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SufU Is an Essential Iron-Sulfur Cluster Scaffold Protein in *Bacillus subtilis*▽

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Bacteria use three distinct systems for iron-sulfur (Fe/S) cluster biogenesis: the ISC, SUF, and NIF machineries. The ISC and SUF systems are widely distributed, and many bacteria possess both of them. In *Escherichia coli*, ISC is the major and constitutive system, whereas SUF is induced under iron starvation and/or oxidative stress. Genomic analysis of the Fe/S cluster biosynthesis genes in *Bacillus subtilis* suggests that this bacterium's genome encodes only a SUF system consisting of a *sufCDSUB* gene cluster and a distant *sufA* gene. Mutant analysis of the putative Fe/S scaffold genes *sufU* and *sufA* revealed that *sufU* is essential for growth under minimal standard conditions, but not *sufA*. The drastic growth retardation of a conditional mutant depleted of SufU was coupled with a severe reduction of aconitase and succinate dehydrogenase activities in total-cell lysates, suggesting a crucial function of SufU in Fe/S protein biogenesis. Recombinant SufU was devoid of Fe/S clusters after aerobic purification. Upon *in vitro* reconstitution, SufU bound an Fe/S cluster with up to ~1.5 Fe and S per monomer. The assembled Fe/S cluster could be transferred from SufU to the *apo* form of isopropylmalate isomerase Leu1, rapidly forming catalytically active [4Fe-4S]-containing *holo*-enzyme. In contrast to native SufU, its D43A variant carried a Fe/S cluster after aerobic purification, indicating that the cluster is stabilized by this mutation. Further, we show that *apo*-SufU is an activator of the cysteine desulfurase SufS by enhancing its activity about 40-fold *in vitro*. SufS-dependent formation of *holo*-SufU suggests that SufU functions as an Fe/S cluster scaffold protein tightly cooperating with the SufS cysteine desulfurase.

Iron-sulfur (Fe/S) clusters are one of the most ubiquitous and versatile cofactors employed by nature for catalyzing a variety of redox reactions or for serving as redox sensors in a broad range of regulatory processes (10). Iron and sulfide are toxic for the cells in concentrations needed for spontaneous chemical Fe/S protein maturation. Hence, cells have developed complex biosynthesis machineries which are essential *in vivo* to assemble Fe/S proteins. Three phylogenetically distinct biosynthesis systems have been found in bacteria: ISC (*i*ron-*s*ulfur *c*luster), SUF (*suf*ur mobilization), and NIF (*ni*trogen *f*ixation) (9, 11, 24). The ISC machinery is the most widely distributed bacterial Fe/S cluster biogenesis system and is also present in eukaryotes (31). In *Escherichia coli* a second system for Fe/S cluster assembly, SUF, is induced under conditions of iron limitation and/or oxidative stress, thus replacing the housekeeping ISC system for assembly of Fe/S proteins. In contrast, SUF was found as the exclusive Fe/S biogenesis system in mycobacteria (22) and *Enterococcus faecalis* (39) and hence may also serve as a constitutive system. Furthermore, SUF is present in plastids of green plants, resembling the situation found in their cyanobacterial ancestors (25, 53). The NIF system is responsible for the dedicated maturation of the complex Fe/S protein nitrogenase involved in nitrogen fixation, e.g., in *Azotobacter vinelandii*. Some NIF genes are associated with anaerobic or microaerobic growth in *Helicobacter pylori* and *Entamoeba histolytica* (24).

Common principles for Fe/S protein assembly in each system have been defined (30). The *de novo* assembly of an Fe/S cluster occurs on scaffold proteins which transiently bind the Fe/S cluster before transfer to target apoproteins. Cysteine desulfurases such as IscS and SufS serve as sulfur donors, which acquire sulfur from free L-cysteine by pyridoxal-5′-phosphate-dependent desulfuration. The sulfur is transiently bound in the form of a persulfide to an active-site cysteine of the desulfurase and is subsequently transferred to the scaffold protein. Several SUF systems contain SufE, which specifically forms a complex with the cysteine desulfurase SufS (36, 42). SufE enhances SufS activity significantly and assists the sulfur transfer to scaffold proteins. In this case the persulfide is transiently bound to SufE and not to the desulfurase. Recent studies show that the *E. coli* cysteine desulfurase CsdA is able to complement the SUF system and interacts with SufE if SufS is inactivated (50). A general iron donor involved in Fe/S cluster assembly is not known yet; however, frataxin homologs in prokaryotes and eukaryotes are postulated to deliver iron to the scaffold protein IscU in the ISC system (5, 9, 31, 34).

Several components have been suggested to act as scaffold proteins. U-type scaffold proteins such as bacterial NifU, IscU, and eukaryotic Isu1 preferentially bind [2Fe-2S] clusters. However, the assembly of [4Fe-4S] clusters was described to proceed by reductive coupling of two [2Fe-2S] clusters that bind

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype/description	Reference or source
Strains		
Bacillus subtilis		
168	*trpC2 sfp*^{0}	2
AA02	168 *amyE*::pX*sufU*	This study
AA04	168 Δ*sufU*::*kan amyE*::pX*sufU*	This study
AA01	168 Δ*sufA*::*kan*	This study
Escherichia coli		
TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 deoR nupG recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL*(Str^r) *endA1*	Invitrogen
BL21(DE3)	F$^-$ *ompT gal dcm lon hsdS*$_B$(r_B^- m_B^-) (DE3)	Novagen
Plasmids		
pX	*amyE* site integration vector; antibiotic resistance cassette: Cm^r	26
pET28a(+)	Expression vector	Novagen
pTrcHis-TOPO	Expression vector, containing topoisomerase for TOPO cloning	Invitrogen
pET28a_sufU	pET28a(+) containing an N-terminal His$_6$ tag fusion of SufU	This study
pET28a_sufU_d43a	pET28a(+) containing an N-terminal His$_6$ tag fusion of SufU with aspartate residue 43 changed to alanine	This study
pTrcHis_sufS	pTrcHis-TOPO containing N-terminal His$_6$ tag fusion of SufS	This study

successively to an IscU dimer (1). A-type scaffolds like bacterial SufA or IscA can bind [2Fe-2S] clusters in their monomeric state and were found to be involved in the maturation of [4Fe-4S] proteins such as aconitase (17, 32, 47). Overproduced IscA was also shown to bind mononuclear iron which could be used for Fe/S cluster assembly on IscU *in vitro*.

The ISC system characteristically contains the molecular chaperone pair HscA and HscB that are involved in Fe/S cluster transfer from the IscU scaffold protein to the target proteins. The Hsp70-type HscA specifically binds to a highly conserved LPPVK motif located near the third strictly conserved cluster-binding cysteine in IscU. Specific IscU-HscA complex formation was found to be necessary and sufficient to stimulate the ATPase activity of HscA (12, 21, 48). The SUF system, in contrast, does not contain HscA and HscB chaperones in the *suf* gene cluster. Instead it comprises the SufBCD proteins. The SufC protein has intrinsic ATPase activity and forms a complex with SufB, a putative scaffold protein, and SufD (29). The precise molecular function of the ATP-hydrolyzing SufBCD complex is not yet clear.

The best-characterized SUF system from *E. coli* contains the gene cluster *sufABCDSE* (5). However, many bacteria, in particular members of the phylum *Firmicutes*, contain a different *suf* gene cluster encoding *sufCDSUB*, which has been studied so far only by bioinformatic approaches (39, 46). While SufS and SufBCD in the two gene clusters appear to be similar proteins, SufE is lacking in most *Firmicutes* and also in the mycobacterial and *Thermotoga maritima* SUF machineries (22, 24). The additionally present SufU shares similarities with IscU of the ISC system (39). The protein contains all three conserved cysteine residues involved in Fe/S cluster association and yet characteristically lacks the LPPVK motif, consistent with the absence of HscA and HscB proteins in *sufCDSUB* species.

