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SPECIAL FEATURE: REVIEW

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"Hot" acetogenesis

Mirko Basen[1] · **Volker Müller**[1]

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Abstract Thermophilic microorganisms as well as acetogenic bacteria are both considered ancient. Interestingly, only a few species of bacteria, all belonging to the family *Thermoanaerobacteraceae*, are described to conserve energy from acetate formation with hydrogen as electron donor and carbon dioxide as electron acceptor. This review reflects the metabolic differences between *Moorella* spp., *Thermoanaerobacter kivui* and *Thermacetogenium phaeum*, with focus on the biochemistry of autotrophic growth and energy conservation. The potential of these thermophilic acetogens for biotechnological applications is discussed briefly.

Keywords Acetogenesis · Thermophiles · *Thermoanaerobacter kivui* · *Moorella thermoacetica* · *Thermacetogenium phaeum* · Wood-Ljungdahl pathway · CODH/ACS

Abbreviations

WLP	Wood-Ljungdahl pathway
CODH/ACS	CO dehydrogenase/acetyl-CoA synthase
Fd	Ferredoxin
Ech	Energy converting hydrogenase

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✉ Volker Müller
vmueller@bio.uni-frankfurt.de

[1] Department of Molecular Microbiology and Bioenergetics, Institute of Molecular Biosciences, Johann Wolfgang Goethe University, Frankfurt Am Main, Germany

Introduction

Thermophilic life is very old. The origins of life probably go back to a period of somewhat around 4 billion years ago (Lindahl and Chang 2001; Lunine 2006), when temperatures had cooled off enough that a stable atmosphere and ocean had formed. At that time, temperatures in the Hadean atmosphere and ocean may still have been elevated between 55 and 80 °C (Lunine 2006). In case of more moderate conditions (Kasting and Howard 2006), locally the temperatures could have been much higher due to volcanic activity, e.g., at hydrothermal vents. Thermophiles are defined as a group of organisms that thrive at elevated temperatures, with temperature optima higher than 45 °C, often higher than 55 °C, in distinction from hyperthermophiles which optimally grow above 80 °C (Gottschal and Prins 1991; Wiegel 1998). Thermophiles occur in both domains of prokaryotes, *Archaea* and *Bacteria*. While many genera of bacteria belong to the thermophiles, hyperthermophiles are mostly archaea. Thermophily is not considered a monophyletic trait, and while likely the first archaeal and bacterial cells evolved independently (Martin and Sousa 2016), thermophilic organisms of both domains branch off deeply (early) in phylogenetic trees (Pace 1997; Stetter 2006). This indicates that many of the phyla (or their ancestors) existed over billions of years already, which is consistent with geological records from 3.33 Ga old volcanic rocks (Westall et al. 2015).

Acetogenic microorganisms may also have been among the first microorganisms. The early atmosphere harbored CO_2 in addition to N_2, but also traces of reduced gases such as H_2, CH_4, CO, NH_3 and reduced sulfurous gases (Russell et al. 2010). The Hadean ocean was likely saturated with CO_2 and rich in transition metals (Russell et al. 2010). The fluid released from hydrothermal vents, e.g., the off-ridge

vents of the Lost City hydrothermal field (Kelley et al. 2005), putatively providing conditions that supported the evolution of the first cells (Russell et al. 2010; Sousa et al. 2013), are enriched in reduced gases and transition metals. In his famous experiments, Wächtershäuser showed that under conditions that may have supported hyperthermophilic life the co-precipitated metal sulfides NiS and FeS catalyze the condensation of methanethiol and CO to an acetyl thioester (Huber and Wächtershauser 1997), a reaction very similar to that catalyzed by the key enzyme for carbon fixation in acetogens, CO dehydrogenase/acetyl-CoA synthase (CODH/ACS). Taken together, these observations gave rise to the thought that the chemoautotrophy may have developed before heterotrophy (Martin and Russell 2003). Thereafter, the earliest chemolithoautotrophs derived their biomass from CO_2 or CO, used molecular hydrogen (or CO) as source of reductant and exploited existing chemical gradients (e.g., of protons or sodium ions) for energy conservation (Sousa et al. 2013). They may have been ancestors of acetogenic bacteria on the one hand and ancestors of methanogenic archaea on the other hand, forming two independent lineages for the evolution of the life. In this regard, the recent discovery of a putative acetogenic archaeon based on metagenome data from a deep biosphere environment is of interest (He et al. 2016). In any case, the reduction of CO_2 with inorganic electron donors to acetate (Eq. 1) or methane (Eq. 2) are exergonic reactions that can be coupled to energy conservation (Thauer et al. 1977).

$$4\,H_2(g) + 2\,CO_2(g) \rightarrow CH_3COO^- + H^+ + 2\,H_2O \quad \Delta G'_0 = -95\,\text{kJ mol}^{-1}\,\text{at}\,25\,^\circ\text{C} \tag{1}$$

$$4\,H_2(g) + CO_2(g) \rightarrow CH_4(g) + 2\,H_2O \quad \Delta G'_0 = -131\,\text{kJ mol}^{-1}\,\text{at}\,25\,^\circ\text{C} \tag{2}$$

Acetogens are capable of chemolithoautotrophic growth and use the Wood-Ljungdahl pathway (WLP) for carbon fixation (Schuchmann and Müller 2014), which is presumably one of the oldest biochemical pathways. First heterotrophs may have thrived by fermentation of the biomass constituents of the chemoautotrophs: peptides, purine/pyrimidine bases and ribose (Schönheit et al. 2016). Many scientists argue that autotrophs were first, but Wächtershäuser's experiments also show that small organic molecules such as acetate could have been formed chemically under primordial conditions (Huber and Wächtershauser 1997). The chemical synthesis of amino acids from NH_3, H_2 and CO under similar conditions is at least thermodynamically favorable (Amend and Shock 1998). According to thermodynamic calculations, acetate and other short-chain fatty acids may have been the most prevalent form of carbon in mixtures of seawater and hydrothermal vent fluids at 100 °C (Amend et al. 2013). In a scenario where heterotrophy came before autotrophy, acetogens or their common ancestors may still be very old, as they could have evolved as acetate-oxidizers. Chemically formed acetyl-CoA or a similar acetylated thioester may have been disproportionated into a CO and a methyl moiety by a CODH/ACS- like enzyme, followed by CO oxidation to CO_2. Acetate may have been completely oxidized via the Wood-Ljungdahl pathway to CO_2, given that hydrogen was readily removed by hydrogen-consuming reactions or cells. Interestingly, there is a described thermophilic microorganism, *Thermacetogenium phaeum*, that either forms acetate from H_2 and CO_2 or oxidizes acetate, depending on hydrogen partial pressure (Hattori et al. 2000). Alternatively, in the primordial world, acetate may have served as electron acceptor, coupled to, e.g., CO oxidation, as shown for a genetically engineered strain of the hyperthermophilic archaeon *Pyrococcus furiosus* (Basen et al. 2014) and as recently suggested for the closely related acetogens *Clostridium ljungdahlii* and *Clostridium autoethanogenum* (Mock et al. 2015).

