Hyperproduction of Recombinant Ferredoxins in *Escherichia coli* by Coexpression of the ORF1-ORF2-*iscS-iscU-iscA-hscB-hscA-fdx*-ORF3 Gene Cluster[1]

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Fe-S proteins acquire Fe-S clusters by an unknown post-translational mechanism. To study the *in vivo* synthesis of the Fe-S clusters, we constructed an experimental system to monitor the expressed ferredoxin (Fd) as a reporter of protein-bound Fe-S clusters assembled in *Escherichia coli*. Overexpression of five Fds in a T7 polymerase-based system led to the formation of soluble apoFds and mature holoFds, indicating that assembly of the Fe-S cluster into apoFd polypeptides is a rate-limiting step. We examined the coexpression of the *E. coli* ORF1-ORF2-*iscS-iscU-iscA-hscB-hscA-fdx*-ORF3 gene cluster, which has recently been suggested to be involved in the formation or repair of Fe-S protein [Zheng, L., Cash, V.L., Flint, D.H., and Dean, D.R. (1998) *J. Biol. Chem.* 273, 13264-13272], with reporter Fds using compatible plasmids. The production of all five reporter holoFds examined was dramatically increased by the coexpression of the gene cluster, and apparent specificity to the polypeptides or to the type of Fe-S clusters was not observed. The increase in holoFd production was observed under the coexpression conditions in all culture media examined, with either 2×YT medium or Terrific broth, and with or without supplemental cysteine or iron. These results indicate that the proteins encoded by the gene cluster are involved in the assembly of the Fe-S clusters in a wide variety of Fe-S proteins.

Key words: assembly, *Escherichia coli*, expression, ferredoxin, iron-sulfur cluster.

Fe-S clusters contained in Fe-S proteins have been found to play a number of biological roles, including electron transfer, catalysis, stabilization of proteins, and as sensors of iron, dioxygen, and superoxide (for recent reviews, *1-5*). Although a considerable amount of information has been accumulated about the physicochemical properties and physiological significance of Fe-S proteins, very little is known about the *in vivo* synthesis of protein-bound Fe-S clusters. For example, it is not known whether Fe-S clusters are synthesized spontaneously or with the involvement of specific proteins. Chemical reconstitution of the Fe-S cluster has been demonstrated since the 1960s (*6*) on various Fe-S proteins including ferredoxin (Fd). ApoFd can be reconstituted to holoFd by chemical reaction with sulfide and ferric or ferrous ion in the presence of dithiothreitol or mercaptoethanol. It is uncertain, however, whether an analogous reaction is involved in the *in vivo* assembly of the Fe-S clusters (*7, 8*). Several protein factors have been suggested in the biosynthetic process of Fe-S proteins. Cysteine sulfur was incorporated into a [2Fe-2S] cluster of Fd in isolated, intact chloroplasts (*9*). In chloroplasts, the assembly of the Fe-S cluster was found to be a stroma-located process (*10*), consisting of two separate steps: NADPH-stimulated liberation of sulfide from cysteine, followed by the ATP-dependent incorporation of sulfide into the Fe-S cluster (*11*). Studies on *nif* mutants of *Azotobacter vinelandii* identified *nifS* as essential for nitrogenase activity (*12*). The NifS protein is a pyridoxal-phosphate dependent cysteine desulfurase (*13*) and found to catalyze the *in vitro* reconstitution of Fe-S clusters of several proteins including the nitrogenase iron protein component (*14*). A NifS-like protein was also isolated from *Escherichia coli* and found to reactivate the [4Fe-4S] cluster of dihydroxy acid dehydratase *in vitro* (*15*). Nevertheless, direct involvement of NifS and its homologue in the assembly of the Fe-S cluster has yet to be established, because both catalyze cysteine cleavage in the absence as well as in the presence of apoprotein, and insertion of sulfide into the Fe-S cluster is not coupled with these enzymes. Other sulfur-producing enzymes, 3-mercaptopyruvate sulfur-transferase (*16*), rhodanese (*17*), *O*-acetylserine sulfhydrylase, β-cystathionase (*8*), and cysteine/cystine *C-S*-lyase (*18*) have also been demonstrated to be effective in purified *in vitro* reconstitution

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Abbreviations: BtFd, *Bacillus thermoproteolyticus* ferredoxin; EcFdx, *Escherichia coli* ferredoxin encoded by the *fdx* gene; EcFdxN, *E. coli* ferredoxin encoded by the *fdxN* gene; Fd, ferredoxin; IPTG, isopropyl-1-thio-β-D-galactopyranoside; ORF, open reading frame; PbFdxH, *Plectonema boryanum* ferredoxin encoded by the *fdxH* gene; PbPetF, *P. boryanum* ferredoxin encoded by the *petF* gene; RcFdII, *Rhodobacter capsulatus* ferredoxin II encoded by the *fdxA* gene.

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systems, comprising apoprotein, thiol, ferric or ferrous ion, and the respective sulfur-containing substrates.

Recent progress in the field of molecular biology has made it possible to clone the genes for Fe-S proteins from a wide variety of organisms and express them in foreign hosts such as *E. coli*. Genes encoding adrenodoxin and plant-type Fds have been efficiently expressed in *E. coli*, which resulted in high-level production of the holoproteins with correctly assembled [2Fe-2S] clusters (*19-21*). Similarly, a number of Fe-S proteins containing [2Fe-2S], [3Fe-4S], and [4Fe-4S] clusters have been synthesized in holoform with their authentic Fe-S clusters in the heterogeneous expression system (*22-30*). The correct assembly of the Fe-S clusters into foreign proteins suggests several possibilities for *in vivo* cluster formation. The Fe-S cluster may be assembled spontaneously from apoprotein, sulfide, and iron by a mechanism similar to that reported in earlier *in vitro* chemical reconstitutions. Alternatively, enzymes may be involved in the assembly process. If the assembly is enzymatic, the enzyme responsible must have broad specificity for protein acceptors or be involved in preassembly of the Fe-S cluster moiety followed by its spontaneous incorporation into apoproteins.

