SI Appendix

Energy conservation by a hydrogenase-dependent chemiosmotic mechanism in an ancient metabolic pathway

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Supplementary Materials and Methods

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Supplementary References

Organism and cultivation

T. kivui LKT-1 (DSM 2030) was routinely cultivated on complex medium at 60°C. The medium was prepared using the anaerobic techniques described previously (1, 2). Cultivation of *T. kivui* was carried out in 1-l-flasks (Müller-Krempel) containing 200 ml complex medium for cultivation on CO + CO_2 (50% CO, 10% CO_2, 40% N_2 at 2.0 × 10^5 Pa) or H_2 + CO_2 (50% H_2, 20% CO_2, 30% N_2 at 1.5 × 10^5 Pa), or in 1-l-flasks (Müller-Krempel) containing 500 ml complex medium for cultivation on 25 mM glucose + CO_2 (20% CO_2, 80% N_2 at 1.3 × 10^5 Pa). Complex medium contained: 50 mM NaH_2PO_4, 50 mM NaH_2HPO_4, 1.2 mM K_2HPO_4, 1.6 mM KH_2PO_4, 4.7 mM NH_4Cl, 1.7 mM $(NH_4)_2SO_4$, 7.5 mM NaCl, 0.37 mM $MgSO_4$, 42 µM $CaCl_2$, 7.2 µM Fe(II)SO_4, 54 mM $KHCO_3$, 3 mM cysteine-HCl, 0.2% [w/v] yeast extract, 1.0% [v/v] trace element solution (DSM 141), 1.0% [v/v] vitamin solution (DSM 141) and 4 µM resazurin. Growth was determined by measuring the optical density at 600 nm (OD_{600}).

Preparation and experiments with resting cells

T. kivui was cultivated on CO + CO_2, H_2 + CO_2 or glucose and the preparation of resting cells was performed under strictly anoxic conditions in an anaerobic chamber (Coy Laboratory Products). Cells were harvested by centrifugation (11,500 × g, 10 min, 4°C) at OD_{600} 0.2, 0.4 or 1.8 – 2.2, washed with resting cell buffer (50 mM imidazole (pH 7.0) containing 50 mM $KHCO_3$, 20 mM $MgSO_4$, 20 mM KCl, 20 mM NaCl, 4 mM DTE and 4 µM resazurin), resuspended in 1 ml and kept in a gas-tight Hungate tube under a 100% N_2 atmosphere. The protein concentration was determined according to (3). The experiments with resting cells were carried out in crimped sealed 120-ml serum bottles or 8-ml-glass vials at a final liquid volume of 1 or 20 ml. The assays were composed of 1 ml H_2 evolution buffer I (50 mM Tris buffer (pH 7.0) containing 4 mM DTE and 4 µM resazurin) containing 1 mg/ml protein with 100% N_2 for H_2 evolution (Fig. 1*A*), 20 ml resting cell buffer containing 1 mg/ml protein with N_2+CO_2 (80:20 [v/v]) for ATP measurements (Fig. 1*B*) or 1 ml H_2 evolution buffer II (I + 5 mM NaCl) containing 0.5 mg/ml protein with 100% N_2 for H_2 evolution with uncouplers (Fig. 1*C*). The

assays were preheated to a temperature of 60°C for 10 min in a water bath and the reaction was started by flushing the vials with 100% CO for 20 s. 50 µl gaseous samples were withdrawn and directly injected into the gas chromatograph (Fig. 1*A, C*). The intracellular ATP content was detected (Fig. 1*B*) as described previously (4).

To analyse the effect of Na^+, CO-pregrown cells were incubated in 8-ml-glass vials containing a 100% N_2 gas atmosphere and 1 ml H_2 evolution buffer I (50 mM Tris buffer (pH 7.0) containing 2 mM DTE and 4 µM resazurin (*SI Appendix,* Fig. S2) or 10 mM MES/MOPS/Tris buffer (pH 6.0 (●), 7.0 (○) or 8.0 (▲)) containing 2 mM DTE and 4 µM resazurin (*SI Appendix,* Fig. S3). NaCl (■) or KCl (□) was added as indicated. The assays were treated as described above.