As likely both, origins of thermophiles and acetogens trace back to early evolution of life, it is a logical assumption that many genera of thermophilic acetogenic bacteria should have been isolated and described; however, that is surprisingly not the case. Our knowledge of "hot" acetogenesis is mainly based on less than a handful of genera, i.e., species of the genus *Moorella*, *Thermoanaerobacter kivui* and *Thermacetogenium phaeum*, which all belong to the class *Clostridia*. This review article focusses on our present knowledge of acetogenesis in these thermophilic microorganisms. Acetogenesis in general with additional focus on mesophilic model organisms has been extensively reviewed by several authors in the past, gathered in a book (Drake 1994). Novel aspects have been discussed more recently (Schuchmann and Müller 2014, 2016), and we would like to refer to of one of these sources for deeper insights into the ecology, physiology and biochemistry of acetogens.

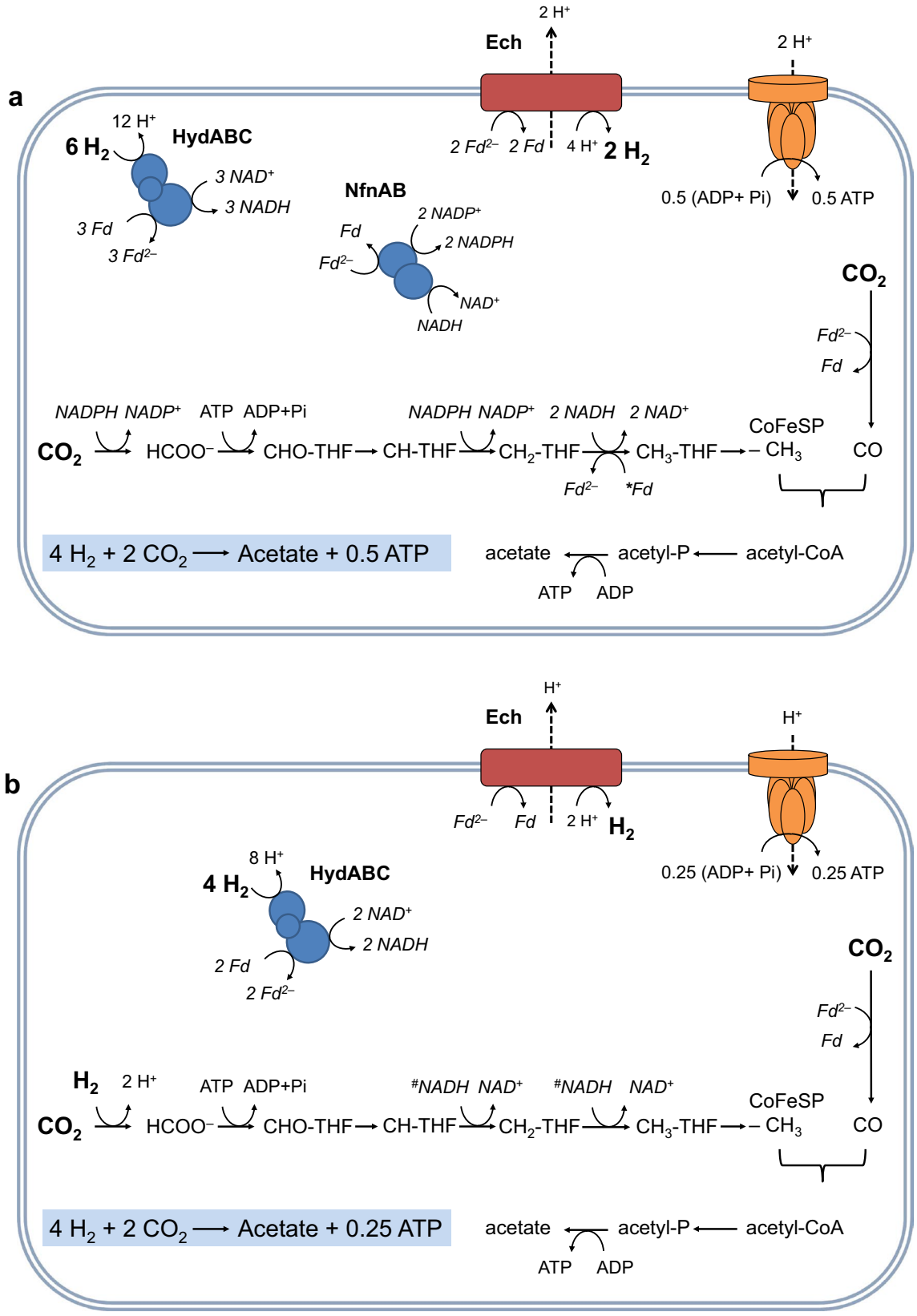
Carbon fixation and energy metabolism in acetogenic bacteria

Acetate is a common end product of carbohydrate fermentations (by many different microorganisms) or ethanol oxidation (by acetic acid bacteria). However, the term "acetogens" is restricted to microorganisms that are capable of producing acetate from two molecules of CO_2 via the WLP. In the following, the catabolism of acetogens will be discussed as modular. The first "module" consists of the electron-donating reactions, while the second "module" connects redox cofactor recycling to energy conservation. The third module is the terminal electron-accepting pathway. It

is a unique property of acetogenic microorganisms to use the WLP not only as the carbon fixation pathway in anabolism, but also as the terminal electron sink.