To study the *in vivo* process, we developed an experimental system to monitor the expressed Fd as a reporter of protein-bound Fe-S cluster assembled in *E. coli*. Plasmids were constructed for the overexpression of five reporter Fds carrying several types of Fe-S clusters. When transcription was driven by a T7 promoter, the overexpression led to the accumulation of soluble apo- and holo-Fds, which were clearly distinguishable on nondenaturing PAGE. A quantitative estimation of holoFd was carried out from bacterial extracts on an anion-exchange HPLC system, which allowed specific detection of the holoFd peak based on its absorbance at 400 nm. This system enabled us to examine various effects, such as culture conditions and mutation or overexpression of other genes, on the assembly of the protein-bound Fe-S cluster in *E. coli* cells.

Recently, Zheng and coworkers identified a gene cluster composed of nine ORFs, ORF1-ORF2-*iscS* (*nifS*-like gene)-*iscU* (*nifU*-like gene)-*iscA*-*hscB*-*hscA* (*dnaK*-like gene)-*fdx* ([2Fe-2S] Fd)-ORF3, which is conserved in *Azotobacter vinelandii* (*31*), *E. coli* (*32*), and *Haemophilus influenzae* (*33*). The occurrence of *nif*-like genes in non-nitrogen fixing organisms led them to propose that these might play a role in the formation or repair of Fe-S proteins and to designate them as *isc* (iron-sulfur cluster) genes (*31*). It appears likely that most of the genes are cotranscribed and encode proteins with coupled functions. To investigate this proposed role, we examined the coexpression of the entire gene cluster with the reporter Fds from compatible plasmids. In this report, we demonstrate that the increased dosage of the *isc* gene cluster dramatically promotes the assembly of the Fe-S clusters on reporter Fds overproduced in *E. coli*.

MATERIALS AND METHODS

Materials—*E. coli* C41(DE3) was provided by J.E. Walker. Enzymes for DNA manipulation were obtained from New England Biolabs, Inc. or TaKaRa Shuzo. Bacterial growth media components were from Difco, and other reagents were from Sigma Chemical, Wako Pure Chemical, or Nacalai Tesque.

Construction of Plasmids—Recombinant DNA techniques were carried out according to the established procedures (*34*). The *petF* and *fdxH* genes both encoding [2Fe-2S] Fd (*21, 35*) were prepared by PCR amplification of genomic DNA isolated from a cyanobacterium, *Plectonema boryanum* strain IAM-M101. Primers used for *petF* amplification were 5′-CATATGCCTAGTTTTAAGGTCACA-3′ (forward primer, the underlined bases indicate an *Nde*I site) and 5′-CTC*GAGCTC*AGTAGAGATCTTCTTCTTG-GT-3′ (reverse primer, the underlined bases indicate a *Xho*I site, and italic bases indicate a *Sac*I site), and those for *fdxH* were 5′-CATATGGCTACTTACCAAGTTCG-3′ (forward primer, the underlined bases indicate an *Nde*I site) and 5′-CTC*GAGCTC*AAACTAGGTAAGCTTCTTG-ATG-3′ (reverse primer, the underlined bases indicate a *Xho*I site, and italic bases indicate a *Sac*I site). The *fdxN* gene identified in the genome sequence of *E. coli* (*32*) may code for 2[4Fe-4S] Fd with a sequence similarity to bacterial-type Fds. The *fdxN* gene and the *fdx* gene, which is known to encode [2Fe-2S] Fd (*36*), were prepared by PCR amplification of genomic DNA isolated from *E. coli* strain DH5α and HB101, respectively. Primers used for *fdxN* amplification were 5′-CCATGGCGTTGTTAATCAC-TAA-3′ (forward primer, the underlined bases indicate an *Nco*I site) and 5′-GAATTCTATTAAATTTTATCCGCG-TG-3′ (reverse primer, the underlined bases indicate an *Eco*RI site), and those for *fdx* were 5′-CATATGCCAAAG-ATTGTTATTTTG-3′ (forward primer, the underlined bases indicate an *Nde*I site) and 5′-GAGCTCAATGCTCA-GGCGCATGG-3′ (reverse primer, the underlined bases indicate a *Sac*I site). The *fdxA* gene encoding [3Fe-4S] [4Fe-4S] FdII in *Rhodobacter capsulatus* (*37, 38*) was prepared by PCR amplification of the pKFB1S8 plasmid (*38*) using the primer set of 5′-CATATGACCTATGTCGT-CACCGA-3′ (forward primer, the underlined bases indicate an *Nde*I site) and 5′-GAGCTCAGTCGCCCGTGCC-3′ (reverse primer, the underlined bases indicate a *Sac*I site). PCR was carried out using LA Taq polymerase (TaKaRa) under the conditions recommended by the manufacturer. The PCR products were inserted into the PCR2.1-TOPO vector (Invitrogen) by the TA cloning method, and their correct sequences were confirmed by the dideoxynucleotide chain-termination method using an Applied Biosystems 373A sequencer. The *petF* and *fdxH* genes were cloned into the *Nde*I/*Xho*I sites of the pET-21a(+) vector (Novagen) to make pET21-PbPetF and pET21-PbFdxH plasmids, respectively. The *fdx* and *fdxA* genes were cloned into *Nde*I/*Sac*I sites of pET-21a(+) vector to make pET21-EcFdx and pET21-RcFdII plasmids, respectively. The *fdxN* gene was cloned into the *Nco*I/*Eco*RI sites of the pET-21d(+) vector to make pET21-EcFdxN plasmid. The gene coding for *Bacillus thermoproteolyticus* [4Fe-4S] Fd was synthesized from oligonucleotides (Saeki K., unpublished) based on the published protein sequence (*39*) and cloned into the *Nde*I/*Sac*I sites of the pET-21a(+) vector to make pET21-BtFd plasmid. The *lacZ* gene was excised from the Control B plasmid (Novagen) by *Nco*I and *Hin*dIII digestion and ligated into *Nco*I/*Hin*dIII sites of pET-21d(+) vector to make pET21-lacZ plasmid.