Determination of H_2, CO and acetate

The concentration of H_2, CO and acetate was determined as described previously (4).

Preparation of inverted membrane vesicles

T. kivui was grown on CO as described above, harvested by centrifugation (14,300 × g, 15 min, 4°C) and washed in vesicle buffer (60 mM KP_i buffer (pH 6.0) containing 420 mM sucrose, 20 mM $MgSO_4$, 8 mM DTE and 4 µM resazurin). For the cell disruption, the cells were suspended in the same buffer, supplemented with 100 µM PMSF and a few crystals of DNase. Cells were passed through a French pressure cell at 41 MPa, which resulted in the formation of crude vesicles. The remaining unbroken cells as well as cell debris were removed by centrifugation (6,250 × g, 45 min, 4°C). This step was repeated twice. The supernatant was then ultracentrifuged at 208,000 × g for 45 min at 4°C to collect IMVs. When indicated, the IMVs were washed, and finally resuspended in buffer (depending on the subsequent experiment) to a protein concentration of 3.0-5.0 mg/ml. The protein concentration was determined as described previously (5).

To determine whether the IMVs are bioenergetically coupled, energization with an artificial ammonium diffusion potential was tested. For this, a small portion of the IMV preparation was

resuspended in aerobic 500 mM NH_4Cl-containing buffer (10 mM Tris buffer (pH 8.0) containing 500 mM NH_4Cl, 420 mM sucrose and 5 mM $MgCl_2$). Next, 5 µl of IMVs were diluted in 500 µl buffer containing no NH_4Cl (10 mM Tris buffer (pH 8.0) containing 500 mM choline chloride, 420 mM sucrose and 5 mM $MgCl_2$) and 4 µM ACMA (from a 2 mM ethanolic stock solution). Fluorescence was measured in a fluorescence cell (High Precision Cell, 3 × 3 mm light path; Hellma Analytics) in a fluorescence spectrophotometer (F-4500; Hitachi) with an excitation at 410 nm and emission at 490 nm.

Measurement of Ech activity at IMVs

Ech activity was investigated by measuring H_2 evolution using CO, or the artificial electron donors sodium dithionite or titanium (III) citrate as reductant. The assays were carried out in crimp sealed 8-ml-glass vials at a final liquid volume of 1 ml. The vials were filled with IMV buffer (50 mM MES buffer (pH 6.0) containing 420 mM sucrose, 10 mM $MgCl_2$, 8 mM DTE and 4 µM resazurin with or without 5 mM NaCl and with 100 mM KCl where indicated) in a 100% N_2 gas atmosphere. IMVs were added, supplemented with ferredoxin as indicated and, if applicable, 20 µM K^+ ionophore valinomycin or 30 µM protonophore TCS or Na^+ ionophore ETH2120. The assays were preheated to 60°C in a water bath for 10 min and the reaction was started by flushing the vials with 100% CO for 20 s, or by the addition of 1.5 mM sodium dithionite or 1 mM titanium (III) citrate. In control experiments, the uncouplers were substituted with solvents (DMSO for valinomycin or ethanol for TCS and ETH2120). 50 µl gaseous samples were continuously withdrawn and directly injected into the gas chromatograph.

Measurement of $^{22}Na^+$ translocation

The experiments were performed under anaerobic conditions in rubber stopper-sealed 3.5-ml-glass vials at a final liquid volume of 500 µl. 0.5 mg/ml IMVs were incubated in IMV buffer with contaminating Na^+ concentration of 280 µM, as determined by an Orion Star A214 sodium electrode (Thermo Fisher Scientific). The assay was supplemented with 30 µM ferredoxin and, when applicable, 20 µM ETH2120 or 20 µM TCS. $^{22}NaCl$ (final activity 1 µCi/ml carrier-free)

was added and incubated for 30 min to ensure Na^+ equilibrium before the reaction was started. After 4 min, 1 mM titanium (III) citrate was added or the vial was flushed with 100% CO for 10 s. Control experiments received ethanol instead of the uncoupler. The samples were taken and radioactivity was measured as described previously (6).