The key enzyme of the WLP is CODH/ACS, an NiFeS-protein with $\alpha_2\beta_2$ confirmation that condenses a methyl group and a carbonyl group with coenzyme A to form acetyl-CoA (Ragsdale 2004). The methyl moiety and the carbonyl moiety are both derived from CO_2 (Fig. 1) (Schuchmann and Müller 2014). In the methyl branch, CO_2 is initially reduced to formate (Schuchmann and Müller 2014). The standard redox potential (Thauer et al. 1977) for this reaction is so low ($E_0' = -432$ mV) that it cannot be coupled to NADH oxidation ($E_0' = -320$ mV; $E' = -280$ mV based on intracellular concentrations). The electron donors and responsible enzymes for that reaction differ among the acetogens. The mesophilic "model" acetogen *Acetobacterium woodii* uses hydrogen, which is oxidized by a recently described soluble hydrogen-dependent carbon dioxide reductase (HDCR) (Schuchmann and Müller 2013; Schuchmann et al. 2016). In another mesophilic species, *C. autoethanogenum*, the physiological electron donors for formate dehydrogenase are likely reduced ferredoxin (Fd) and NADPH, as the enzyme forms a functional complex with an electron-bifurcating hydrogenase (Wang et al. 2013). Formate is subsequently bound to the cofactor tetrahydrofolate (THF) in an ATP-dependent reaction. The resulting formyl-THF is in turn dehydrated by formyl-THF cyclohydrolase to form methenyl-THF, which serves as substrate for two consecutive reduction steps. Methenyl-THF is first reduced by methylene-THF dehydrogenase to methylene-THF. This reaction is NADH-dependent in *A. woodii* (Ragsdale and Ljungdahl 1984) and NADPH-dependent in *C. autoethanogenum* (Mock et al. 2015). The subsequent reduction of methylene-THF to methyl-THF is catalyzed by methylene-THF reductase. This reaction is the most thermodynamically favorable in the WLP (Schuchmann and Müller 2014), which lead to the idea that the enzyme might be a site of chemiosmotic energy conservation. In *A. woodii*, however, the heterotrimeric enzyme could be purified from the cytosolic fraction (Bertsch et al. 2015). It is only NADH-dependent and not electron-bifurcating. The physiological electron donor for methylene-THF reductase in *C. autoethanogenum* has not been identified yet (Mock et al. 2015). The methyl group is subsequently transferred to a corrinoid iron sulfur protein by a methyl transferase. In the carbonyl branch of the WLP, CO_2 is initially reduced to enzyme-bound CO. The standard redox potential of the reaction is very low ($E_0' = -558$ mV) and the physiological electron donor is Fd. The standard redox potential of the involved Fd in many acetogenic bacteria is not known, but likely between –450 mV and –500 mV (Schuchmann and Müller 2014). Determined midpoint redox potentials of two iron sulfur clusters in ferredoxin II (–454 and –487 mV) from the thermophilic acetogenic bacterium *Moorella thermoacetica* fall in that range (Bender and Ragsdale 2011). Finally, CODH/ACS receives the methyl moiety from the CoFeS and catalyzes its condensation with the enzyme-bound [CO] and coenzyme A to acetyl-CoA. Acetyl-CoA subsequently serves as precursor for cell biomass synthesis, but during autotrophic growth, a significant fraction is further metabolized to acetate by the reactions of phosphotransacetylase and acetate kinase. The latter reaction is important for energy metabolism as ATP is formed via substrate-level phosphorylation. Some acetogens such as *C. ljungdahlii* and the closely related species *C. autoethanogenum* also form ethanol during autotrophic growth, either by reduction of acetate or acetyl-CoA (Köpke et al. 2010; Mock et al. 2015). As mentioned above, the WLP is the terminal electron accepting pathway, however, some acetogens use different electron acceptors in addition to CO_2. For example, *A. woodii* preferentially uses caffeate (Tschech and Pfennig 1984), while *M. thermoacetica* contains cytochromes and respires thiosulfate, nitrite and nitrate (Pierce et al. 2008).

Electron donors for autotrophic growth are CO and H_2. CO provides electrons via CODH. The redox potential of the couple CO_2/CO is very low, low enough to allow reduction of Fd ($E_0' = -558$ mV). Alongside HDCR, the key enzyme for H_2 oxidation in acetogenic microorganism is an electron-bifurcating hydrogenase. In *A. woodii*, the enzyme contains four subunits (HydABCD), harbors iron-sulfur centers and a flavin, and catalyzes the oxidation of hydrogen coupled to the concomitant reduction of Fd and NAD in a 1:1 stoichiometry (Schuchmann and Müller 2012). In *C. autoethanogenum*, the enzyme uses Fd and NADP simultaneously as electron acceptor (Wang et al. 2013). Bifurcating hydrogenase, operating in reverse, may also be important for redox balancing during heterotrophic growth, thereby becoming a confurcating enzyme. In fact, a Fd-dependent NADH-oxidizing hydrogenase has been described as hydrogen evolving enzyme in the thermophilic fermentative bacterium *Thermotoga maritima* (Schut and Adams 2009). Besides CO and H_2, the variety of electron donors for acetogens is huge. Heterotrophic growth of acetogens and its physiological and ecological significance has recently been reviewed (Schuchmann and Müller 2016). For example, acetogenic bacteria grow on formate, methanol, hexoses, pentoses, alcohols, diols, methylated and methoxylated compounds, organic acids such as lactate or pyruvate, acetoin and two-carbon compounds including oxalate and glycolate. It should be noted that single acetogenic species are usually capable of using a variety of electron donors from the list above. This metabolic diversification may thereby explain why they occur in the same environments as methanogenic archaea or sulfate-reducing


a
2 H+
Ech
2 H+
12 H+
6 H2
HydABC
3 NAD+
3 NADH
3 Fd
3 Fd2–
2 Fd2– 2 Fd 4 H+ 2 H2
0.5 (ADP+ Pi) 0.5 ATP
NfnAB
2 NADP+
Fd
2 NADPH
Fd2–
NAD+
NADH
CO2
Fd2–
Fd
NADPH NADP+ ATP ADP+Pi
NADPH NADP+ 2 NADH 2 NAD+
CO2 HCOO– CHO-THF CH-THF CH2-THF CH3-THF
CoFeSP – CH3
CO
Fd2– *Fd
4 H2 + 2 CO2 Acetate + 0.5 ATP
acetate acetyl-P acetyl-CoA
ATP ADP
b
H+
Ech
H+
Fd2– Fd 2 H+ H2
0.25 (ADP+ Pi) 0.25 ATP
8 H+
4 H2
HydABC
2 NAD+
2 NADH
2 Fd
2 Fd2–
CO2
Fd2–
Fd
H2 2 H+
ATP ADP+Pi
#NADH NAD+ #NADH NAD+
CO2 HCOO– CHO-THF CH-THF CH2-THF CH3-THF
CoFeSP – CH3
CO
4 H2 + 2 CO2 Acetate + 0.25 ATP
acetate acetyl-P acetyl-CoA
ATP ADP

