A genome-guided analysis of energy conservation in the thermophilic, cytochrome-free acetogenic bacterium *Thermoanaerobacter kivui*

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**Abstract**

**Background:** Acetogenic bacteria are able to use CO₂ as terminal electron acceptor of an anaerobic respiration, thereby producing acetate with electrons coming from H₂. Due to this feature, acetogens came into focus as platforms to produce biocommodities from waste gases such as H₂ + CO₂ and/or CO. A prerequisite for metabolic engineering is a detailed understanding of the mechanisms of ATP synthesis and electron-transfer reactions to ensure redox homeostasis. Acetogenesis involves the reduction of CO₂ to acetate via soluble enzymes and is coupled to energy conservation by a chemiosmotic mechanism. The membrane-bound module, acting as an ion pump, was of special interest for decades and recently, an Rnf complex was shown to couple electron flow from reduced ferredoxin to NAD⁺ with the export of Na⁺ in *Acetobacterium woodii*. However, not all acetogens have *rnf* genes in their genome. In order to gain further insights into energy conservation of non-Rnf-containing, thermophilic acetogens, we sequenced the genome of *Thermoanaerobacter kivui*.

**Results:** The genome of *Thermoanaerobacter kivui* comprises 2.9 Mbp with a G + C content of 35% and 2,378 protein encoding orfs. Neither autotrophic growth nor acetate formation from H₂ + CO₂ was dependent on Na⁺ and acetate formation was inhibited by a protonophore, indicating that H⁺ is used as coupling ion for primary bioenergetics. This is consistent with the finding that the c subunit of the F₁F₀ ATP synthase does not have the conserved Na⁺ binding motif. A search for potential H⁺-translocating, membrane-bound protein complexes revealed genes potentially encoding two different proton-reducing, energy-conserving hydrogenases (Ech).

**Conclusions:** The thermophilic acetogen *T. kivui* does not use Na⁺ but H⁺ for chemiosmotic ATP synthesis. It does not contain cytochromes and the electrochemical proton gradient is most likely established by an energy-conserving hydrogenase (Ech). Its thermophilic nature and the efficient conversion of H₂ + CO₂ make *T. kivui* an interesting acetogen to be used for the production of biocommodities in industrial microbiology. Furthermore, our experimental data as well as the increasing number of sequenced genomes of acetogenic bacteria supported the new classification of acetogens into two groups: Rnf- and Ech-containing acetogens.

**Keywords:** Acetogen, *Thermoanaerobacter kivui*, Energy conservation, Genome sequence, H⁺ transport, Ech

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Background

Acetogenic bacteria represent an ecologically important group of anaerobes that are ubiquitous in nature [1]. They constitute the penultimate limb in the anaerobic food web and convert a number of substrates exclusively to acetate, which is then converted by methanogenic archaea to methane, the final product of anaerobic food webs [2]. Acetogens convert hexoses to three moles of acetate according to:

$$\text{1 C}_6\text{H}_12\text{O}_6 \rightarrow 3 \text{CH}_3\text{COOH} \quad (1)$$

indicating the presence of a pathway that is able to reduce two moles of CO$_2$ to acetate [3]. This pathway has been elucidated mainly in the thermophilic species *Moorella thermoacetica* (formerly *Clostridium thermoaceticum*) and is named according to its discoverers as the Wood-Ljungdahl pathway (WLP) [4]. It involves two branches and each of them contributes to the reduction of one molecule of CO$_2$. In the carbonyl branch, CO$_2$ is reduced to enzyme-bound CO by the CO dehydrogenase/acytetyl-CoA synthase (CODH/ACS). Electrons for this reduction are provided by reduced ferredoxin [5-7]. In the methyl branch of the WLP, another molecule CO$_2$ is reduced first to formate, which is then activated to formyl-THF in an ATP-dependent reaction [8,9]. Subsequently, formyl-THF is converted to methenyl-THF and then reduced stepwise to methyl-THF via methylene-THF. Eventually, the methyl group and the enzyme-bound CO are joined to form acetyl-CoA, catalyzed by the key enzyme of the WLP, the CODH/ACS [5-7,10-12]. Subsequently, acetyl-CoA is converted to acetyl-phosphate and acetate [13,14]. The latter step involves the production of ATP by an acetate kinase. However, since one mole of ATP is consumed during activation of formate, there is no net ATP gain by substrate level phosphorylation in the WLP. The WLP also enables lithotrophic growth of acetogens on H$_2$ + CO$_2$ according to:

$$\text{4 H}_2 + 2 \text{CO}_2 \rightarrow \text{CH}_3\text{COOH} + 2 \text{H}_2\text{O} \quad (2)$$

and, therefore, additional energy must be conserved by a chemiosmotic mechanism. The chemiosmotic process of energy conservation was recently uncovered in *Acetobacterium woodii*. A soluble, electron bifurcating, ferredoxin- and NAD$^+$- reducing hydrogenase oxidizes molecular hydrogen and generates a reduced electron carrier with a very low redox potential, ferredoxin ($E^\text{pH} \approx -500 \text{mV}$); a redox potential in this range has to be assumed since a ferredoxin is the electron donor for the CO$_2$/CO couple $E^\text{pH} \approx -520 \text{mV}$). Reduced ferredoxin is then oxidized by a membrane-bound ferredoxin:NAD$^+$-oxidoreductase encoded by the *rnf* genes and therefore also termed Rnf complex. The Rnf complex is composed of six subunits that harbor (covalently-bound) flavins and iron sulfur centers as electron carriers, and the electrons are transferred to the acceptor NAD$^+$ [15]. Electron transfer from reduced ferredoxin to NAD$^+$ is exergonic and this electron transfer is used to expel sodium ions from the cytoplasm thus generating an electrochemical sodium ion gradient across the membrane [16]. The electrochemical Na$^+$ gradient then drives the synthesis of ATP via a Na$^+$$\text{F}_1\text{F}_0$ ATP synthase [17,18]. The energy-conserving module in *A. woodii* thus comprises only one coupling site, the Rnf complex, and a Na$^+$$\text{F}_1\text{F}_0$ ATP synthase. Acetogenesis in *A. woodii* therefore has a modular appearance: An energy-conserving module that is connected to the WLP module by soluble electron carriers such as pyridine nucleotides and ferredoxin. The WLP module is not energy conserving, it serves the function to reoxidize the end products of the anaerobic respiration and to provide acetyl-CoA for biomass synthesis [19].

The use of two different modules for energy conservation and re-oxidation of reduced electron carriers (WLP) opens interesting possibilities for biotechnological applications. In principal, any reductive pathway can be coupled to the energy-conserving module and indeed, acetogens are known that reduce nitrate [20,21], phenylacrylates [22,23] and fumarate [24,25], or acetyl-CoA to ethanol [26] or butyrate [27,28]. Very recently, the genetic coupling of a butanol-production pathway to the energy-conserving module proved successful in *Clostridium ljungdahlii* [29].