Measurement of H^+ translocation

The experiments were performed at 40 or 60°C in a fluorescence cell (High Precision Cell, 3×3 mm light path; Hellma Analytics) at a final liquid volume of 250 µl with a 100% CO gas atmosphere. 0.5 mg/ml IMVs were incubated in IMV buffer with a contaminating Na^+ concentration of 280 µM in the presence of 4 µM ACMA. 20 µM protonophore TCS or 20 µM sodium ionophore ETH2120 and 150 mM NaCl were additionally added where indicated. After equilibration, 30 µg CODH (purified from *A. woodii* according to (7)) and 30 µM ferredoxin were added as indicated. The quench was abolished by addition of 10 µl of 90% $(NH_4)_2SO_4$. Fluorescence was measured in a fluorescence spectrophotometer (Fluorolog-3, HORIBA Scientific) with excitation at 410 nm and emission at 490 nm.

Determination of relative transcript levels

To analyze gene expression of *ech1* and *ech2*, cultures *of T. kivui* were grown on CO+CO_2, H_2+CO_2 or glucose and harvested by centrifugation (10,000 × g, 10 min, 4°C) at OD_{600} 0.3, 0.4 or 0.8, frozen in liquid nitrogen and stored at - 80°C until RNA extraction. Total RNA was isolated using the InviTrap Spin Cell RNA Mini kit (Stratec Molecular) with some modifications. After lysozyme treatment, the lysis solution was added to the cells where they were fully lysed in a cell disrupter (Retsch) at 30 Hz for 5 min. The cell debris was separated by centrifugation (14,000 × g, 10 min, 4°C) and the supernatant was used as described in the kit protocol. 10 µg nucleic acids were used for DNaseI treatment (Promega) in the presence of Rnasin (Promega). The nucleic acids were precipitated using 2.5 volumes 100% ethanol and 1/10 volume 3 M sodium acetate (pH 6.5). Subsequently, the sample was and incubated at - 20°C for 1 h and centrifuged (11,000 × g, 30 min, 4°C). The precipitate was washed in 500 µl ice-

cold 70% ethanol and centrifuged (11,000 × g, 30 min, 4°C). The supernatant was discarded and the pellet was air-dried and then solved in 20 µl RNase-free MQ water. The RNA was checked using an agarose gel (1% (w/v) in 1×TBE buffer). Afterwards, 1 µg RNA was used for reverse transcription using M-MLV Reverse Transcriptase according to the manufacturer's protocol (Promega). All quantitative PCRs (qPCRs) were performed in triplicate, using SYBR Green qPCR Kits (2×without ROX) (Thermo Fisher Scientific). Expression of the *ech* clusters was analyzed using primers raised against the first genes of each cluster, *ech1A* and *ech2D*. The two genes encoding the gyrase subunit B (*gyrB*) and the aconitase (*acn*) were used as housekeeping genes and changes in mRNA expression levels were calculated according to (8) and (9). Primers used for qPCR are listed in Tab. S2. The relative mRNA levels were calculated from two biological independent experiments.

