. Many *sufC*-like genes were excluded from the table due to the large number of paralogous genes encoding the ATPase subunits of ABC transporters. Likewise, several *hscA*-like genes encoding an HSP70-type molecular chaperone were excluded, when they were found as isolated genes in the respective genome sequences. X, unidentified genes; ND, not detected. Data for the completely sequenced microbial genomes were retrieved from NCBI with the BLAST software (http://www.ncbi.nlm.nif.gov/BLAST/). The phylogenic tree shown on left is based on the sequences of small ribosomal RNA, in which putative origins of the SUF, NIF, and ISC systems are indicated.

since wild-type (MG1655) cells harboring these plasmids grew equally well even in the presence of PMS (data not shown). Rather, it appears most likely that the NIF system is hypersensitive to ROS, and that this sensitivity brought about the growth defects in the presence of $O_2^{-\bullet}$.

The addition of H_2O_2 revealed additional phenotypic differences between the transformants (Fig. 6B). Growth inhibition was most pronounced in the cells expressing the ISC system. In these cells, the doubling time was 117 ± 13 min in the presence of 1 mM H_2O_2, *i.e.* 4-fold longer compared to that in the absence of H_2O_2. A relatively small effect was observed for UT109/pRKHpSU cells expressing the NIF system. Thus, susceptibility differed in these cells depending on the Fe-S assembly system (NIF or ISC) and ROS ($O_2^{-\bullet}$ or H_2O_2). This presumably reflects mechanistic diversity in the assembly of labile intermediate Fe-S clusters and/or a subsequent transfer step toward apo Fe-S protein targets. Interestingly, UT109/pRKSUF106 cells expressing the SUF system were not significantly affected by H_2O_2 up to 2 mM.

We also examined iron-limited growth conditions by inclusion of the ferrous iron chelator 2,2′-dipyridyl (DIP). The effect of iron-limitation was relatively small for the cells expressing the ISC and SUF systems, both of which did not show significant differences from the wild-type cells (Fig. 6C). In contrast, the UT109/pRKHpSU cells stopped growing after the addition of 200 μM DIP. These results might suggest specific donation of iron to the ISC and SUF systems in *E. coli* cells rather than the foreign NIF system, but the proteins involved in iron supply are currently unknown. Alternatively, it is plausible that the intermediate Fe-S cluster on the NifU scaffold is more exposed and susceptible to DIP than that in the ISC and SUF systems.

DISCUSSION

In this study, we constructed an *E. coli* mutant in which the chromosomal *suf* and *isc* operons were both inactivated by deletion. Using the plasmid replacement technique, we have succeeded in engineered 'lateral gene-

transfer' that replaced the *E. coli suf* and *isc* operons with the *H. pylori nif* operon, and clearly demonstrated a "housekeeping" function for the NIF system. This experimental system also allowed us to compare the SUF and ISC systems expressed from the same promoter, which revealed subtle but significant difference between these operons depending on the growth conditions. These results demonstrate the versatile roles of the Fe-S cluster assembly systems as well as selective sensitivity against environmental stress.

Evolutionary Aspects of the SUF System—In the microbial genome databases, we found a number of *suf*-, *isc*-, and *nif*-related genes, some of which are clustered in the genome sequences, probably as operons (Fig. 7). Since *sufBC*-like genes are found in many species from each of the major domains of life, we speculate that they are ancient genes and were present in the common ancestor of bacteria and Archaea. It seems likely that a primitive SUF system composed of SufB and SufC has evolved with the recruitment of other components such as SufS, SufD, SufA, and SufE. The wide distribution of SufS/IscS/NifS–family proteins may suggest that cysteine desulfurase also originated early in the evolution of life. Paralogous enzymes found in many bacteria may reflect the versatile roles of this family of proteins that are required for sulfur-transfer not only to Fe-S clusters, but also to other sulfur-containing compounds such as thiamine and molybdopterin, as well as the thiolation of tRNA (*15*, *41*). SufD exhibits limited similarity to SufB in its C-terminal domain (<30% identity), suggesting that *sufD* was the result of gene duplication of *sufB* and subsequent domain shuffling. It is not surprising, therefore, that some organisms including *Thermoplasma acidophilum* and *Bacillus subtilis* possess a SufB-SufB pair instead of a SufB-SufD pair. IscU, an ISC-specific component in *E. coli*, is also found in the *suf*-related operons in several organisms such as *B. subtilis*. Judging from our preliminary results, the *sufCBS-iscU-sufB* operon cloned from *B. subtilis* (*yurYXWVU*) also complemented the *E. coli* mutant UT109.

Distribution and Unique Properties of the NIF System—The NIF system is composed of only two proteins, NifS and NifU, the latter being generated through the fusion of *iscU* with other genes corresponding to the second and third domains (*6*). Although the origin of the NIF system is difficult to determine, the distribution of *nifSU* in nitrogen-fixing cyanobacteria (*e.g*. *Anabaena* sp. PCC 7120) and γ-proteobacteria (*e.g*. *A. vinelandii*; not shown in Fig. 7) as well as non-nitrogen-fixing ε-proteobacteria (*H. pylori* and *C. jejuni*) and an anaerobic protozoan *Entamoeba histolytica* (*47*) might be a consequence of lateral gene-transfer. It is noteworthy that *H. pylori* and *C. jejuni* are both pathogenic microaerophiles and are unable to grow under atmospheric oxygen. It is also well known that nitrogen-fixation is carried out under anaerobic conditions due to the instability of nitrogenase in the presence of oxygen. Thus, these organisms employ the NIF system under low oxygen pressure.