For the coexpression experiments in *E. coli*, we used an IncP1-type vector. The pRK415 vector (*40*) was digested with *Eco*RI and ligated with an oligonucleotide linker,

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5′-AATTACTCGAGTTTAAACGGATCCTTAAT-TAAGCTAGCT-3′. The construct, containing additional *Xho*I, *Pme*I, *Bam*HI, *Pac*I, and *Nde*I cloning sites instead of the *Eco*RI site of pRK415, was designated pRKNMC and checked by sequencing using the dideoxy chain-termination method. To generate a new *Eco*RI restriction site, pRKNMC was digested with *Sac*I, blunt-ended with T4 DNA polymerase, and ligated with a 5′-CCGAATTCGG-3′ linker to make pRKNSE plasmid. The 2.2-kb fragment containing ORF1 and ORF2 was excised from Kohara clone #430 (*41, 42*) by *Bst*1107I and *Eco*RI digestion. The resulting DNA fragment was ligated into the pRKNSE vector, which was digested with *Kpn*I, blunt-ended with T4 DNA polymerase, and cut with *Eco*RI. The resulting plasmid was designated pBsE40. The 5.4-kb *Eco*RI fragment containing the *iscS-iscU-iscA-hscB-hscA-fdx*-ORF3 gene cluster excised from Kohara clone #430 was then ligated into an *Eco*RI site of pBsE40, and correct orientation was checked by digestion with several restriction enzymes. The resulting plasmid was designated pRKISC.

Expression and Purification of Fds—Competent *E. coli* strain C41(DE3) (*43*) was transformed either with pET21-PbPetF, pET21-PbFdxH, pET21-EcFdxN, pET21-BtFd, pET21-RcFdII, pET21-EcFdx, pET21-lacZ, or pET-21a-(+). For the coexpression of the *isc* gene cluster, *E. coli* strain C41(DE3) harboring one of the pET21-derived plasmids was transformed with pRKISC. The pRKNMC plasmid was also introduced into the cells for control experiments. The bacteria were cultivated in 4 ml of Terrific broth or 2×YT medium containing 100 μg/ml ampicillin and 10 μg/ml tetracycline to an absorbance of approximately 0.5 at 600 nm, and expression was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). The cells were then grown aerobically for 18 h at 28°C. Where indicated, the culture media were supplemented with 0.1 mg/ml ferric ammonium citrate or 1 mM cysteine. The cells were pelleted and suspended in a solution containing 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 2 mM EDTA, 0.1% Triton X-100, and 0.1 mg/ml lysozyme at a cell density represented by $A_{600}=30$. After incubation at 25°C for 5 min, the cells were disrupted by sonication. The suspension thus obtained was centrifuged at 15,000×g for 15 min at 4°C, and the resulting supernatant was subjected to the subsequent analysis.

Recombinant Fd proteins were purified from the soluble extracts of cells cultivated in 200 ml of Terrific broth containing 100 μg/ml ampicillin, 10 μg/ml tetracycline, and 0.1 mg/ml ferric ammonium citrate. Purification was carried out essentially as described elsewhere (*20, 37*). Briefly, the bacterial extract was loaded onto a DEAE-cellulose column and washed, and the brown Fd-containing band was eluted with 0.7 M NaCl. The eluate was fractionated with 50% ammonium sulfate, and the supernatant was concentrated with a small DEAE-cellulose column. The preparation was subjected to further chromatography on an anion-exchange column (Shodex IEC QA-824; Showa Denko, Tokyo) and a hydrophobic column (Shodex HIC PH-814; Showa Denko) equipped with an HPLC system (Nanospace SI-1; Shiseido, Tokyo).

Analytical Procedures—Conventional electrophoresis was carried out at 4°C under nondenaturing conditions on 20% polyacrylamide mini slab-gels with the buffer system of Davis (*44*). The gels were fixed in 10% TCA solution to prevent diffusion of polypeptides, stained with Coomassie Blue, and subjected to densitometric analysis using a Molecular Dynamics Computing Densitometer. For the determination of Fds in *E. coli* extracts, the extracts were diluted with 4 volumes of the solution containing 25 mM Tris-HCl, pH 7.5, and 50 mM NaCl, filtered through 0.22 μm Durapore membrane (Ultrafree-MC; Millipore), then applied on an anion-exchange column (Shodex IEC QA-824) equilibrated with 25 mM Tris-HCl, pH 7.5, and 50 mM NaCl. The column was developed at a flow rate of 1 ml/min with a 25-min linear gradient from 50 to 480 mM NaCl using an HPLC system (Nanospace SI-1; Shiseido). The elution of Fd was monitored by recording the absorbance at 400 nm and quantified by peak integration. The calibration curve was obtained using purified Fds as standards. The absorption spectra were recorded on a Shimadzu model UV-3101PC spectrophotometer. Fd concentrations were based on absorbance values in the visible region and calculated on the basis of their Fe-S clusters. These values were confirmed by Lowry protein determination (*45*) using spinach Fd as standard. β-galactosidase activity was assayed by the method of Miller (*46*).

RESULTS

We attempted to examine the effect of the coexpression of *E. coli* ORF1-ORF2-*iscS-iscU-iscA-hscB-hscA-fdx*-ORF3 gene cluster (designated as the *isc* gene cluster in this study) on the production of reporter Fds. The pET21 vector, which contains a T7 promoter and a pBR322-based replicon, was used to express reporter Fds. Genes encoding five Fds carrying several types of Fe-S clusters were prepared for this purpose. They are [2Fe-2S] PetF from the cyanobacterium *P. boryanum* (PbPetF) (*35*), [2Fe-2S] FdxH from *P. boryanum* (PbFdxH) (*21*), 2[4Fe-4S] FdxN from *E. coli* (EcFdxN) (*32*), [4Fe-4S] Fd from *B. thermoproteolyticus* (BtFd) (*39*), and [3Fe-4S][4Fe-4S] FdII from *R. capsulatus* (RcFdII) (*37, 38*). The *isc* gene cluster was inserted into the pRKNMC vector, which contains an IncP1 replicon and tetracycline resistance marker, in the same direction as the *lac* promoter. The generated plasmid, pRKISC, allowed cotransformation with the pET21-derived plasmid to produce various reporter Fds based upon plasmid compatibility.