Production of biocommodities at high temperatures has several advantages such as lower costs for cooling and distillation or lower risk of contaminations. The genome of the thermophilic acetogen *M. thermoacetica* ($T_{\text{opt}}$: 55-60°C) has been sequenced [30] and the way(s) of energy conservation can be predicted from the genome as well as experimental analyses [31]. Unfortunately, this thermophilic model strain grows poorly on H$_2$ + CO$_2$ (doubling times of up to 24 h [32]), a substrate used for 3rd generation biotechnology, thus limiting its use in industrial microbiology. In contrast, the acetogen *Thermoaerobacter kivui* grows very fast on H$_2$ + CO$_2$ with doubling times around 2 h and has an even higher $T_{\text{opt}}$ of 66°C [33]. Moreover, its acetate to biomass ratio was about half compared to *M. thermoacetica* indicating a more efficient way to conserve energy [34]. This is also exemplified by yield measurements: 97% more biomass is produced from one mole of H$_2$ in *T. kivui* compared to *M. thermoacetica* [34]. This prompted us to sequence the genome of *T. kivui* to predict by bioinformatic analyses followed by experimental analyses how this acetogen couples acetogenesis to chemiosmotic energy conservation.

Methods

Growth conditions

*T. kivui* LKT-1 (DSM 2030) was grown at 65°C in medium that was prepared as described by Leigh *et al.*
Preparation of membranes from \textit{T. kivui}

Cells were grown to an optical density at 600 nm of 1.9 – 2.4 as described in 2 × 500 ml complex medium in 1-L flasks (Glasgerätebau Ochs, Bovenden-Lengern, Germany) with 28 mM glucose as carbon source and harvested anaerobically by centrifugation at 11,500 × g for 10 min at 4°C. Cells were washed once in buffer A (50 mM Tris, 20 mM MgSO$_4$, 20% glycerol, 2 mM DTE, 4 μM resazurin, pH 7.5) and, after another centrifugation step, resuspended in 5 – 10 ml buffer A. Cells were disrupted by a single passage through a French press (110 MPa). Cell debris and whole cells were removed by a centrifugation step for 30 minutes at 23,700 × g (150,000 × g, 2 h, 4°C). The cell extract was separated into the cytoplasmic and membrane fraction by ultracentrifugation.

Finally, the membranes were resuspended in ~ 5 ml buffer A and used immediately for the measurement of \( \text{Fdred} : \text{NAD}^+ \) oxidoreductase activity. Protein concentrations were determined as described previously [36].

Measurement of \( \text{Fd}_{\text{red}} : \text{NAD}^+ \) oxidoreductase activity

Measurement of electron transfer from reduced ferredoxin to NAD$^+$ was performed as described [37] at 60°C in anaerobic cuvettes filled with 1 ml 20 mM Tris–HCl buffer (pH 7.7) containing 20 mM NaCl, 2 mM DTE and 4 μM resazurin at a pressure of 0.5 × 10$^5$ Pa CO. Ferredoxin (30 μM; purified from \textit{C. pasteurianum} as described [38]), CODH/ACS (30 μg/ml) purified from \textit{A. woodii} as described [37], and washed membranes (150 μg/ml) were added. The reaction was started by addition of NAD$^+$ (4 mM). Formation of NADH was measured at 340 nm.

Isolation of chromosomal DNA

Chromosomal DNA of \textit{T. kivui} was isolated according to the procedure described [39] and modified by Ausubel et al. [40].

Sequencing strategy

The genome of \textit{T. kivui} was sequenced with a combined approach using the 454 GS-FLX Titanium XL system (Titanium GS70 chemistry, Roche Life Science, Mannheim, Germany) and the MiSeq (Illumina, San Diego, CA). Shotgun libraries were prepared according to the manufacturer’s protocols, resulting in 110,391 reads for 454 shotgun sequencing and 5,916,460 150-bp paired-end reads for Illumina sequencing. All of the 454 shotgun reads and 1 Mio 150-bp paired-end Illumina reads were used for the initial hybrid \textit{de novo} assembly with MIRA 3.4 [41] and Newbler 2.8 (Roche Life Science, Mannheim, Germany). The final assembly contained 42 contigs with an average coverage of 89.20. For scaffolding and contig ordering tasks we used the Move Contigs tool of the Mauve Genome Alignment Software [42] and the genomes of \textit{T. mathranii mathranii} A3, DSM 11426, \textit{T. brockii finii} Ako-1, DSM 3389 and \textit{T. wiegeli} R8.B1 as references. Additionally, contigs that could not be ordered with Mauve were examined \textit{via} Gene Ortholog Neighborhoods based on bidirectional best hits implemented at the IMG-ER (Integrated Microbial Genomes-Expert Review) system [43,44], and with multiplex PCR [45]. Sequence gaps were closed in the Gap4 (v.4.11) software of the Staden Package [46] by PCR-based techniques and primer walking with conventional Sanger sequencing, using BigDye 3.0 chemistry on an ABI3730XL capillary sequencer (Applied Biosystems, Life Technologies GmbH, Darmstadt, Germany).

Gene prediction and annotation analysis

The software tool prodigal (Prokaryotic Dynamic Programming Genefinding Algorithm) [47] was used for automatic gene prediction, while identification of rRNA and tRNA genes was performed withRNAmmer and tRNAscan, respectively [48,49]. Automatic annotation was carried out with the IMG-ER (Integrated Microbial Genomes-Expert Review) system [43,44] and with multiplex PCR [45]. Sequence gaps were closed in the Gap4 (v.4.11) software of the Staden Package [46] by PCR-based techniques and primer walking with conventional Sanger sequencing, using BigDye 3.0 chemistry on an ABI3730XL capillary sequencer (Applied Biosystems, Life Technologies GmbH, Darmstadt, Germany).

Experiments with resting cells and acetate determination

\textit{T. kivui} was grown as described in 2 × 500 ml complex medium with 28 mM glucose as carbon source to an OD$_{600}$ of 1.9 to 2.4. The culture was centrifuged anaerobically at 11,500 × g and 4°C for 10 min. Cells were washed twice in imidazole buffer (50 mM imidazole,
20 mM MgSO₄, 20 mM KCl, 4 mM DTE, 4 μM resazurin, pH 7.0). After the last centrifugation step, cells were resuspended in 2 ml imidazole buffer. The protein concentration was determined according to [52], being approx. 100 mg/ml. In order to determine the conversion of H₂ + CO₂ to acetate, 100-ml-serum flasks (Glasgerätebau Oechs GmbH, Bovenden-Lengler, Germany) were filled with 10 ml imidazole buffer containing 50 mM KHCO₃. If indicated, NaCl was added to a concentration of 20 mM. If applied, the ionophores ETH2120 and TCS were added to a concentration of 30 μM each. Subsequently, the gas phase of the serum flasks was exchanged to 1 bar H₂ + CO₂ (80:20 [v/v]) and then preheated to a temperature of 65°C in a water bath. The reaction was started by addition of resting cells to a final concentration of 1 mg/ml. Henceforth, 500-μl-samples were taken at time points as indicated. Samples were centrifuged immediately at 18,000 × g for 1 min and the supernatant was stored at −20°C. Determination of the acetate concentration of all samples was carried out using a commercially available kit (Acetic acid, Co. R-Biopharm, Darmstadt, Germany).

Nucleotide sequences and accession number
The sequence data described here have been deposited in GenBank under Accession No. CP009170 [GenBank: CP009170].