In this study, we made use of the Gram-positive bacterium *Bacillus subtilis* to initiate functional analysis of the *sufCDSUB* genes in Fe/S cluster biosynthesis by genetic and biochemical approaches. In particular, since no functional information is available for SufU, we tested its putative role as an Fe/S scaffold protein. SufU was found to be crucial for cell viability and for Fe/S-dependent enzyme activities in crude cell lysates. *In vitro* cluster reconstitution with recombinant SufU indicated that SufU binds a labile Fe/S cluster which can be transferred to *apo*-Leu1 in a fast and efficient way, fully activating its catalytic function as a [4Fe-4S] cluster-dependent isopropylmalate isomerase. The *B. subtilis* SufU was found to activate the desulfurase activity of purified *B. subtilis* SufS. Our results suggest that SufS and the SufU scaffold protein closely act together in the Fe/S cluster biogenesis in *B. subtilis.*

MATERIALS AND METHODS

Strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Antibiotics for the selection of *E. coli* strains were used at the indicated concentrations: kanamycin, 50 μg/ml; ampicillin, 100 μg/ml. For the selection of *B. subtilis* strains, antibiotics were used at the indicated concentrations: kanamycin, 10 μg/ml; chloramphenicol, 5 μg/ml; tetracycline, 20 μg/ml. Single colonies of *B. subtilis* strains were grown on LB or DSM (Difco sporulation medium) agar medium. For liquid culture experiments, strains were grown in Belitsky minimal medium without citrate (BOC-MM) using glucose or fructose as carbon source (45). Due to the tryptophan (Trp) auxotrophy of *B. subtilis* 168, 0.005% (wt/vol) Trp was added to the minimal medium. For growth of the conditional mutants with integrated pX vector, 0.5% or 1% (wt/vol) xylose was added to the transformation and overnight cultures. *E. coli* strains were grown on LB agar and in LB medium. DNA manipulations and transformations were carried out as described previously (20, 28, 41).

Mutant construction. For construction of *B. subtilis* knockout mutants, the PCR synthesis method of marker cassettes with long flanking homologous regions was used (51). For generation of *B. subtilis* conditional mutants, the pX vector (26) was used for target insertion into the *amyE* site. The genes *sufU* and *sufB* (formerly *yurV* and *yurU*) were amplified using Phusion HF DNA polymerase (New England Biolabs), introducing a 5′ SpeI and a 3′ BamHI restriction site into the PCR product. The products were digested with BamHI and SpeI, ligated into the pX vector, and transformed into *E. coli* TOP10 cells. The vector with insert was transformed into *B. subtilis* 168 without digestion. Correct insertion of the constructs into the *amyE* site and deletion of the original loci were verified by PCR. The list of primers is available by request.

Cloning, heterologous expression, and purification of SufU and SufS. The genes *sufU* (*yurV*) and *sufS* (*csd*) were amplified via PCR from the chromosomal DNA of *B. subtilis* 168. The *sufU* gene was amplified with Phusion HF DNA polymerase (New England Biolabs), introducing a 5′ BamHI and a 3′ XhoI restriction site into the PCR product. The product was digested with BamHI and XhoI, ligated into the pET28a(+) vector, and transformed into *E. coli* TOP10 cells, to obtain a *sufU* construct with an N-terminal His$_6$ tag.

The *sufS* gene was amplified without introduction of restriction sites with Phusion HF polymerase. After purification the PCR product was treated for 15 min with *Taq* polymerase and dATP at 72°C to introduce A overhangs. The product was then cloned into the pTrcHis-TOPO vector (Invitrogen) following the manufacturer's instructions and transformed into *E. coli* TOP10 cells to obtain a *sufS* construct with an N-terminal His_6 tag. The correct sequences of all plasmid inserts were confirmed by DNA sequencing (GATC Biotech).

For expression the constructs were transformed into *E. coli* BL21(DE3) cells. Recombinant proteins were overproduced by inducing the cultures at an optical density at 600 nm (OD_{600}) of 0.5 to 0.7 with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h at 37°C. After harvesting, the cells were resuspended in buffer A (50 mM HEPES, 300 mM NaCl, pH 8.0) and disrupted using a French press (Sim Aminco) at 10,000 lb/in^2. After the cell debris was removed (centrifugation at 34,000 × *g* at 4°C), the supernatant was loaded on a nickel-nitrilotriacetic acid (Ni-NTA) column (Qiagen). Elution was performed with a linear gradient (0 to 100%) of buffer B (50 mM HEPES, 300 mM NaCl, 250 mM imidazole, pH 8.0), at a flow rate of 1 ml per min over 50 min using a fast-performance liquid chromatography (FPLC) system (Amersham Pharmacia Biotech) or an Äkta Prime or Äkta purifier system (GE Healthcare). The sample fractions were analyzed by SDS-PAGE, and the fractions with the highest protein yields were pooled. The pooled fractions were concentrated and dialyzed using Amicon Ultra-15 centrifugal filter units (Millipore) with a 10-kDa (SufU) or 30-kDa (SufS) molecular mass cutoff. The protein concentrations were determined using Bradford solution with bovine serum albumin (BSA; Roth) as an internal standard.

The yeast Leu1 protein was overproduced and purified as described previously (35).

Site-directed mutagenesis of SufU. The introduction of an aspartate (D)-to-alanine (A) mutation in position 43 of *B. subtilis* SufU was performed using a modified QuikChange protocol (55). In addition to the desired mutation, a silent mutation introducing a HindIII restriction site was introduced for mutant selection. The modified plasmid was transformed into *E. coli* TOP10, and the correct mutation was confirmed by DNA sequencing (GATC Biotech). Recombinant $SufU_{D43A}$ was expressed in *E. coli* BL21(DE3) cells. Cells were grown at 37°C to an OD_{600} of 0.5 to 0.7, after which the temperature was shifted to 18°C and the expression was induced with 0.1 mM IPTG overnight. The protein was purified as described above.

Aconitase activity assay. Cells were grown in minimal medium to an OD_{600} of 0.4. Fifty milliliters of the culture was pelleted, resuspended in 1 ml buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl), and disrupted by sonication. Cell debris was removed by centrifugation (16,000 × *g*, 4°C), and the clear lysate was used for activity measurement. Aconitase activity was tested using a coupled assay with isocitrate dehydrogenase (from porcine heart; Sigma-Aldrich). The reaction was performed four times independently at microscale using a Tecan Infinite M200 microplate reader. To a solution of *cis*-aconitate (0.2 mM), isocitrate dehydrogenase (0.4 U/ml), and NADP (1 mM) in buffer (100 mM triethanolamine, pH 8.0, 1.5 mM $MgCl_2$, 0.1% [vol/vol] Triton X-100), ~10 μg of total crude extract protein was added to a total reaction volume of 100 μl. The activity was measured by the absorption of converted NADPH at 340 nm (13, 19, 40).

Malate dehydrogenase activity assay. Cells were grown in minimal medium to an OD_{600} of 0.4. Fifty milliliters of the culture was pelleted, resuspended in 1 ml buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl), and disrupted by sonication. Cell debris was removed by centrifugation (16,000 × *g*, 4°C), and the clear lysate was used for activity determination. The activity of malate dehydrogenase was measured by following the consumption of NADH spectroscopically at 340 nm. The reaction was performed four times independently at microscale using a Tecan Infinite M200 microplate reader. The total reaction volume was 100 μl. To 0.1 mg/ml NADH and 0.05 mg/ml oxaloacetate in buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl), ~10 μg of total crude extract protein was added, and the decrease in absorption at 340 nm was measured (27).