The cells grown under the conditions coexpressing reporter Fds and the *isc* gene cluster were dark brown, suggesting overproduction and accumulation of reporter holoFds. To examine the correct assembly of the Fe-S clusters, we purified Fd proteins from cells harboring both pRKISC and pET21-derived plasmid containing the coding region of Fds. Isolation of the recombinant Fds yielded 5.2 mg of PbPetF, 5.8 mg of PbFdxH, 3.8 mg of EcFdxN, 14.3 mg of BtFd, and 0.4 mg of RcFdII from cells grown in 200 ml of Terrific broth containing 0.1 mg/ml ferric ammonium citrate. As shown in Fig. 1, purified PbPetF and PbFdxH show absorption spectra characteristic of [2Fe-2S] Fds, with peaks at 277, 333, 423, and 464 nm ($A_{423}/A_{277}=0.60$) and 275, 333, 422, and 468 nm ($A_{422}/A_{275}=0.73$), respectively, which are similar to those reported by Schrautemeier *et al.* (*21*). The absorption spectrum of purified RcFdII is identical to that previously reported (*37*), with a peak at 283 nm and a broad shoulder near 400 nm ($A_{400}/A_{283}=0.56$). The absorption spectrum of BtFd shows peaks

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at 280, 306, and 389 nm ($A_{389}/A_{280}=0.69$) and is similar to that of *B. stearothermophilus* Fd (*47*), which has only two amino acid substitutions compared with BtFd (*39*). EcFdx-N was purified for the first time and shows an absorption spectrum with peaks at 283 and 387 nm and a shoulder near 310 nm ($A_{387}/A_{283}=0.69$), which is typical of 2[4Fe-4S] Fd judging from its tyrosine and tryptophan content. Thus, the reporter Fds coexpressed with the *isc* gene cluster have authentic Fe-S clusters. The *fdx* gene contained in the *isc* gene cluster codes for [2Fe-2S] Fd (EcFdx) (*36*). The coding region for EcFdx was also cloned into the pET-21a(+) vector and expressed in *E. coli* in the absence of the pRKISC plasmid. The absorption spectrum of purified EcFdx is similar to that reported by Ta and Vickery (*36*), exhibiting peaks at 280, 339 (broad), 415, and 459 nm.

To estimate the level of reporter Fds produced in *E. coli* cells, bacterial extracts were analyzed by nondenaturing PAGE, taking advantage of the fact that the Fds are small and highly acidic proteins which run ahead of most *E. coli* proteins. In addition, nondenaturing PAGE allowed us to clearly separate the holoFds from apoFds, the latter migrating much more slowly. Figure 2 shows that in cells in which the expression of reporter Fds was induced with IPTG, recombinant PbPetF, PbFdxH, EcFdxN, BtFd, and RcFdII accumulated in large amounts in both holo- and apo-forms (lanes 2, 5, 8, 12, and 15). Similarly, overproduction of apo- and holo-EcFdx was observed when expressed from the pET21-EcFdx plasmid (lane 18). When EcFdxN was expressed from the pRKISC plasmid, however, the production of EcFdx was limited and could not be clearly detected. Therefore, under the coexpression conditions with other reporter Fds, the production of EcFdx was almost negligible (lanes 2, 5, 8, 12, and 15). We then analyzed the effect of the coexpression of the *isc* gene cluster on the production of reporter Fds. C41(DE3) cells harboring one of the pET21-derived plasmids were transformed with either pRKISC plamid or pRKNMC control vector, and the resulting protein extracts were analyzed by

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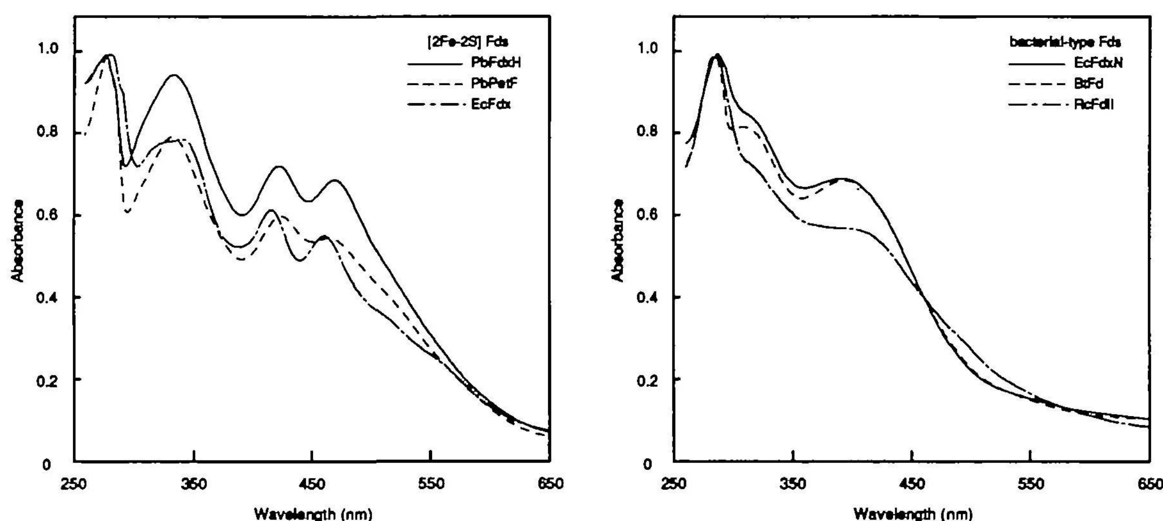

Fig. 1. **Absorption spectra of recombinant Fds purified from *E. coli* C41(DE3).** Samples were dissolved in 50 mM Tris-HCl buffer, pH 7.5, containing 0.5 M NaCl. The spectra were recorded at room temperature using a Shimadzu model UV-3101PC spectrophotometer and normalized to give peak absorbance near 280 nm of 1.0 to facilitate comparisons.

