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Review

# *Moorella thermoacetica*: A promising cytochrome- and quinone-containing acetogenic bacterium as platform for a $CO_2$-based bioeconomy

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ABSTRACT

The negative effect of fossil-based industrial processes on the environment, especially the contribution to global warming by emitting greenhouse gases such as $CO_2$ causes a global threat to mankind. Therefore, technologies are demanded by the society for a sustainable and environmentally friendly economy. The biotechnological use of sugar-based feedstocks to produce valuable products are in conflict with, for example, food production. In order to overcome this issue, waste products such as syngas ($H_2$, CO and $CO_2$) or $CO_2$ taken from the atmosphere are of increasing interest for biotechnological applications. Acetogenic bacteria are already used at industrial scale to produce sustainable and environmentally friendly biofuels from syngas. A promising candidate due to its physiological flexibility is the thermophilic acetogen *Moorella thermoacetica*. In contrast to most acetogens *M. thermoacetica* is not restricted to one energy conserving system. In addition to the Ech complex, cytochromes and quinones may be involved in energy conservation by, for example, DMSO respiration. The extra energy conserved can be used to form highly valuable but energy demanding products. In this review we give insights into the physiology of this acetogen, the current state of the art of *M. thermoacetica* as a platform for biotechnological applications and discuss future perspectives.

## 1. Introduction

Climate change and the resulting global warming is one of the most severe problems of the 21st century. Fossil resource-based economy contributes to the climate change due to the emission of greenhouse gases such $CO_2$ but also some CO [1]. Therefore, the development of new concepts for energy supply and storage as well as the production of basic compounds for chemical industry beyond fossil resources is of major interest [2,3]. Microbes provide a sustainable solution to use waste products such as $CO_2$ to produce valuable products [4]. Several groups of microorganisms such as cyanobacteria, methanogenic archaea (methanogens) or acetogenic bacteria (acetogens) are capable to use waste products such as CO and/or $CO_2$ as feedstock [5–8]. Whereas the photosynthetic cyanobacteria require solar energy for their autotrophic lifestyle, methanogens and acetogens need an inorganic electron donor such as $H_2$ (when growing on $CO_2$) [5,6,9]. Waste gases released by combusting fossil resources contain mainly 30–60% CO, 5–15% $CO_2$ and 25–30% $H_2$. This combination is known as synthesis gas or syngas. $H_2$ + $CO_2$, CO or syngas are used by acetogens *via* the Wood-Ljungdahl pathway (WLP) as a central metabolic pathway [5,10,11]. The WLP consists of two branches, the carbonyl and the methyl branch [12]. The carbonyl branch consists of one enzyme that catalyses the reduction of one molecule $CO_2$ to enzyme-bound CO under consumption of two electrons [12]. In the methyl branch, another two electrons are used in the first reaction to reduce $CO_2$ to formate. In the next reaction, adenosine triphosphate (ATP) is required to form formyl-tetrahydrofolate from formate and tetrahydrofolate (THF) [13–15]. Formyl-THF is further metabolized to methenyl-THF which is further reduced to methylene-THF and subsequently to methyl-THF and each step requires two electrons [16,17].

*Abbreviations:* ATP, adenosine triphosphate; C, cytochrome *b*; [CO], enzyme-bound carbon monoxide; CoA, coenzyme A; CODH/ACS, CO dehydrogenase/acetyl-CoA synthase; CdS, cadmium sulfide nanoparticle; CoFeSP, corrinoid/iron sulfur protein; DMSO, dimethyl sulfoxide; Ech complex, energy converting hydrogenase; Ech-MTHFR complex, energy converting hydrogenase- methylene-THF reductase complex; $EM_{red}$, electron mediator reduced; $EM_{ox}$, electron mediator oxidized; Fd, ferredoxin; $Fd^{2-}$, reduced ferredoxin; Fix complex, electron-transferring flavoprotein-menaquinone oxidoreductase; [H], redox equivalents; HDCR, hydrogen-dependent $CO_2$ reductase; $K_m$, Michaelis-Menten constant; MTHFR, methylene-THF reductase; NADH, nicotinamide adenine dinucleotide; NDH, NADH dehydrogenase; NADPH, nicotinamide adenine dinucleotide phosphate; Nfn, NADH-dependent reduced ferredoxin:$NADP^+$ oxidoreductase; Q, menaquinone; $QH_2$, OD, menaquinoloptical density; Rnf, *Rhodobacter* nitrogen fixation complex; THF, tetrahydrofolate; $V_{max}$, maximum velocity; WLP, Wood-Ljungdahl pathway

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In the last step of the methyl branch methyl-THF is converted to methyl-CoFeSP [18]. The methyl-group is then combined with CO to form acetyl-CoA, the final product of the WLP. This reaction is catalysed by the key enzyme of the pathway, the CO dehydrogenase/acetyl-CoA synthase (CODH/ACS) [19–23].