Succinate dehydrogenase activity assay. Cells were grown in minimal medium to an OD_{600} of 0.4. Fifty milliliters of the culture was pelleted, resuspended in 1 ml buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl), and disrupted by sonication. The whole-cell crude extract containing the membrane fraction was used. To measure the activity of succinate dehydrogenase, the artificial electron acceptor dichlorophenol-indophenol (DCPIP) was used. The reaction was performed four times independently at microscale using a Tecan Infinite M200 microplate reader. To a solution of 0.25% (wt/vol) succinate in buffer (50 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 1 mM KCN, 70 μM DCPIP, 0.1% [vol/vol] Triton X-100), ~10 μg of total crude extract protein was added to a total reaction volume of 100 μl, and the absorption change at 600 nm was measured (18). The specificity of the reaction was confirmed by adding malonate to a final concentration of 0.25% (wt/vol) as inhibitor.

Cysteine desulfurase assay. Cysteine desulfurase activity of SufS was measured by the amount of released sulfide from cysteine. Sulfide detection was performed with *N,N*-dimethyl-*p*-phenylenediamine sulfate (DMPD) and $FeCl_3$ as previously described (44). An 0.5 μM concentration of SufS was preincubated without or with 10 μM SufU in Csd buffer (100 mM Tris-HCl, pH 8.0, 30 mM KCl, 0.5 mM dithiothreitol [DTT]) for 5 min. The reaction was started by the addition of L-cysteine to a final concentration of 2.5 mM in a total reaction volume of 800 μl. The reaction was stopped after 10 min by the addition of 100 μl DMPD (20 mM in 7.2 M HCl) and 100 μl $FeCl_3$ (30 mM in 1.2 M HCl). The mixture was incubated for 30 min in the dark, and the absorption was measured at 650 nm.

Anaerobic work. Anaerobic work was performed in an anaerobic chamber (Coy Laboratories) with a forming gas (95% N_2/5% H_2) atmosphere. Solutions were made anoxic by purging them with forming gas overnight. Solutions and plastic ware were allowed to equilibrate >6 h inside the chamber before usage. DTT (final concentration range from 0.5 to 5 mM) was added in all buffered solutions.

Iron-sulfur cluster reconstitution. SufU (50 μM, 1 mg/ml) was reduced anaerobically with 5 mM DTT in PD-10 buffer (25 mM Tris-HCl, 100 mM NaCl, pH 8.0) for 1 h, prior to the addition of Fe^{3+} or S^{2-}. After this pretreatment, $FeCl_3$ or ferric ammonium citrate was added in a stoichiometry of 5:1, 4:1, 2:1, or 1:1 (iron to protein) and incubated for approximately 10 min until the observed color change to red was stable. An equal amount of Li_2S was added slowly, and the sample was incubated for 15 min before unbound iron and sulfide were removed via chromatography over a PD-10 column equilibrated in PD-10 buffer with 0.5 mM DTT.

The UV/visible (UV/Vis) spectra were recorded on a Jasco V-550 spectrometer, and the determination of bound iron and acid-labile sulfide was carried out as described elsewhere (7).

Reconstitution of the Fe/S cluster on SufU with SufS, iron, and cysteine was carried out by preincubating 50 μM SufU with 0.5 μM SufS in reconstitution buffer with 5 mM DTT for 1 h. Ferric ammonium citrate was added to a final concentration of 250 μM and incubated until the color change was stabilized. Then, L-cysteine was added to a final concentration of 5 mM and the mixture was incubated for 30 min. Cluster formation was detected spectrophotometrically.

Leu1 activation assay. The *apo* form of isopropylmalate isomerase (*apo*-Leu1) (25 μM) was reduced with 5 mM DTT in the anaerobic chamber for at least 1 h before the assay was started. The Leu1 activation assay mixtures contained 2.45 μM Leu1, 5 mM DTT, 50 mM Tris-HCl, pH 8.0, and 150 mM NaCl in a total volume of 250 or 500 μl. The concentration of reconstituted SufU was determined, and *holo*-SufU was added in a molar ratio of 2:1 to Leu1 to initiate the activity assays. Ten-microliter aliquots of the assay mixture were taken at various time points and tested for the ability to convert (2*R*,3*S*)-3-isopropylmalate to dimethylcitraconate (ε_{235} = 4.35 mM^{-1} cm^{-1}) (16). In a parallel experiment, the same amount of Fe^{3+} and S^{2-} (13.3 μM each) present in the reconstituted and desalted SufU protein was added to *apo*-Leu1 and tested for Leu1 enzyme activity reactivation.

RESULTS

Genetic organization of the *suf* operon in *B. subtilis*. The *B. subtilis* genome was searched by pBLAST analysis with the protein sequences of the *E. coli* ISC and SUF systems in order to identify homologous components for Fe/S cluster biogenesis. According to a recent report (39), only a *suf* gene cluster is present encoding proteins with high homology to these components (Table 2). Hence, the *yurY-yurX-csd-yurV-yurU* genes of this cluster were named *sufCDSUB* (Fig. 1A). In contrast to the *E. coli suf* gene cluster, no *sufE* gene was found in *B. subtilis*. The SufU (YurV) protein sequence shows significant differences from alignments of IscU-type scaffold protein sequences (24). First, the LPPVK motif essential for HscA interaction was found to be absent. Second, a 19-amino-acid insertion is present between the second and third conserved cysteine residues which discriminates SufU from IscU and NifU sequences. Third, a conserved lysine replaces the IscU typical histidine directly at the N-terminal side of the third conserved cysteine. A further *suf*-related gene, *yutM*, with low

TABLE 2. Homologs of the *E. coli* Fe/S cluster biogenesis machinery in *B. subtilis*

E. coli protein (length in aa[a])	*B. subtilis* homolog (length in aa)	No. of identical residues/total no. of residues (%)	No. of similar residues/total no. of residues (%)
SufA (122)	YutM (120)	34/104 (32)	59/104 (56)
SufB (495)	YurU (465)	194/477 (40)	283/477 (59)
SufC (248)	YurY (261)	126/246 (51)	180/246 (73)
SufD (432)	YurX (437)	39/115 (33)	71/115 (61)
SufS (406)	Csd (YurW) (406)	193/397 (48)	271/397 (68)
IscU (128)	YurV (147)	43/136 (30)	74/136 (54)

[a] aa, amino acids.

but significant homology with *sufA*, is located outside the *sufCDSUB* cluster (Table 2). Thus, *B. subtilis* has at least two potential Fe/S cluster scaffold proteins: SufU and SufA (*yutM*). We therefore compared the functional importance of these two genes.

The *sufU* gene is crucial for growth of *B. subtilis*. Since direct deletion of the *sufU* gene was not possible, indicating its essentiality, a *sufU* conditional mutant was constructed. After integration of an intact gene version under the control of the *xylR*-dependent promoter into the *amyE* site (26), deletion of *sufU* at its original locus was possible. The conditional mutant showed a drastically reduced growth in minimal medium when inoculated from fresh LB plates. This phenotype could be reverted by the addition of 1% (wt/vol) xylose (Fig. 1B). The growth in LB medium was also strongly diminished (60 to 80% of wild-type [WT] growth rate) but, however, not upon addition of xylose (not shown). Thus, the strictly xylose-dependent growth of the mutant revealed that *sufU* fulfills an essential function under vegetative conditions.

In contrast, deletion of *sufA* was successful. The resulting Δ*sufA* mutant did not show any significant growth defect compared to wild-type cells (Fig. 1C). It was previously shown that, under aerobic growth conditions, *E. coli* SufA is required for assembly of [4Fe-4S] proteins such as ThiC, endonuclease III, and aconitase B (47). In the case of aconitase, which is necessary to provide α-ketoglutarate as a precursor for glutamate, supplementation with glutamate restored wild-type growth. As shown in Fig. 1C, omission of glutamate in *B. subtilis* Δ*sufA* mutant cultures had no significant effect on growth, excluding an essential function of *B. subtilis sufA* in aconitase maturation. Thus, *B. subtilis sufA* was not found to be essential under minimal growth conditions, distinguishing it from the *E. coli* A-type scaffold proteins.

