Electronic Supplementary information for:

Turning around the electron flow in an uptake hydrogenase. EPR spectroscopy and *in vivo* activity of a designed mutant in HupSL from *Nostoc punctiforme*

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Contents:

Experimental

C12P f-HupS construct

The plasmid vector pET431HupS[1] was used as a template for site-directed mutagenesis using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene Cloning Systems, CA) and the primers FWC12P and RVC12P (all primer sequences are listed in Table S1; Fig. S2, A), according to the manufacturer's instructions. The resulting mutant plasmid, pET431HupS_C12P was transformed into DH5α cells and plated on LB-agar plates containing 50 μg mL^{-1} ampicillin. Colonies were cultivated overnight at 37°C in 5 mL LB containing 50 μg mL^{-1} ampicillin each, and plasmid DNA was purified using the GeneJET Plasmid Miniprep Kit (Fermentas). The mutation was confirmed by sequencing.

Cloning and plasmid construction for knockout mutant in Nostoc punctiforme

Genomic DNA of *Nostoc punctiforme* ATCC 29133 (WT *N. punctiforme*; a complete list of *N. punctiforme* strains used in this work is listed in Table 1, main manuscript) was isolated as previously described.[2] PCR amplifications were carried out using the high fidelity DNA polymerase Phusion (Finnzymes) creating PCR fragments of 1.2 kb from the *hupS* upstream region using primers fUpst_HupS and rUpst_HupS, and 1.4 kb from the *hupL* downstream region using primers fDown_HupL and rDown_HupL (Table S1). The two PCR fragments were fused through overlapping PCR and the resulting 2.6 kb DNA fragment was cloned in pRL278 plasmid in the SpeI and BglII sites creating the vector pRL278HupSLΔ (Fig. S1, A). The erythromycin resistance gene was introduced in the SmaI site of pRL278HupSLΔ creating the vector pRL278HupSLΔEm (Fig. S1, A), which was transformed in WT *N. punctiforme* through conjugation.[3] Transformants were selected by screening for resistance to erythromycin (Fig. S1, B). The fully segregated Δ*hupSL* mutant was denominated ΔHupNp. Isolated genomic DNA from both WT and ΔHupNp cells was extracted and used for PCR amplification of the *hupSL* region using primers HupSf and HupLr (Table S1) in order to monitor the segregation of the Δ*hupSL* mutation. The PCR products were verified by agarose gel electrophoresis (Fig. S1, B).

hupSL *genes complementation in* Nostoc punctiforme

PCR amplification was performed using primers HupOf and HupOr (Table S1) creating a fragment of 3.4 kb carrying the *hupSL* operon. This fragment was then cloned in the ClaI and PstI restriction sites in the self-replicative vector pSUN119,[4] making the pSUN119HupSL

vector (Fig. S1, C). Vector pSUN119HupSL carrying either a wild-type version of *hupS* or the C12P point mutation (performed as for pET431HupS) was re-introduced in ΔHupNp through electroporation. The resulting strains were HupNp, containing wild-type *hupSL*; and C12PNp, containing the *hupS* C12P mutant (Table 1, main manuscript).

Tables and Figures

Table S1 – List of primers used in this work.

Primer name	Sequence
fUpst_HupS	5'-CCGACTAGTTTCGTCATAAGCAGCGATCGCACG-3'
rUpst_HupS	5'-CAATCCTCCCGGGAGCCTGTCTTGTACGTTAAGAAATATCAGCACTAGCC-3'
fDown_HupL	5'-AGGCTCCCGGGAGGATTGTTTATGAATTAGTGTAGACGTGTAGTGG-3'
rDown_HupL	5'-GGCAGATCTAGGAAATGAGGATGAAGGAACGCGAG-3'
HupSf	5'-GGAATTCCCGCCATGTCACCCAACCCCAG-3'
HupLr	5'-CCACCCAGTGAGATAAAGAACCCCGCGC-3'
HupOf	5'-CTGCCCGGGCTAGTGCTGATATTTCTTAACG-3'
HupOr	5'-GCGCTGAGCAGTTATCAGTTTCATCATTACC-3'
FWC12P	5'-GCTACAAGGTGGTGCTCCTTCAGGCAACACCATG-3'
RVC12P	5'-CATGGTGTTGCCTGAAGGAGCACCACCTTGTAGC-3'

Table S2 – HupS and HupL detected by mass spectrometry in samples from WT *N. punctiforme* and in the complemented HupNp and C12PNp strains. Overexpression of both proteins in the two latter strains is demonstrated by markedly increased values of the identification scores, amounts of detected peptides, precursor ion intensities and spectral counts.