Acetyl-CoA is then phosphorylated to acetyl phosphate while releasing CoA, and in the last step of acetogenesis, acetyl phosphate is metabolized to acetate, releasing one molecule ATP [18,24,25]. In summary, the fixation of two molecules $CO_2$ to one molecule acetate *via* the WLP requires eight electrons and one ATP but the last reaction of acetogenesis gains one ATP, leading to an overall ATP gain of zero (Fig. 1) [12,25,26].

Acetogens and methanogens produce acetyl-CoA from $H_2$ + $CO_2$ and acetyl-CoA is the precursor of biosynthetic reactions. Acetyl-CoA is carboxylated to pyruvate and further used *via* the usual routes to synthesize cellular constituents such as proteins, lipids and nucleic acids [27,28]. Whereas acetate is formed by acetogens as end product, the highly flammable methane is produced by methanogens [6,12]. Methane as storable energy carrier produced from waste is of great interest but faces a major problem due to its flammability. A substance of similar interest as energy carrier is formate. Formate is the anionic form of the simplest carbonic acid, formic acid, which can be formed by acetogens and methanogens *via* formate dehydrogenases [12,29]. Within the last decade a remarkable enzyme has been found, purified and characterized from the acetogenic bacteria *Acetobacterium woodii* and *Thermoanaerobacter kivui*, the hydrogen-dependent $CO_2$ reductase (HDCR) [30,31]. The HDCR is capable to reduce $CO_2$ using $H_2$ to form formate as well as oxidize formate to $H_2$ and $CO_2$ without the need of additional soluble electron carriers [2]. In a recent study, the structure of HDCR found in *T. kivui* was solved at a resolution of 3.4 Å [32]. The HDCR forms filaments that are bundled together and seems to be anchored to the cytoplasmic membrane [32]. Furthermore, it was demonstrated that filamentation increased the activity [33]. The outstanding activity of the HDCR is also of great interest for biotechnological applications since no other enzyme nor chemical catalyst reaches comparable reaction rates [30]. Due to this remarkable efficiency a study was conducted demonstrating for the first time the storage and release of $H_2$ in a bioreactor over multiple cycles using whole cells of *A woodii* [2]. Briefly, in this study, day-night periods were simulated, with *A. woodii* being used as biocatalyst to convert $H_2$ + $CO_2$ to formate in the day period. In the subsequent night period, the gas mixture used was changed from $H_2$ + $CO_2$ to $N_2$, which led *A. woodii* to oxidize the formate formed in the day period to $H_2$ + $CO_2$ [2]. Beyond energy storage and production of acetate by acetogens, the production of other valuable products for industrial applications such as lactate, various alcohols or other chemicals such as acetone by acetogens is possible and of high interest [34]. In industrial applications mainly *Clostridium autoethanogenum* is used to produce ethanol from syngas [35]. Other acetogenic bacteria such as *A. woodii* or *Clostridium ljungdahlii* play a minor role. Interestingly, the new species *Clostridium muellerianum* produces caproate, hexanol and butyrate in addition to ethanol and acetate from $H_2$ + $CO_2$ [36]. To our knowledge no thermophilic acetogenic bacteria are used in industrial application despite their advantages compared to mesophilic acetogenic bacteria. Such advantages include the lower risk of contamination, increased metabolic and diffusion rates, lower cost for cooling and in case of products with low boiling points, reduced costs of product recovery [37,38].

Since acetogens are limited in the amount of ATP gained during autotrophic growth, the production of energy-demanding products such as, for example, isoprene, isobutene or even acetone are challenging [34]. The question arose how acetogens can overcome energetic barriers in order to produce such energy demanding products [34]. One approach is to increase the amount of ATP formed but how can this be accomplished? There is a second way of synthesizing ATP in acetogens, by a chemiosmotic mechanism. Thereby, ATP synthesis is coupled to an electrochemical ion gradient across the cytoplasmic membrane, which is build up by respiratory enzymes [5]. So far, two respiratory enzymes have been found in acetogenic bacteria, the ferredoxin:NAD oxidoreductase (Rnf complex) and the ferredoxin:$H^+$ oxidoreductase (Ech complex) and a given species has only the one or the other enzyme. These enzymes do not contain cytochromes. However, cytochromes have been found very early in *M. thermoacetica* and since they are typical compounds of respiratory chains a cytochrome-containing respiratory chain was postulated. Therefore, one approach could be the design of an acetogen with multiple respiratory enzymes. Beside a Rnf complex and a Ech complex as respiratory complex, cytochromes and quinones are known for their role in respiratory chains [39–45]. However, even 50 years after the discovery of cytochromes and quinones in *M. thermoacetica*, there role is still unclear [6,45]. In general, cytochromes and quinones are rather rare in the group of acetogens [47]. In recent publications the contribution of cytochromes and quinones in energy conservation were investigated in *M. thermoacetica* and *Sporomusa ovata* and, therefore, acetogens such as *M. thermoacetica* capable to use cytochromes and quinones in energy-conserving processes may have additional ways to synthesize ATP and are of interest for biotechnological applications because energy barriers could be overcome, and more valuable products maybe be formed [48,49]. In this review we give insights into the remarkable physiology and bioenergetics of *M. thermoacetica*, the current state of the art of *M. thermoacetica* as a platform microorganism for biotechnological applications and discuss future perspectives.