◂**Fig. 1** Model of acetogenesis from hydrogen and carbon dioxide in *Moorella thermoacetica* and in *Thermoanaerobacter kivui* (modified after Schuchmann and Müller 2014; Hess et al. 2014). **a** In *M. thermoacetica*, energy conservation during autotrophic growth is dependent on an energy-converting hydrogenase (Ech). The membrane-bound complex couples the exergonic oxidation of reduced ferredoxin (Fd) to proton reduction, thereby translocating protons across the membrane. The exact stoichiometry of protons translocated per Fd oxidized is unknown. Reduced Fd and NADH are provided by an electron-bifurcating hydrogenase. The electron-bifurcating transhydrogenase NfnAB couples the oxidation of Fd^{2-} and NADH to the co-concomitant reduction of $NADP^+$. Alternatively, NADPH could be provided by a soluble hydrogenase. NADPH is consumed by formate dehydrogenase and methylene-THF dehydrogenase. Methylene-THF reductase is NADH-dependent and described to be electron-bifurcating, but its second electron acceptor is unknown. For simplification, Fd is included in the model. In case the second electron acceptor with a putatively very negative redox potential can either reduce Fd or substitute it in one of the Fd-dependent reactions (Ech or CODH), 0.5 mol ATP is conserved per mole acetate formed, based on a stoichiometry of one H^+ per oxidized Fd and 4 H^+ per formed ATP. **b** *T. kivui* also depends on proton translocation by energy-converting hydrogenase (Ech), of which two are encoded in its genome. Unlike in *M. thermoacetica*, CO_2 reduction to formate is likely catalyzed by a soluble hydrogen-dependent carbon dioxide reductase similar as in *A. woodii* (HDCR; Schuchmann and Müller 2013). The electron donors of methylene-THF dehydrogenase and methylene-THF reductase are unknown, therefore, NADH was included in the model (indicated with #). Also, methylene-THF reductase is likely not electron-bifurcating, explaining the lower ATP yield of 0.25 mol ATP per mole acetate formed based on a stoichiometry of 4 H^+ per ATP

bacteria, as they avoid direct competition by simultaneously using "left-over" electron donors or several substrates (Schuchmann and Müller 2016).

Heterotrophic growth is thermodynamically favorable over autotrophic growth on H_2 and CO_2, and that manifests in the conservation of more ATP, e.g., via substrate-level phosphorylation in glycolysis and by acetate kinase during growth on hexoses (Schuchmann and Müller 2016). Considering autotrophic growth, it is striking that the WLP itself does not account for any net ATP gain, as the activation of formate requires one ATP, balancing out the ATP formed by acetate kinase. The search for a coupling site remained unsuccessful for decades. In 2010, it was found that the membranes of *A. woodii* catalyze the reversible electron transfer from reduced Fd to NAD^+ thereby translocating sodium ions across the membrane (Biegel and Müller 2010). A membrane-bound enzyme with NADH dehydrogenase activity had been partially purified before and two proteins in the preparation were identified as Rnf subunits (Biegel et al. 2009). *Rnf* genes had been found before in *Rhodobacter capsulatus* ("*Rhodobacter* nitrogen fixation"), where it had been postulated to operate in reverse direction, consuming a membrane gradient to reduce Fd with NADH for nitrogen fixation (Jouanneau et al. 1998; Schmehl et al. 1993). Based on the abovementioned biochemical properties of bifurcating hydrogenase, HDCR and methylene-THF reductase, a model of bioenergetics for autotrophic growth of *A. woodii* can be deduced (Schuchmann and Müller 2014). The WLP consumes 1 mol of hydrogen (HDCR), 1 mol of reduced Fd (CODH/ACS) and 2 mol of NADH (methylene-THF dehydrogenase and methylene-THF reductase) to reduce 2 mol of CO_2 to acetyl-CoA. Bifurcating hydrogenase oxidizes 3 mol of H_2, providing reducing equivalents (electrons) to 1.5 mol of NAD and Fd each. Rnf oxidizes the remaining 0.5 mol of reduced Fd with NAD as electron acceptor, a reaction which is coupled to the export of 1 mol of Na^+. The F_1F_o ATP synthase of *A. woodii* is Na^+-dependent (Brandt et al. 2016; Heise et al. 1991), and generates 1 mol ATP per 3.3 mol of Na^+ ions, which is equivalent to 0.3 mol ATP per mole acetate synthesized (according to Eq. 1). The low ATP yield is consistent with the $\Delta G'$ of about –40 kJ mol^{-1} under physiological conditions (Schuchmann and Müller 2014). This model of bioenergetics for *A. woodii* underlines the essential function of the Rnf complex in this model acetogen for redox homeostasis and energy conservation. The essential role has recently been demonstrated in another mesophilic acetogen, *C. ljungdahlii*. In *C. ljungdahlii,* the Rnf complex is proton-translocating, and the disruption of the *rnf* operon lead to a mutant that was not capable of autotrophic growth anymore (Tremblay et al. 2013). Interestingly, *M. thermoacetica* and *T. kivui* do not rely on the Rnf complex for energy conservation, and models for autotrophic acetogenesis in these thermophilic acetogenic bacteria will be discussed in the following paragraphs.

Thermophilic acetogens

Moorella spp.