	HupL			**HupS**		
Strain	WT	HupNp	C12PNp	WT	HupNp	C12PNp
Protein ID score	300	1011	1268	47	217	203
Amount of detected peptides	7	28	31	2	9	6
Area (sum of peptide intensities)	2e6	1.1e8	1.9e8	1.6e5	1.3e6	1e6
Sum of peptide spectral counts	7	63	79	2	10	6

Table S3 – Hydrogen production, hydrogen uptake and nitrogenase activities in *N. punctiforme* strains, with respective standard errors of the mean.

	Hydrogen production activity nmol H_2 (µg Chl-*a*)$^{-1}$ h^{-1}			**Hydrogen uptake activity** 10^{-4} nmol H_2 (µg Chl-*a*)$^{-1}$ h^{-1}			**Nitrogenase activity** 10^{-2} nmol C_2H_2 (µg Chl-*a*)$^{-1}$ h^{-1}		
Strain	**Day 2**	**Day 4**	**Day 6**	**Day 2**	**Day 4**	**Day 6**	**Day 2**	**Day 4**	**Day 6**
ΔHupNp	1.12±0.12	1.34±0.14	0.86±0.04	0	0	0	1.65±0.06	1.78±0.01	0.94±0.04
HupNp	0.06±0.02	0.98±0.10	0.39±0.05	0.81±0.17	4.51±0.16	2.18±0.19	1.86±0.07	2.55±0.17	0.86±0.02
C12PNp	1.18±0.24	1.23±0.08	1.33±0.08	0	0	0	2.13±0.04	1.99±0.03	1.34±0.06

