Accelerated Publication

A Third Bacterial System for the Assembly of Iron-Sulfur Clusters with Homologs in Archaea and Plastids*[S]

Yasuhiro Takahashi and Umechiyo Tokumoto
From the Department of Biology, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan

The assembly of iron-sulfur (Fe-S) clusters is mediated by complex machinery. In several proteobacteria, this process involves ISC (Fe-S cluster assembly) machinery composed of at least six components also conserved in mitochondria from lower to higher eukaryotes. In nitrogen-fixing bacteria, another system, termed NIF (nitrogen fixation), is required for the maturation of nitrogenase. Here we report the identification of a third system, designated the SUF machinery, the components of which are encoded in *Escherichia coli* by an unassigned operon, *sufABCDSE*. We have analyzed spontaneous pseudorevertants isolated from a mutant strain lacking all the components of the ISC machinery. The suppressor mutations in the revertants have been localized to the regulatory region of the *suf* operon; overexpression of this operon restores the growth phenotypes and activity of Fe-S proteins in mutant cells lacking ISC. Disruption of the *suf* operon alone does not cause any major defects, but synthetic lethality was observed when both the *isc* and *suf* operons were inactivated. These results indicate that proteins encoded by the *suf* operon participate in the ISC-independent minor pathway for the assembly of Fe-S clusters. The genes homologous to *sufBC* are present in a wide range of bacteria, Archaea, and plastids, suggesting that this type of system is almost ubiquitous in nature.

Iron-sulfur (Fe-S) clusters are cofactors of proteins that perform a number of biological roles, including electron transfer, redox and non-redox catalysis, and sensing for regulatory processes (1). The complex mechanism by which Fe-S clusters are assembled is now becoming elucidated by genetic and biochemical methods, which led to the identification of two distinct systems, termed NIF (nitrogen fixation) and ISC (iron-sulfur cluster). In a pioneering report, Dean’s group demonstrated the roles of two *nif* gene products in the assembly of Fe-S clusters in the nitrogenase proteins in *Azotobacter vinelandii* (2). NifS is a pyridoxal phosphate-dependent cysteine desulfurase that initiates Fe-S cluster formation by producing elemental sulfur from cysteine (3). NifU appears to serve as a scaffold for the assembly of transient Fe-S clusters before delivery to other apoFe-S proteins (4).

In contrast to the NIF machinery that specifically deals with the maturation of nitrogenase, the ISC machinery is involved in the general pathway of biosynthesis of numerous Fe-S proteins (5). Genetic experiments support a crucial role for the proteins encoded by the so-called *isc* operon (*iscSUAB-CSFdx*), because the mutations in these genes in *Escherichia coli* decrease the activity of many Fe-S proteins (6–8). Several similarities have been identified between the ISC and NIF systems. First, IscS bears similarity to NifS in its sequence and function as a cysteine desulfurase (5, 9, 10). Second, IscU corresponds to the N-terminal domain of NifU and contains three conserved cysteine residues that are essential for its function as a scaffold for intermediate Fe-S clusters (11–15). Third, IscA is closely related to its NIF counterpart (NifIscA), both of which were shown to acquire a labile Fe-S cluster and have been proposed as an alternative scaffold in Fe-S cluster biosynthesis (16–18). The ISC machinery contains at least three additional components, HscB, HscA, and Fdx, which appear to take part in a series of reactions that have yet to be fully characterized (19–21).

Homologous components of the NIF machinery are found in several nitrogen-fixing bacteria as well as non-diazotrophic *e*-proteobacteria including *Helicobacter pylori* (22). On the other hand, the components of the ISC machinery are conserved from bacteria to higher eukaryotes, although a complete set of the components appears to be confined to α-, β-, and γ-proteobacteria and mitochondria (23). Intriguingly, some Archaea such as *Methanococcus jannaschii* and *Thermoplasma acidophilum* lack all the homologous components, despite the fact that they possess genes encoding many proteins that require Fe-S clusters for proper function. Thus, our understanding of the mechanism by which Fe-S clusters are assembled is limited.