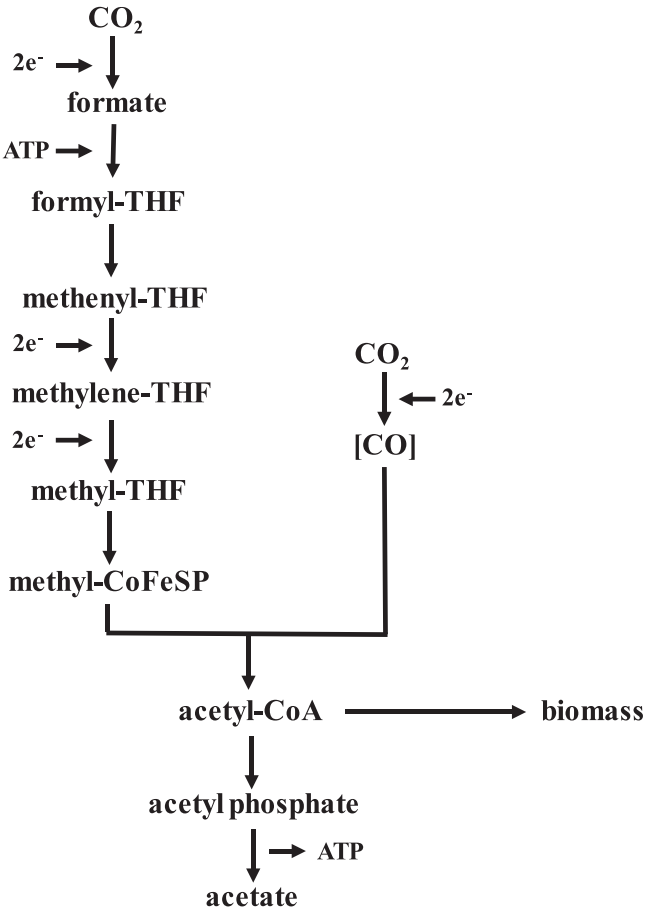


**Fig. 1.** Schematic overview of acetogenesis. In the WLP, two mol of $CO_2$ are reduced to one mol of acetyl-CoA at the expense of eight electrons. Acetyl-CoA is either used in the generation of biomass or further metabolized to acetate. THF, tetrahydrofolate; CoFeSP, corrinoid/iron sulfur protein; [CO], enzyme-bound carbon monoxide; CoA, coenzyme A.
(modified after [12])

## 2. Bioenergetics of acetogenic bacteria

Acetogenesis from $H_2$ + $CO_2$ does not go along with the net synthesis of ATP by substrate-level-phosphorylation, therefore additional ATP synthesis by a chemiosmotic mechanism has to occur [12]. Indeed, acetogens gain ATP by a chemiosmotic mechanism with either the Ech or Rnf complex as a respiratory enzyme to establish a $H^+$- or $Na^+$ gradient, which then fuel $F_1F_0$ ATP synthases [40,42,43,50,51]. Since the bioenergetics of *M. thermoacetica* is not well studied, a brief description of the bioenergetics of *T. kivui* as an Ech-containing model organism growing on $H_2$ + $CO_2$ will be given first. In general, acetogens oxidize four mol of $H_2$ and reduce two mol of $CO_2$ to one mol of acetate [52]. First, hydrogen is oxidized and nicotinamide adenine dinucleotide phosphate (NADP) and ferredoxin (Fd) are reduced by an electron-bifurcating FeFe-hydrogenase [53]. This enzyme drives the endergonic reduction of Fd ($E_0' \approx -450$ mV) with $H_2$ ($E_0' = -414$ mV) by simultaneous exergonic electron transport from $H_2$ to $NADP^+$ ($E_0' = -320$ mV). Whereas NADPH is the reductant for the reduction of methenyl-THF to methylene-THF, $Fd^{2-}$ is the reductant for the methylene-THF reductase (MTHFR) [54]. It is assumed that methylene-THF reduction is performed by the membrane-coupled Ech-MTHFR complex, coupled to translocation of protons [54]. Another mol of $Fd^{2-}$ is required by the CO dehydrogenase, Fd is reduced by the Ech complex with $H_2$ as reductant and this endergonic reaction is driven by the transmembrane electrochemical ion potential [55]. In this bioenergetic model, Ech complexes are the key players. Actually, *T. kivui* has two different Ech complexes with different subunit composition [56]. Ech2 has been purified and shown to be a respiratory enzyme that translocates protons coupled to proton reduction to $H_2$ with $Fd^{2-}$ as electron donor [43]. It is postulated that one Ech complex drives the backward reaction, reduction of Fd with $H_2$ as reductant, whereas the other Ech complex is coupled to the methylene-THF reductase and catalyses the exergonic electron transfer from $Fd^{2-}$ to methylene-THF, coupled to $H^+$ export from the cell [55]. The number of $H^+$ translocated must be higher than the number of $H^+$ consumed by the other Ech complex in order to have protons left for ATP synthesis (Fig. 2A). Comparing the composition of the ATP synthases of *T. kivui* and *Clostridium paradoxum* suggests a $H^+$/ATP ratio of 3.6, indicating a $Fd^{2-}$/ATP ratio of 0.28 [43].