Supplementary Figures

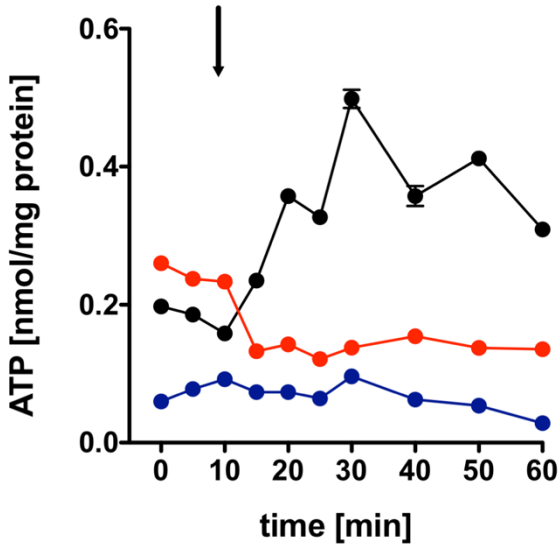

Fig. S1. ATP synthesis is inhibited by ionophores in resting cells. *T. kivui* cells were harvested from CO-grown cultures in the exponential growth phase, washed and resuspended to a protein concentration of 1 mg/ml in 20 ml of buffer (50 mM imidazole (pH 7.0) containing 50 mM $KHCO_3$, 20 mM $MgSO_4$, 20 mM KCl, 20 mM NaCl, 4 mM DTE and 4 µM resazurin) in 120-ml-serum bottles containing an N_2+CO_2 (80:20 [v/v]) gas atmosphere. Cells were preincubated for 10 minutes at 60°C. The assay contained no ionophore (●), 30 µM protonophore TCS (●) or 30 µM sodium ionophore ETH2120 (●). Vials were flushed with CO for 20 seconds, as indicated by the arrow. The ATP content was measured by using the luciferin-luciferase assay (10). Shown is the average of two measurements from one representative out of two experiments. Some of the data was already published (4).

Supplementary Figures

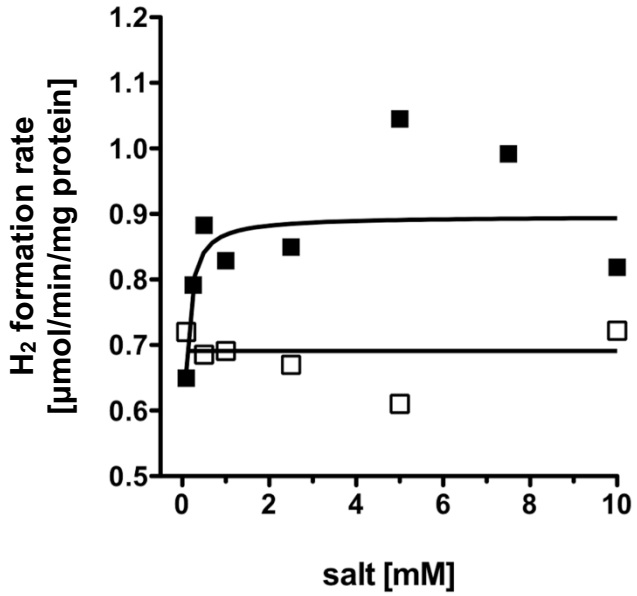

Fig. S2. H_2 formation from CO is stimulated by Na^+. Resting cells (0.5 mg/ml) pregrown on CO evolved H_2 from CO. NaCl (0.25-10 mM; ■) but not equal amounts of KCl (□) stimulated this activity. Curve fitting was performed using GraphPad Prism Version 4.03 and the Michaelis-Menten equation.