In an earlier study, we described an *E. coli* mutant, YT1014, in which the entire *isc* operon has been deleted (8). The mutant grows very poorly and exhibits a marked decrease in the activity of Fe-S proteins, 2–10% when compared with wild-type cells. Thus, the ISC machinery serves a predominant function in the biosynthesis of Fe-S proteins. On the other hand, the residual modest activity of Fe-S enzymes raises interesting questions regarding the existence of alternative assembly machinery. In the present study, we isolated several pseudorevertants from YT1014 and analyzed the suppression mutations with respect to the expression of the *sufABCDSE* operon. Although *suf*-encoded proteins have been proposed to be involved in S, Se, Fe or Fe-S metabolism and the oxidative stress response (the name was given after mobilization of sulfur), there has been no direct evidence for the function of any specific proteins (24–28). We show functional redundancy between the *isc* and *suf* operons by mutational experiments, in which five *suf* genes are essential for viability in the mutant background of the *isc* operon. From these observations, we propose that the

* This work was supported in part by Grants-in-aid for Scientific Research 13680690 from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by a grant from the Japan Foundation for Applied Enzymology. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains Supplemental Tables S1 and SII and Fig. S1.

‡ To whom correspondence should be addressed. Tel.: 81-6-6850-5423; Fax: 81-6-6850-5425; E-mail: ytaka@bio.sci.osaka-u.ac.jp.

The abbreviations used are: Fe-S, iron-sulfur; Gm′, gentamycin-resistant; Km′, kanamycin-resistant; nt, nucleotide(s).

© 2002 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

The Journal of Biological Chemistry
Vol. 277, No. 32, Issue of August 9, pp. 28380–28383, 2002
This paper is available online at http://www.jbc.org
proteins encoded by the suf operon work in concert in a novel system that mediates the biogenesis of Fe-S proteins. We also show that suf homologs are present in a wide range of bacteria, Archaea, and plastids.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Growth Media—** E. coli strains and plasmids used in this study are listed in the supplementary data. Luria-Bertani (LB) medium was used as the standard medium. When required, kanamycin, tetracycline, chloramphenicol, and gentamicin were added. E. coli strains are listed in Table S1. Tryptone, yeast extract, and glucose were added to a final concentration of 2% (w/v). Free casein hydrolysate (0.1%) was supplemented with glucose (20 mM), thiamine (1 μg/ml), and vitamin-free casein hydrolysate (0.1%).

**Molecular Techniques—** Oligonucleotides used in this study are listed in Table S1. Genomic DNA was isolated from the pseudorevertant YT2001, partially digested with Sau3AI, and the termini partially filled in with dATP and dGTP. The fragments were ligated into the Sali site of pUC119, which had been partially filled in with dCTP and dTTP and then introduced into YT1014 cells. The plasmid pU628 was isolated from a transformant growing on minimal media in the absence of nicotinic acid. The plasmid was re-introduced into YT1014 cells to confirm the ability to suppress the growth defect. Genomic DNA fragments corresponding to the −983 to +32 nt relative to the sufA start codon were amplified by PCR using the primers 

**Targeted Mutagenesis—** A sufABCDEF deletion strain, YT2512, was constructed by replacing the entire operon with a Gm’ cassette. For this purpose, the 975-bp fragment corresponding to the 5’ upstream region of sufA (sufA-5’) and the 1015-bp fragment in the 3’ downstream region of sufR (sufR-3’) were amplified by PCR using the SuUF/SuUR2 and YnhGF/YnhGR2 primer sets, respectively. The Gm’ cassette was amplified from pUGCM (30) using the primers Gm’F and Gm’R. The PCR products were initially cloned into pCR2.1-TOPO (Invitrogen), and the fragments were amplified by PCR using primer sets containing Bsal and XbaI restriction sites and cloned into the XbaI/NheI sites of pKNSE (29).