Results
General features of the T. kivui genome
The complete genome of T. kivui (accession number CP009170) consists of a circular chromosome with a size of 2.397 Mbp and an overall G + C content of 35.06 mol%. General features of the genome are listed in Table 1. We could identify 2378 putative protein-coding genes, three complete rRNA clusters and 58 tRNA genes. The tRNA necessary for incorporation of selenocystein is also present, but this organism is probably not able to assemble selenoproteins, since the selABC gene cluster coding for essential proteins could not be identified, only a very short fragment of selB is present. 76.05% (1810) of the open reading frames (ORF) could be assigned to a putative function, 568 ORFs (23.86%) were annotated as hypothetical proteins and 35 ORFs as pseudo genes. Approximately 61% (1551 ORFs) of all protein-encoding genes could be assigned to at least one of the 21 functional COGs (Cluster of Orthologous Groups). The two most abundant categories were “general function” and “amino acid transport and metabolism”, to which 10.24% and 9.18%, respectively, could be assigned to, followed by “function unknown”, “translation, ribosomal structure and biogenesis”, “coenzyme transport and metabolism”, “replication, recombination and repair” and “energy production and conversion” with 9.06%, 8.30%, 6.83%, 6.65% and 6.00%, respectively.

As mentioned before, “replication, recombination and repair” is one of the most abundant COG category in the genome of T. kivui. Proteins which were assigned to this category are Cas proteins (CRISPR-associated sequences) and the encoding genes are often located adjacent to CRISPR loci (Clustered Regularly Interspaced Short Palindromic Repeats). The CRISPR/Cas system protects the genome against invading mobile elements such as plasmids or phages and consists of contiguous repeats with different length (20 to 47) and different numbers [53-55]. The genome of T. kivui harbors 3 CRISPR loci with 35, 48 and 21 repeats, respectively. The first CRISPR locus is flanked by four genes which could be assigned to a III-B/polymerase-RAMP module CRISPR/Cas system subtype (TKV_c14620-TKV_c14650), the second locus is upstream flanked by a type III-B/polymerase-RAMP module subtype (TKV_c23500-TKV_c23560) and downstream by a hybrid cluster consisting of I-B/Teanap-Hmari and again a III-B/polymerase-RAMP module subtype where the cmr2 gene is interrupted by a transposase (TKV_c23590-TKV_c23770), while a second I-B/Teanap-Hmari subtype CRISPR/Cas system is located adjacent to the third CRISPR locus (TKV_c24261). In addition we could identify a complete subtype III-A CRISPR/Cas system (TKV_c21730-TKV_c217390), which is not associated with a CRISPR locus. All CRISPR/Cas clusters were annotated according to the polythetic classification of CRISPR/Cas systems [56]. There is no complete prophage located on the chromosome of T. kivui.

Substrate utilization
T. kivui can not only grow autotrophically on H₂ + CO₂ (or formate) but also heterotrophically on glucose, fructose, mannose, and pyruvate [33]. Correspondingly, genes coding for all enzymes for glycolysis were found in the T. kivui genome with only the hexokinase and the aldolase being encoded twice (Table 2). Despite the gene cluster TKV_c16300-TKV_c16340 (coding for the conversion of

| Table 1 General features of the T. kivui genome |
| GenBank: CP009170 |
| Genome size | 2397289 bp |
| Protein encoding orfs | 2378 |
| Pseudo genes | 35 |
| Percent coding (%) | 87.55 |
| G + C content (mol%) | 35.06 |
| rRNA | 10 |
| tRNA | 58 |
| CDS with function prediction | 2024 |
| CDS without assigned function | 356 |
| CDS assigned to COGs | 1559 |
| CRISPR count | 3 |
Table 2 Genes encoding enzymes of the Embden-Meyerhof-Parnas pathway

| Enzyme                                | Locus tag               |
|---------------------------------------|-------------------------|
| Hexokinase                            | TKV_c00920, TKV_c17910  |
| Glucose-6-P isomerase                  | TKV_c16780              |
| 6-phosphofructokinase                  | TKV_c16900              |
| Aldolase                              | TKV_c01430, TKV_c04080  |
| Triose-P isomerase                     | TKV_c16320              |
| Glycerinaldehyde-3-P dehydrogenase     | TKV_c16340              |
| Phosphoglycerate kinase                | TKV_c16330              |
| Phosphoglycerate mutase                | TKV_c16310              |
| Enolase                               | TKV_c16300              |
| Pyruvate kinase                       | TKV_c16890              |

genes involved in the Wood-Ljungdahl pathway

The genes encoding the key enzymes in the Wood-Ljungdahl pathway of T. kivui are shown in Figure 1A. The first step in the methyl branch of the WLP is the reduction of CO₂ to formate by a formate dehydrogenase. A corresponding gene cluster very similar to the one of A. woodii was found on the chromosome of T. kivui (TKV_c19950-TKV_c19990). In A. woodii, the cluster consists of 7 genes [57], with fdhF1 being an isogene of fdhF1 encoding a selenium-containing Fdh. A gene encoding an electron transfer protein (hydB) follows each fdh gene. In T. kivui, only a selenium-free Fdh (fdhF/TKV_c19990) is encoded in the cluster. This gene is followed by hydC3 (TKV_c19980) and hydC4 (TKV_c19970), both encoding small FeS proteins. Adjacent to hydC4 is a gene (hydA2/TKV_c19960) encoding a hydrogenase subunit. Both clusters also encode a gene annotated as fdhD. Since the enzyme purified from A. woodii did not contain an FdhD subunit [58], its function remains to be elucidated. In A. woodii, this enzyme complex was recently shown to catalyze the hitherto unknown reduction of CO₂ with electrons coming directly from molecular hydrogen and was therefore named hydrogen-dependent CO₂ reductase (HDCR) [58]. Thus, it is likely that T. kivui reduces CO₂ to formate with H₂ as well. This is in contrast to the close phylogenetic neighbor M. thermoacetica, which was shown to reduce CO₂ with electrons coming from NADPH [59]. Many of the genes encoding functions of the WLP are located in one main gene cluster (Figure 1B), which lies in close proximity to the genes coding for the hydrogen-dependent CO₂ reductase. The product of the first gene of the operon, fhs (TKV_c19930), probably catalyzes the initial ATP-dependent activation of formate to formyl-THF. Subsequent to fhs is an open reading frame (orf1), whose product is similar to thymidylate syntheses. Adjacent to orf1 are genes encoding the methenyl-THF cyclohydrolase (fhaA), methylene-THF dehydrogenase (fod) as well as two subunits of the methylene-THF reductase (metV and metF). Those are followed by a dihydrodiphenyldehydrogenase (pdhD), a maturation factor of the CO dehydrogenase (cooC), subunit beta (acsD) and subunit alpha (acsC) of the corrinoid iron-sulfur protein. The putative operon is completed by genes encoding a methyltransferase (acscE), an acetyl-CoA synthase (acscB) and protein H of a glycine cleavage system (acscH). A possible function of GcvH within the WLP is unclear, but since this gene is also found within this gene cluster in A. woodii [57] and M. thermoacetica [30], one cannot exclude a so far undiscovered function of this enzyme within the pathway. Thus, as in A. woodii, proteins for the conversion of formate to methyl-THF are encoded by one gene cluster. However, in A. woodii folD and metV do not lie adjacent to each other but are separated by the gene rnfC2, probably coding for a third subunit of the methylene-THF reductase in this organism [57]. In M. thermoacetica, the genes coding for proteins of the methyl branch of the WLP are spread all over the genome. Only recently, MetF and MetV were shown to form a complex with the proteins HdrABC and MvhD [31]. Such genes were not found in T. kivui, implicating that the soluble methylene-THF reductase is composed of only 2 subunits. In A. woodii and M. thermoacetica, a separate gene cluster coding for the methyltransferase AcsE as well as subunits of the CODH/ACS is located somewhere else on the genome. In T. kivui, only two genes encoding a CO...
dehydrogenase (acsA/TKV_c20100 and cooS/TKV_c08080), a phosphotransacetylase and an acetate kinase are encoded elsewhere on the genome. Thus the WLP gene cluster is outstanding, since it encodes all proteins (despite AcsA, which is separated by 10 genes) that are required for the conversion of CO$_2$ to acetyl-CoA.