SufU is essential for the activity of proteins containing Fe/S cofactors. To investigate the influence of a SufU deficiency on the activities of Fe/S-dependent proteins, three enzymes of the Krebs cycle were tested: aconitase, containing a [4Fe-4S] cluster; succinate dehydrogenase, containing [2Fe-2S], [3Fe-4S], and [4Fe-4S] clusters; and the non-Fe/S enzyme malate dehydrogenase. Enzyme activities were determined in cell lysates of the WT and the SufU-depleted mutant. As shown in Fig. 2, the Fe/S-dependent enzyme activities were found to be two- to fourfold lower in the *sufU* mutant than in wild-type cell lysates. As a control, the malate dehydrogenase activity was not significantly affected in the *sufU* conditional mutant lysate. In contrast, the enzyme activities in the *sufA* mutant were not found to be lower than those in the wild type. Thus, only SufU deficiency had drastic consequences for the activities of Fe/S cluster-containing proteins of *B. subtilis*, suggesting a crucial function of SufU in Fe/S protein biogenesis.

SufU binds a labile Fe/S cluster under anaerobic conditions *in vitro*. SufU was recombinantly produced in *E. coli* as a fusion protein with an N-terminal His_6 tag and purified using Ni-NTA columns. About 15 mg of purified SufU per liter bacterial culture was obtained. The SufU protein with the expected molecular mass on SDS-PAGE (20 kDa [not shown]) was

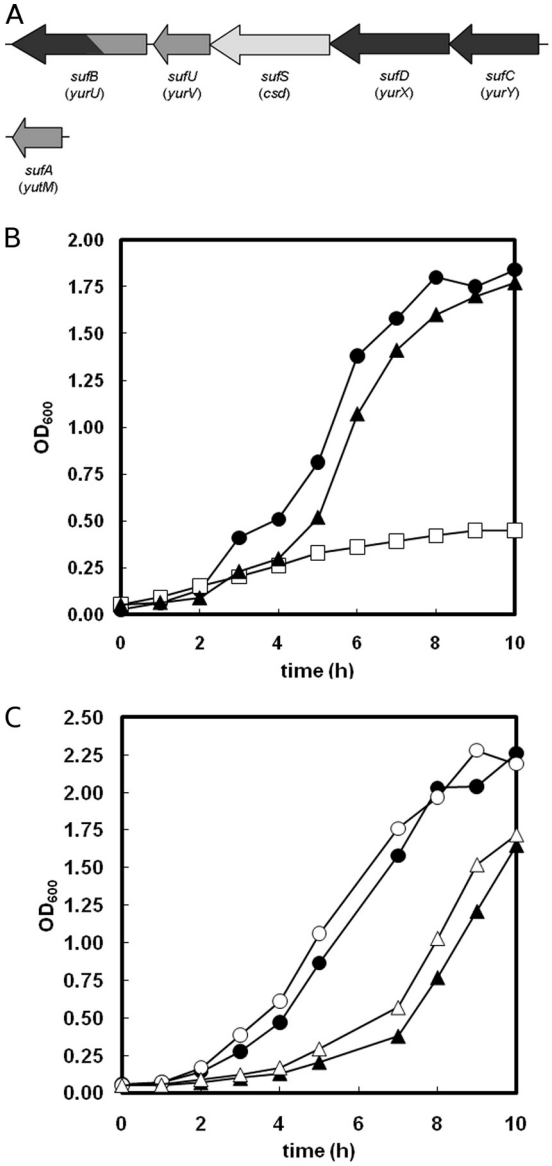

FIG. 1. Phenotypes of *B. subtilis* scaffold protein mutants. (A) Scheme of the *B. subtilis suf* gene cluster (black, energy-producing system; medium gray, putative scaffold; light gray, cysteine desulfurase). (B) Growth of WT (●) without xylose and *sufU* conditional mutant without xylose (□) and with 1% xylose induction (▲) in Belitsky minimal medium. (C) Growth in minimal medium of WT in the presence (●) or absence (▲) of glutamate compared with the *sufA* mutant in the presence (○) or absence (△) of glutamate.

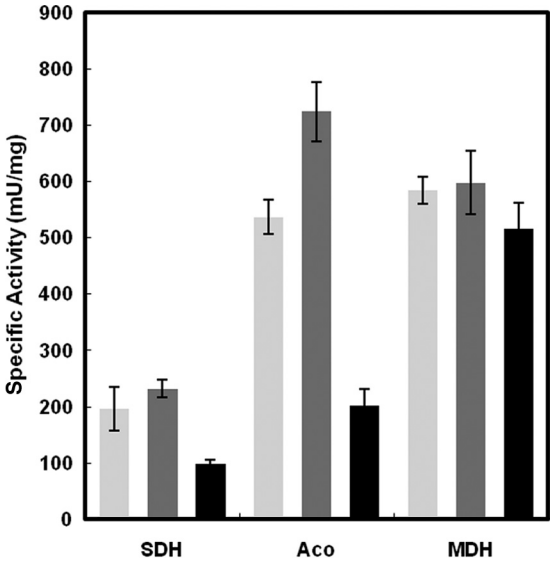

FIG. 2. Activities of the Fe/S cluster proteins aconitase (Aco) and succinate dehydrogenase (SDH) and the non-Fe/S protein malate dehydrogenase (MDH) in cell lysates of the wild type (light gray), the Δ*sufA* mutant (dark gray), and the *sufU* conditional mutant (black) without xylose induction. Error bars indicate standard deviations from four independent experiments.

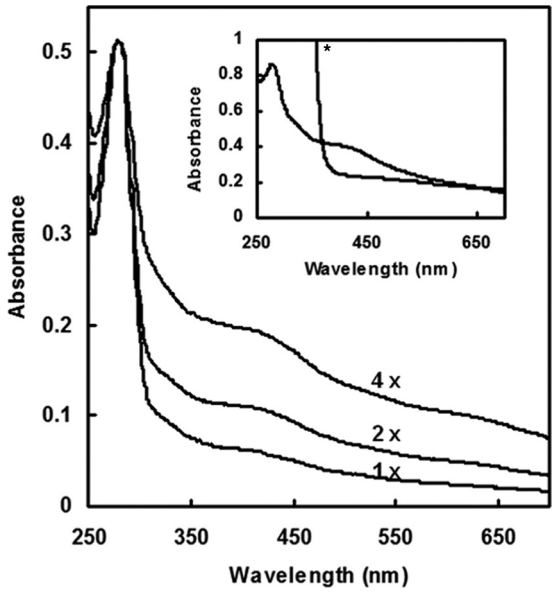

FIG. 3. UV/visible absorption spectrum of reconstituted *holo*-SufU shows spectral features similar to those for known Fe/S cluster proteins. SufU was reconstituted by using $FeCl_3$ and Li_2S in one- to fourfold molar excesses over SufU as indicated in 50 mM Tris, 150 mM NaCl, pH 8.0. The UV/visible absorption spectra were recorded. The inset shows spectra of SufU after reconstitution without and with reduction (*) with 2 mM sodium dithionite.

colorless, and chemical analysis did not detect any iron or sulfide. In order to test the capability of SufU to assemble an Fe/S cluster, purified SufU was chemically reconstituted with $FeCl_3$ and Li_2S. The UV/visible absorption spectrum of SufU reconstituted with 4 molar equivalents of Fe^{3+} and S^{2-} per monomer showed a dominant broad shoulder at 400 nm and slight shoulders at 325 and 650 nm (Fig. 3). The broad shoulder at 400 nm, a typical feature of $[4Fe\text{-}4S]^{2+}$ clusters, disappeared almost completely upon reduction with 2 mM dithionite (not shown). The same spectroscopical features were also present, albeit with decreased intensity, when the reconstitution was carried out using less of Fe^{3+} and S^{2-} (1 or 2 equivalents; Fig. 3). The type of Fe/S cluster could not be determined by electron paramagnetic resonance (EPR), probably due to the instability of the cluster upon reduction with dithionite, even after rapid exposure and shock freezing. Also Mössbauer spectroscopy could not be pursued, since the reconstituted protein could not be concentrated without precipitation.