*A. woodii* is a well-studied Rnf-containing model organism. *A. woodii* uses an electron-bifurcating hydrogenase to oxidize three mol of $H_2$ and subsequently reduces 1.5 mol of Fd and 1.5 mol of nicotinamide adenine dinucleotide (NAD) [57]. One mol of $Fd^{2-}$ is used in the carbonyl branch to reduce $CO_2$ to CO and the remaining 0.5 $Fd^{2-}$ is used by the Rnf complex to reduce $NAD^+$ to NADH [5,40,41]. The two mol of NADH and one mol of $H_2$ are used as reductants in the methyl branch of the WLP [5]. The Rnf complex is a ferredoxin:$NAD^+$ reductase that consists of 6 subunits [56]. The Rnf complex oxidizes $Fd^{2-}$ ($E_0' \approx -450$ mV) with reduction of $NAD^+$ ($E_0' = -320$ mV) [40,58]. This reaction provides enough energy ($\Delta G_0' = -25$ kJ) to translocate $Na^+$ across the membrane [59–61]. The $Na^+$ gradient is used to fuel a $Na^+$ $F_1F_0$-ATP synthase to form one mol of ATP per 3.3 $Na^+$ (Fig. 2B) [62–64].

## 3. Insights into the bioenergetics of *M. thermoacetica*

The bioenergetics of acetogenesis in *M. thermoacetica* is still an enigma although important enzymes such as the ATP synthase, the electron-bifurcating hydrogenase or the Nfn complex have been found and characterized [51,65,66]. As already discussed, molecular hydrogen is used by the electron-bifurcating hydrogenase to reduce Fd and $NAD^+$ which are used as reductants in the WLP. The purified electron-bifurcating hydrogenase of *M. thermoacetica* consists of three subunits HydABC, which catalyses the oxidation of hydrogen with $NAD^+$ and Fd as electron acceptors with a specific rate of 57.5 U/mg at 45 °C and pH 7.5 [66]. Since only NADH and $Fd^{2-}$ are provided by HydABC, another enzyme has to supply the cell with NADPH as reductant. Two enzymes seem to be involved. First, a putative NADP-reducing hydrogenase activity was measured in cell extracts of *M. thermoacetica* [65]. This hydrogenase was only detectable when cells were grown on $H_2$ + $CO_2$ and absent under heterotrophic condition [65]. The second enzyme capable of supplying the cell with NADPH is the electron-bifurcating, NADH-dependent reduced ferredoxin:$NADP^+$ oxidoreductase (Nfn). This enzyme was heterologously produced in *Eschericha coli* and further characterized [65]. The Nfn complex consists of two subunits NfnA and NfnB which catalyse the reduction of $NADP^+$ with $Fd^{2-}$ and NADH as