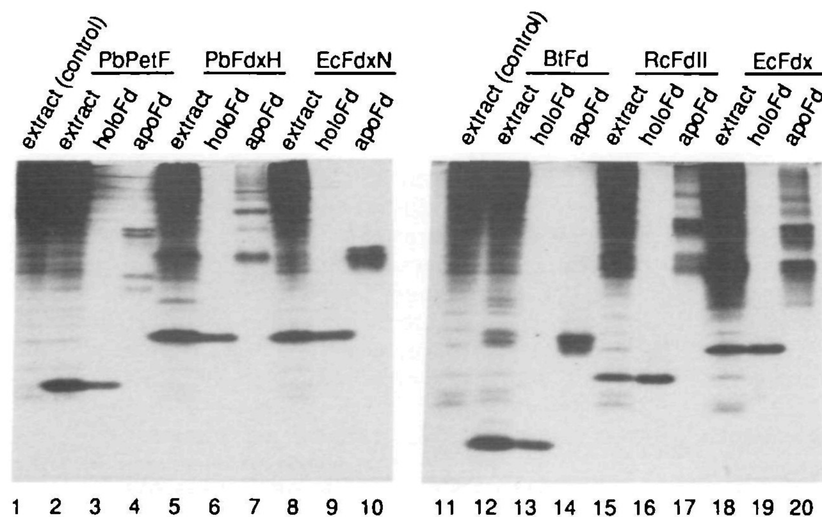

Fig. 2. **Nondenaturing gel electrophoresis of Fds expressed in *E. coli*.** Electrophoresis was performed in 20% nondenaturing polyacrylamide gels. Bacterial extracts were normalized with respect to the cell density of each culture. After purification, 1 μg of holoFds and 3 μg of heat-denatured apoFds were analyzed. Control extract was prepared from C41(DE3)(pET-21a).

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Culture medium	2 x YT												Terrific broth											
Reporter Fd	-	-	PbPetF		PbFdxH		EcFdxN		BtFd		RcFdII		-	-	PbPetF		PbFdxH		EcFdxN		BtFd		RcFdII	
Coexpression of the *isc* gene cluster	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+

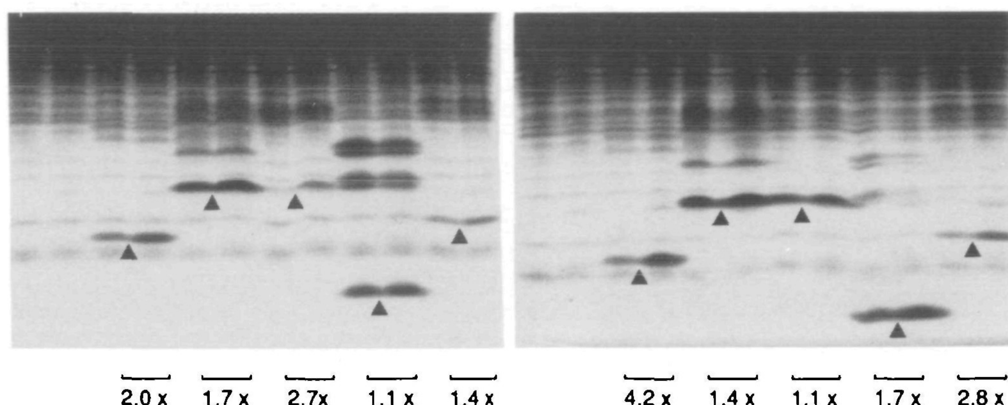

Fig. 3. **Electrophoretic analysis of the effect of coexpression of the *isc* gene cluster on the production of reporter Fds.** *E. coli* C41(DE3) strains bearing pET21-derived plasmid for the expression of reporter Fd and pRKISC plasmid for the coexpression of the *isc* gene cluster were grown in 2×YT medium or Terrific broth, and expression was induced with 1 mM IPTG. The cells harboring pET-21a(+) or pRKNMC vector were used in the control experiments. Bacterial extracts were normalized with respect to their cell density (A_{600}=30), and aliquots (10 μl) were subjected to the nondenaturing PAGE. Positions of the holoFds are shown by filled triangles. After staining with Coomassie blue, holoFds were determined by densitometric analysis, and their increase under the coexpression of the *isc* gene cluster is shown at the bottom of the figure.

nondenaturing PAGE. As shown in Fig. 3, the production of holo-PbPetF (lanes 3, 4, 15, and 16), -PbFdxH (lanes 5, 6, 17, and 18) and -RcFdII (lanes 11, 12, 23, and 24) was significantly increased by the coexpression of the *isc* gene cluster, which could be clearly observed irrespective of the culture medium. Increased production of holo-EcFdxN (lanes 7 and 8) and -BtFd (lanes 21 and 22) by the co-expression of the *isc* gene cluster was obvious when cells were cultivated in 2×YT medium and Terrific broth, respectively. Therefore, the coexpression promotes the production of all five reporter holoFds examined. In contrast, the production of apo-BtFd was clearly decreased by the coexpression of the *isc* gene cluster (lanes 21 and 22), while the contents of apo-PbPetF, -PbFdxH, -EcFdxN, and -RcFdII were not significantly changed under the coexpression conditions. Thus, the overproduction of the reporter holoFds was not correlated with the production of apoFds.