Transfer of reducing equivalents by soluble enzymes

In all acetogens examined, ferredoxin and NAD(P) serve as common electron carriers in the WLP and in energy conservation. Reducing equivalents required in the WLP of *T. kivui* may be delivered from molecular hydrogen by a soluble electron bifurcating hydrogenase HydABC complex (TKV_c19580-TKV_c19600) similar to the electron bifurcating hydrogenases of *A. woodii* and *M. thermoacetica*, which reduce NAD$^+$ and ferredoxin in equimolar amounts [60-62]. HydA1 (TKV_c19850) of *T. kivui* shows 56% and 49% identity to the corresponding subunits of *M. thermoacetica* and *A. woodii*, respectively. The identities for HydB (TKV_c19590) to the homologous proteins of *M. thermoacetica* and *A. woodii* are 54% and 59%, the ones for HydC (TKV_c19600) amount 42% and 47%, respectively. Apart from HydA2 of the HDCR and the HydABC complex, there is no other soluble hydrogenase encoded in the genome of *T. kivui*.

As also found in *M. thermoacetica* [32], the genome of *T. kivui* encodes an electron bifurcating transhydrogenase (NfnAB complex), which is encoded by the genes TKV_c22270 and TKV_c22280. TKV_c22270 shows 57% and TKV_c22280 69% identity to NfnA (Moth_1518) and NfnB (Moth_1517), respectively, from *M. thermoacetica*. In *M. thermoacetica*, this enzyme complex coupled the endergonic transfer of electrons from NADH to NADP$^+$ to the exergonic reduction of NADP$^+$ with Fd$_{red}$ [32].

**F$_{1}$F$_{0}$ ATP synthase of *T. kivui***

ATP synthases consist of a soluble head domain, that catalyzes ATP synthesis or hydrolysis and a membrane domain that is responsible for ion translocation [63]. Genes encoding all the subunits of a F$_{1}$F$_{0}$ ATP synthase
were found in the genome of *T. kivui* in one gene cluster (TKV_c06410-TKV_c06480). Interestingly, the cluster does not code for the gene *atpI*, which is the first gene of most bacterial *atp* operons and was shown to be essential for the assembly of the c ring in *A. woodii* [64]. The genes flanking the ATP synthase gene cluster of *T. kivui* apparently do not code for proteins that are required for ATP synthesis.

The membrane-integral c subunit of F$_1$F$_0$ ATP synthases determines the ion specificity of the enzyme. To check whether the c subunit of *T. kivui* has a conserved Na$^+$ binding site, sequence alignments of several c subunits of Na$^+$-dependent ATP synthases along with the one of *T. kivui* (atpE/TKV_c06420) were performed. As can be seen in Figure 2, the Na$^+$-dependent F$_1$F$_0$ ATP synthases from *Ilyobacter tartaricus*, *Propionigenium modestum* and *A. woodii* have the conserved Na$^+$ binding motif consisting of the amino acids Q…ES/T [65,66]. This motif cannot be found in the c subunit of *T. kivui* (Figure 2). Here, the glutamine (Q) is changed to an isoleucine (I) and instead of the serine (S) or threonine (T), there is an alanine (A). Since the proton-binding site (E55) of the c subunit is well conserved in *T. kivui*, this finding suggests that the F$_1$F$_0$ ATP synthase of *T. kivui* is H$^+$-dependent. This finding is in line with the hypothesis that the bioenergetics of *T. kivui* is not based on a sodium ion but proton current across its cytoplasmic membrane. To substantiate this hypothesis, the effect of Na$^+$ on growth and acetate formation was analyzed.

### Acetate formation from H$_2$ + CO$_2$ is H$^+$-dependent

In order to identify the nature of the coupling ion in acetogenesis of *T. kivui* irreversibly, cell suspensions of glucose-grown cells were prepared and acetate formation from H$_2$ + CO$_2$ was determined. As evident from Figure 4A, the presence of NaCl had almost no effect on the final amount and rate of acetate formation, arguing against Na$^+$ as coupling ion. In contrast, the addition of NaCl even led to a slightly decreased acetate production rate. Without NaCl present in the system, the final amount of acetate was about 42 mM, whereas only 35 mM acetate were produced in the presence of 20 mM NaCl. The contaminating amount of Na$^+$ was 350 ± 20 μM.

Although the experimentally determined Na$^+$ concentration in the assay without added NaCl is well below the $K_M$ for Na$^+$ during acetate formation in *A. woodii*, it may well be that the $K_M$ for Na$^+$ in *T. kivui* is lower than the contaminating Na$^+$ concentration in the assay. To determine whether acetogenesis in *T. kivui* relies on a transmembrane Na$^+$ or H$^+$ gradient, the effect of different ionophores on acetate formation was monitored. The Na$^+$ ionophore ETH2120 had almost no effect on acetate formation (in the absence or presence of sodium ions) (Figure 4B), whereas the protonophore TCS led to a complete inhibition of acetate formation. Here, the final amount of acetate was only 5 mM (Figure 4C). The experiments with resting cells of *T. kivui* give strong evidence that *T. kivui* does not belong to the Na$^+$-dependent acetogens, but that H$^+$ is the coupling ion.

### Membrane-embedded electron transfer coupled to the formation of a transmembrane proton gradient

In *A. woodii*, the Na$^+$-translocating Rnf complex is the only coupling site [15,16,37]. However, inspection of the genome and the absence of Fd$_{ox}$NAD$^+$ oxidoreductase activity at membranes of *T. kivui* (data not shown) revealed that *T. kivui* does neither possess an Rnf complex nor a complex with similar function. Furthermore, no genes encoding for cytochrome synthesis could be detected in the genome. Therefore, the genome of *T. kivui* was sought for genes encoding potential ion pumping membrane-bound oxidoreductases. As in *M. thermoacetica*, two gene clusters with

**Figure 2** Sequence alignment of c subunits of Na$^+$-dependent ATP synthases and the subunit c (TKV_c06420) of *T. kivui*. The Na$^+$ binding motif is highlighted in bold.
The product of the ech1B cluster, as well as 20% to ech1C, codes for the small hydrogenase subunit, probably involved in electron transfer. The identity to M. barkeri EchC is 38%, to M. thermoacetica Moth_2184 36% and to Moth_0978 25%. The fourth gene of the cluster, ech1F, encodes another subunit of Ech complexes. It has 24% identity to the corresponding subunit of M. barkeri and 26% to the homologous subunits (Moth_2185 and Moth_0982, respectively) from M. thermoacetica. It is predicted to have two 4Fe-4S clusters and therefore might be involved in electron transfer from ferredoxin. The following gene (jycB1/TKV_c01270) encodes a small FeS protein with four 4Fe-4S clusters that shows homology to HycB from Escherichia coli (33% identity) and CooF from Rhodospirillum rubrum (32% identity). Its identity to the homologous protein from M. thermoacetica (Moth_2192) is 36%. The protein products of the next two genes do not have any homologues in known Ech-type complexes, but show sequence similarity to the N-terminus of MetV, the small subunit of the methylene-THF reductase of the Wood-Ljungdahl pathway. The sequence identity to each other is 22%, the identity to MetV of T. kivui is 23% (for TKV_c01280) and 20% (for TKV_c01290), respectively. Both proteins might coordinate one 4Fe-4S cluster.