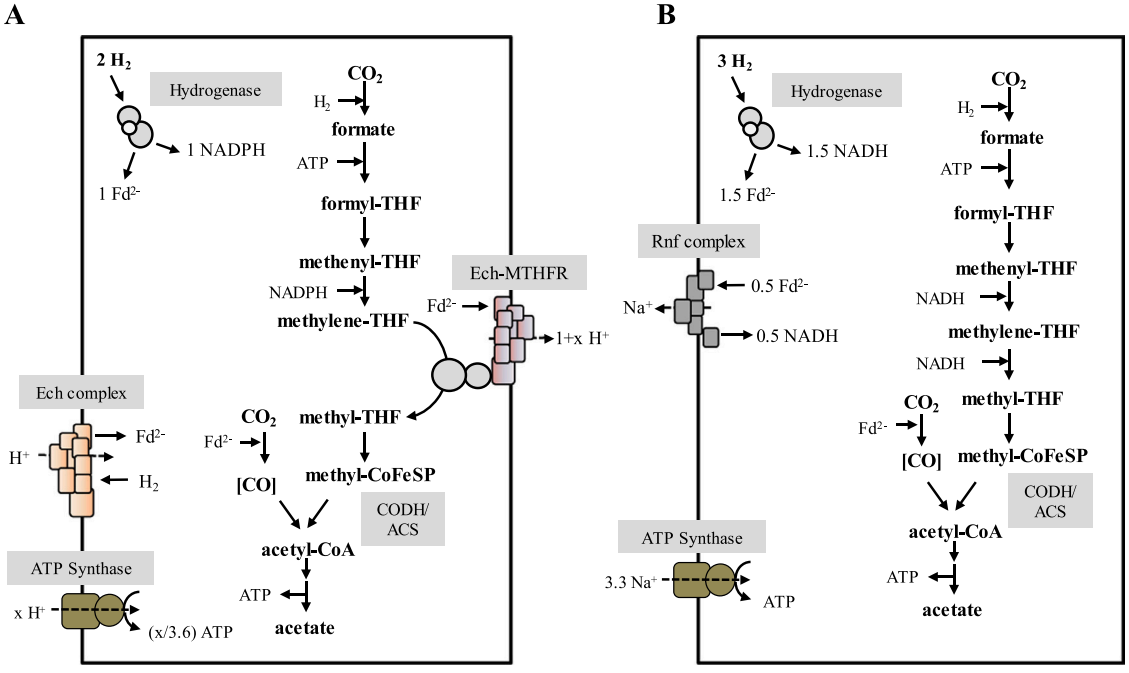


**Fig. 2.** Models of the bioenergetics of acetogenesis from $H_2$ + $CO_2$ in *T. kivui* (A) and *A. woodii* (B). For Ech, a $H^+/e^-$ stoichiometry of 0.5 is assumed, the $H^+$/ATP stoichiometry is assumed to be 3.6 (A). For Rnf, a $Na^+/e^-$ stoichiometry of 1 is assumed, the $Na^+$/ATP stoichiometry is 3.3 (B). For further explanations, see text. (modified after [55])

electron donors with a specific activity of 22.4 U/mg (Fig. 3) [65]. Chemiosmotic energy conservation involves a $H^+$-dependent $F_1F_0$ ATP synthase [50]. Highly speculative is the nature of the respiratory enzyme. *M. thermoacetica* has a gene cluster encoding an Ech complex which encodes in the same cluster a formate dehydrogenase as well as a formate transporter [67]. These genes are absent in the Ech gene cluster of *T. kivui* but the gene cluster is similar to the Fdh-Mbh of the archaeon *Thermococcus onnurineus* [68,69]. Currently, no experimental data are available addressing the question whether or not formate is oxidized in *M. thermoacetica* by a membrane-bound enzyme complex. Based on these enzymes, a model of energy conservation can be built.

Briefly summarized, *M. thermoacetica* oxidizes hydrogen *via* an electron-bifurcating hydrogenase to reduce $NAD^+$ and Fd. Since NADPH is required as reductant for the reduction of $CO_2$ to formate and later for the reduction of methenyl-THF to methylene-THF, either the Nfn or the NADP-reducing hydrogenase is required to supply the cell with NADPH. Unlike the MTHFR, present in *A. woodii* or *T. kivui*, NADH is the electron donor, and methylene-THF and a second unknown electron acceptor are the oxidants. In the carbonyl branch, $Fd^{2-}$ is required as the reductant for the CO dehydrogenase. Similar to *T. kivui*, *M. thermoacetica* is assumed to use the Ech complex to transport protons across the membrane. It is suggested that two protons are translocated per $Fd^{2-}$, resulting in the formation of 0.5 mol ATP by ATP synthase per acetate formed during growth on $H_2$ + $CO_2$ (Fig. 3) [5].

In addition, genes potentially encoding a NADH dehydrogenase (NDH) are present in *M. thermoacetica* [67]. This finding is in contrast to all other acetogens known, that have only one respiratory enzyme, either Rnf or Ech [47]. The putative NDH is similar to the "headless" NDH complex of *Thermosynechococcus elongatus*, which does not use NADH but like the enzyme from *T. elongatus* uses $Fd^{2-}$ as electron donor and a quinone as electron acceptor [70]. Like the enzyme from *T. elongatus*, the enzyme from *M. thermoacetica* apparently lacks subunits involved in NADH oxidation [67]. The Ech as well as the NDH complex are probable $H^+$ translocating enzyme complexes and fuel the $H^+$-dependent $F_1F_0$ ATP synthase [51]. Furthermore, the genome encodes a putative cytochrome *b*-dependent formate/nitrate reductase [67]. This formate/nitrate reductase has neither been purified nor characterized (Fig. 4).