HoloFds expressed in *E. coli* could not be quantified precisely by nondenaturing PAGE because endogenous *E. coli* proteins comigrated with the holoFds. Therefore, reporter holoFds were quantified using an HPLC system based on their absorption at 400 nm. When bacterial lysates were chromatographed on an anion-exchange column, recombinant reporter holoFds were eluted at the same retention time as purified holoFds and background absorbance was negligible (Fig. 4). Thus, Fds were specifically quantified by the integration of the absorption peak. In the experiments, *E. coli* cells were grown in Terrific broth supplemented with 0.1 mg/ml ferric ammonium citrate and 1 mM IPTG, and the amount of holoFds was normalized with respect to the bacterial density of each culture. As shown in Fig. 5 (left), the strains coexpressing the *isc* gene cluster in the presence of pRKISC plasmid gave a higher yield of reporter holoFds. The expression of holo-PbPetF was increased 7.9-fold by the coexpression of the *isc* gene cluster. Similarly, holoproteins of PbFdxH, EcFdxN, BtFd, and RcFdII were increased 2.6-, 2.1-, 1.3-, and 3.1-fold, respectively. The content of the reporter holoFd is estimated to be 24.6±4.8 mg/liter of culture of C41(DE3)(pET21-PbPetF)(pRKNMC), 185±31 mg/liter of C41(DE3)(pET21-PbPetF)(pRKISC), 78.8±9.4 mg/liter of C41(DE3)(pET21-PbFdxH)(pRKNMC), 204±43 mg/liter of C41(DE3)(pET21-PbFdxH)(pRKISC), 48.4±18.9 mg/liter of C41(DE3)(pET21-EcFdxN)(pRKNMC), 107±17 mg/liter of C41(DE3)(pET21-EcFdxN)(pRKISC), 114±7.3 mg/liter of C41(DE3)(pET21-BtFd)(pRKNMC), 162±42 mg/liter of C41(DE3)(pET21-BtFd)(pRKISC), 4.7±0.4 mg/liter of C41(DE3)(pET21-RcFdII)(pRKNMC), and 16.1±2.6 mg/liter of C41(DE3)(pET21-RcFdII)(pRKISC). These results indicate that the amount of reporter holoFds expressed and accumulated in *E. coli* cells is dependent on the coexpression of the *isc* gene cluster. Stimulation was observed irrespective of reporter Fd used, and specificity to the polypeptide or the type of Fe-S clusters was not detected. Figure 5 (right) shows control experiments using β-galactosidase as a reporter. The host strain C41(DE3) has the intrinsic *lacZ* gene, and the activity of β-galactosidase was increased about 25-fold when the expression was directed from the T7 promoter of the pET21-lacZ plasmid. The coexpression of the *isc* gene cluster did not promote the production of active β-galactosidase, showing rather an inhibitory effect. Therefore, the overexpression of reporter holoFds might be attributed to the elevated activity for the assembly of Fe-S clusters under the coexpression of the *isc* gene cluster.

During the course of this study, we noticed that the production of holoFds in *E. coli* varied depending on culture medium, 2×YT or Terrific broth, and the presence or

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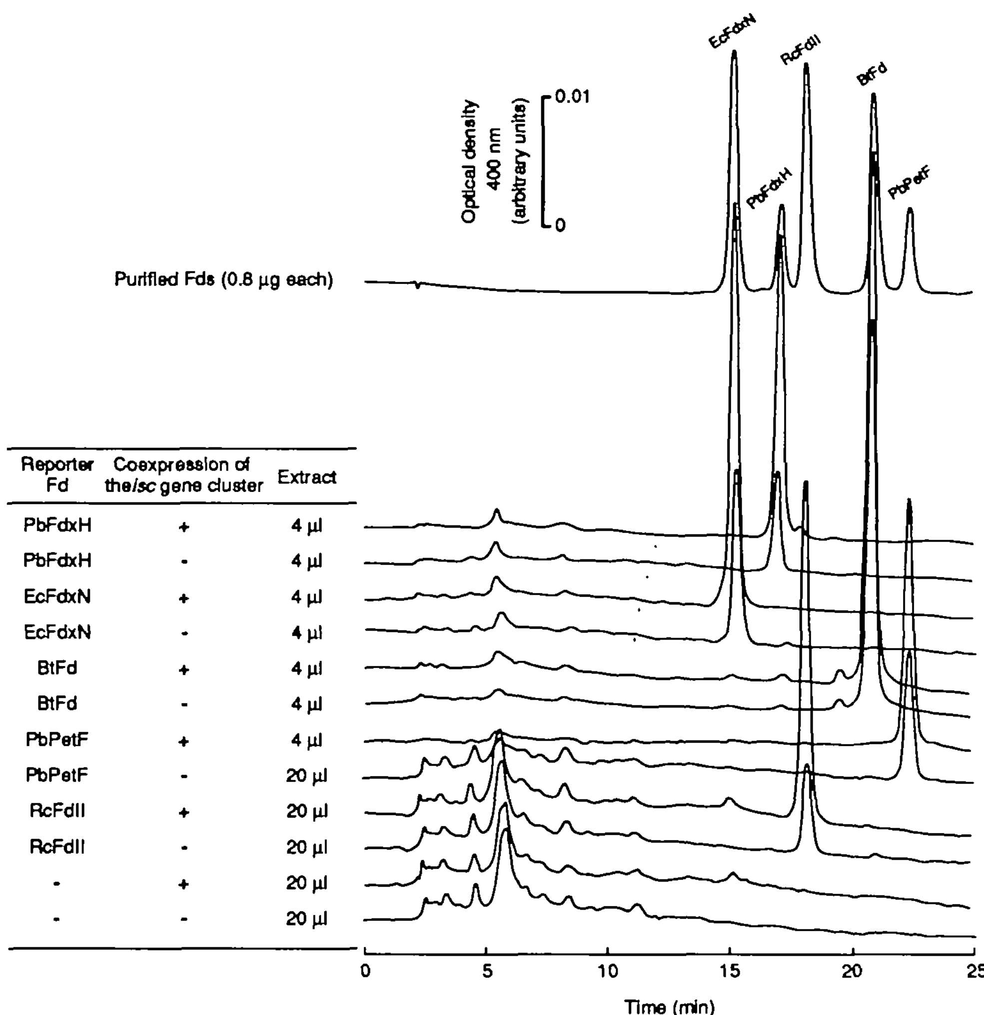

Fig. 4. **Comparative anion-exchange HPLC of protein extracts from *E. coli* cells expressing reporter Fds.** *E. coli* C41(DE3) strains harboring several plasmids were grown in Terrific broth supplemented with 0.1 mg/ml ferric ammonium citrate, and expression was induced with 1 mM IPTG. Bacterial extracts were normalized with respect to their cell density ($A_{600}=30$), and the volume indicated was subjected to Shodex QA-824 ion-exchange column (8×75 mm). The column was developed at a flow rate of 1 ml/min with a 25-min linear gradient from 50 to 480 mM NaCl, and elution of Fd was monitored by recording the absorbance at 400 nm. Elution profiles of the purified Fds are shown at the top.

