

SUPPORTING MATERIAL

Iron Sulfur Cluster Biosynthesis. Human NFU Mediates Sulfide Delivery to ISU in the Final Step of [2Fe-2S] Cluster Assembly.

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MATERIALS & METHODS

General Chemicals. All restriction enzymes were from Invitrogen (Carlsbad, CA). Qiaquick gel extraction kit and Ni-NTA columns were from Qiagen (Valencia, CA). BL21 (DE3) cells and pET 28 vectors were from Novagen (Madison, WI). Primers were from Integrated DNA Technologies Inc. (Coralville, IA) and CM 32 and DE 52 ion exchange resins were from Whatman (Aston, PA). Homogenous-20 precast polyacrylamide gels and G-25 resin were obtained from Pharmacia (Peapack, NJ).

Cloning and Expression of human NFU. PCR amplification of the human NFU was achieved by use of a high fidelity PCR buffer (1X), a 0.2 mM dNTP mixture containing 2 mM MgSO₄, 100 ng human genome DNA, 0.2 μM amount of each primer and 1.0 unit platinum Taq DNA polymerase following a procedure in the Invitrogen's manual. Primers were: 5'-GGC CAT ATG TTT ATT CAA ACA CAA GAT ACC CC-3' and 5'-GCG GGA TCC TTA AGG TGA GTT TGC TTC TTT TT-3', where the underlined regions are Nde I and BamH I sites. The PCR product was digested with 10 unit of BamH I and 20 unit of Nde I in 1X React 3 buffer (Invitrogen). Vector pET-28 was similarly digested. After digestion, the samples were purified from agarose gel with the Qiagen gel extraction kit. The extracted samples were ligated according to the T4 ligase manual (Invitrogen). Subsequently the ligation mixture was transformed into DH5α competent cells and screened with restriction enzyme Pst I. The positive clone was confirmed by nucleotide sequencing at the Ohio State University Plant-Microbe Genomics Facility. An N-terminal His-tag was introduced to the construct for purification purpose.

BL21 Lysozyme plus (DE3) was used for protein expression. A 50ml Luria-Bertani culture (supplemented with 30 μg/ml kanamycin and 30 μg/ml chloramphenicol) was grown overnight as the starter culture. 10ml of the starter culture was used as an inoculum for a 1 L culture, which was grown to OD₆₀₀ ~ 0.6 and 1 mM IPTG was added to induce protein expression. Cells were harvested after 6 h induction and stored at -80 °C for future use.

Protein Purification of human NFU. Cell pellets were resuspended in five volumes of 50 mM Tris-HCl, (pH 7.5) and lysed by sonication. The cell lysate was centrifuged at 15,000 rpm for 30 min and the supernatant subsequently applied to a Ni-NTA column equilibrated with binding buffer (50 mM NaH₂PO₄, pH 7.9, 5 mM imidazole, 300 mM NaCl). The sample was then washed with 5 volumes of binding buffer + 15 mM imidazole, and the protein subsequently eluted with binding buffer + 295 mM imidazole. The His-tagged protein was exchanged with 50 mM sodium phosphate (pH 7.5) via repeated ultrafiltration (Amicon). The sample was loaded

onto an anion exchange column (DE-52) and the column washed with 5 column volumes of 50 mM sodium phosphate (pH 7.5) before elution with 50 mM sodium phosphate containing 300 mM NaCl (pH 7.5). All fractions with λ_{max} (280 nm) were pooled and concentrated via ultrafiltration. The concentrated sample was confirmed to be pure human NFU by SDS-PAGE (Figure S1). Mass spectrometry (ESI) data was used to confirm the identity of the human NFU. The protein sample was either stored at -80 °C (long term) or 4 °C (short term).

Circular Dichroism of NFU. The secondary structure of human NFU was characterized by circular dichroism with an 0.85 μM solution of human NFU in 20 mM phosphate buffer, pH 7.5. Spectra were collected on an Aviv model 202 circular dichroism spectrometer at 25 °C at 1 nm resolution in a 10 mm path length cuvette. The wavelength range was from 200nm to 280nm for each spectrum collection. The data was obtained five times and averaged. A background spectrum was subtracted (Figure S2) and secondary structure evaluated by use of the on-line software K2D (<http://www.embl-heidelberg.de/~andrade/k2d/>).