Another big difference of *M. thermoacetica* compared to *A woodii*, *T. kivui* and others is the presence of cytochromes and quinones in the former (Fig. 4) [46]. Their presence has been known for decades but their contribution to energy conservation or metabolism is rather unclear. Recently, it was suggested that menaquinone is a putative electron acceptor for an electron-bifurcating electron-transferring flavoprotein-menaquinone oxidoreductase (EtfABCX, alternatively FixABCX complex) [71]. The Fix complex has also been found in nitrogen-fixing microorganisms such as *Azotobacter vinelandii* as well as in microorganisms that do not fix nitrogen such as *Thermotoga maritima* [72,73]. The Fix complex of *Thermotoga maritima* was recently characterized and its structure was solved at high resolution [73]. It forms a dimer with two membrane-associated subunits, which contains two bound menaquinones. The complex uses NADH as electron donor to reduce Fd as well as menaquinone simultaneously [73]. The Fix complex is present in several acetogenic bacteria such as different *Moorella* and *Sporomusa* species including *M. thermoacetica* and *S. ovata*, but the role of this complex was proposed so far only in lactate metabolism of *M. thermoacetica* [47,71]. *M. thermoacetica* is hypothesized to use the Fix complex to oxidize NADH, thus lowering the NADH concentration which allows to overcome the thermodynamic barrier to reduce $NAD^+$ ($E_0' = -320$ mV) with electrons coming from the oxidation of lactate to pyruvate ($E_0' = -190$ mV), but experimental data supporting this hypothesis are lacking [71]. Furthermore, menaquinol is suggested as electron donor for the DMSO reductase present in *M. thermoacetica* based on the finding that anthraquinone-2,6-disulfonate, a quinone analogue that is used by the DMSO reductase as electron donor [49]. The DMSO reductase present in *M. thermoacetica* consists of three subunits and is similar to the one from *E. coli* [49]. Nitrate is as well

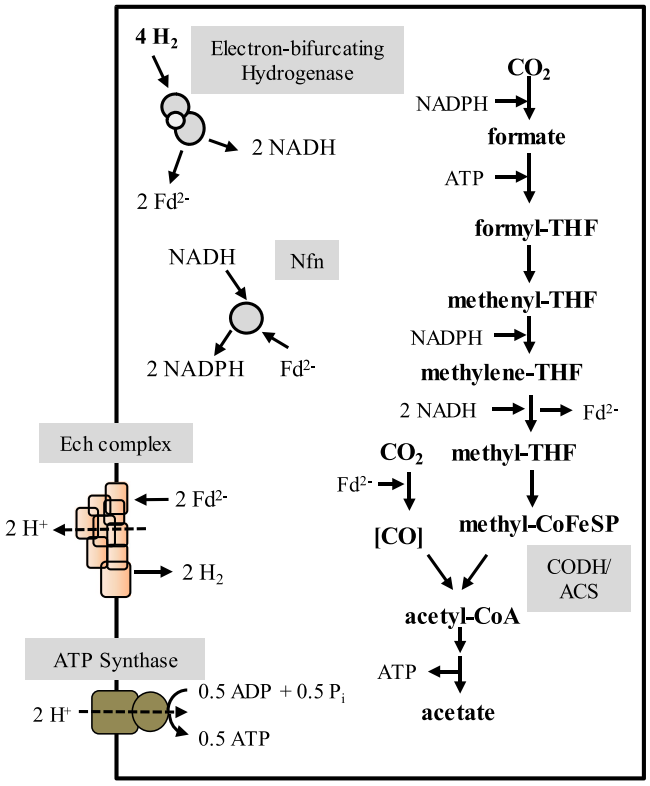


**Fig. 3.** Model of the bioenergetics of acetogenesis from $H_2$ + $CO_2$ by *M. thermoacetica*. The model is based on predictions from the genome sequence. The hypothetical $H^+$ transport by the Ech complex is assumed to be 0.5 $H^+/e^-$. The $H^+$/ATP ratio of the ATP synthase is assumed to be 4.
Modified from Schuchmann and Müller [5].

reduced by *M. thermoacetica* but almost nothing is known about the biochemistry and bioenergetics of nitrate reduction (Fig. 4) [74–77].

## 4. *Moorella thermoacetica* – the model microorganism for acetogenesis

Acetogenesis was first observed by Fischer and coworkers since 1930s, albeit the microorganisms involved were not well understood [78]. The Dutch microbiologist K.T. Wieringa accomplished the isolation of *Clostridium aceticum* and further experiments demonstrated the formation of acetate under growth condition using $H_2$ + $CO_2$ as substrate [79]. Unfortunately, cultures of *C. aceticum* were lost for decades, until the German microbiologist Gerhard Gottschalk found a tube of *C. aceticum* spores in the collection of H.A. Barker. The spores of *C. aceticum* were used to recultivate the microorganism for further investigations [80]. In the meantime, a successful enrichment and isolation of a thermophilic acetogen was accomplished by F.E. Fontaine, the microorganism was originally named *Clostridium thermoaceticum* but was later reclassified and renamed to *Moorella thermoacetica* [81,82].