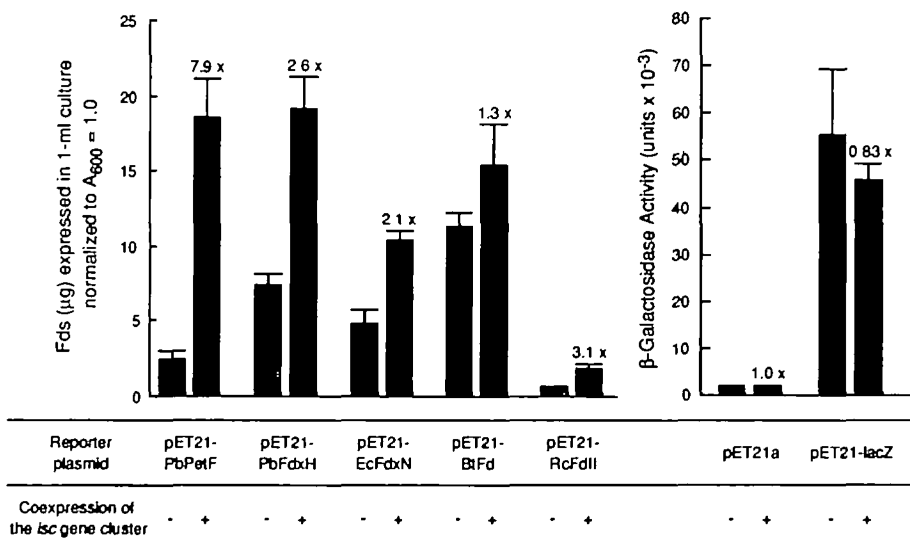

Fig. 5. **Effect of coexpression of the *isc* gene cluster on the production of reporter holoFds.** Fds were quantified by integration of the peak absorbance at 400 nm shown in Fig. 4 and normalized with respect to the bacterial density of each culture. Measurements of β-galactosidase activity were obtained in crude extracts prepared from the indicated strains, and units of the activity were normalized with respect to the bacterial density (*46*). Experiments were carried out using at least three independent transformants, and values are the mean±SD of triplicate measurements. Increase in holoFd or β-galactosidase production by the coexpression of the *isc* gene cluster is shown in the figure.

absence of supplemental iron. Hence, the effect of the coexpression of the *isc* gene cluster was examined using various culture media shown in Fig. 6. The supplement of cysteine was also examined, because the sulfur source for the Fe-S clusters has been identified as cysteine (*48*). As shown in Fig. 6, Terrific broth was superior to the 2×YT

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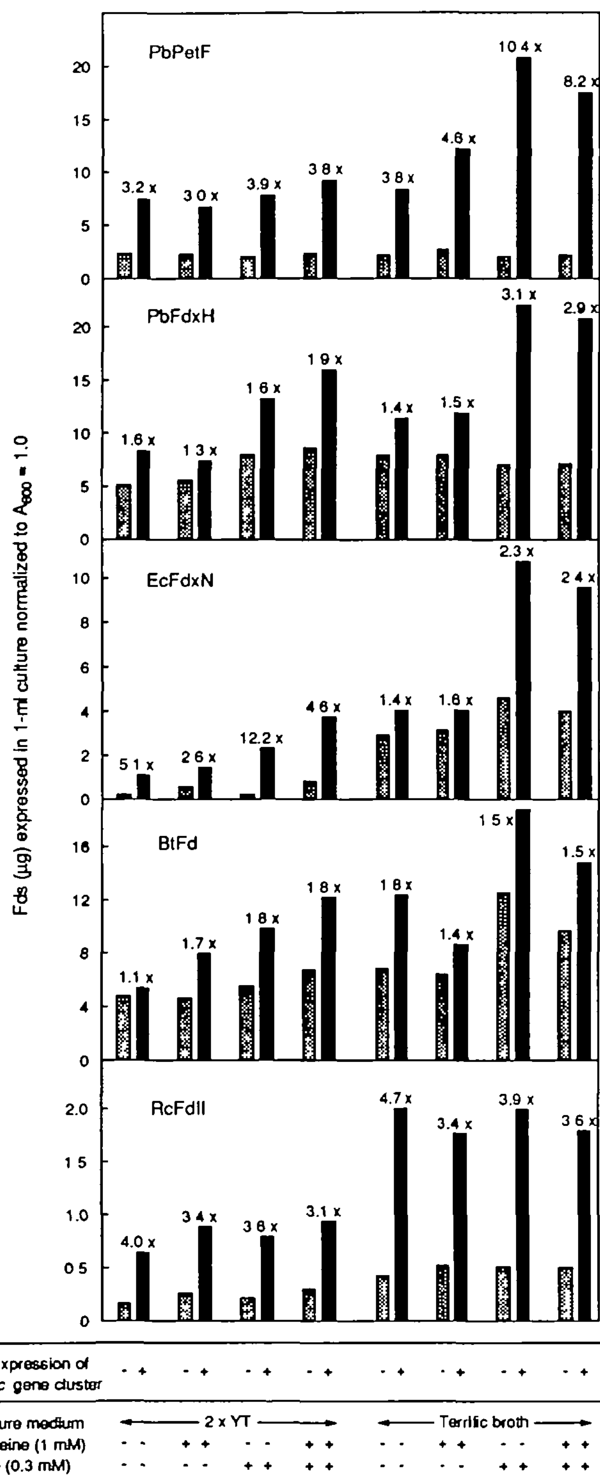

Fig. 6. **Effect of the coexpression of the *isc* gene cluster on the production of holoFds under various growth conditions.** *E. coli* C41(DE3) strains harboring several plasmids were grown in 2×YT medium or Terrific broth in the presence or absence of supplemental cysteine (1 mM) and/or ferric ammonium citrate (0.1 mg/ml, about 0.3 mM), and expression was induced with 1 mM IPTG. Fds were determined as described in the legends of Figs. 4 and 5 and normalized with respect to the bacterial density of each culture. Increase in holoFd production under the coexpression of the *isc* gene cluster is shown.

medium for the overproduction of the five reporter holoFds examined. Iron supplement in the culture media increased the yield of holo-PbPetF, -PbFdxH, -EcFdxN, and -BtFd, but not that of holo-RcFdII. Addition of cysteine to the culture media was less effective on the overproduction of holoFds. In all culture media examined, increased production of reporter holoFds was observed under the coexpression of the *isc* gene cluster. The results indicate that proteins encoded by the *isc* gene cluster are not involved in the transport of iron or cysteine into the cells; rather, it seems plausible that the gene products have functions directly related to the assembly of the Fe-S clusters.