Determination of nonheme iron and acid-labile sulfide in reconstituted and subsequently desalted SufU showed that the total amount of bound Fe/S cluster increased as a function of the molar excess of Fe^{3+} and S^{2-} used in the reconstitution assay up to 1.5 iron and 1.5 sulfide ions per monomer SufU (Table 3). We conclude that SufU can bind an Fe/S cluster in a labile fashion.

Recombinant $SufU_{D43A}$ protein can be purified with an Fe/S cluster. It is known that in truncated NifU (and IscU) (15, 33, 38, 54) substitution of the conserved aspartate residue 37 near the first conserved cysteine residue (position 35) leads to more-stable Fe/S cluster association and higher resistance to oxygen damage. In order to analyze the effect of substitution of this conserved residue in *B. subtilis* SufU, the respective mutant protein ($SufU_{D43A}$) was recombinantly produced in *E. coli* and purified with a yield of about 6 mg protein per liter culture. Interestingly, when purified under aerobic conditions, $SufU_{D43A}$ was found to carry an Fe/S cluster (Fig. 4) showing a higher Fe/S cluster stability. The iron and sulfide content was 0.15 per monomer, indicating an incomplete Fe/S cluster loading during heterologous production. The protein was found to precipitate during concentration or desalting when salt concentrations in the buffer were below 300 mM or when imidazole or DTT was omitted. Even in the presence of DTT or imidazole, the protein is not stable and precipitates after a short time. This suggests that imidazole or DTT could stabilize the Fe/S cluster in $SufU_{D43A}$ by supplying an external cluster ligand, since similar phenomena were observed after Ni-NTA purification or chemical reconstitution with buffer containing DTT in cysteine mutants of some Fe/S proteins (4). *In vitro* reconstitution of $SufU_{D43A}$ did not result in clearly interpretable UV/Vis spectra. Thus, despite the increased Fe/S cluster stability it was impossible also for this protein to determine the type of Fe/S cluster. The instability of the mutant protein prevented further activity assays.

TABLE 3. Absolute amounts of iron and sulfide bound to the SufU protein determined after chemical reconstitution with different equivalents of Fe^{3+} and S^{2-}

Molar eq of Fe^{3+} and S^{2-}/SufU monomer	Fe^{2+}/monomer	S^{2-}/monomer
0	<0.05	<0.05
1	0.19	0.12
2	0.54	0.66
4	1.48	1.54

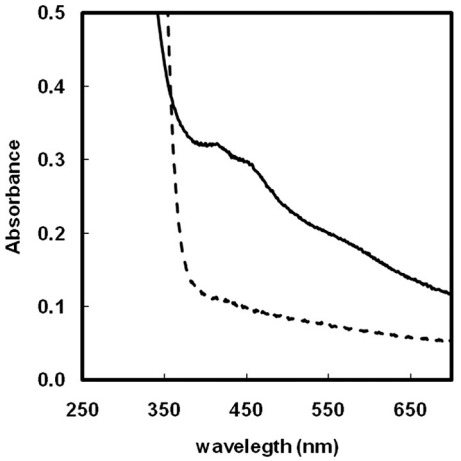

FIG. 4. The UV/visible absorption spectrum of purified $SufU_{D43A}$ shows spectral features similar to those for known Fe/S cluster proteins. $SufU_{D43A}$ was purified aerobically, and a UV/visible spectrum was recorded (solid line). The dashed curve represents the spectrum of $SufU_{D43A}$ after reduction with 0.5 mM sodium dithionite.

The *holo* form of SufU can activate Leu1 isopropylmalate isomerase. To analyze if *holo*-SufU was able to donate its chemically assembled Fe/S cluster to an Fe/S-dependent target protein, *in vitro* cluster transfer assays were performed. The isopropylmalate isomerase Leu1 from *Saccharomyces cerevisiae* requires a [4Fe-4S] cluster to convert (2*S*)-2-isopropylmalate into (2*R*,3*S*)-3-isopropylmalate (23, 35). Incubation of inactive *apo*-Leu1 with reconstituted SufU led to rapid and efficient reconstitution of Leu1 activity (Fig. 5A). The efficacy of SufU-dependent reconstitution is particularly evident from a comparison with the reconstitution rates and efficiencies of chemical Leu1 reconstitution, which is slow and, even after longer incubation times, inefficient (5% compared to SufU). In a different experiment, *apo*-Leu1 incubation with SufU reconstituted with one-, two-, or fourfold molar equivalents of Fe^{3+} and S^{2-} was analyzed. As shown in Fig. 5B, reconstitution of Leu1 activity was dependent on the amount of Fe/S cluster present on SufU. Increasing the amounts of Fe and S resulted, as observed by UV/Vis spectrometry, in substantial [4Fe-4S] cluster reconstitution on Leu1. These results suggest that SufU binds a labile Fe/S cluster which can be rapidly and efficiently transferred to a suitable acceptor. SufU therefore fulfills the criteria of an Fe/S scaffold protein.

SufU functionally cooperates with the cysteine desulfurase SufS. To investigate the possible interaction of SufU with its predicted sulfur donor protein, the cysteine desulfurase SufS, a 49.8-kDa protein, was recombinantly produced in *E. coli* as an N-terminal His_6 tag fusion and purified with yields up to 8 mg/liter culture. Analysis of the UV/Vis absorption spectrum revealed that the pyridoxal-5′-phosphate cofactor was bound to the protein (not shown). The activity of SufS was determined by measuring the amount of sulfide released from L-cysteine 10 min after the start of the reaction. The amount of released sulfide was determined by using *N*,*N*-*p*-phenylenediamine, which results in formation of methylene blue as previously described (44). While SufS alone exhibited only a weak enzymatic activity, the specific activity was drastically increased when *apo*-SufU was added to the assay (Fig. 6A). In contrast, *holo*-SufU led only to much weaker activation of SufS. Thus, *apo*-SufU stimulates the cysteine desulfurase activity of SufS, suggesting that SufS could act as a cognate partner of *apo*-SufU during Fe/S cluster assembly. To test this, we tested whether cluster reconstitution on SufU was possible in a SufS-dependent manner. SufU was preincubated with iron and then incubated with L-cysteine and SufS, which indeed resulted in Fe/S cluster formation (Fig. 6B). Thus, in the presence of excess cysteine, SufS can act as a sulfide donor for SufU during Fe/S cluster assembly *in vitro*.