The genus *Moorella* is the most well-known among the thermophilic acetogenic microorganisms. Phylogenetically, the genus is part of the family *Thermoanaerobacteraceae* within the order *Thermoanaerobacterales* of the class *Clostridia* (Wiegel 2009). It is currently comprised of at least seven species, *M. thermoacetica*, Moorella *thermoautotrophica, M. mulderi, M. glycerini, M. stamsii* (Alves et al. 2013) and *M. humiferrea* and *M. perchloratireducens*, with additional isolates not been described as species yet. *Moorella* species have a broad substrate spectrum, with variation among the different species. Within the genus, hexoses, pentoses, lactate, pyruvate, glycerol, CO and H_2 are among the used electron donors. CO_2, nitrate, nitrite, thiosulfate, AQDS, humic acids, Fe(III) and perchlorate are used as electron acceptors (Alves et al. 2013; Balk et al. 2008, 2003; Wiegel 2009). Importantly, not all of the seven species grow on H_2 and CO_2. *Moorella* species contain quinones and cytochromes, which led to the original classification of acetogens with and without cytochromes. This has recently been revised and replaced by a new classification based on the coupling

site for energy conservation (Rnf *vs.* Ech) (Schuchmann and Müller 2014). *M. thermoacetica* (before: *Clostridium thermoaceticum*), the type strain, was isolated from horse manure and described as first homoacetogenic fermentative organism in 1942 (Fontaine et al. 1942). It grows at 45–65 °C (T_{opt} 55–60 °C), using hexoses, pentoses, methoxylated compounds, several two-carbon compounds, formate, CO, H_2 and CO_2. Using extracts and cells of *M. thermoacetica*, Harland G. Wood and Lars Ljungdahl deciphered the pathway of acetogenesis, which was later named after them (Ljungdahl 2009). Growing heterotrophically on sugars, e.g., glucose, *M. thermoacetica* glycolytically produces pyruvate, which is in turn oxidatively decarboxylated by pyruvate:ferredoxin oxidoreductase (POR), producing reduced Fd and 2 mol of acetyl-CoA (Wiegel 2009). Acetyl-CoA is then converted via acetyl-phosphate to acetate, which yields ATP via substrate-level phosphorylation. Per mole of glucose, 2 mol of acetate, 2 mol of CO_2 and eight reducing equivalents (electrons) are produced, with four of the reducing equivalents derived from the oxidation of glyceraldehyde 3-phosphate in glycolysis. The latter, bound in Fd_{red} and NAD(P)H, are transferred to the WLP, to reduce the 2 mol of CO_2 to a third molecule of acetate, explaining the name "homoacetogenic" bacteria. Interestingly, it took over 40 years longer to discover the ability of *M. thermoacetica* to autotrophically grow with H_2 and CO_2 (Daniel et al. 1990; Wiegel 2009). The WLP and reactions for energy conservation in *M. thermoacetica* are different than in *A. woodii* (Fig. 1a; Schuchmann and Müller 2014). *M. thermoacetica* does not depend on Na^+-ions. *M. thermoacetica* does not have an Rnf complex but a membrane-bound, putative energy-converting hydrogenase (Ech). The electron donor for the Ech is reduced Fd provided by a bifurcating hydrogenase. The first step in the methyl branch is catalyzed by a putatively membrane-associated NADPH-dependent formate dehydrogenase that may form a complex with the Ech hydrogenase and that may work as formate-hydrogen lyase in reverse (Pierce et al. 2008; Yamamoto et al. 1983). Methylene-THF dehydrogenase is NADPH-dependent as well. NADPH is likely provided by a NADH-dependent reduced ferredoxin:NADP oxidoreductase (NfnAB) (Huang et al. 2012) or by a soluble NADPH-dependent hydrogenase (Mock et al. 2014). Methylene-THF reductase is likely a bifurcating enzyme, using NADPH as electron donor, and an unknown second electron acceptor. Notably, Fd from *Clostridium pasteurianum* is not reduced. The enzyme is part of a complex with predicted subunits of a heterodisulfide reductase (Mock et al. 2014). Taken together, the reduction of 2 mol of CO_2 requires 2 mol of NADPH, 2 mol of NADH (for methylene-THF reductase, with 1 mol of an unknown acceptor produced), and 1 mol Fd for CO_2 reduction to CO. Two moles of NADPH could be provided by NfnAB or by a soluble hydrogenase. Under the assumption that the unknown electron acceptor possesses a low redox potential similar to ferredoxin and being able to provide reducing equivalents for one of the Fd-dependent reactions (the reduction of CO by CODH or to hydrogen production by Ech), the overall ATP gain is 0.5 mol/mole acetate, if the Ech complex translocates one proton per molecule of hydrogen formed (Fig. 1a; Schuchmann and Müller 2014).

Thermoanaerobacter kivui

Thermoanaerobacter kivui belongs to the genus *Thermoanaerobacter*, which is as *Moorella* spp. part of the family *Thermoanaerobacteraceae* within the order *Thermoanaerobacterales* of the class *Clostridia*. It was isolated from Lake Kivu and first described under the name *Acetogenium kivui* (Leigh et al. 1981; Leigh and Wolfe 1983). It grows optimally around 66 °C within a range of 55–70 °C and at a pH of 6.4, using sugars, pyruvate, formate, $H_2 + CO_2$ and CO (Weghoff and Müller 2016). The doubling time for growth on $H_2 + CO_2$ is about 2 h in defined medium without vitamin addition (Leigh et al. 1981), so it grows about ten times faster as *M. thermoacetica* and the growth yield per H_2 is 0.91 g mol^{-1} is approximately twice as much (Daniel et al. 1990). *T. kivui* has recently been adapted to grow on CO with a doubling time of about 10 h at 70 % CO (Weghoff and Müller 2016), which is of significance as CO is a major constituent of syngas (see section "Possible biotechnological applications" below). The reactions of the WLP and energy conservation differ from those described above for *M. thermoacetica* and *A. woodii*. While many enzymes have not been biochemically characterized yet, physiological experiments and the genome sequence allow some conclusions towards a model of bioenergetics for autotrophic growth (Hess et al. 2014). As *M. thermoacetica*, *T. kivui* uses protons and not Na^+ for ATP synthesis. The coupling site is likely an Ech complex, of which two are encoded in the genome, while Rnf genes are missing (Fig. 1b). It is unclear which of the two Ech complexes or if both complexes are important for energy conservation. Hydrogen oxidation is catalyzed by a bifurcating hydrogenase, yielding reduced Fd and NADH. The genome also encodes for NfnAB, possibly providing NADPH for biosynthetic reactions. Initial CO_2 reduction is likely catalyzed by a soluble HDRC, as in *A. woodii.* The electron donors of methylene-THF dehydrogenase and methylene-THF reductase are not yet determined, but there is no genomic evidence that methylene-THF reductase is bifurcating. Considering that both methylene-THF dehydrogenase and methylene-THF reductase use NADH or an energetic equivalent of NADH as electron donor, and Ech translocating one proton per molecule of H_2 formed, 0.25 ATP is conserved per acetate formed, only half as much as in *M. thermoacetica*, due to the bifurcating methylene-THF reductase (Hess et al. 2014). Remarkably, *T. kivui* is the only species among the characterized *Thermoanaerobacter* species that