Purification of *T. maritima* D40A IscU and NifS. *T. maritima* IscU was purified as previously reported with the following minor modifications.¹ In brief, cell pellets containing the His-tagged *T. maritima* D40A IscU were resuspended in five volumes of 50 mM Tris-HCl (pH 7.5) and lysed by sonication. Centrifugation (15000 rpm) was used to remove the insoluble material. The supernatant was collected and incubated at 85 °C for 0.5 h and centrifuged at 15,000 rpm for 10 min. The cleared supernatant was loaded to Ni-NTA column equilibrated with binding buffer (50 mM NaH_2PO_4 , pH 7.9, 5 mM imidazole, 300 mM NaCl) and washed with 5 volumes of binding buffer + 15 mM imidazole. Subsequently the His-tagged *T. maritima* D40A IscU was eluted with binding buffer + 295 mM imidazole. Excess imidazole and NaCl was removed by repeated ultrafiltration (Amicon).

The non-His tagged *T. maritima* D40A IscU cell lysate was centrifuged at 15,000 rpm and the cleared supernatant was heated up to 85 °C for 0.5 h. The denatured protein was removed by centrifuging at 15,000 rpm for 10 min. The supernatant was applied to a cation exchange column (CM32), which was equilibrated with 50 mM sodium phosphate (pH 7.5). The column was washed with 2 volumes of sodium phosphate buffer. The flowthrough and washed fractions were pooled and passed through an anion exchange column (DE-52). The column was washed with 3 volumes of 50 mM sodium phosphate buffer (pH 7.5). The flowthrough and wash fractions were combined and concentrated. The concentrated non-His tagged D40A IscU was loaded to G-75 gel filtration column, the fractions containing the non-His tagged D40A IscU were collected and concentrated. The protein was stored at 4 °C for future use.

T. maritima NifS was purified as previously described.² The cultured cells were harvested, lysed and centrifuged (15,000 rpm) to remove cell debris. The cleared supernatant was heated to 85 °C and incubated for 20 min. The insoluble precipitate was removed by centrifugation at 15,000 rpm. Subsequently 1% of streptomycin sulfate was added and the precipitate was removed by centrifugation. Ammonium sulfate (1.5-3 M) was used to precipitate the *T. maritima* NifS. The protein pellet was resuspended in 50 mM sodium phosphate (pH 7.5) and reconstituted with Pyridoxal-5'-Phosphate (PLP) for one hour. Subsequently the sample was loaded to G-75 gel filtration column, and the fractions with maximum absorbance at 280 nm were pooled and concentrated by an amicon stirred cell concentrator. The protein was stored at -80 °C for future use.