Supplementary Figures

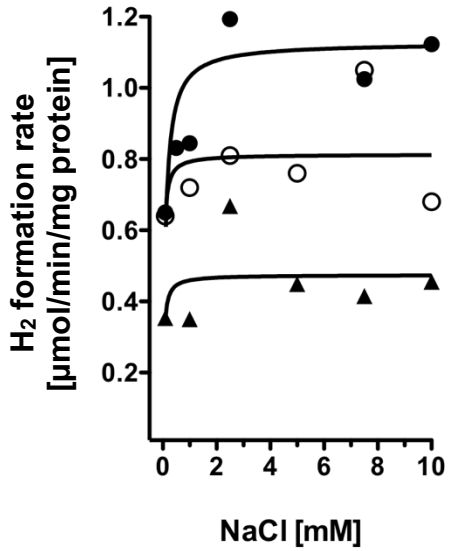

Fig. S3. H_2 formation is stimulated by Na^+ in resting cells. *T. kivui* was grown on CO. Cells were harvested, washed and resuspended to a protein concentration of 0.5 mg/ml in 1 ml of buffer (10 mM MES/MOPS/Tris buffer (pH 6.0 (●), 7.0 (○) or 8.0 (▲)) containing 2 mM DTE and 4 µM resazurin). The assay was carried out in 8-ml-glass vials containing a 100% N_2 gas atmosphere. After an incubation of the cells at 60°C for 15 minutes, the reaction was started by flushing the vial with CO for 20 seconds. The data shown are representative of two experiments using independent preparations. Curve fitting was performed using GraphPad Prism Version 4.03 and the Michaelis-Menten equation.

Supplementary Figures

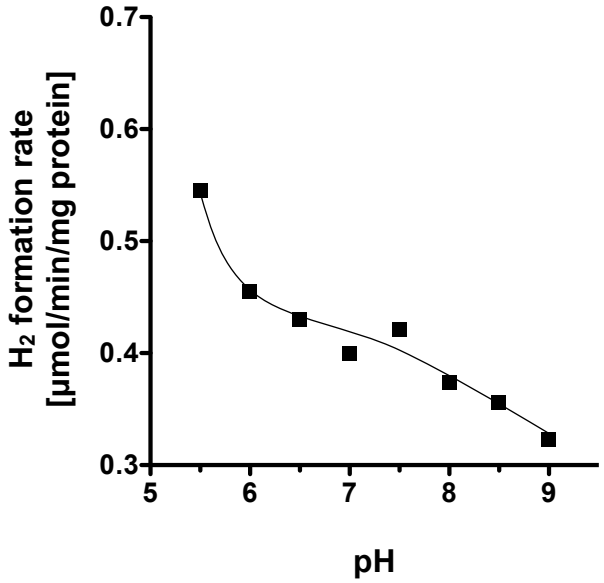

Fig. S4. pH optimum of CO-dependent H_2 evolution in resting cells. *T. kivui* was grown on CO. Cells were harvested in the exponential growth phase, washed and resuspended to a protein concentration of 1 mg/ml in 1 ml of buffer (10 mM MES/MOPS/Tris-HCl, 5 mM NaCl, 2 mM DTE and 4 µM resazurin, pH 5.5-9.0). The assay was carried out in 8-ml-glass vials with a 100% N_2 gas atmosphere. After an incubation of the cells at 60°C for 15 minutes, the reaction was started by flushing the vial with CO for 20 seconds. Shown is the average of two measurements from one representative experiment out of two independent replicates.