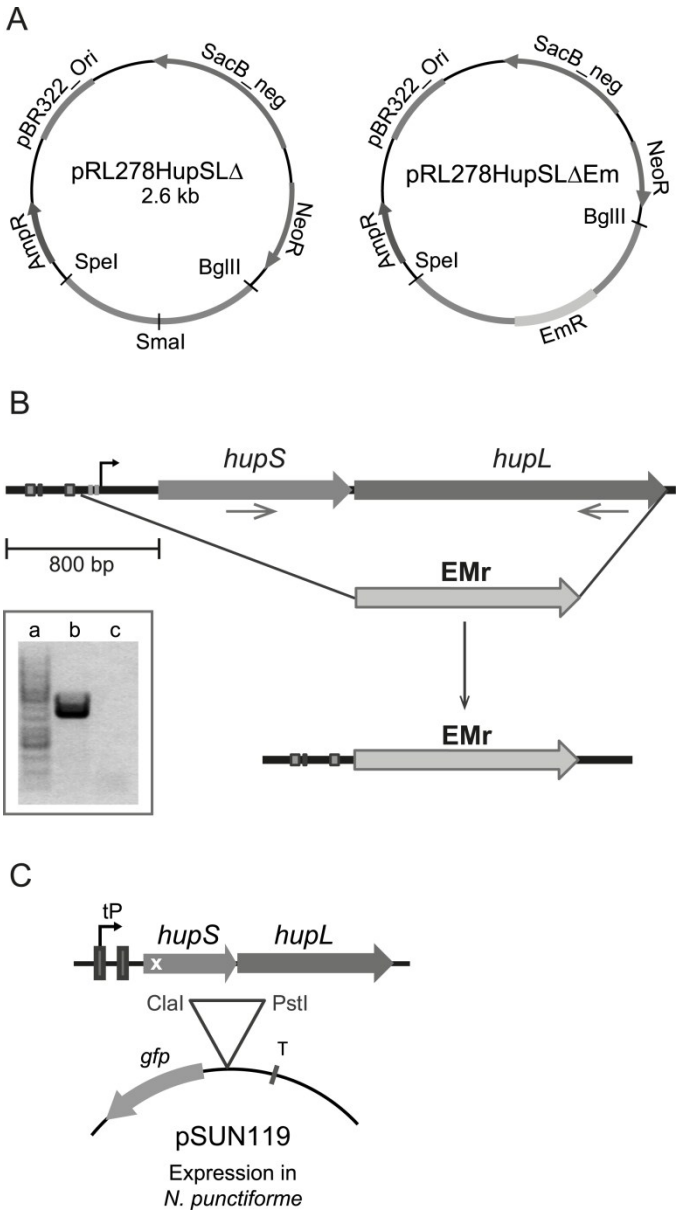

Fig. S1 - Construction of engineered *N. punctiforme* strains (see Table 1 for complete list). (A) Plasmids for constructing the ΔHupNp strain. pBR322_Ori: origin of replication (recognized only in *E.coli*). SacB_neg: for expression of the gene *sacB* that is lethal to the cyanobacteria in the presence of sucrose. AmpR: ampicillin resistance cassette. NeoR: neomycin resistance cassette. EmR: erythromycin resistance cassette. (B) The region containing the uptake hydrogenase structural genes *hupS* and *hupL* in *N. punctiforme* was completely replaced by an erythromycin cassette. Angled arrow denotes transcription start. Grey arrows show primer binding sites (primers HupSf and HupLr) used for PCR shown in inset. *Inset*: agarose gel electrophoresis of PCR demonstrating full knock-out segregation. *Lane a:* 1 kb DNA ladder, *lane b:* WT *N. punctiforme*, *lane c:* ΔHupNp. (C) A fragment containing the *hupSL* structural genes, as well as a heterocyst-specific promoter, was cloned into the plasmid vector pSUN119 for expression of HupSL and C12P HupSL (after site-directed mutagenesis) in ΔHupNp. The white cross denotes the location of the point mutation.