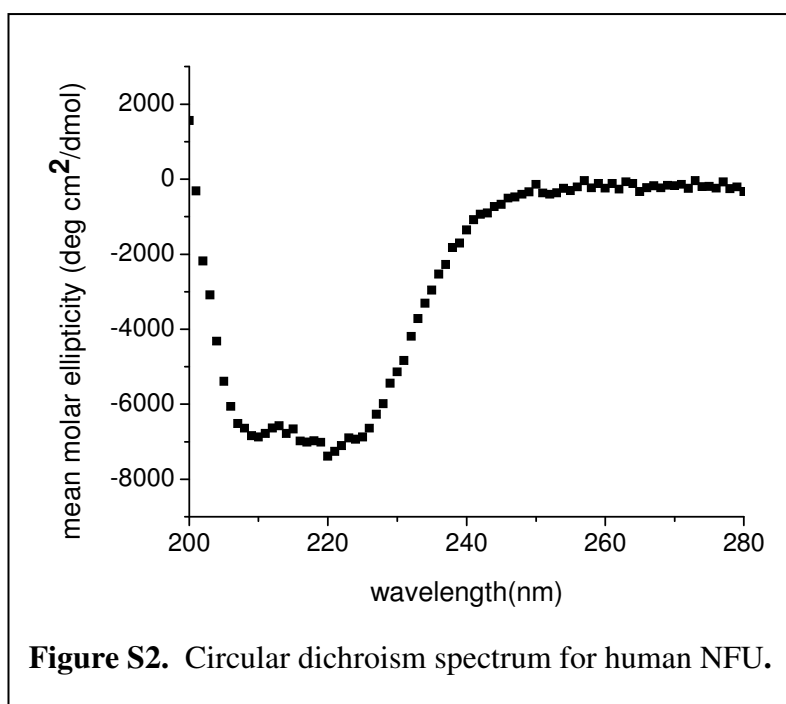
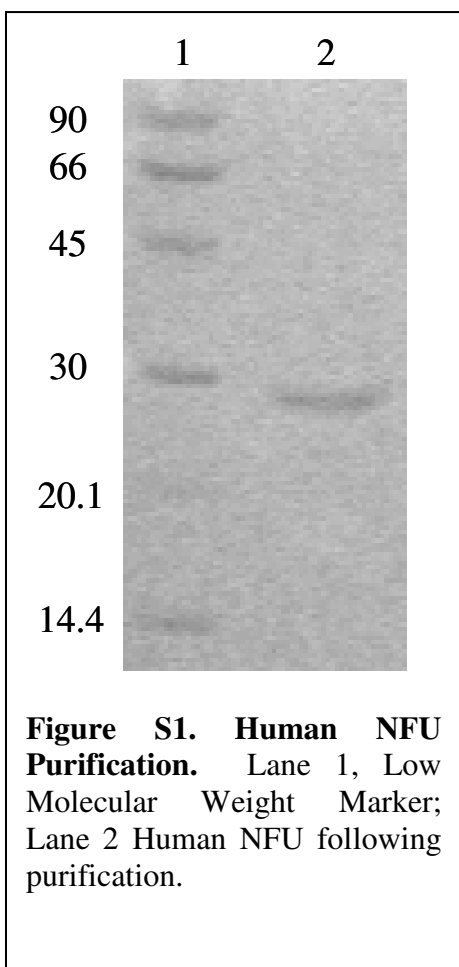
Purification of human D37A ISU. Human ISU was purified as described previously.³ The cells were thawed on ice and resuspended in binding buffer (5 mM imidazole, 300 mM NaCl, 50 mM NaH₂PO₄, pH 7.9) and lysed by sonication. Centrifugation at 15,000 rpm at 4 °C for 20 min was used to remove cellular debris, and the supernatant was applied to a Ni-NTA column (Qiagen) already equilibrated with binding buffer. After loading, the column was washed with 10 volumes of wash buffer (20 mM imidazole, 300 mM NaCl, 50 mM pH 7.9) and eluted with elution buffer (400 mM imidazole, 300 mM NaCl, 50 mM NaH₂PO₄, pH 7.9). Fractions containing D37A ISU as judged by SDS-PAGE were pooled. D37A ISU was incubated with 1 mM EDTA overnight to remove residual iron sulfur cluster bound to ISU. Then the ISU was passed through G-25 column equilibrated with 50 mM Tris-HCl (pH 7.5) to remove any EDTA-complexed iron and sulfide. ISU was concentrated for future use.

Oxygen sensitivity of human NFU. Purified human NFU was treated with excess DTT in the absence of air, incubated for 15 min, and excess DTT subsequently removed by repeated ultrafiltration. Free thiol content was determined by incubation with DTNB solution and the absorbance at 412nm was converted to moles of free thiol based on published extinction coefficients, as previously described.⁴ The ratio of moles of free thiol to moles of NFU was evaluated at various time intervals to determine the stability of the reduced NFU.

Methylene blue assay of sulfide reduction. NFU-promoted sulfide release from the persulfide labeled Tm NifS was evaluated by use of the methylene blue assay described by Siegel.⁵ Reactions were carried out in glass tubes capped with serum stoppers. Human NFU was treated with DTT anaerobically for 15 min, and excess DTT subsequently removed by repeated ultrafiltration. Assay samples contained 600 nM Tm NifS and 1 μM pyridoxal phosphate, to promote the formation of the persulfide-labeled NifS following abstraction of sulfur from cysteine,² and reaction was initiated by addition of 1 mM L-cysteine and 600 μM human NFU in 50 mM HEPES (pH 7.5) buffer. As a positive control, a separate reaction with addition of 1 mM L-cysteine and 600 μM DTT was also carried out. As a negative control a reaction lacking human NFU was performed. In all cases the assay was carried out under anaerobic conditions under argon in a septum-stoppered glass vial. Following incubation the reaction was terminated by addition of 100 μl of 20 mM N,N-dimethyl-p-phenylenediamine and 100 μl of 30 mM ferric chloride. Product was determined by measuring methylene blue formation at 670 nm,⁵ and comparison with a standard curve generated by testing known concentrations of sulfide with the methylene blue assay.