Supplementary Figures

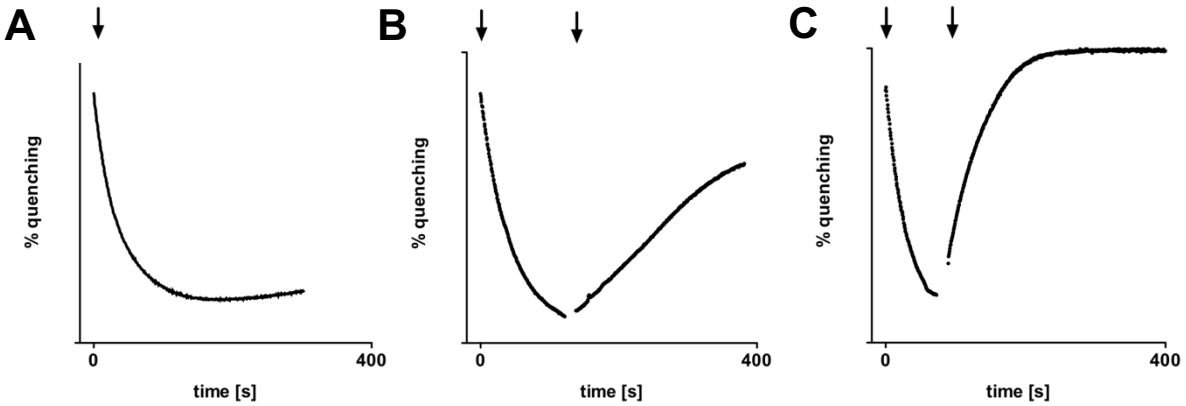

Fig. S5. Establishment of an artificial pH gradient in IMVs. The artificial pH gradient was formed upon addition (first arrow) of 20 µg washed IMVs loaded with 500 mM NH_4Cl to a final liquid volume of 500 µl composed of 10 mM Tris buffer (pH 8.0) containing 420 mM sucrose, 500 mM choline chloride, 5 mM $MgCl_2$ and 4 µM ACMA in a fluorescence cell under aerobic conditions at RT. The quench was maintained for 5 minutes (*A*) or could be relieved (second arrow) by addition of 30 µM TCS (*B*) or 20 µl 1-butanol (*C*). Fluorescence was measured in a fluorescence spectrophotometer with an excitation of 410 nm and an emission of 490 nm.

Supplementary Figures

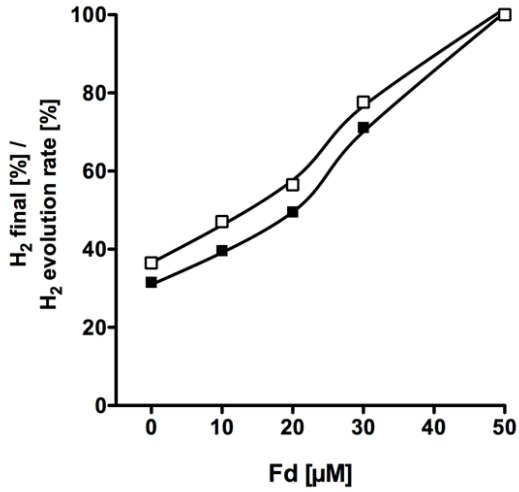

Fig. S6. Stimulation of CO-dependent H_2 evolution by ferredoxin. 60 µg washed IMVs were incubated in an 8-ml-glass vial containing buffer (60 mM KP_i (pH 6.0) containing 420 mM sucrose, 20 mM $MgSO_4$, 8 mM DTE and 4 µM resazurin) at an overall liquid volume of 1 ml under a 100% N_2 gas atmosphere at 60°C. The assays were supplemented with 0-50 µM ferredoxin. The reaction was started by flushing the assay with CO for 20 seconds. The final amount of H_2 evolved (▪) and the H_2 evolution rate (□) is plotted in % against the ferredoxin concentration added to the assay.

Supplementary Figures

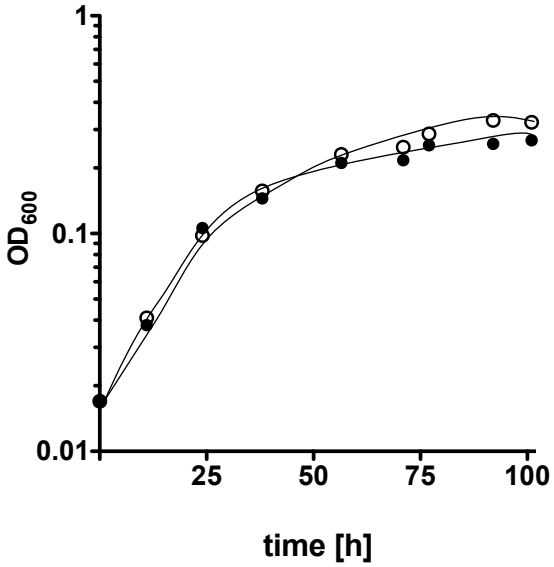

Fig. S7. Effect of Na^+ on autotrophic growth with CO. *T. kivui* was cultivated on CO (50% CO, 40% N_2 and 10% CO_2 at 2.0 × 10^5 Pa) in 20 ml Na^+ enriched (●; 56 mM NaCl) or Na^+-deprived (○; 56 mM KCl) minimal medium. Precultures were grown for three transfers in the same medium. Two biological duplicates were analysed and one representative growth curve is depicted.