*M. thermoacetica* is described as a Gram-positive, rod-shaped, peritrichous flagellated and endospore-forming bacterium [83]. This thermophilic acetogen grows at temperatures of 45–65 °C, but has its optimal growth at a temperature of 55–65 °C. The pH optimum is 6.8, but a pH range of 5.7–7.7 is tolerated. *M. thermoacetica* can use a variety of carbon sources such as $CO_2$, CO, monosaccharides, organic acids, alcohols or methoxylated aromatic compounds [83]. Fontaine demonstrated that glucose is fermented to acetate in a ratio of 1:2.5, which was an exciting finding because the classical glucose fermentation does not allow a glucose:acetate ratio higher than 2.0 [81]. A few years later, Barker and Kamen were able to demonstrate in the first ever conducted $^{14}C$ experiment that *M. thermoacetica* is capable to reduce $CO_2$ to acetate [84]. However, the underlying metabolic pathway remained

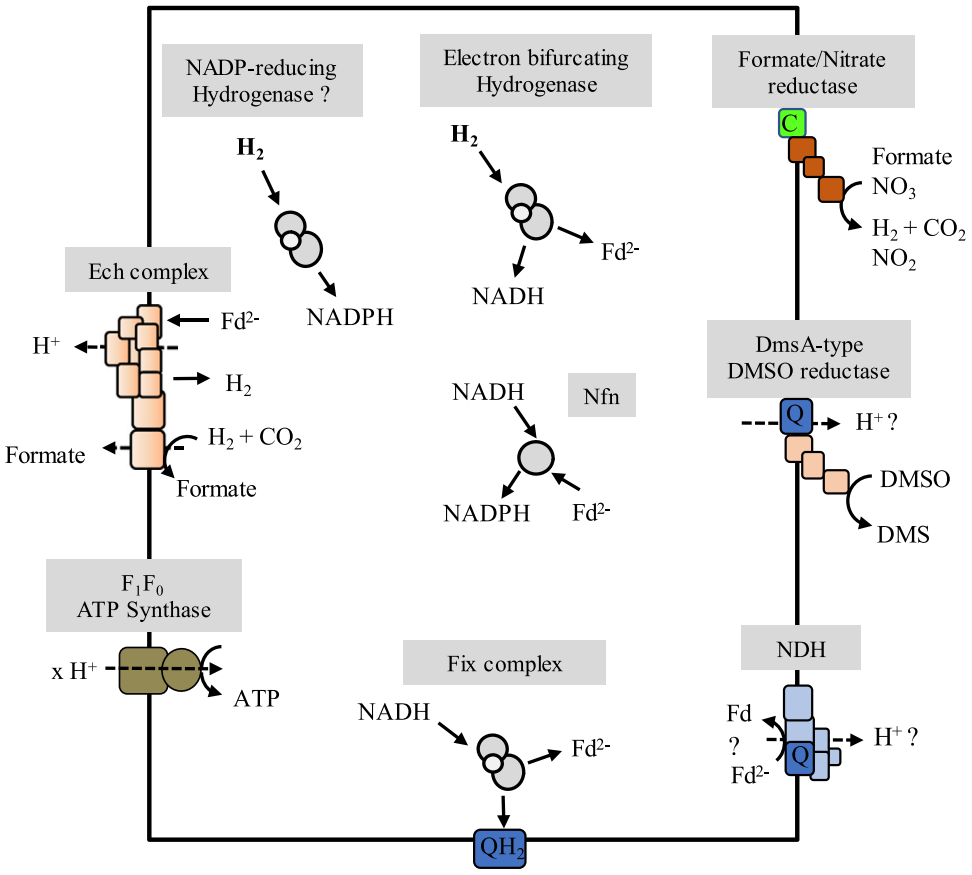


**Fig. 4.** Enzymes in *M. thermoacetica* potentially involved in redox balancing and/or energy conservation. C, cytochrome *b*; Q, menaquinone; Fix complex, electron-transferring flavoprotein-menaquinone oxidoreductase; NDH, NADH dehydrogenase; DMSO, dimethyl sulfoxide; Nfn, NADH-dependent reduced ferredoxin:$NADP^+$ oxidoreductase, Ech complex, energy converting hydrogenase; Fd, ferredoxin; $Fd^{2-}$, reduced ferredoxin; $QH_2$, menaquinol. The hypothetical $H^+$ transport by the Ech complex is assumed to be 0.5 $H^+/e^-$. The $H^+$/ATP ratio of the ATP synthase is assumed to be 4. For further explanations, see text.

enigmatic for decades. The pathway was then elucidated by the two biochemists, Harland G. Wood and Lars G. Ljungdahl and their co-workers. Due to their outstanding work this pathway is named Wood-Ljungdahl pathway (alternative reductive acetyl-CoA pathway).