**RESULTS AND DISCUSSION**

**Isolation and Characterization of Pseudorevertants—** E. coli YT1014 has a defined deletion of the isc operon (ΔiscRSUA- hscBA-fdx-ORF3::Km’) and lacks all the components of the ISC machinery. The mutant shows auxotrophy toward nicotinic acid, thiamine, methionine, isoleucine, and valine, exhibiting very poor growth on rich medium even when these compounds are supplied. This phenotype has been attributed primarily to a defect in the assembly of Fe-S clusters because the activities of Fe-S enzymes are decreased markedly in the mutant cells (8). To identify alternative systems mediating Fe-S cluster assembly that may contribute to the modest amount of the residual Fe-S protein activity, spontaneous pseudorevertants of YT1014 were isolated. Such revertants appeared on minimal medium lacking nicotinic acid at a frequency of 10−7 to 10−6. Five independent revertants (YT2001, YT2002, YT2003, YT2006, and YT2007) were chosen for further study. Their growth rate in liquid LB medium was about 2–3 times slower than wild-type cells but significantly faster than the parental YT1014 cells (Fig. 1A). Activity measurement of succinate dehydrogenase, an Fe-S enzyme, showed that the enzyme level also lies between those found in wild-type and YT1014 cells. In addition, auxotrophy toward nicotinic acid, thiamine, methionine, isoleucine, and valine were partially relieved in the revertants (data not shown), indicating that activities of various Fe-S proteins were restored. Because the iscRSUA-hscBA-fdx-ORF3 allele in YT1014 is a nonreverting null mutation, the revertants must result from extragenic suppressor mutations.

In an attempt to clone the gene conferring the pseudorevertant phenotype, we made a genomic library from YT2001 and isolated a plasmid selected for its ability to complement the nicotinate auxotrophy and slow growth phenotype of YT1014. Sequencing of the obtained plasmid pU628 revealed a 788-bp chimeric DNA fragment composed of truncated IS2 (459 bp) and incomplete sufA (127 and 202 bp for upstream and coding regions, respectively). The results were surprising because: 1) the transposable element IS2 is not located in the corresponding region of MG1655 sequence; 2) both IS2 and sufA are truncated; and 3) only a few transformants gave a revertant phenotype, at a frequency of 10−3 to 10−2, when pU628 was re-introduced into YT1014 cells. Thus, we analyzed the 5’ upstream region of chromosomal sufA (−983 to +32 nt relative to the sufA start codon) by PCR and found that a 2.3-kb fragment was amplified from the YT2001 allele rather than the 1.0-kb fragment amplified from wild-type and YT1014 cells (Fig. 1B). The 2.3-kb fragment was used as a template for direct sequencing, which revealed the complete 1331-bp IS2 sequence containing 41-bp imperfect inverted terminal repeats. Duplication of 5-bp direct repeats in the target DNA (CTATG; −123 to −127 nt relative to the sufA start codon) clearly indicates that IS2 was transposed and inserted into the upstream region of sufA in YT2001. Next, the corresponding region was analyzed in YT1014 cells transformed with the pU628 plasmid. From the

Fig. 1. Pseudorevertants isolated from E. coli YT1014 lacking the ISC machinery. A, restoration of growth rate and succinate dehydrogenase (SDH) activity. The value for MG1655 (wild-type) was set to 100%. Results and error bars represent means and standard deviations of at least three independent experiments, respectively. B, PCR analysis of the 5’ upstream region of sufA. Lane 1, MG1655; lane 2, YT1014; lane 3, YT2001; lane 4, YT1014 harboring pU628; lane 5, size marker (a DNA-StyI digest); lane 6, YT2006. C, mutations in the 5’ upstream region of sufA in the five pseudorevertants. Homology scores (32) for the potential promoter sequences are indicated in parentheses.
Fig. 2. Functional redundancy between ISC and SUF. A, plasmids carrying the suf operon. The gene clusters were amplified by PCR (thick lines) with the preceding ribosome binding sequences and cloned into the pRKNS vector under the control of the lac promoter. Filled triangles denote the insertion of a PstI linker that contains stop codons in all reading frames. B, heterologous complementation of YT1014 by the suf-containing plasmids shown in A. The relative growth rate and succinate dehydrogenase (SDH) activity are indicated as described in the legend to Fig. 1. C, combination of mutations tested for synthetic lethality. YT2512 cells were transformed with the suf-containing plasmids shown in A, and then the chromosomal isuSUA allele was deleted. Synthetic lethality was measured by failure to lose chloramphenicol resistance for over 300 sucrose-resistant colonies. The results were confirmed by PCR.

slow growing cells, a 1.0-kb fragment was amplified by PCR, similar to that observed for wild-type and YT1014, whereas an additional 4.9-kb fragment was amplified from the fast growing cells (Fig. 1B, lane 4). Partial sequencing of the purified 4.9-kb fragment revealed the junction between pUC119 and truncated IS2 or sufA. Thus, the plasmid pUS28 (3950 bp) is most likely integrated into the chromosomal sufA region via a single crossover homologous recombination, which may be responsible for the pseudorevertant phenotype.