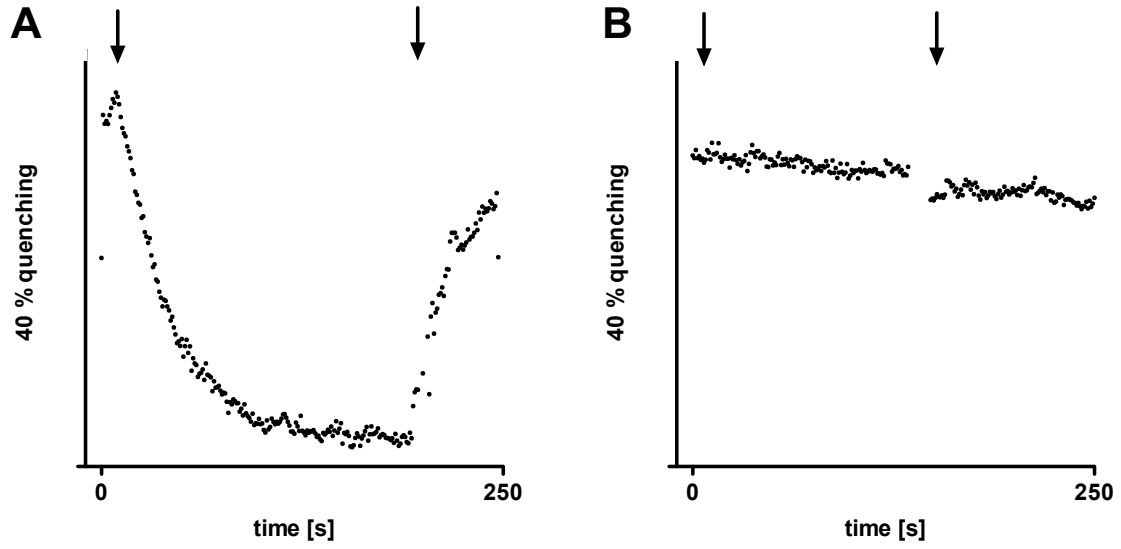

Fig. S8. ATP-driven H^+ transport into IMVs. 125 µg (*A*) or no (*B*) washed IMVs were incubated in 250 µl buffer (50 mM MES buffer (pH 6.0) containing 420 mM sucrose, 10 mM $MgCl_2$, 8 mM DTE, 281 µM NaCl and 4 µM resazurin), with 4 µM ACMA, in a fluorescence cuvette under aerobic conditions at 60°C. The reaction was started by addition of 2 mM MgATP, as indicated by the arrow. The quench was abolished by addition of 10 µl 90% $(NH_4)_2SO_4$, as indicated by the second arrow. The measurement was carried out at an excitation- and emission wavelength of 410 nm and 490 nm, respectively. The data shown are representative of two experiments using independent IMV preparations.