Figure 3 Effect of Na⁺ on autotrophic growth of T. kivui. Cultures grown on H₂ + CO₂ were transferred into Na⁺-enriched (●) and Na⁺-deficient (○) minimal medium. The curves shown are representative for three independent experiments. Precultures were grown for four transfers in the same medium.

similarities to Ech-type complexes were found: TKV_c01230-TKV_c01280 (Figure 5A) and TKV_c19750-TKV_c19680 (Figure 6A). Ech complexes are thought to be the ancestor of complex I [67] and although final proof with a purified enzyme is still pending, strong indications were given that Ech complexes in methanogens couple the electron transfer from reduced ferredoxin to H⁺ with the translocation of H⁺ across the cytoplasmic membrane [68,69]. However, in M. thermoacetica, one Ech-type complex might be coupled to a formate dehydrogenase (Moth_2183) and the second does not possess all residues essential for a catalytic [NiFe] domain in the large hydrogenase subunit EchE (Moth_0980).

The first gene of the first ech gene cluster of T. kivui, ech1A (TKV_c01230), codes for a transmembrane protein with 19 predicted transmembrane helices with a weak similarity (17%) to EchA from Methanosarcina barkeri. The identity to homologous proteins of M. thermoacetica is 21% to Moth_2191, 23% to Moth_2188 and Moth_2187 (encoded by the first ech cluster), as well as 20% to Moth_0985 and 21% to Moth_0986 and Moth_0987 (encoded by the second ech cluster). The product of the following gene, ech1B, has 8 transmembrane helices and shows 21% identity to M. barkeri EchB, the identity to homologous proteins from M. thermoacetica is 22% for Moth_2190 and 26% for Moth_0981. Both subunits are thought to be involved in H⁺ transport [70,71]. ech1C encodes for the small hydrogenase subunit, probably involved in electron transfer. The identity to M. barkeri EchC is 38%, to M. thermoacetica Moth_2184 36% and to Moth_0978 25%. The fourth gene of the cluster, ech1F, encodes another subunit of Ech complexes. It has 24% identity to the corresponding subunit of M. barkeri and 26% to the homologous subunits (Moth_2185 and Moth_0982, respectively) from M. thermoacetica. It is predicted to have two 4Fe-4S clusters and therefore might be involved in electron transfer from ferredoxin. The following gene (jycB1/TKV_c01270) encodes a small FeS protein with four 4Fe-4S clusters that shows homology to HycB from Escherichia coli (33% identity) and CooF from Rhodospirillum rubrum (32% identity). Its identity to the homologous protein from M. thermoacetica (Moth_2192) is 36%. The protein products of the next two genes do not have any homologues in known Ech-type complexes, but show sequence similarity to the N-terminus of MetV, the small subunit of the methylene-THF reductase of the Wood-Ljungdahl pathway. The sequence identity to each other is 22%, the identity to MetV of T. kivui is 23% (for TKV_c01280) and 20% (for TKV_c01290), respectively. Both proteins might coordinate one 4Fe-4S cluster. 170 bp downstream of TKV_c01290 is a gene encoding the subunit Ech1D with 27% identity to the corresponding subunit from M. barkeri. It has no predicted cofactors and its function in the complex is unknown so far. The genome of M. thermoacetica does not code for a homologous protein. Adjacent lies the gene ech1E, encoding the catalytic NiFe hydrogenase subunit. The protein sequence shows 40% identity to EchE from M. barkeri and 39% and 33% to Moth_2186 and Moth_0980, respectively, from M. thermoacetica. A model of the protein complex Ech1 encoded by this ech1 cluster is shown in Figure 5B. Electrons coming from reduced ferredoxin may enter the complex at subunit Ech1F and may be transferred via the FeS proteins HycB1, TKV_c01280 and TKV_c01290 onto the catalytic hydrogenase subunit Ech1E that catalyzes H₂ formation. Subunits Ech1A and Ech1B mediate H⁺ transport across the cytoplasmic membrane.

The second gene cluster potentially encoding an Ech-type hydrogenase is TKV_c19750-TKV_c19680 (Figure 6A). The product of the first gene, ech2D, encodes a 14 kDa protein (Figure 6B) and is 19% identical to EchD from M. barkeri. The sequence identity to its homologue (TKV_c0 1300) in the Ech1 complex is only 8%. The succeeding gene, ech2E, codes for the large catalytic NiFe hydrogenase subunit and the deduced protein product shows 37% identity to M. barkeri EchE, 40% to Moth_2186 and 32% to
Moth_0980 from *M. thermoacetica*, as well as 45% to TKV_c01310/Ech1E from *T. kivui*. Thereafter lies a gene encoding a 13 kDa protein (HycB2) with four predicted 4Fe-4S clusters. It has 35% identity to the FeS protein HycB1 (TKV_c01270) of the above described Ech-type complex and is homologous to CooF from *R. rubrum* (36%)

**Figure 4** Acetate formation from H$_2$ + CO$_2$ by resting cells of *T. kivui* is inhibited by TCS. Whole cells of *T. kivui* were incubated with H$_2$ + CO$_2$ in buffer containing 50 mM imidazole, 20 mM MgSO$_4$, 20 mM KCl, 50 mM KHCO$_3$ and 4 mM DTE (pH 7.0). **A**: 350 ± 20 μM (▲) or 20 mM (▲) NaCl. **B**: 20 mM NaCl with (▼) or without (▲) 30 μM ETH2120. **C**: 350 ± 20 μM NaCl with (▲) or without (■) 30 μM TCS. The final protein concentration of the resting cells in the assay was 1 mg/ml. All values are mean from three replicates.
identity) and HycB from *E. coli* (39% identity), as well. The next two genes, TKV_c19720 and TKV_c19710, both encode proteins similar to the membrane-bound subunit EchA (with 22% and 21% identity to *M. barkeri* EchA, respectively and 18 to 27% identity to the six EchA homologues from *M. thermoacetica*) and were therefore named *ech2A1* and *ech2A2*. The homologue of Ech2A1 in complex I is NuoL, whereas the homologue of Ech2A2 is NuoM. Ech2C is encoded by the gene TKV_c19700. It has one hydrophobic stalk and represents the small hydrogenase subunit. Its identity to EchC from *M. barkeri* is 43%, *M. thermoacetica* Moth_2184 and Moth_0978 are 48% and 37% identical. The gene *ech2B* encodes another membrane-integral subunit with 7 transmembrane helices but no known cofactors. Its identity to Moth_0981 is 25%, sequence identity to Moth_2190 is only 17%. The last gene encodes another small electron transfer protein with two 4Fe-4S clusters and is similar to EchF, the identity to the corresponding proteins from *M. thermoacetica* is 28% (Moth_2185) and 24% (Moth_0982). A postulated subunit composition of the Ech2 complex

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**Figure 5** Arrangement of genes in the cluster (A) and model (B) of the Ech-type complex Ech1. Electron flow from reduced ferredoxin to H⁺ and the coupled export of protons is shown. FeS clusters are indicated. *, predicted transmembrane protein (number of transmembrane helices); Fd, ferredoxin; orf1/orf2, encoding small FeS containing proteins (TKV_c01280 and TKV_c01290) with similarity to the N-terminus of MetV.