PCR and DNA sequence analysis were carried out on the 5’ upstream region of sufA from 5 pseudorevertants, resulting in identification of the following distinct mutations: 1) transposition of IS2 at position –128 relative to the sufA start codon in YT2001; 2) transposition of IS1 at position –181 in YT2006; 3) C to T base substitution at position –149 in YT2002; 4) insertion of A at position –51 in YT2003; and 5) insertion of T at position –51 in YT2007 (Fig. 1C). All of the mutations lie in the non-coding region and, in fact, are confined between a putative ρ-independent terminator and the sufA start codon. In this region of DNA, no base differences were observed for YT1014 or MG1655. The sufA gene transcript begins 32 nt upstream of the sufA start codon (28). Nearly consensus –35 and –10 elements of an ρ-dependent promoter are present upstream of the transcription start site, which overlaps with a Fur box sequence for recognition by the iron-sensing transcriptional regulator Fur (24, 26). Two of our mutations, insertion of A or T at position –51, disrupt the consensus sequence of the Fur box. Furthermore, these insertions alter the promoter sequence to match the consensus 17-bp spacing between the –35 and –10 elements. Improvement of the promoter was confirmed with computer programs by the Homology Score Method (32) and the Neural Network Method. Although the original promoter sequence remains unaffected by other mutations, we found several promoter-like sequences created by the base substitution and IS transpositions (Fig. 1C). Taken together, these data suggest that the mutations in the regulatory region may increase the expression of sufA and downstream genes in the pseudorevertants.

Pseudorevertant Phenotype Is Afforded by Overexpression of the sufABCFSE Operon—The sufA gene is the first member of the sufABCFSE operon, located at 38 min in the E. coli MG1655 genome (Fig. 2A). The sufS gene product is a paralog of IscS and catalyzes elimination of sulfur from l-cysteine and selenium from l-selenocysteine (10, 25). Although gene products of the remaining five suf genes were not characterized, SufA is 47% identical to IscA and SufC shows homology to ATPase subunits of ABC transporters (24, 26, 27). These observations have led to the suggestion that suf-encoded proteins are involved in S, Se, Fe, or Fe-S cluster metabolism, although no direct evidence for its functional role has been attained. To determine if increased expression of the suf operon could be responsible for the phenotypes of our isolates, the operon, without the promoter, was amplified by PCR, cloned into a low copy number vector, pUC119, and provided no complementation of YT1014 (data not shown). The growth phenotypes of the transformants were not affected by addition of isopropyl-β-D-thiogalactopyranoside, indicating that basal expression from the uninduced lac promoter is sufficient for the complementation. When the operon was cloned into high copy number vector, pUC119, it provided no complementation of YT1014 and rather had an inhibitory effect on growth. Hence, excessive expression of the suf operon appears toxic, which explains why we could not clone the entire operon in the initial screening from the revertant library.

Functional Redundancy between the isc and suf Operons—The possibility of redundant roles between isc and suf has been investigated by inactivating the corresponding operons, either singly or in combination. The sufABCDSE operon was deleted by substituting the entire coding region with a Gm’ cassette.

2 www.fruitfly.org/seq_tools/promoter.html.
cointegrates that formed after plasmid insertion into chromosome could not be resolved. Growth of mucoid colonies on sucrose plates and a failure to lose chloramphenicol resistance provided by pKO3 were indicative of merodiploidy (pseudodisruptant) (31), suggesting that the suf operon may be essential for viability in the mutant background of the isc operon.