Quantitation of iron binding to human NFU by Fluorimetry. The intrinsic tryptophan fluorescence was measured by use of a Perkin-Elmer LS50B luminescence spectrometer with excitation at 291 nm and emissions monitored at 341 nm. For ferric ion binding, 100 μM of the human NFU was first treated anaerobically with 1 mM DTT for 10 min, and excess removed by ultrafiltration. After desalting, the iron titration was performed with 15 μM NFU under anaerobic conditions. A modest decrease of fluorescence intensity was observed with the addition of higher concentrations of ferric ion. Similarly, the titration was repeated with ferrous ion. All samples were prepared in argon-purged 100 mM HEPES, pH 7.5. The binding function r was obtained from the decrease of fluorescence intensity and plotted (Figure S3) against the concentration of [Fe²⁺] or [Fe³⁺] to generate the binding constant as previously described.^{6,7}

Figures for Supplementary Material.



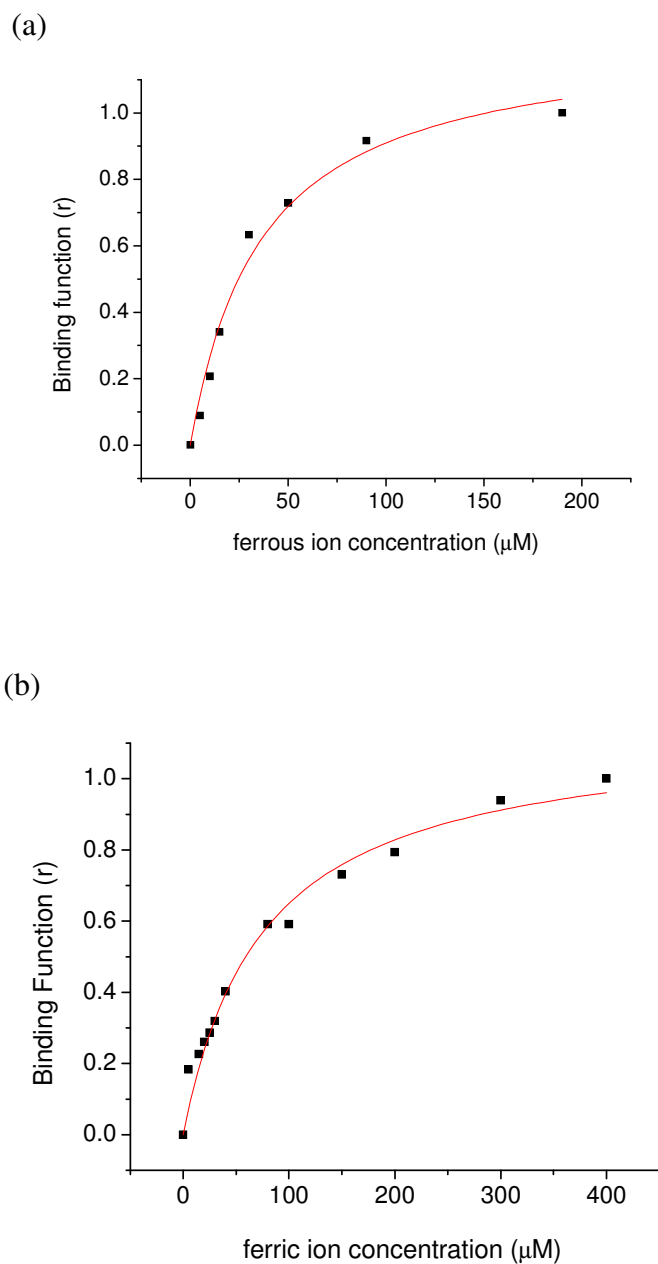


Figure S3. Quantitation of iron binding to human NFU by fluorimetry. (a) Ferrous ion binding to human NFU. (b) Ferric ion binding to human NFU.