**Figure 6** Arrangement of genes in the gene cluster (A) encoding a second potential Ech-type complex Ech2 (B). FeS clusters are indicated. *, predicted transmembrane protein (number of transmembrane helices); Fd, ferredoxin.
and electron flow from reduced ferredoxin to \( H^+ \) with concomitant \( H^+ \) export is shown in Figure 6B. Thus, in contrast to the above-described Ech-type complex, the second has one more membrane-integral subunit that might be involved in \( H^+ \) transport, probably allowing for a more beneficial stoichiometry of the electron:\( H^+ \) ratio. On the other hand, the small FeS proteins with similarity to MetV are missing. Sequence identities of the homologous proteins of the two Ech-type complexes to each other are summarized in Table 3.

**Discussion**

**Model of energy conservation during autotrophic growth of \( T. kivui \)**

The genomic data allow to propose a model for electron and carbon flow during acetogenesis from \( H_2 + CO_2 \) in \( T. kivui \). Electrons coming from molecular hydrogen are transferred to the electron carriers NAD\(^+\) and ferredoxin by the soluble, electron-bifurcating hydrogenase HydABC. In the carbonyl branch of the WLP, the reduced ferredoxin serves as electron donor for the reduction of one molecule of CO\(_2\) to CO, catalyzed by the CODH/ACS. In the methyl branch of the pathway, another CO\(_2\) is reduced to formate with electrons coming directly from molecular hydrogen. This reaction is catalyzed by the hydrogen-dependent CO\(_2\) reductase (HDCR), as it was also shown for \( A. woodii \). The electron donor for the subsequent reduction of methenyl-THF cannot be predicted, since both NADH and NADPH are used in other acetogens and bioinformatic analyses do not allow to discriminate between the possibilities. However, since NADH is more common, it was used as electron donor for the \( T. kivui \) model. The following step, reduction of methylene-THF is still under debate for an indirect role in energy conservation. The redox potential \( (E^0) \) of the methylene-THF/methyl-THF pair is \(-200 \text{ to } -130 \text{ mV} \) [72] and electron transfer from NADH \( (E^0 = -320 \text{ mV}) \) to methylene-THF is highly exergonic. Therefore, this reaction was assumed already in 1977 to be involved in energy conservation [73]. One scenario is that methylene-THF reduction is coupled to ferredoxin reduction by electron bifurcation, as recently suggested for \( M. thermoacetica \) [31]. In \( T. kivui \), MetV and MetF are encoded in the WLP operon (see Figure 1B), however, the genes encoding the putatively bifurcating Hdr subunits of \( M. thermoacetica \) are lacking in the \( T. kivui \) genome. Therefore, the situation is more similar to \( A. woodii \), which is also lacking Hdr-encoding genes and in which evidence for electron bifurcation was not obtained (Bertsch J, Öppinger C, Hess V, Langer JD, Müller V: A heterotrimeric NADH-oxidizing methylenetetrahydrofolate reductase from the acetogenic bacterium Acetobacterium woodii, in preparation). Actually, MetV and MetF of \( T. kivui \) are the minimal subunit composition of an acetogenic, non-electron-bifurcating methylene-THF reductase. Since cell-free extract of \( T. kivui \) does not catalyze NADH- or NADPH-dependent methylene-THF reduction (data not shown) we assume an electron carrier in that redox range which may be, for example, a flavodoxin, which is energetically equivalent to NADH. If we take into account a non-electron-bifurcating methylene-THF reductase that uses the electrons derived from a carrier energetically equivalent to NADH we can conclude a quantitative scheme for the bioenergetics of acetogenesis in \( T. kivui \) (Figure 7). According to this model, \( T. kivui \) can synthesize 0.25 ATP via the \( F_1F_0 \) ATP synthase per acetate formed.

The ATP:acetate ratio in the phylogenetically close neighbor \( M. thermoacetica \) is predicted to be 0.5 [19], thus in comparison, \( T. kivui \) conserves only half as much energy from 4 \( H_2 + 2 \text{ CO}_2\). Both organisms have an electron bifurcating hydrogenase HydABC that couples initial \( H_2 \) oxidation to the reduction of NAD\(^+\) and ferredoxin. The latter is the electron donor for the energy conserving reaction, the electron flow to \( H^+ \) via the Ech complex that concomitantly translocates protons across the cytoplasmic membrane. The above-mentioned difference in ATP production per acetate can be explained by the postulated additional coupling of methylene-THF reduction to the reduction of ferredoxin (or an energetic equivalent) in a second electron bifurcating reaction in \( M. thermoacetica \). Hence this bacterium can transfer four electrons onto the Ech complex, thereby translocating two protons across the cytoplasmic membrane, whilst \( T. kivui \) uses the Ech complex only once per acetate formed.

As described in this paper, all data achieved are in accordance with \( H^+ \) based bioenergetics in \( T. kivui \). Based on bioinformatic and experimental data, we could show that the ATP synthase is \( H^+ \)-dependent and the electrochemical ion gradient is probably formed by a \( H^+ \)-translocating Ech type complex. However, these data are in contrast to the results obtained by Yang et al. in 1990 [35], who found a strict Na\(^+\) dependence when \( H_2 \) was the electron donor. In order to assure our diverging results, we prepared our Na\(^+\)-deficient medium strictly according to the instructions as given in [35]. We measured a contaminating Na\(^+\) concentration of

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**Table 3 Sequence identities of homologous subunits of the two Ech-type complexes from \( T. kivui \)**

| Locus tag 1 | Locus tag 2 | Protein subunit | Identity [%] |
|------------|------------|----------------|-------------|
| TKV_c19720 | TKV_c01230 | EchA           | 22          |
| TKV_c19710 | TKV_c01230 | EchA           | 26          |
| TKV_c19720 | TKV_c19710 | EchA           | 18          |
| TKV_c19690 | TKV_c01240 | EchB           | 28          |
| TKV_c19700 | TKV_c01250 | EchC           | 50          |
| TKV_c19750 | TKV_c01300 | EchD           | 8           |
| TKV_c19740 | TKV_c01310 | EchE           | 45          |
| TKV_c19680 | TKV_c01260 | EchF           | 21          |
| TKV_c19730 | TKV_c01270 | HycB           | 35          |
160 μM, while Yang and Drake reported 200 μM NaCl in their medium. Thus a different amount of Na⁺ in the deficient medium can be excluded as a source of the discrepancy. We reproduced this experiment several times and could never see an effect of Na⁺ on growth. Unfortunately, we were not able to find a reason for the discrepancy, but the additional data obtained in cell suspensions of T. kivui as well as the absence of a sodium ion binding site in the c subunit of the F₁Fₐₐₜ ATP synthase are consistent with the classification of T. kivui as H⁺-dependent.