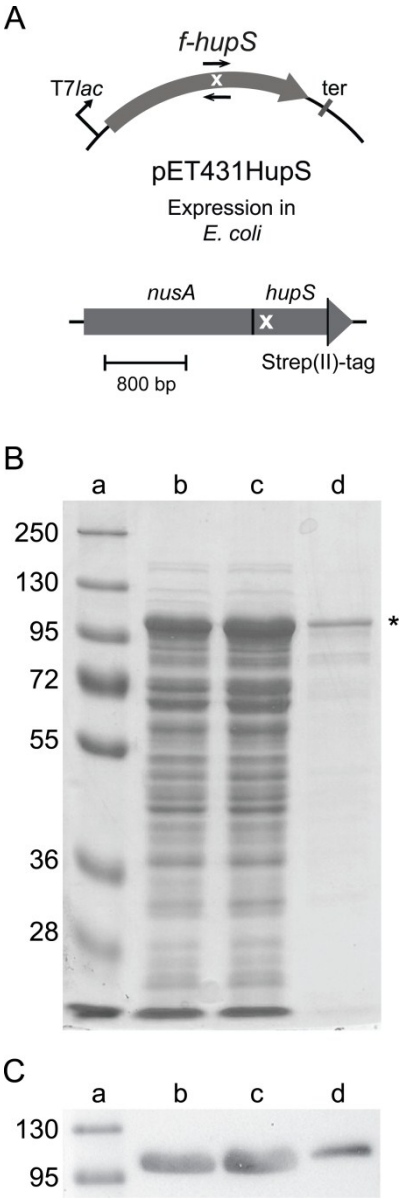

Fig. S2 – Expression of C12P f-HupS in *E. coli*. (A) *Top:* The pET431HupS plasmid vector was a target for site-directed mutagenesis for expression of C12P f-HupS in *E. coli* BL21(DE3). *Bottom:* A detailed scheme of the organization of the NusA-HupS-Strep(II)-tag fusion construct. The white cross denotes the location of the point mutation. (B) 10% SDS-PAGE, after staining with PageBlue. *Lane a*: molecular weight markers (size shown in kDa). *Lane b*: soluble cell extract from BL21(DE3) expressing WT f-HupS. *Lane c*: soluble cell extract from BL21(DE3) expressing C12P f-HupS. *Lane d*: C12P f-HupS after purification. *Asterisk* indicates the band corresponding to WT f-HupS or C12P f-HupS. (C) Western blot after chemiluminescence detection with HRP-conjugated Strep-Tactin. Lane contents are as in panel B; lanes b and c contain 50 µg total protein each, lane d contains 5 µg total protein. Note that the expected molecular weight for the f-HupS fusion protein is 97.4 kDa, from the sum of the molecular weights of the Nus-tag (60.4 kDa), the HupS protein (34.9 kDa), and the Strep(II)-tag and linker peptide (2.1 kDa).

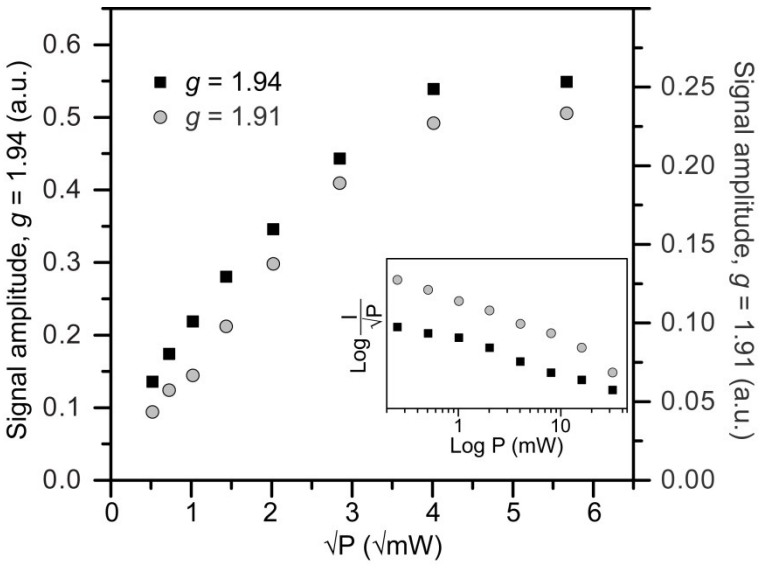

Fig. S3 – Applied microwave power dependence of g = 1.94 (*black squares*) and 1.91 (*gray circles*) resonances in reduced C12P f-HupS at T = 10 K. *Inset*: power dependence normalized to spectral intensity, plotted as described by Rupp *et al.*[5]

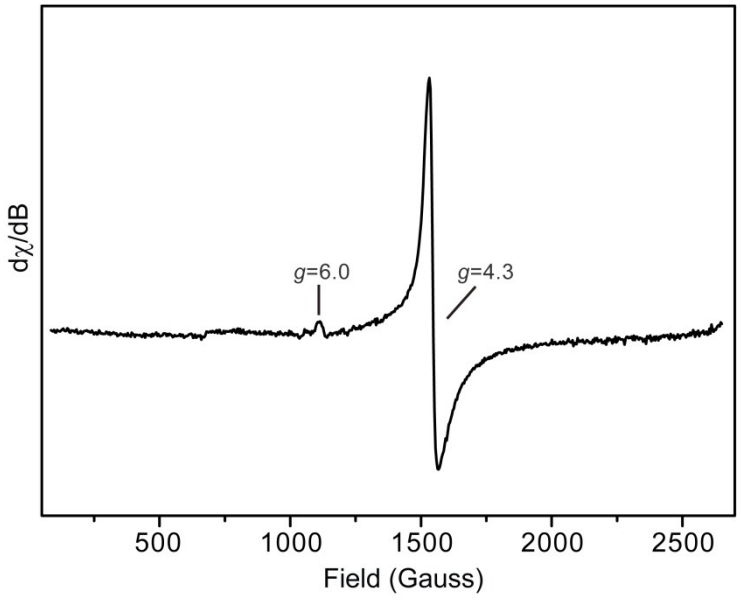

Fig. S4 – Low-field spectrum of C12P f-HupS. Indicated g values are characteristic of high-spin (S =5/2) Fe(III) and are not thought to be specifically-bound to the protein.

References

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