Table 1 Presence of genes encoding key enzymes involved in carbon catabolism, carbon fixation and energy metabolism in the genome of the acetogenic *T. kivui* in comparison to different *Thermoanaerobacter* species representing the three Clades (Verbeke et al. 2013; Hess et al. 2014)

	T. kivui	*T.* sp. strain X514 (Clade 1)	*T. mathranii* susp. *mathranii A3* (Clade 2)	*T. wiegelii* Rt8.B1 (Clade 3)
GAPDH (glycolysis)	*TKV_c16340*[d]	*Teth514_1306*	*Tmath_1562*	*Thewi_1689*
Pyruvate: Fd oxidoreductase (POR)[a]	*TKV_c04340*[d]	*Teth514_0384*	*Tmath_0499*	*Thewi_0500*
Aldehyde/Alcohol dehydrogenase (bifunctional)	**n.f.**	*Teth514_0627*[d]	*Tmath_2110*	*Thewi_2535*
Aldehyde:Fd oxidoreductase (AOR)	**n.f.**	*Teth514_1380*[d]	**n.f.**	*Thewi_0375*
Primary alcohol dehydrogenase[b]	***TKV_c02600***	*Teth514_ 0564*[d]	*Tmath_2093*	*Thewi_0723*[d]
Acetate kinase	*TKV_c13960*[d]	*Teth514_1731*	*Tmath_1338*	*Thewi_1435*
Bifurcating hydrogenase	*TKV_c19580*[d]*–TKV_ c19600*	*Teth514_2138–Teth514_2142*	*Tmath_0865–Tmath_0869*	*Thewi_0976–Thewi_0980*
Ni–Fe hydrogenase (putative Ech)[c]	*TKV_c01230ff*[d] *TKV_c19680ff*	**n.f.**	*Tmath_1603–Tmath_1608*	*Thewi_0134–Thewi_0139*
Rnf complex	**n.f.**	*Teth514_0079–Teth514_0084*	*Tmath_0169–Tmath0173*	**n.f.**
CODH/ACS (beta subunit)	*TKV_c19820*[d]	**n.f.**	**n.f.**	**n.f.**
HDCR	*TKV_c19950–TKV_ c19990*[d]	**n.f.**	**n.f.**	**n.f.**
Methylene:THF reductase subunit MetV	*TKV_c19890*[d]	**n.f.**	**n.f.**	**n.f.**

Italics, present; Bold, absent; Bolditalics, unclear

n.f. not found, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase, *Fd* ferredoxin, *Rnf* Rhodobacter nitrogen fixation (energy-conserving Fd:NAD oxidoreductase), *CODH/ACS* carbon monoxide dehydrogenase/acetyl-CoA synthase, *HDCR* hydrogen-dependent carbon dioxide reductase

[a] All species contain several genes encoding for POR . Shown here are the closest homologues by sequence identity to characterized POR from *Thermoanaerobacterium saccharolyticum* (*Tsac_0046*). For more information see Verbeke et al. (2013)

[b] *T.* sp. strain X514, *T. mathranii* susp. *mathranii* and *T. wiegelii* Rt8.B1 contain several alcohol dehydrogenases, with mostly uncharacterized substrate spectrum and cofactor specificity. Shown here the closest homologue (based on amino acid identity) to Teth514_0564, a NADPH-dependent primary alcohol dehydrogenase from *T.* sp. strain X514 (Basen et al. 2014). TKV_c02600 annotated as NADH-dependent alcohol dehydrogenase, but only 37 % sequence identity to Teth514_0564

[c] The *T. kivui* genome contains two clusters of Ech-type hydrogenases. For more information, see Hess et al. (2014)

[d] Used for BLASTp search (http://blast.ncbi.nlm.nih.gov/Blast.cgi)

contains the WLP and fixes CO_2 (Table 1). Therefore, it is tempting to speculate whether *T. kivui* acquired CODH/ACS genes via lateral gene transfer or whether the other fermentative *Thermoanaerobacter* lost the corresponding genes. However, it is striking that *T. kivui* seems phylogenetically most related to *Thermoanaerobacter* species of Clade 3 (e.g., *Thermoanaerobacter wiegelii*) (Oehler et al. 2012; Verbeke et al. 2013), so if genes were secondarily lost, that event would have taken place independently in the three Clades. CODH/ACS (beta subunit, TKV_c19820) of *T. kivui* is most similar to CODH/ACS of non-acetogenic thermophilic species *Thermoanaerobacteraceae* (e.g., *Thermosediminibacter oceani* DSM 16646, 80 % sequence identity). The second interesting observation is that *T. kivui* is the only *Thermoanaerobacter* species that, while capable of heterotrophic growth on sugars, does not have the potential to produce ethanol. Unlike *M. thermoacetica*, it neither contains aldehyde dehydrogenase nor aldehyde:ferredoxin oxidoreductase, important enzymes for the production of ethanol from acetyl-CoA or acetate (Basen et al. 2014; Olson et al. 2015). Interestingly, all other *Thermoanaerobacter* spp. are described as glycolytic bacteria, producing ethanol, acetate, H_2 and CO_2 in sugar fermentation, with some species producing almost exclusively ethanol as end product (Olson et al. 2015; Verbeke et al. 2013).