In the early history of acetogenic bacteria, M. thermoaacetica was used as a model organism to unravel the mechanism of energy conservation in H⁺-dependent acetogens. The detection of cytochromes [74] led to the assumption that H⁺ translocation in M. thermoaacetica is cytochrome-based [75-77]. However, despite many decades of intensive research, the participation of cytochromes in H⁺ translocation of M. thermoaacetica could never be confirmed and their involvement in energy conservation became questionable. Instead, a role of cytochromes in O₂ reduction was discussed in recent years [32]. With our finding that T. kivui and M. thermoaacetica seem to use very similar mechanisms for energy conservation and that the genome of T. kivui does not encode any proteins necessary for cytochrome synthesis, it becomes more and more evident that the function of cytochromes in M. thermoaacetica is indeed not in energy conservation coupled to the WLP. Instead, we have recently proposed to classify the acetogens bioenergetically in Rnf- and Ech-containing acetogens [19] and each class has a subclass with Na⁺- and H⁺-dependent species. T. kivui thus belongs to the subclass of H⁺-depending, Ech-containing acetogens.

By further comparing the two acetogens T. kivui and M. thermoaacetica, it is remarkable that, despite similar mechanisms for energy conservation, their growth behavior on H₂ + CO₂ is quite different. As mentioned earlier, the doubling time of M. thermoaacetica when growing autotrophically is about 24 h [32], while T. kivui grows 10-times faster [33]. But how can this discrepancy be explained in spite of the similar genetic configuration of the two bacteria? Since a weaker coupling of ATP synthesis and autotrophic acetate formation in M. thermoaacetica was already excluded, the only explanation left is that at least one reaction of the Wood-Ljungdahl pathway is rate limiting. As M. thermoaacetica is postulated to use three reactions that do not seem to be necessary in T. kivui (the transhydrogenase reaction for production of NADPH, reduction of CO₂ with NADPH, and a bifurcating reaction in the course of methylene-THF reduction), one of those might well be the limiting factor. However, in order to irrevocably identify the bottleneck, extensive biochemical studies based on experimental data need to be carried out in future.

Conclusions

Sequencing the genome of T. kivui allowed for a better insight into the reactions enabling the autotrophic reduction of CO₂ to acetate. Since this process is thought to be
one of the first pathways that evolved on the early earth [78], its detailed understanding is of great relevance.

The thermophilic acetogen *T. kivui* oxidizes the electron donor H$_2$ by use of an electron bifurcating hydrogenase HydABC. The reduced ferredoxin then fuels the energy conserving membrane module that is coupled to the WLP: the genome of *T. kivui* harbors two gene clusters, which both encode Ech-type complexes. Although the deduced protein subunit composition of the two complexes alter slightly, both enzymes have all cofactors and catalytic domains that are required to catalyze ferredoxin oxidation as well as H$^+$ reduction. The proton gradient formed by Ech can be used by a H$^+$-dependent F$_1$F$_0$ ATP synthase to drive phosphorylation of ADP.

**Abbreviations**

CODH/ACS: CO dehydrogenase/acetyl-CoA synthase; CoFeSP: Corrinoid/FeS protein; Ech: Energy conserving hydrogenase; ETH2120: Sodium iodophore III/NNA/N'-Tetracyclosyd-1,2-phenylenedioxyacetamide; Fd: Ferredoxin; PPS: Phosphotransferase system; TCS: 4',5'-tetrachlorosalicylanilide; THF: Tetrahydrofolate; WLP: Wood-Ljungdahl pathway.

**Competing interests**
The authors declare that they have no competing interests.

**Authors’ contributions**
AP and RD planned the genome sequencing, AP did the genome sequencing, AP did the genome annotations, VH and MCW analyzed the data, VH and VM conceived and designed the experiments, VH provided the experimental data, VH, AP and VM wrote the manuscript. All authors read and approved the final manuscript.

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**References**

1. Drake HL, Gössner A, Daniel SL. Old acetogens, new light. *Ann N Y Acad Sci* 2008, 1128:100–128.
2. Müller V, Freirsch J. Acetogenic bacteria. In et al. Edited by Battista J. Chichester: John Wiley & Sons Ltd, 2013 (doi:10.1002/978047015902. a0200868.pub2).
3. Ljungdahl LG. The acetyl-CoA pathway and the chemiosmotic generation of ATP during acetogenesis. In *Acetogenesis*. Edited by Drake HL. New York, Chapman & Hall, 1994:63–87.
4. Ragsdale SW. Enzymology of the Wood-Ljungdahl pathway of acetogenesis. *Ann N Y Acad Sci* 2008, 1128:129–136.
5. Pezzack E, Wood HG. Role of carbon monoxide dehydrogenase in the autotrophic pathway used by acetogenic bacteria. *Proc Natl Acad Sci U S A* 1984, 81:6261–6265.
6. Raybuck SA, Bastian NR, Orme-Johnson WH, Walsh CT. Kinetic characterization of the carbon monoxide-acetyl-CoA (carbonyl group) exchange activity of the acetyl-CoA synthesizing CO dehydrogenase from *Clostridium thermoaceticum*. *Biochemistry* 1988, 27:7698–7702.
7. Seravalli J, Kumar M, Lu WP, Ragsdale SW. Mechanism of carbon monoxide oxidation by the carbon monoxide dehydrogenase/acetyl-CoA synthase from *Clostridium thermoacetivorans*. *Biochemistry* 1997, 36:11241–11251.
8. Hicks RH, Harmony JA. *Formyltetrahydrofolate synthetase*. *Ccr Rev Bch Mol* 1973, 1:501–535.
9. Lovell GR, Prybyla A, Ljungdahl LG. Cloning and expression in *Escherichia coli* of the *Clostridium thermoacetivorans* gene encoding thermostable *formyltetrahydrofolate synthetase*. *Arch Microbiol* 1988, 149:283–285.
10. Ragsdale SW, Ljungdahl LG. *Devrientian* DV. *EPR evidence for nickel-substrate interaction in carbon monoxide dehydrogenase from Clostridium thermoaceticum*. *Biochim Biophys Acta* 1982, 1085:658–663.
11. Ragsdale SW, Ljungdahl LG. *Devrientian* DV. Isolation of carbon monoxide dehydrogenase from *Acetobacter woodii* and comparison of its properties with those of the *Clostridium thermoacetivorans* enzyme. *J Bacteriol* 1983, 155:1224–1237.
12. Ragsdale SW, Wood HG. Acetate biosynthesis by acetogenic bacteria. Evidence that carbon monoxide dehydrogenase is the condensing enzyme that catalyzes the final steps in the synthesis. *J Biot Chem* 1985, 260:3970–3977.
13. Schaupp A, Ljungdahl LG. Purification and properties of acetate kinase from *Clostridium thermoacetivorans*. *Arch Microbiol* 1974, 100:121–129.
14. Eden G, Fuchs G. Total synthesis of acetyl coenzyme A involved in autotrophic CO$_2$ fixation in *Acetobacter woodii*. *Arch Microbiol* 1982, 133:66–74.
15. Biegel E, Schmidt S, Gonzalez JM, Müller V. Biochemistry, evolution, and physiological function of the Rnf complex, a novel ion-motive electron transport complex in prokaryotes. *Cell Mol Life Sci* 2011, 68:613–634.
16. Biegel E, Müller V. Bacterial Na$^+$-translocating ferredoxin:NAD$^+$ oxidoreductase. *Proc Natl Acad Sci U S A* 2010, 107:18138–18142.
17. Heine R, Reidlinger J, Müller V, Gottschalk G. A sodium-stimulated ATP synthase in the acetogenic bacterium *Acetobacter woodii*. *FEBS Lett* 1991, 295:119–122.
18. Müller V, Aufarth S, Rahafs S. The Na$^+$ cycle in *Acetobacter woodii*: identification and characterization of a Na$^+$-translocating F$_{1}$F$_{0}$-ATPase with a mixed oligomer of 8 and 16 kDa proteolipids. *Biochim Biophys Acta* 2001, 1505:108–120.
19. Schuchmann K, Müller V. Autotrophy at the thermodynamic limit of life: a model for energy conservation in acetogenic bacteria. *Nat Rev Microbiol* 2014, 12:809–821.
20. Frölt JH, Seifritz C, Drake HL. Effect of nitrate on the autotrophic metabolism of the acetogens *Clostridium thermoautotrophicum* and *Clostridium thermoaceticum*. *J Bacteriol* 1996, 178:4597–4603.
21. Seifritz C, Daniel SL, Gössner A, Drake HL. Nitrate as a preferred electron sink for the acetogen *Clostridium thermoacetivorans*. *J Bacteriol* 1993, 175:8008–8013.
22. Dilling S, Imkamp F, Schmidt S, Müller V. Regulation of caffeate respiration in the acetogenic bacterium *Acetobacter woodii*. *Appl Environ Microbiol* 2007, 73:3630–3636.
23. Misoph M, Daniel SL, Drake HL. Bidirectional usage of ferulate by the acetogen *Peptostreptococcus productus* U-1: CO$_2$ and aromatic acrylate groups as competing electron acceptors. *Microbiology-Uk* 1996, 142:1983–1988.
24. Gössner A, Daniel SL, Drake HL. Acetogenesis coupled to the oxidation of aromatic aldehyde groups. *Arch Microbiol* 1994, 161:126–131.
25. Matthes C, Freiberger A, Drake HL. Fumarate dissimilation and differential reductant flow by *Clostridium formicoacetatum* and *Clostridium aceticum*. *Arch Microbiol* 1993, 160:273–278.
26. Tanner RS, Miller LM, Yang Z. *Clostridium ljungdahlii* sp. nov., an acetogenic species in clostridial rRNA homology Group-I. *Int J Syst Bacteriol* 1993, 43:232–236.
27. Liou JS, Balkwill DL, Drake GR, Tanner RS. *Clostridium carboxidivorans* sp. nov., a solvent-producing clostridium isolated from an agricultural settling lagoon, and reclassification of the acetogen *Clostridium scotorenogenes* strain SL1 as *Clostridium drakei* sp. nov. *Int J Syst Evol Microbiol* 2005, 55:2085–2091.
28. Schiel-Bengelsdorf B, Düre P. Pathway engineering and synthetic biology using acetogens. *FEBS Lett* 2012, 586:2191–2198.
29. Köpke M, Mihakea C, Liever T, Tizard JH, All MS, Conolly J, Ali-Sinawi B, Simpson SD. 2,3-butanediol production by acetogenic bacteria, an alternative route to chemical synthesis, using industrial waste gas. *Appl Environ Microbiol* 2011, 77:5467–5475.
2014, 53. Horvath P, Barrangou R: MIRA: an automated genome and EST assembler. FEMS Microbiol Lett 2010, 305:169–171.