DISCUSSION

We have demonstrated that the coexpression of the *isc* gene cluster dramatically increased the production of all five reporter holoFds examined in *E. coli*. In general, such factors as transcriptional and translational efficiency, stability of the mRNA transcript, protein stability and degradation by endogenous protease, and formation of inclusion bodies are often considered as possible determinants of the yield of proteins expressed in *E. coli*. However, the situation is somewhat different in the case of Fds used in this study, as a high level of expression was achieved using the T7 polymerase-based system, and the recombinant Fds were soluble and fairly stable in the *E. coli* C41(DE3) host cells. The fact that reporter Fds were expressed and accumulated in both apo- and holo-forms (Figs. 2 and 3) strongly supports the idea that assembly of the Fe-S clusters into the apoFd polypeptides is a rate-limiting step. The overwhelming efficiency of the T7 polymerase-dependent machinery probably exceeds the capacity of the endogenous system responsible for the synthesis and insertion of the Fe-S clusters. Increased dosage of the *isc* gene cluster should accelerate the assembly of the Fe-S clusters and thus increase the content of holoFds. This function of the *isc* gene cluster presumably mimics its role in host cells. The apparent lack of specificity to the polypeptides of reporter Fds or to the type of Fe-S clusters (Fig. 5) indicates that the gene products are involved in the assembly of Fe-S clusters in a wide variety of Fe-S proteins in *E. coli*.

A survey of reports on the heterologous expression of Fds in *E. coli* indicates that [2Fe-2S] Fds from cyanobacteria, chloroplasts, mitochondria, and bacteria are produced in higher yield than 2[4Fe-4S] Fds or [3Fe-4S][4Fe-4S] Fds (*19, 22-24, 27, 28, 49*). Although the difference may primarily depend on the expression system employed, it is worth noting that RcFdII failed to be overproduced irrespective of the expression system (*49*). In our experiments, the production of holo-RcFdII was increased more than 3-fold by the coexpression of the *isc* gene cluster, but it was still less than 20% of that of the other reporter Fds (Fig. 5). One possible reason for its low-level production is that the assembly and insertion of the [3Fe-4S] cluster is a critical rate-limiting factor for the synthesis of [3Fe-4S][4Fe-4S] Fd. This seems unlikely, however, since [3Fe-4S] Fd from *Desulfovibrio gigas* has been produced in *E. coli* in a large amount (*28*). Another possibility is that the long carboxy-terminal extension of RcFdII compared to the common bacterial-type Fds might disturb the formation of the Fe-S cluster or prevent correct folding. Consistently, it has been

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suggested that the nascent polypeptide of RcFdII might fold incorrectly and form a secondary structure which is likely to hinder the assembly of the Fe-S cluster (*49*).

In addition to the dosage of the *isc* gene cluster, the iron contained in the culture medium is another limiting factor for the overproduction of holo-PbPetF, -PbFdxH, -EcFdxN, and -BtFd (Fig. 6). Iron limitation of the assembly of the Fe-S cluster has also been demonstrated in the overproduction of the NQO2 subunit of *Paracoccus* NDH-1 in *E. coli* (*30*). In the case of holo-RcFdII production, stimulation by added iron was not observed, probably because the iron content was sufficient for the low-level production of holo-RcFdII. Similarly, the fact that the overproduction of holoFds was not increased by the supplemental cysteine indicates that the culture medium contained sufficient sulfur compounds for the overproduction of Fe-S clusters. Comparison of Terrific broth and 2×YT medium (Fig. 6) suggests that the former contains the unknown limiting factor in greater abundance than the latter. Alternatively, the high level of holoFds expressed in Terrific broth might be related to the metabolic regulation. From this point of view, glycerol contained in Terrific broth might serve as a good substrate for respiratory growth, which induces a number of Fe-S proteins involved in the respiratory pathway. Consistent with this idea, preliminary experiments indicate that the production of reporter holoFds was suppressed under the fermentative growth conditions in the presence of available sugars. Whatever the case, these findings will be useful for producing large amounts of Fe-S proteins in *E. coli* for structural and molecular biological studies.

The *isc* gene cluster contains nine ORFs, ORF1-ORF2-*iscS*-*iscU*-*iscA*-*hscB*-*hscA*-*fdx*-ORF3, whose physiological functions have not been elucidated except for that of *iscS*. The IscS protein is a homologue of NifS, and pyridoxal phosphate-dependent cysteine desulfurase activity has been demonstrated for both proteins (*13, 15*). Thus, IscS is the source of the sulfide used for the Fe-S cluster assembly. Recently, Chen and coworkers examined the effect of coexpression of *nifS* from *A. vinelandii* on the production of glutamine phosphoribosylpyrophosphate amide-transferase from *Bacillus subtilis* in *E. coli* and showed that the coexpression elevates the maturation of the *Bacillus* enzyme by several fold (*50*). Because the amide-transferase requires two maturation steps, assembly of a [4Fe-4S] cluster and cleavage of an 11-residue amino-terminal propeptide, the increased activity of cysteine desulfurase might accelerate the synthesis and insertion of the [4Fe-4S] cluster into the enzyme. In contrast, our preliminary experiments indicate that the full overproduction of reporter Fds was not achieved by coexpression of the *iscS* gene alone and requires several genes located in the *isc* gene cluster. Consistent with our observations, recent studies on the suppressor mutants of superoxide dismutase (*SOD1*) deficiency in *Saccharomyces cerevisiae* have revealed that mutations in yeast homologues of IscS (NFS1), HscA (SSQ1), and HscB (JAC1) resulted in severe reduction of mitochondrial Fe-S proteins such as aconitase and succinate dehydrogenase (*51*). Thus, the question remains of how many components are required for the assembly of the Fe-S clusters. Investigations into this question are currently in progress.

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