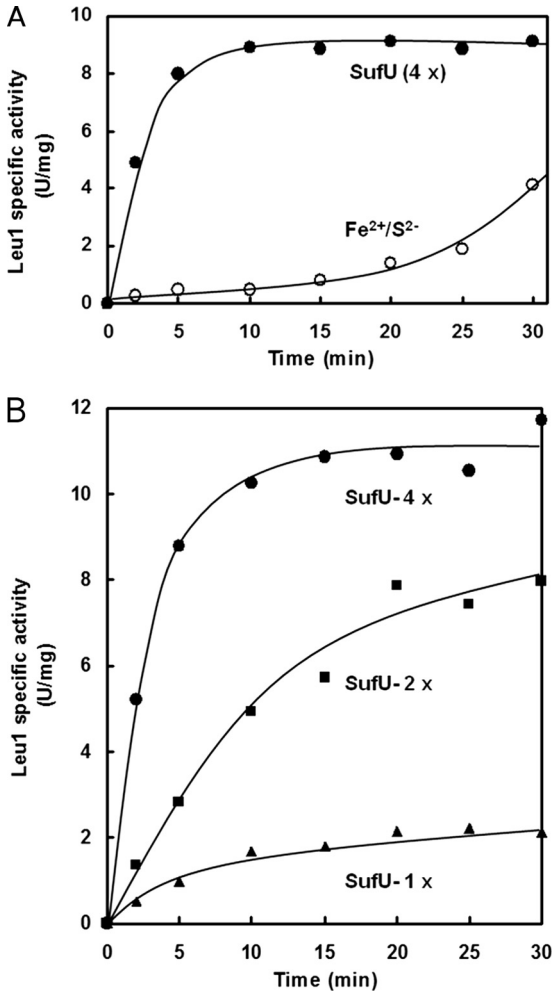

FIG. 5. *holo*-SufU activates the isopropylmalate isomerase Leu1. (A) Time course of Leu1 activation by the addition of *holo*-SufU reconstituted with fourfold (●) molar excesses of Fe and S and 13.3 μM (final concentration after reconstitution) concentrations of $FeCl_3$ and Li_2S in the absence of SufU (○). (B) Time course of Leu1 activation by the addition of *holo*-SufU reconstituted with the indicated molar excess of Fe and S. All Leu1 activation reaction mixtures contained 2.9 μM *apo*-Leu1 in 50 mM Tris, 150 mM NaCl, pH 8.0. Leu1 activity was measured at the indicated time points by the formation of isopropylmalate in 20 mM Tris, pH 7.4, and 50 mM NaCl by measuring the absorption at 235 nm. The reaction was started by the addition either of *holo*-SufU or of Fe^{3+} and S^{2-} at the same concentrations.

DISCUSSION

In this study, the SUF system for Fe/S cluster biogenesis was identified and characterized as an essential system in *B. subtilis*. In contrast to the *E. coli* SUF system, which is under the control of

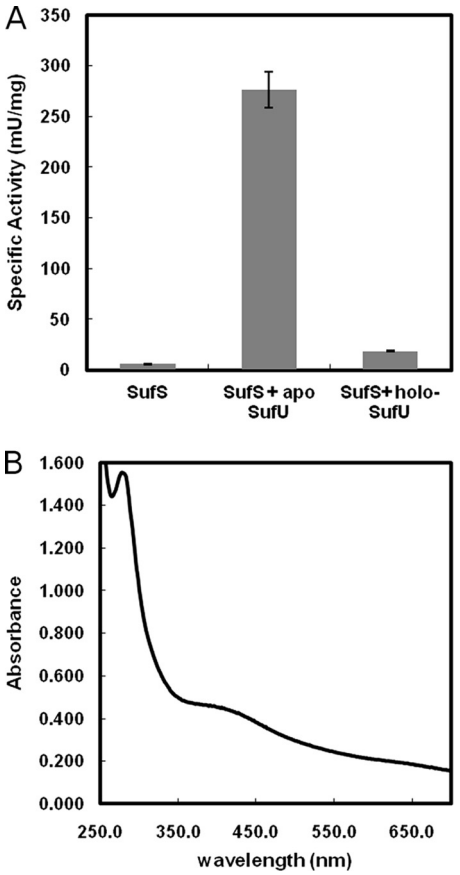

FIG. 6. SufU activates the cysteine desulfurase SufS. (A) Specific activity of SufS in the absence and presence of SufU. (B) SufS-dependent Fe/S cluster reconstitution on SufU. SufU (50 μM) and SufS (0.5 μM) were preincubated with 5 mM DTT for 1 h. After preincubation, 250 μM ferric ammonium citrate was added and incubated until color stability. Then 2.5 mM cysteine was added and incubated for 30 min.

the iron-dependent Fur regulator (14), SUF seems to represent the "housekeeping" Fe/S biogenesis system in *B. subtilis*, since the *B. subtilis* SUF system was identified as the only complete Fe/S biosynthesis machinery present in the genome and was further not found to be part of the Fur regulon, which was characterized previously (6). So far, the factors involved in regulation of the SUF system in *Bacillus subtilis* are unknown.

Interestingly, the exclusive presence of SUF seems to be a general feature within the *Firmicutes* phylum of Gram-positive bacteria, as recently indicated (39). Regarding its components, the *B. subtilis* SUF machinery resembles that which is encoded in *Thermotoga maritima*, *Enterococcus faecalis*, and mycobacterial genomes, consisting of a SufU scaffold protein, a SufS cysteine desulfurase, and the SufBCD energy complex (22, 37, 39). A SufE component is not present in these bacteria which, in contrast, is essential in *E. coli* for sulfide transfer to their scaffold protein(s) (29, 36). Thus, interestingly, SufE and SufU components were not found to be encoded together in the same genome so far, which may indicate distinct phylogenetic routes during the evolution of SUF systems. Further, regulatory proteins like IscR or SufR, which are part of the ISC or cyanobacterial SUF systems, are generally missing in SUF systems of *Firmicutes* genomes (39).

In addition to the SUF system, there are other potential Fe/S cluster biogenesis proteins encoded in the *B. subtilis* genome such as cysteine desulfurases NifS and NifZ involved in NAD and thiamine biosynthesis, respectively. Furthermore, the partial NfuA homolog YutI, which might act as an additional scaffold protein (3, 8), and YrvM, a homolog of CsdL that is possibly involved in Fe/S cluster biogenesis in *E. coli* (50), can be found.

Genetic analysis of the *B. subtilis* SUF system revealed that deletion of the *sufU* gene causes a lethal phenotype. Without addition of xylose, growth of the *sufU* conditional mutant was not observed in minimal medium. In LB medium a reduced growth was observed. In contrast, mutational studies of scaffold proteins in the *E. coli* ISC and SUF systems revealed that no single gene deletion of either *iscA*, *iscU*, or *sufA* led to a lethal phenotype. An Δ*iscA*/Δ*sufA* mutant did not grow on minimal medium under aerobic conditions but was able to grow under anaerobic conditions (32), while an Δ*iscSUA* mutant could be rescued on minimal medium by addition of nicotinic acid and thiamine, and this phenotype was essentially associated with the deletion of *iscS* (49). This indicates that the Fe/S biogenesis machinery in *E. coli* is more flexible in a way that several components from the different systems may complement each other. The essentiality of distinct components seems to be closely associated with environmental conditions such as aerobic or anaerobic growth or sensitivity to oxidative stress or iron limitation. In *B. subtilis*, the absolute requirement for *sufU* reflects the constitutive role of the SUF machinery in this bacterium, in particular since no further Fe/S biogenesis components independent of the SUF machinery were found in its genome. Still we cannot exclude the existence of further systems which are activated under conditions interfering with Fe/S assembly such as oxidative stress or iron limitation.

Although U-type scaffold proteins from ISC systems have been characterized in previous studies, no biochemical characterization was reported for a SufU-type scaffold. The present study shows that the SufU scaffold protein is both similar to and characteristically different from other U-type scaffolds such as *E. coli* and *A. vinelandii* IscU. All U-type scaffolds can bind an Fe/S cluster after chemical reconstitution. In the case of SufU the UV/Vis spectra and Leu1 cluster transfer assays suggest the binding of a [4Fe-4S] cluster, but the lack of further spectroscopic data prevents a final definition of the cluster type. The $SufU_{D43A}$ variant, but not the wild-type protein, was purified aerobically with an Fe/S cluster, which indicates that the mutation led to stabilization of the Fe/S cluster, similarly to what was found for NifU and IscU. The remaining Fe/S cluster instability of $SufU_{D43A}$, on the other hand, may indicate that its binding is possibly dependent on external ligands, such as imidazole or DTT.