Thermacetogenium phaeum

Thermacetogenium phaeum is a rod-shaped Gram-positive bacterium. Like *Moorella* spp. and *Thermoanaerobacter kivui* it belongs to the order *Thermoanaerobacterales* (Oehler et al. 2012). Within that, it is phylogenetically

relatively isolated, with the mesophilic syntrophic acetate oxidizing bacterium *Syntrophaceticus schinkii* as closest relative (<92 % sequence identity based on 16S rRNA gene). It has been isolated from an acetate-oxidizing methanogenic co-culture, using pyruvate (Hattori et al. 2000). In the co-culture, it oxidizes acetate producing CO_2 and H_2 in a reversal of the acetogenic reaction (Eq. 1) thereby relying on hydrogen scavenging by a strain of the methanogen *Methanobacterium thermoautotrophicus*. It grows optimally at pH 6.8 and at a temperature of 58 °C (within a range of 40–65 °C), with a doubling time of about 70 h in syntrophic co-culture. In pure culture, it forms acetate as major end product, using a variety of electron donors including CO, H_2 (autotrophic growth), formate, pyruvate, different alcohols, methoxylated aromatic compounds such as vanillate, glycine, cysteine, but interestingly not hexose sugars. It has been reported to oxidize acetate with thiosulfate or sulfate as electron acceptor (Hattori et al. 2000), though these results could not be confirmed in another study (Oehler et al. 2012). Physiological characteristics have been deduced from its genome (Oehler et al. 2012). It does not possess genes for cytochrome biosynthesis. The genome encodes for all necessary enzymes of the WLP. It contains several genes encoding formate dehydrogenase including a putative membrane-bound formate hydrogen lyase. Energy conservation likely takes place via an Ech, but there is no evidence for a Rnf complex. Unfortunately, relatively little physiological and biochemical data for *T. phaeum* is available, despite its distinct phylogenetical position and its unique ability among the thermophilic acetogens for the reversal of the acetogenic pathway. Therefore, we decided not to construct a model of energy conservation for *T. phaeum* during autotrophic acetogenic growth.

Physicochemical effects of elevated temperatures

As described above, few thermophilic acetogenic species are described, but why? Of course, that could simply originate in below-average interest of researchers in thermophiles and especially thermophilic acetogens in the past, and many more acetogenic thermophiles are still awaiting their discovery. For example, the metagenome of a deep-branching bacterium assembled from DNA isolated from a hot aquifer in Japan recently revealed the presence of all genes necessary for an acetogenic lifestyle (Takami et al. 2012). However, there is a non-negligible temperature effect on the overall thermodynamic of the reactions, which may at least in part explain why few thermophilic acetogens have been described to date. There is a long-term discussion about why hydrogen-consuming acetogens are able to occur anyway, as they compete with methanogens for the same substrates (H_2 and CO_2), and acetogenesis releases lower Gibb's free energy of the overall reaction (Eq. 1 and 2) under standard conditions. In conclusion, hydrogenotrophic methanogens tolerate lower hydrogen partial pressures than acetogens. A temperature increase to 70 °C, which is 4 °C above the optimal growth temperature of *T. kivui*, causes an increase of Gibb's free energy under standard conditions for acetogenesis from H_2 and CO_2 by +9 kJ mol^{-1} (from –170 to –161 kJ mol^{-1}), while Gibb's free energy for hydrogenotrophic methanogenesis increases by +7 kJ mol^{-1} (from –194 to –187 kJ mol^{-1}, calculated for all reactants in the aqueous state according to Amend and Shock (2001)). The impact of gas solubilities on the ΔG values (under environmental conditions), however, may be profound. Solubility of hydrogen is moderately temperature dependent, while the solubility of CO_2 drastically decreases with temperature (Amend and Shock 2001). As acetogens need two molecules of CO_2, they are more affected by a decrease in CO_2 concentration, which in turn increases the hydrogen partial pressure at which energy conservation is still possible. While during industrial fermentations partial pressures of the gases can be kept high, lower availability in the environment will affect microbial communities. It should be considered, however, that on top of thermodynamic effects, lower partial pressures of the gases may trigger the activation or repression of gene expression. For example, expression of a bifunctional aldehyde/alcohol dehydrogenase and a Fd-dependent [FeFe]-hydrogenase may be regulated depending on the hydrogen partial pressure by a PAS-domain containing hydrogenase HydS in the fermentative gut bacterium *Ruminococcus albus* (Zheng et al. 2014). A BLASTp search (https://blast.ncbi.nlm.nih.gov/Blast.cgi), revealed that the genomes of *Acetobacterium woodii* and *Eubacterium limosum* KIST612 each contain a gene encoding a putative regulatory hydrogenase (Awo_c14160 and ELI_1214) with 38 % amino acid sequence identity to HydS (RumaI_3405) from *R. albus*, however, they are not located in close proximity to any genes obviously involved in hydrogen metabolism such as those encoding for bifurcating hydrogenase or HDCR. Based on a stricter criterion of at least 90 % query coverage, including the putative sensory PAS domain, and 50 % amino acid sequence identity, no homologues to HydS were found in the genomes of the acetogenic genera *Acetobacterium*, *Sporomusa*, *Moorella* and the acetogenic species *Thermoanaerobacter kivui*, *Thermacetogenium phaeum Eubacterium limosum*, *Clostridium autoethanogenum* and *Clostridium ljungdahlii*.

While gas concentration determines the overall energetics of a reaction, the diffusivity of the gases may influence metabolic processes as well. Thermophiles have higher metabolic rates and, therefore, CO_2 consumption may be faster than CO_2 diffusion, limiting the product formation and growth rates. In that case, metabolic rates depend on the diffusion coefficient, which for CO_2 is more than twice as high at 60 °C compared to 25 °C (Tamimi et al. 1994). As discussed below, higher metabolic rates and diffusivities

at elevated temperatures represent an intrinsic advantage of thermophiles for biotechnological applications (Taylor et al. 2009).

Higher temperatures also affect the membrane composition. In general, membranes become leakier to protons than to sodium ions, which favors Na^+-based bioenergetics (Mayer and Müller 2014; Grüber et al. 2014). Therefore, the observation that *M. thermoacetica* and *T. kivui* both apparently use protons as coupling ion is interesting, but clearly not without precedence among thermophiles.