Supplementary Tables

Tab. S1. Comparison of Ech subunits from *T. kivui* with corresponding subunits of other related complexes.

subunit	cofactor/ TMH	proposed function	*E. coli*			*M. barkeri*		*C. h.*		*P. furisosus*	
Ech1			**Nuo**	**Hyc**	**Hyf**	**Ech**	**Fpo**	**Coo**	**Nuo**	**Mbh**	**Mbx**
1A	19 TMHs	anchor, ion transport	M/L 26/28	C 32	B/C 32/30	A* 26	M/L/N 30/29/28	M 29	L 28	H 25	H'/H 30/26
1B	8 TMHs	anchor, ion transport	H 29	C/D 32/27	-	B 27	H 29	K 46	H 28	M 33	M 32
1C	1[Fe-S]	small hyd, e^- transfer	B 32	G 37	I 34	C 45	B 39	L 57	B 37	J 51	J 33
1D	-	stability	-	-	-	-	-	U 29*	-	-	-
1E	[NiFe]	large hyd, H_2 conversion	C 28	E 35	G 35	E 40	D 38	H 52	D 36	L 39	L 32
1F	2[Fe-S]	e^- input	n.r.	F 29	H 28	F 32*	I 31*	X 33	I 34*	N 36	N 37
HycB1	4[Fe-S]	e^- transfer	n.r.	n.r.	n.r.	-	-	F 36*	n.r.	n.r.	n.r.
1280	1[Fe-S]	e^- transfer	-	-	-	-	-	-	-	-	-
1290	1[Fe-S]	e^- transfer	-	-	-	-	-	-	-	-	-
Ech2			**Nuo**	**Hyc**	**Hyf**	**Ech**	**Fpo**	**Coo**	**Nuo**	**Mbh**	**Mbx**
2A1	15 TMHs	anchor, ion transport	L 33	C 30	D/B 39/28	A 32	L/M 34/27	M 31	L 35	H 30	H'/H 25/28
2A2	10 TMHs	anchor, ion transport	M 25*	D 28*	F 30*	A 26*	M 27*	M 26*	N 30*	H 30	H'/H 29/28
2B	7 TMHs	anchor, ion transport	M 25*	C 25*	D/F 27*/30*	A 26	M 27	M 26*	N 30*	H 30*	H'/H 29/26
2C	1[Fe-S]	small hyd, e^- transfer	-	C/D 29*/27*	-	B 28*	-	K 34	H 22*	H 30*	M 30
2D	-	stability	-	-	-	-	-	-	-	-	-
2E	[NiFe]	large hyd, H_2 conversion	C 29	E 39	G 36	E 37	D 38	H 45	D 36	L 38	L 33
2F	2[Fe-S]	e^- input	I 31*	F 28*	F 24*	F 33*	I 33*	X 28*	I 37*	N 33*	N 41
HycB2	4[Fe-S]	e^- transfer	n.r.	n.r.	n.r.	n.r.	n.r.	F 34*	n.r.	n.r.	n.r.

*Cofactors and TMHs were determined using InterProScan and TMHMM Server v. 2.0. BLASTP 2.2.26+ was used for homology analyses. The numbers reflect the amino acid identities in %. Genomic data was retrieved for *E. coli* K-12 MG1655, *M. barkeri* Fusaro DSM 804, *C. hydrogenoformans* Z-2901 (C. h.), *P. furiosus* DSM 3638 from IMG/M ER. E-value > 1e-5; (*); E-values 1e-5 >< 1e-20; (n.r.), hits not related to corresponding complex; (-), no hits; hyd, [NiFe] hydrogenase.

Supplementary Tables

Tab. S2. List of primers used in this study.

Primer name	**5'→3' sequence**
acn_for_qRT T.k.	GCTTGTGGACCTTGTATTGG
acn_rev_qRT T.k.	AAACTGACGCTGACTTGG
ech1A_for_qRT T.k.	CCTCCTTTGCCGGTGTAATGAGTAAGG
ech1A_rev_qRT T.k.	AAGCATGGTAAACGCACCCAAC
ech2D_for_qRT T.k.	CAATTGAAGCCTGAGATGTC
ech2D_rev_qRT T.k.	AGCAGAATGGGCAGAAAG
gyrB_for_qRT T.k.	CCAGTTGTGCTTCCTTCTCGATTTCC
gyrB_rev_qRT T.k.	GCGACAATGCCATCTATGACTTCTCC

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