The distribution of the NIF system corresponds well with our data demonstrating that the *nifSU* operon cloned from *H. pylori* was sufficient for the anaerobic biosynthesis of Fe-S proteins in an *E. coli* mutant lacking both the ISC and SUF systems. In contrast to the ISC and SUF systems, however, the NIF system is not fully functional under aerobic conditions, probably due to its susceptibility to oxygen. Hypersensitivity to $O_2^{-\bullet}$ was also demonstrated by the growth defects in the presence of PMS. Recently, we also reported that the NIF-like system from the obligatory anaerobic protozoan *E. histolytica* is functional only under anaerobic conditions (*47*). Considering the limited distribution of the *nifSU* genes described above, oxygen-sensitivity appears to be an intrinsic property of the NIF system, and this sensitivity may have prevented the NIF system from being distributed to aerobic organisms. Nonetheless, it is surprising that only the two components of the NIF system, NifS and NifU, are capable of almost completely substituting for the ISC and SUF systems, both of which are composed of at least six components. Most likely cysteine desulfurase NifS acts as a specific sulfur-donor to NifU on which an intermediate Fe-S cluster seems to be assembled and subsequently transferred to a number of target apo Fe-S proteins. It should be noted that, in *A. vinelandii*, the "true" NIF system involved in the maturation of nitrogenase is unable to substitute for the ISC function (*7*). At present, the molecular mechanisms underlying this target specificity are not clear.

SUF vs. ISC in E. coli—The multicomponent systems examined here, *i.e*. ISC and SUF, appear to be advantageous over the NIF system under aerobic conditions and especially in the presence of $O_2^{-\bullet}$. The ISC system is composed of six proteins, IscS, IscU, IscA, HscB, HscA, and Fdx, which might have originated through gene duplication of the components recruited from the SUF system and other metabolic pathways. The progenitor of the *hscA-hscB* pair is the *dnaK-dnaJ* pair encoding an HSP70-type molecular chaperone and co-chaperone, respectively, in which the three-helix bundle domain of HscB replaced the C-terminal domain of DnaJ (*48*). Fdx is a member of the [2Fe-2S]ferredoxin family (*49*). The ISC system appears to have been established in the common ancestor of α-, β-, and γ-proteobacteria, and subsequently gene loss has occurred in this clade (Fig. 7). Many of these species lost the *suf* operon, whereas the converse is observed for other bacteria including *Xylella fastidiosa* and *Mesorhizobium loti*, which retained the *suf* but lost the *isc* operon. Interestingly, *E. coli* and its close relatives, *Y. pestis* and several *Salmonella* species, retained both *suf* and *isc*.

Mutant strain UT109 also provides a useful system for the comparative analysis of cells expressing either the *isc* or *suf* operon from the same promoter in an isogenic background. Although these cells exhibited no significant differences in most functional tests (*i.e*. growth rate, nutritional requirements and activities of Fe-S proteins), susceptibility to H_2O_2 was remarkable in the cells expressing *isc*. Thus, the SUF system appears to be more advantageous for Fe-S assembly in the presence of H_2O_2. The H_2O_2-resistant function of the SUF system is in agreement with the expression profile of the *suf* operon, *i.e.* the chromosomal *suf* operon is markedly induced by OxyR upon treatment with H_2O_2 (*36*). Conversely, expression of the *isc* operon is controlled by one of its products, IscR, and an attractive model for the feedback regulation by the [2Fe-2S] form of IscR has been proposed (*43*). We speculate from these observations that the differential

regulation of the *isc* and *suf* operons is beneficial for the fine-tuning of Fe-S cluster synthesis. Such control would allow *E. coli* cells to satisfy the cellular needs for Fe-S clusters under a broad range of environmental conditions. This regulation also seems to be a prerequisite to avoid an excess supply of Fe-S clusters**.**

The mitochondrial components required for Fe-S cluster formation in higher organisms are similar to the bacterial ISC proteins and apparently were inherited from a prokaryotic progenitor such as *Rickettsia prowazekii*, the putative closest relative to eukaryotic mitochondria (*32*, *33*). Similarly, the cyanobacterial SUF machinery appears to have been adopted by chloroplasts and other plastids. However, we also found that there are many unusual patterns of gene distribution, such as the presence of *iscU* and *fdx* homologs, and the absence of *suf* genes in *Aquifex aeolicus* (*50*), a deep-branching species on the bacterial tree. Some Archaea possess only a limited number of known *suf* genes. Furthermore, the genome sequences of *Borrelia burgdorferi* and several *Mycoplasma* species were found to contain no identifiable genes for Fe-S proteins. These species have also lost the genes encoding Fe-S assembly systems, although genes for cysteine desulfurase and IscU (weak homology) are still retained. We believe that the analysis reported here can serve as a starting point for experimental studies on Fe-S cluster biogenesis in a wide variety of species as well as for an increased understanding of the complex mechanism of Fe-S assembly.

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