The synthetic lethality was examined further by introducing the ΔiscSUA::KmR mutation into YT2512 (ΔsufABCDSE::GmR). As shown in Fig. 2C, we obtained the double mutant when the recipient cells carried the suf-containing plasmid pRKSUF017 but not in control cells without the plasmid. To determine which suf gene is essential for growth, we modified pRKSUF017 such that genes were inactivated independently by truncation or by introducing translational stop codons (Fig. 2A). The results of a second ΔiscSUA::KmR mutagenesis indicate that all suf genes but one (sufD) are indispensable (Fig. 2C). Although viable, cells in which sufD was inactivated displayed a severe growth defect in the mutant background of ΔiscSUA; the strain grew very slowly in LB medium with a doubling time of about 230 min and reached a lower final density of about one-fifth, as measured by A660. The sufD− ΔiscSUA cells were unable to grow on minimal media, even when nicotinic acid, thiamine, and casamino acids were supplemented. These results taken together provide additional evidence for the functional redundancy between the isc and suf operons. Most probably, the six proteins encoded by the suf operon work in concert as the components of a novel apparatus (here termed the SUF machinery), which participates in the maturation of Fe-S proteins. A recent microarray expression analysis has shown that suf operon is regulated by OxyR and induced upon treatment with hydrogen peroxide (28). Because damage to Fe-S clusters is a major consequence of oxidative stress, expression of the suf operon may be essential for Fe-S cluster formation.

sufBC-Related Operons Are Also Present in Archaea and Plastids—In the data bases of completely sequenced microbial genomes, we found a number of suf-, isc-, and nif-related genes (see supplemental data, Fig. S1). Some of these genes are clustered in their respective genome sequences, probably as operons, indicating several models for the genetic organization of Fe-S cluster assembly systems. First, E. coli and its close relatives (Yersinia pestis and several Salmonella species) retain both the suf and isc operons, whereas other species possess only one of the two. This provides additional evidence for the redundant role of the SUF and ISC systems that operate in separate, parallel pathways. In A. vinelandii, the iscS and hscA genes are essential for viability (5), which might be explained by the lack of the SUF machinery. Second, the ε-proteobacteria (H. pylori and Campylobacter jejuni) contain the NIP machinery similar to that observed in several nitrogen-fixing bacteria (22). Third, iscU is not only found in the isc operon of proteobacteria but also in the suf-related operon of other species, including Thermotoga maritima and Bacillus subtilis, suggesting that the gene originated as a component of the SUF system. Fourth, the sufBC-like genes are present in a number of species, including Archaea, suggesting that they play central roles in the SUF machinery. In eukaryotic genome sequences, sufBC-like genes are present in the plastid genomes of the red algae, including Guillardia theta and Odontella sinensis (27, 33). Although these genes are absent from the plastid genomes of higher plants, sufABCDSE-like genes are all encoded in the nuclear DNA of Arabidopsis thaliana, with potential transit peptides for import into chloroplasts.

In conclusion, we have identified an operon encoding a novel bacterial system for the assembly of Fe-S clusters. The available evidence suggests that SUF-like systems operate also in Archaea and plastids, raising the possibility that this type of system is almost ubiquitous in nature.

Acknowledgments—We thank H. Ohmori for technical assistance and Mrs. K. Fukuyama and K. Saeki for valuable suggestions and critical reading of the manuscript.