Possible biotechnological applications

Acetogenic microorganisms have recently attracted more interest as source of biocatalysts or as whole-cell biocatalysts for biotechnological applications. The HDCR from *A. woodii* reversibly converts H_2 and CO_2 to formate, which is easier to store than the biofuel H_2, at higher rates than chemical catalysts (Schuchmann and Müller 2013). Thermophilic acetogens could also be used as whole-cell catalysts for bioconversions. *T. kivui* has been shown to produce acetate from cellulose in co-culture with the cellulose-hydrolyzing *Clostridium thermocellum* (Le Ruyet et al. 1984), which points towards a potential application of *T. kivui* in second generation biofuel formation. Recently, acetogens have also been considered as model microorganisms for the conversion of reduced gases (H_2 and CO) to acetyl-CoA as precursor for third-generation biofuels or valuable chemicals. Additionally, there is evidence for acetogenesis using cathodes as electron donors in "electrosynthesis chambers" (Gildemyn et al. 2015; Nevin et al. 2011; Rosenbaum and Henrich 2014). CO, H_2 and CO_2 are the main constituents of syngas, which is e.g. produced by incomplete burning of biomass or as off-gas from steel mills. Using thermophilic acetogens in syngas fermentations or processes fueled by H_2 or CO would represent a novel approach. Fermentations, in the sense of steered, contained biotechnological processes in "fermenters", at elevated temperatures may be advantageous over fermentations at 37 °C. Advantages include a lower risk of contamination, higher metabolic and diffusion rates as well as, most importantly, reduced cost of cooling (Taylor et al. 2009), as process heat could be exploited by the thermophiles. Considering syngas as off-gases of steel mills, the heat from the gas stream could be used to fuel fermenters at elevated temperatures. However, it needs to be considered that thermophilic acetogens do not necessarily produce products of interest, such as a biofuel or high-value chemical. Nevertheless, their acetate yield from sugars or from $H_2 + CO_2$ or syngas renders them interesting for biotechnological applications. Therefore, two strategies may be applied. On the one hand a two-stage process with conversion of biomass-derived sugars to acetate, followed by a thermophilic or mesophilic conversion of acetate to a fuel (e.g., ethanol) or a high-value chemical by a different organism. On the other hand, a single-stage process, however, they need to be genetically engineered. Hence, some requirements for a potential industrial application of thermophilic acetogenic microorganisms as whole-cell biocatalysts are potential high conversion efficiencies (yield), titers and rates; the availability of genetic toolboxes and in depth-knowledge of biochemical properties.

Moorella thermoautotrophica and *M. thermoacetica* have already been used in industrial applications to produce the road deicer calcium magnesium acetate (Wiegel 2009). Recently, *M. thermoacetica* has been shown to produce industrially relevant titers of acetate. Growing on CO and CO_2, it reaches a maximal optical density of 11.3, acetic acid titer of 31 g L^{-1}, and productivity of 0.55 g L^{-1} h^{-1} at a CO mass-transfer rate of 83 mM h^{-1} (Hu et al. 2013). Ethanol formation according to Eq. 3 should be possible, considering that autotrophic growth is possible with a positive balance of 0.5 ATP per 1 acetate formed (Schuchmann and Müller 2014), and considering that *M. thermoacetica* contains functional bifurcating hydrogenase, AOR and alcohol dehydrogenase (Simon et al. 1987). The acetate formed could be reduced by AOR and NADH-dependent alcohol dehydrogenase. Like in *A. woodii*, the process is feasible at no additional ATP expense (Bertsch and Müller 2015), as reduced Fd and NADH were provided in a 1:1 ratio by bifurcating hydrogenase.

$$6\ H_2 + 2\ CO_2 \rightarrow \text{Ethanol} + 0.5\ \text{ATP} \quad (3)$$

As *M. thermoacetica* has been suggested to catalyze the through-reduction of organic acids to their corresponding alcohols (Simon et al. 1987), it is unclear why ethanol is not a major product. It has been reported to be formed as side product during growth on $H_2 + CO_2$ in one *Moorella* strain HUC22-1 (Inokuma et al. 2007). Interestingly, *M. thermoacetica* has recently been used in a very different engineering approach, covered with cadmium nanoparticles exploiting photons to photosynthetically reduce CO_2 to acetate (Sakimoto et al. 2016). A genetic system allowing modifications on the chromosome of *M. thermoacetica* has been developed recently (Kita et al. 2013), enabling further physiological studies towards a promising biotechnological platform.

Thermoanaerobacter kivui has been shown to produce relatively high titers of acetate as well (>600 mM) in fermentations of glucose, with a rate of 17 mM h^{-1} and a yield of >2.5 acetate per glucose (Klemps et al. 1987). Equivalent fermentation studies with H_2 or CO as sole electron donor have unfortunately not been performed yet. Interestingly, *T. kivui* apparently does not have vitamin requirements but grows on mineral medium (Leigh et al. 1981), lowering potential fermentation costs. Another interesting aspect is

that it has recently been shown to simultaneously use H_2 and CO, the reduced constituents of syngas (Weghoff and Müller 2016). It is likely not able to produce ethanol or other alcohols due to the lack of aldehyde dehydrogenase and AOR (Table 1). To shift end products from acetate to a biofuel or a high-value chemical, pathways from other thermophilic microorganisms would have to be implemented by metabolic engineering approaches. In that perspective, it has to be kept in mind that *T. kivui* does not have an Rnf complex, so ideally implemented pathways should re-oxidize Fd and NADH in an equimolar ratio. While currently no genetic system is available for *T. kivui*, related *Thermoanaerobacter* spp. have been shown to naturally take up DNA (Shaw et al. 2010), and genome modifications have been performed in *T. ethanolicus* (Shao et al. 2016) and *T. mathranii* (Yao and Mikkelsen 2010). Enzymes associated with the WLP and energy conservation under autotrophic conditions have not been characterized in *T. kivui* in detail, with the exception of CODH, hydrogenase and ATP synthase (Weghoff and Müller 2016). Thus, a genetic system for *T. kivui* would not only allow metabolic engineering but also facilitate understanding of its bioenergetics.

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