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Human_Nfs      MLLRAAWRRAAVAVTAAPGPKPAAPTRGLRLRVGDRAPQSAVPAD
T.maritima_Nifs -----

Cons

Human_Nfs      TAAAPEVGPVLRPLYMDVQATTPLDPRVLDAMPLPYLINIYGNPHS
T.maritima_Nifs -----MRVYFDNNATTRVDDRVLBEMIVFYREKYGNPNS

Cons          :*:* :*** :* ** : * : : *****

Human_Nfs      RTHAYGWESEAAMERARQQVASLIGADPREIIFTSGATESNNIAI
T.maritima_Nifs -AHGMGIEANLHMEKAREKVKVGLVSPSEIFFTSCATESINWIL

Cons          :* . * * : : ** :*** :* : : * . * ** :*** ** * * :

Human_Nfs      KGVARFYRSRKKHLITTOEHKCVLDSRSLAEGFQVTYLPVQK
T.maritima_Nifs KTVABTFEKRKRTIITPIEHKAVLETMKYLSMKGPKVKYVPVDS

Cons          * * . : . * : : ** :*** :* : : * . : * : * : * : * :

Human_Nfs      SGIIDLKLEEAIIQPDTSLVSVMTVNNEIGVKQPIAEIGRICS--
T.maritima_Nifs RGVVKLEELEKLVDEDTFLVSIMAANNEVGTIQPVEDVTRIVKKK

Cons          * : : * :*** : : * * ** :* : * : * : * : * : * :

Human_Nfs      SRKVYFHTDAAQAVGKIPLDVNDMKIDLMSISGHKIYGPKGVGAI
T.maritima_Nifs NKETLVHVDVAVQTIGKIPPSLEKLEV DYASPSAHKFKHGPKGVGIT

Cons          . : : . * . * . * : : * : * : * : * : * : * : * : * : * :

Human_Nfs      YIRRRPRVVRVEALQSGGGQERGMRSQTVPTPLVVGLGAACEVAQQ
T.maritima_Nifs YIRKQ--VPIRPLIHGGGQERGLRSGTQNVPGIVGAARAMEIAVE

Cons          *** : * : . . * * * * * * * * * * * * . * : * : * : * :

Human_Nfs      EMEYDHRKRISKLSERLIQNIMKSLPDVVMNGDPKHHYPGCINLSP
T.maritima_Nifs ELSEAAKHMEKLRSKLVSGLMNLGAHIITPLEIS--LPNTLSVSP

Cons          * : . * : * : * * : : * : . : * : : : * : * : * : * :

Human_Nfs      AYVEGESL--LMALKDVALSSGSACTSASLEPSYVLRAIGTDEDL
T.maritima_Nifs PNIRGSTLQNL LSGYGIYVSTSSACTSKDERLRHVLDAMGVDRRI

Cons          . : : * : * : * : * : * : * : * : * : * : * : * : * :

Human_Nfs      AHSSIRPGIGRFTTEEEVDYTV EKCIQHVKRLREMSPLWEMVQDG
T.maritima_Nifs AQAIRISLCKYNTTEEEVDYFLKK-----IBBILSFLDLTGNN

Cons          * : : * : * : : : * : * : * : * : * : * : * : * : * :

Human_Nfs      IDLKS IKWTQH
T.maritima_Nifs RR-----

Cons          *
    
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Figure S4. The alignment between Tm NifS and human Nfs. The figure is generated by the on-line software T-COFFEE, on ExPasy server. The red represents the best alignment and yellow part represents the good alignment, the blue represents the bad alignment which is not seen here. The 'Cons' stands for the consensus sequence, (*) is the identical sequence and (l) is the positive alignment.

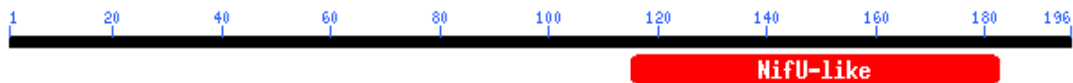


Figure S5. Conserved domain search from the NCBI website. The red indicates the conserved NFU domain present in human full-length NFU. Residue numbers are shown.