REFERENCES

1. Beinert, H., Holm, R. H., and Manec, E. (1997) Science 277, 653–659
2. Jacobson, M. R., Cash, V. L., Weiss, M. C., Laird, N. F., Newton, W. E., and Dean, D. R. (1989) Mol. Gen. Genet. 219, 49–57
3. Zhang, L., White, R. H., Cash, V. L., Jack, R. F., and Dean, D. R. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2754–2758
4. Yovanovich, P., Agar, J. N., Cash, V. L., Johnson, M. K., and Dean, D. R. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 599–604
5. Zhang, L., Cash, V. L., Flint, D. H., and Dean, D. R. (1998) J. Biol. Chem. 273, 13261–13272
6. Takahashi, Y., and Nakamura, M. (1999) J. Biochem. (Tokyo) 126, 917–926
7. Schwartz, C. J., Djamian, O., Inlay, J. A., and Kiley, P. J. P. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 9009–9014
8. Tokumoto, U., and Takahashi, Y. (2001) J. Biochem. (Tokyo) 130, 63–71
9. Flint, D. H. (1996) J. Biol. Chem. 271, 16068–16074
10. Mihara, H., Kurihara, T., Yoshimura, T., and Esaki, N. (2000) J. Biochem. (Tokyo) 127, 559–567
11. Garland, S. A., Hoff, K., Vickery, L. R., and Culotta, V. C. (1999) J. Mol. Biol. 284, 897–907
12. Agar, J. N., Krebs, C., Frazzon, J., Huynh, B. H., Dean, D. R., and Johnson, M. K. (2000) Biochemistry 39, 7856–7862
13. Smith, A. D., Agar, J. N., Johnson, K. A., Frazzon, J., Amster, I. J., Dean, D. R., and Johnson, M. K. (2001) J. Am. Chem. Soc. 123, 11103–11104
14. Urbina, H. D., Silberg, J. J., Hoff, K. G., and Vickery, L. E. (2001) J. Biol. Chem. 276, 44521–44526
15. Kato, S., Mihara, H., Kurihara, T., Takahashi, Y., Tokumoto, U., Yoshimura, T., and Esaki, N. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 5948–5952
16. Ollaguer-de-Choudens, S., Mattioli, T., Takahashi, Y., and Fontecave, M. (2001) J. Biol. Chem. 276, 22604–22607
17. Krebs, C., Agar, J. N., Smith, A. D., Weiss, M. C., Laird, W. E., Newton, W. E., and Dean, D. R. (1997) Mol. Gen. Genet. 258, 657–663
18. Smith, A. D., Agar, J. N., Johnson, K. A., Frazzon, J., Amster, I. J., Dean, D. R., and Johnson, M. K. (2001) Biochemistry 40, 14069–14080
19. Wu, G., Manay, S. S., Hemann, C., Hille, R., Surers, K. K., and Cowan, J. A. (2002) J. Biol. Inorg. Chem. 7, 526–532
20. Silberg, J. J., Hoff, K. G., Tapley, T. L., and Vickery, L. E. (2001) J. Biol. Chem. 276, 1866–1700
21. Kakuta, Y., Horio, T., Takahashi, Y., and Fukuyama, K. (2001) Biochemistry 40, 11097–11102
22. Tokumoto, U., Nomura, S., Minami, Y., Mihara, H., Kato, S., Kurihara, T., Esaki, N., Kanazawa, H., Matsubara, H., and Takahashi, Y. (2002) J. Biochem. (Tokyo) 131, 713–719
23. Olesen, J. W., Agar, J. N., Johnson, M. K., and Maier, R. J. (2000) Biochemistry 39, 16213–16219
24. Muhlenhoff, U., and Lill, R. (2000) Biochim. Biophys. Acta 1459, 370–382
25. Patzer, S. I., and Hantke, K. (1999) J. Bacteriol. 181, 3307–3309
26. Mihara, H., Maeda, M., Fujii, T., Kurihara, T., Hata, Y., and Esaki, N. (1999) J. Biol. Chem. 274, 14768–14772
27. Ellis, K. E., Clough, B., Saldana, J. W., and Wilson, R. J. (2001) Mol. Microbiol. 41, 973–981
28. Zhang, L., Wang, X., Templeton, L. J., Smulek, D. L., LaRossa, R. A., and Storz, G. (2001) J. Bacteriol. 183, 4562–4570
29. Nakamura, M., Saeki, K., and Takahashi, Y. (1999) J. Biochem. (Tokyo) 126, 10–18
30. Schweitzer, H. P. (1999) BioTechniques 15, 831–834
31. Link, A. J., Phillips, D., and Church, G. M. (1997) J. Bacteriol. 179, 6228–6237
32. Mulligan, M. E., Hauley, D. K., Enzien, R., and McClure, W. R. (1984) Nucleic Acids Res. 12, 789–800
33. Wittkopf, C., Kroth-Pancic, P. G., and Strotmann, H. (1996) Plant Sci. 114, 171–179