The function of SufU in Fe/S cluster biogenesis was indicated by several observations of this study. The activity of Fe/S cluster-containing aconitase and succinate dehydrogenase was strongly reduced in *sufU* mutant cell lysates. *In vitro* reconstitution indicated the presence of an Fe/S cluster on SufU which could be transferred to *apo*-Leu1 to gave rise to *in vitro* cluster formation on the [4Fe-4S]-dependent Leu1 and to catalytic activation of the *apo*-enzyme. In conclusion, SufU may preferentially assemble Fe/S clusters under quasianaerobic and reductive conditions *in vivo* and provide them to most of the cellular target enzymes, which likely explains its essential func-

tion. Without further structural knowledge, it is speculative if the cluster is bound equally between the SufU protomers or is bound on one protein while being protected by other SufU units, as described for IscU from *Aquifex aeolicus* (43).

The strong activation of SufS by the presence of *apo*-SufU indicates an interactive mode between these proteins. *B. subtilis* SufU led to a strong activation of the SufS enzymatic function by a factor of 40. No activation was observed when *holo*-SufU was added. In comparison, *E. coli* SufE activates SufS by a factor of 8, and the addition of SufBCD to SufS further increases the activity by a factor of 32, without the addition of a scaffold protein (29, 36, 42). The successful reconstitution of SufU in the presence of SufS as a sulfur donor may give an additional hint that SufU directly interacts with SufS, which is even more likely since a mediator component such as SufE seems not to be present in *B. subtilis*. Since SufU preincubated with iron led to formation of the putative [4Fe-4S] cluster in the presence of active SufS and cysteine, this might lead to the suggestion that iron binding could precede sulfide transfer during the cluster assembly process, supporting a "first Fe, second S" model. However, since investigation of cluster assembly during the "′inverse" experiment by incubating presulfurated SufU with iron was not possible due to FeS precipitation, this does not exclude that the order of assembly could also proceed according to a "first S, second Fe" model.

In contrast to the essential *sufU*, the deletion of *sufA* did not result in any significant phenotype under the tested growth conditions. Also, the activity of the tested iron sulfur proteins was not reduced. Although, for example, in *E. coli* at least one A-type scaffold is necessary for growth, these proteins are generally regarded to assemble specific Fe/S proteins (24, 32, 47, 52). Therefore, it might be possible that the SufA function is crucial under specific stress, growth, or development conditions of the cells.

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REFERENCES

1. **Agar, J. N., C. Krebs, J. Frazzon, B. H. Huynh, D. R. Dean, and M. K. Johnson.** 2000. IscU as a scaffold for iron-sulfur cluster biosynthesis: sequential assembly of [2Fe-2S] and [4Fe-4S] clusters in IscU. Biochemistry **39:** 7856–7862.
2. **Anagnostopoulos, C., and J. Spizizen.** 1961. Requirements for transformation in *Bacillus subtilis.* J. Bacteriol. **81:**741–746.
3. **Angelini, S., C. Gerez, S. Ollagnier-de Choudens, Y. Sanakis, M. Fontecave, F. Barras, and B. Py.** 2008. NfuA, a new factor required for maturing Fe/S proteins in *Escherichia coli* under oxidative stress and iron starvation conditions. J. Biol. Chem. **283:**14084–14091.
4. **Antonkine, M. L., E. M. Maes, R. S. Czernuszewicz, C. Breitenstein, E. Bill, C. J. Falzone, R. Balasubramanian, C. Lubner, D. A. Bryant, and J. H. Golbeck.** 2007. Chemical rescue of a site-modified ligand to a [4Fe-4S] cluster in PsaC, a bacterial-like dicluster ferredoxin bound to photosystem I. Biochim. Biophys Acta **1767:**712–724.
5. **Ayala-Castro, C., A. Saini, and F. W. Outten.** 2008. Fe-S cluster assembly pathways in bacteria. Microbiol. Mol. Biol. Rev. **72:**110–125.
6. **Baichoo, N., T. Wang, R. Ye, and J. D. Helmann.** 2002. Global analysis of the *Bacillus subtilis* Fur regulon and the iron starvation stimulon. Mol. Microbiol. **45:**1613.
7. **Balk, J., A. J. Pierik, D. J. Aguilar Netz, U. Mühlenhoff, and R. Lill.** 2004. The hydrogenase-like Nar1p is essential for maturation of cytosolic and nuclear iron-sulphur proteins. EMBO J. **23:**2105–2115.
8. **Bandyopadhyay, S., S. G. Naik, I. P. O'Carroll, B.-H. Huynh, D. R. Dean, M. K. Johnson, and P. C. D. Santos.** 2008. A proposed role for the *Azotobacter vinelandii* NfuA protein as an intermediate iron-sulfur cluster carrier. J. Biol. Chem. **283:**14092–14099.
9. **Barras, F., L. Loiseau, and B. Py.** 2005. How *Escherichia coli* and *Saccharomyces cerevisiae* build Fe/S proteins. Adv. Microb. Physiol. **50:**41.
10. **Beinert, H., R. H. Holm, and E. Münck.** 1997. Iron-sulfur clusters: nature's modular, multipurpose structures. Science **277:**653–659.
11. **Brzóska, K., S. Męczyńska, and M. Kruszewski.** 2006. Iron-sulfur cluster proteins: electron transfer and beyond. Acta Biochim. Pol. **53:**685.
12. **Cupp-Vickery, J. R., J. C. Peterson, D. T. Ta, and L. E. Vickery.** 2004. Crystal structure of the molecular chaperone HscA substrate binding domain complexed with the IscU recognition peptide ELPPVKIHC. J. Mol. Biol. **342:** 1265–1278.
13. **Drapier, J. C., and J. B. J. Hibbs.** 1996. Aconitases: a class of metalloproteins highly sensitive to nitric oxide synthesis. Methods Enzymol. **269:**26–36.
14. **Fontecave, M., S. Ollagnier de Choudens, B. Py, and F. Barras.** 2005. Mechanisms of iron-sulfur-cluster assembly: the SUF machinery. J. Biol. Inorg. Chem. **10:**713–721.
15. **Foster, M. W., S. S. Mansy, J. Hwang, J. E. Penner-Hahn, K. K. Surerus, and J. A. Cowan.** 2000. A mutant human IscU protein contains a stable [2Fe-2S]$^{2+}$ center of possible functional significance. J. Am. Chem. Soc. **122:**6805–6806.
16. **Gross, S. R., R. O. Burns, and H. E. Umbarger.** 1963. The biosynthesis of leucine. II. The enzymic isomerization of β-carboxy-β-hydroxyisocaproate and α-hydroxy-β-carboxyisocaproate. Biochemistry **2:**1046–1052.
17. **Gupta, V., M. Sendra, S. G. Naik, H. K. Chahal, B. H. Huynh, F. W. Outten, M. Fontecave, and S. Ollagnier de Choudens.** 2009. Native *Escherichia coli* SufA, coexpressed with SufBCDSE, purifies as a [2Fe-2S] protein and acts as an Fe-S transporter to Fe-S target enzymes. J. Am. Chem. Soc. **131:**6148–6153.
18. **Hatefi, Y., and D. Stiggall.** 1978. Preparation and properties of succinate: ubiquinone oxidoreductase (complex II). Methods Enzymol. **53:**21–27.
19. **Hausladen, A., and I. Fridovich.** 1996. Measuring nitric oxide and superoxide: rate constants for aconitase reactivity. Methods Enzymol. **269:**37–41.
20. **Hoch, J. A.** 1991. Genetic analysis in *Bacillus subtilis.* Methods Enzymol. **204:**305–320.
21. **Hoff, K. G., J. R. Cupp-Vickery, and L. E. Vickery.** 2003. Contributions of the LPPVK motif of the iron-sulfur template protein IscU to interactions with the Hsc66-Hsc20 chaperone system. J. Biol. Chem. **278:**37582–37589.
22. **Huet, G., M. Daffé, and I. Saves.** 2005. Identification of the *Mycobacterium tuberculosis* SUF machinery as the exclusive mycobacterial system of [Fe-S] cluster assembly: evidence for its implication in the pathogen's survival. J. Bacteriol. **187:**6137–6146.
23. **Jang, S., and J. A. Imlay.** 2007. Micromolar intracellular hydrogen peroxide disrupts metabolism by damaging iron-sulfur enzymes. J. Biol. Chem. **282:** 929–937.
24. **Johnson, D. C., D. R. Dean, A. D. Smith, and M. K. Johnson.** 2005. Structure, function, and formation of biological iron-sulfur-clusters. Annu. Rev. Biochem. **74:**247–281.
25. **Kessler, D., and J. Papenbrock.** 2005. Iron-sulfur cluster biosynthesis in photosynthetic organisms. Photosynth. Res. **86:**391–407.
26. **Kim, L., A. Mogk, and W. Schumann.** 1996. A xylose-inducible *Bacillus subtilis* integration vector and its application. Gene **181:**71–76.
27. **Kispal, G., P. Csere, B. Guiard, and R. Lill.** 1997. The ABC transporter Atm1p is required for mitochondrial iron homeostasis. FEBS Lett. **418:**346–350.
28. **Klein, C., C. Kaletta, N. Schnell, and K.-D. Entian.** 1992. Analysis of genes involved in biosynthesis of the lantibiotic subtilin. Appl. Environ. Microbiol. **58:**132–142.
29. **Layer, G., S. A. Gaddam, C. N. Ayala-Castro, S. Ollagnier-de Choudens, D. Lascoux, M. Fontecave, and F. W. Outten.** 2007. SufE transfers sulfur from SufS to SufB for iron-sulfur cluster assembly. J. Biol. Chem. **282:**13342–13350.
30. **Lill, R.** 2009. Function and biogenesis of iron-sulphur proteins. Nature **460:**831–838.
31. **Lill, R., and U. Mühlenhoff.** 2008. Maturation of iron-sulfur proteins in eukaryotes: mechanisms, connected processes, and diseases. Annu. Rev. Biochem. **77:**669–700.
32. **Lu, J., J. Yang, G. Tan, and H. Ding.** 2008. Complementary roles of SufA and IscA in the biogenesis of iron-sulfur clusters in *Escherichia coli.* Biochem. J. **409:**535–543.
33. **Mansy, S. S., S.-P. Wu, and J. A. Cowan.** 2004. Biochemical characterization of the conformational dynamics of *Thermotoga maritima* IscU and the relevance for cellular cluster assembly. J. Biol. Chem. **279:**10469–10475.
34. **Mihara, H., and N. Esaki.** 2002. Bacterial cysteine desulfurases: their functions and mechanisms. Appl. Microbiol. Biotechnol. **60:**12–23.
35. **Netz, D. J. A., A. J. Pierik, M. Stümpfig, U. Mühlenhoff, and R. Lill.** 2007.