31. Daniel SL, Hsu T, Dean SI, Drake HJ: Characterization of the H+-dependent and CO-dependent chemolithotrophic potentials of the acetogens Clostridium thermoaceticum and Acetogenium kivui. J Bacteriol 1990, 172:4464–4471.

32. Yang H, Drake HL: Differential effects of sodium on hydrogen- and glucose-dependent growth of the acetogenic bacterium Acetogenium kivui. Appl Environ Microbiol 1990, 56:81–86.

33. Leigh JA, Mayer F, Wolfe RS: Electron bifurcation involved in the energy metabolism of the acetogenic bacterium Clostridium thermoaceticum growing on glucose or H2 plus CO2. J Bacteriol 2012, 194:3689–3699.

34. Leigh JA, Moyer F, Wolfe RS: Acetogenium kivui, a new thermophilic hydrogen-oxidizing, acetogenic bacterium. Arch Microbiol 1981, 129:275–280.

35. Schönhütter P, Wäscher C, Thauer RK: A rapid procedure for the purification of ferredoxin from clostridia using polyethyleneimine. FEBS Lett 1978, 89:219–222.

36. Hess V, Schuchmann K, Müller V: The ferredoxin/NAD\(^+\) oxidoreductase (Rnf) from the acetogen Acetobacterium woodii requires Na\(^+\) and is reversibly coupled to the membrane potential. J Biol Chem 2013, 288:13496–3502.

37. Chevreux B: 468: an automated genome and EST-assembly. Ruprecht-Karls University, Heidelberg, 2005.

38. Murray MG, Thompson WF: Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 1980, 8:4321–4325.

39. Wilson K: Preparation of genomic DNA from bacteria. In Current Protocols in Molecular Biology. Edited by Ausubel FM. Hoboken: John Wiley & Sons, Inc, 2001:7.4.1–7.4.25.

40. Tettelin H, Radue O, Kasif S, Khouri H, Salzberg SL: Complete ion-coordination structure in the rotor ring of Na\(^+\)-F\(_2\) ATP synthase from Acetobacter woodii in Escherichia coli. J Biol Chem 2001, 276:24533–24537.

41. Niederhofer J, Ivey DM, Ljungdahl LG: Carbon monoxide-driven electron transport in Clostridium thermoautotrophicum. J Bacteriol 1987, 169:5845–5847.

42. Zdobnov EM, Apweiler R: PHAST: a fast phage annotation tool. Nucleic Acids Res 2005, 33:W352–W356.

43. Chen MH, Chien Y, Chang CY, Chen WC, Liu CY, Chuang CH: Complete genome sequence of Acetobacterium woodii strain ATCC 9641. J Bacteriol 2001, 183:5584–5597.

44. Meuer J, Bartoschek S, Koch J, Künkel A, Hedderich R: Methanosarcina barkeri, a hyperthermophilic encephalitozoonid. FEBS Lett 2009, 585:167–170.

45. J Bacteriol F-M. Hydrogen, metals, bifurcating electrons, and proton gradients: an ancient pathway combining carbon dioxide fixation with energy metabolism. J Bacteriol 2014, 196:3313–3314.

46. Wang S, Huang H, Kehrt J, Thauer RK: A reversible electron-bifurcating ferredoxin- and NADP+-dependent [FeFe]-hydrogenase (HydABC) in Clostridium thermoaceticum. J Bacteriol 2013, 195:1267–1275.

47. Schuchmann K, Müller V: Direct and reversible hydrogenation of CO\(_2\) to formate by a bacterial carbon dioxide reductase. Science 2013, 342:1382–1385.

48. Blumenthal ML: The iron-hydrogenase of Thermotoga maritima utilizes ferredoxin and NADH synergistically: a new perspective on anaerobic hydrogen production. J Bacteriol 2009, 191:4451–4457.

49. Brandt K, Müller DB, Hoffmann J, Hubert C, Brutschy B, Deckers-Hebestreit G, Müller V: Functional production of the Na\(^+\)-F\(_2\)O ATP synthase from Acetobacter woodii in Escherichia coli requires the native AtpL. J Bioenerg Biomembr 2003, 35:15–23.

50. Winters J, Davis MW: The iron-hydrogenase of Clostridium butylicum characterizes C\(_\text{H}_2\) mobilization. J Bacteriol 2006, 188:2299–2306.

51. Baktir Z: A genome-guided analysis of energy conservation in the thermophilic, cytochrome-free acetogenic bacterium Thermoanaerobacter kivui. BMC Genomics 2014, 15:1139.