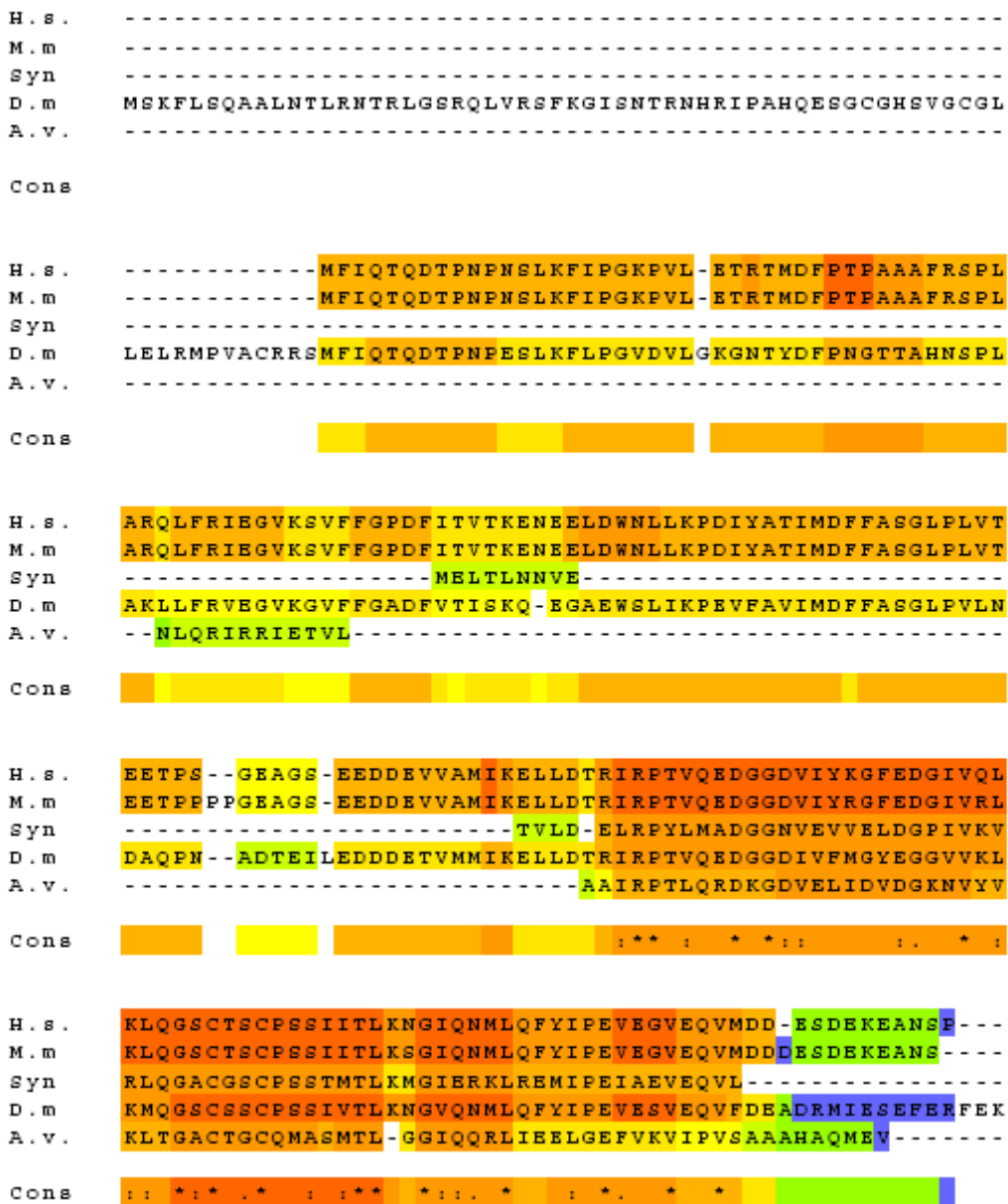


Figure S6. The alignment of NFU-like sequences, showing the C-terminal domain of Av relative to the complete NFU sequences from other organisms. Hs, *Homo sapiens*; Mm, *Mus musculus*; Dm, *Drosophila melanogaster*; Ce, *Caenorhabditis elegans*; Av, *Azotobacter vinelandii*; Sys, *Synechocystis* PCC6803.

References

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