The Cfd1-Nbp35 complex acts as a scaffold for iron-sulfur protein assembly in the yeast cytosol. Nat. Chem. Biol. **3:**278–286.
36. **Outten, F. W., M. J. Wood, F. M. Muñoz, and G. Storz.** 2003. The SufE protein and the SufBCD complex enhance SufS cysteine desulfurase activity as part of a sulfur transfer pathway for Fe-S cluster assembly in *Escherichia coli.* J. Biol. Chem. **78:**45713–45719.
37. **Petrovic, A., C. T. Davis, K. Rangachari, B. Clough, R. J. Wilson, and J. F. Eccleston.** 2008. Hydrodynamic characterization of the SufBC and SufCD complexes and their interaction with fluorescent adenosine nucleotides. Protein Sci. **17:**1264–1274.
38. **Raulfs, E. C., I. P. O'Carroll, P. C. D. Santos, M.-C. Unciuleac, and D. R. Dean.** 2008. In vivo iron-sulfur cluster formation. Proc. Natl. Acad. Sci. U. S. A. **105:**8591–8596.
39. **Riboldi, G. P., H. Verli, and J. Frazzon.** 2009. Structural studies of the *Enterococcus faecalis* SufU [Fe-S] cluster protein. BMC Biochem. **10:**3.
40. **Rose, I. A., and E. L. O'Connell.** 1967. Mechanism of aconitase action. J. Biol. Chem. **242:**1870–1879.
41. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
42. **Sendraa, M., S. Ollagnier de Choudens, D. Lascoux, Y. Sanakis, and M. Fontecave.** 2007. The SUF iron-sulfur cluster biosynthetic machinery: sulfur transfer from the SUFS-SUFE complex to SUFA. FEBS Lett. **581:**1362–1368.
43. **Shimomura, Y., K. Wada, K. Fukuyama, and Y. Takahashi.** 2008. The asymmetric trimeric architecture of [2Fe-2S] IscU: implications for its scaffolding during iron-sulfur cluster biosynthesis. J. Mol. Biol. **383:**133–143.
44. **Siegel, L. M.** 1965. A direct microdetermination for sulfide. Anal. Biochem. **11:**126–132.
45. **Stülke, J., R. Hanschke, and M. Hecker.** 1993. Temporal activation of β-glucanase synthesis in *Bacillus subtilis* is mediated by the GTP pool. J. Gen. Microbiol. **139:**2041–2045.
46. **Takahashi, Y., and U. Tokumoto.** 2002. A third bacterial system for the assembly of iron-sulfur clusters with homologs in archaea and plastids. J. Biol. Chem. **277:**28380–28383.
47. **Tan, G., J. Lu, J. P. Bitoun, H. Huang, and H. Ding.** 2009. IscA/SufA paralogs are required for the [4Fe-4S] cluster assembly in enzymes of multiple physiological pathways in *Escherichia coli* under aerobic growth conditions. Biochem. J. **420:**463–472.
48. **Tapley, T. L., and L. E. Vickery.** 2004. Preferential substrate binding orientation by the molecular chaperone HscA. J. Biol. Chem. **279:**28435–28442.
49. **Tokumoto, U., and Y. Takahashi.** 2001. Genetic analysis of the isc operon in *Escherichia coli* involved in the biogenesis of cellular iron-sulfur proteins. J. Biochem. **130:**63–71.
50. **Trotter, V., D. Vinella, L. Loiseau, S. Ollagnier de Choudens, M. Fontecave, and F. Barras.** 2009. The CsdA cysteine desulphurase promotes Fe/S biogenesis by recruiting Suf components and participates to a new sulphur transfer pathway by recruiting CsdL (ex-YgdL), a ubiquitin-modifying-like protein. Mol. Microbiol. **74:**1527–1542.
51. **Wach, A.** 1996. PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in *S. cerevisiae.* Yeast **12:**259–265.
52. **Yang, J., J. P. Bitoun, and H. Ding.** 2006. Interplay of IscA and IscU in biogenesis of iron-sulfur clusters. J. Biol. Chem. **281:**27956–27963.
53. **Ye, H., M. Pilon, and E. A. H. Pilon-Smits.** 2006. CpNifS-dependent iron-sulfur cluster biogenesis in chloroplasts. New Phytol. **171:**285–292.
54. **Yuvaniyama, P., J. N. Agar, V. L. Cash, M. K. Johnson, and D. R. Dean.** 2000. NifS-directed assembly of a transient [2Fe-2S] cluster within the NifU protein. Proc. Natl. Acad. Sci. U. S. A. **97:**599–604.
55. **Zheng, L., U. Baumann, and J.-L. Reymond.** 2004. An efficient one-step site-directed and site-saturation mutagenesis protocol. Nucleic Acids Res. **32:**e115.