In contrast to most acetogen, all enzymes of the WLP from *M. thermoacetica* have been purified and characterized (Fig. 5). The initial reaction of the methyl branch, the reduction of $CO_2$ to formate with NADPH as reductant is catalyzed by a NADPH-dependent formate dehydrogenase. This tetrameric formate dehydrogenase consists of two subunits, an α subunit with 96 kDa and a β subunit of 76 kDa [13]. The purified enzyme contains two mol of tungsten, two mol of selenium, 36 mol of iron and 50 mol of inorganic sulfur per mol, respectively, and had a specific activity of 1050 U/mg [13]. The Michaelis-Menten constant ($K_m$) values for $NADP^+$ and formate were determined at pH 7.5 and 55 °C to be 117 and 109 µM, respectively [13,85]. Regardless of the unfavourable bioenergetics with NADPH ($E_0$' = −320 mV) as reductant for the reduction of $CO_2$ to formate ($E_0$' = −420 mV) no other electron donor has been found. The E′ value for the NADP/NADPH couple is − 370 mV, making this reaction less endergonic. Furthermore, it is suggested that the high bicarbonate concentration in the growth medium required by *M. thermoacetica* makes the reduction of $CO_2$ to formate energetically feasible [13,85]. Remarkable is the oxygen-sensitivity, a concentration of 7.6 µM of $O_2$ led to a reduction of activity of 50% [13].

Formate is then bound to THF to yield 10-formyl-tetrahydrofolate; this reaction requires hydrolysis of one ATP and is catalysed by a formyltetrahydrofolate synthase. It is a tetrameric enzyme consisting of four identical subunits with a total molecular mass of 240 kDa that catalyses the reaction with a specific activity of 230 U/mg [86,87]. The $K_m$ for ATP, THF and formate determined at pH 8 and 55 °C were 0.63, 1 and 1.67, respectively [86]. The structure of the formyltetrahydrofolate synthase was solved at a 2.5 Å resolution [88].

Next, formyl-THF is converted to 5,10-methenyl-THF by a formyl-THF cyclohydrolase, thereby $H_2O$ is released. The formyl-THF cyclohydrolase was purified and catalyzed in addition to the reduction of methenyl-THF to methylene-THF with NADPH as reductant. The dimeric enzyme had a molecular mass of 60 kDa [16].

The MTHFR catalyzes the reduction of methylene-THF to methyl-THF. The genes *metVF* encoding for the MTHFR form a transcriptional unit together with the genes *hdrCBA-mvhD* that encode for proteins similar to the heterodisulfide reductase in methanogenic archaea [17,67]. The purified protein complex MetFV-HdrABC-MvhD had a molecular mass of around 320 kDa. Furthermore, a flavin mononucleotide was found in the MetF subunit, whereas HdrA contained four iron-sulfur clusters and two flavin adenine dinucleotides. MetF, an iron-sulfur zinc protein is required by MetV for its full activity. The remaining subunits HdrC, HdrB and MvhD contained four iron-sulfur cluster as well as a zinc ion. This enzyme complex did not use NADH or NADPH as reductant for the reduction of methylene-THF regardless of the presence of additional Fd as additional potential electron acceptor for a potential electron bifurcation. Nonetheless, the enzyme had NADH:benzylviologen oxidoreductase activity with specific activities of 190 and 48 U/mg for HdrA and MetFV-HdrABC-MvhD, respectively [17]. Furthermore, methylene-THF reduction with benzyl viologen as reductant was shown in enzymatic assays at 45 °C and pH of 7.5 using MetFV-HdrABC-MvhD (18 U/mg), MetFV (50 U/mg) and MetF (0.7 U/mg) as protein sources. Based on the similarity of the MetFV-HdrABC-MvhD complex to the heterodisulfide reductase of methanogenic archaea, it was hypothesized that MetFV-HdrABC-MvhD is an electron-bifurcating enzyme [17]. Currently, the second electron acceptor is not known, and it was hypothesized that membrane-integral electron carrier such as menaquinone or cytochromes may play a role (Fig. 6A). In addition, a similar mode of action in *T. kivui* can be suggested in which the MTHFR complex maybe coupled to the Ech complex (Fig. 6B).

In the last step of the methyl branch, the methyl group of methyl-THF is transferred to a corrinoid iron-sulphur protein (CoFeSP) by a methyltransferase. The methyltransferase is a dimeric protein of 58.8 kDa and

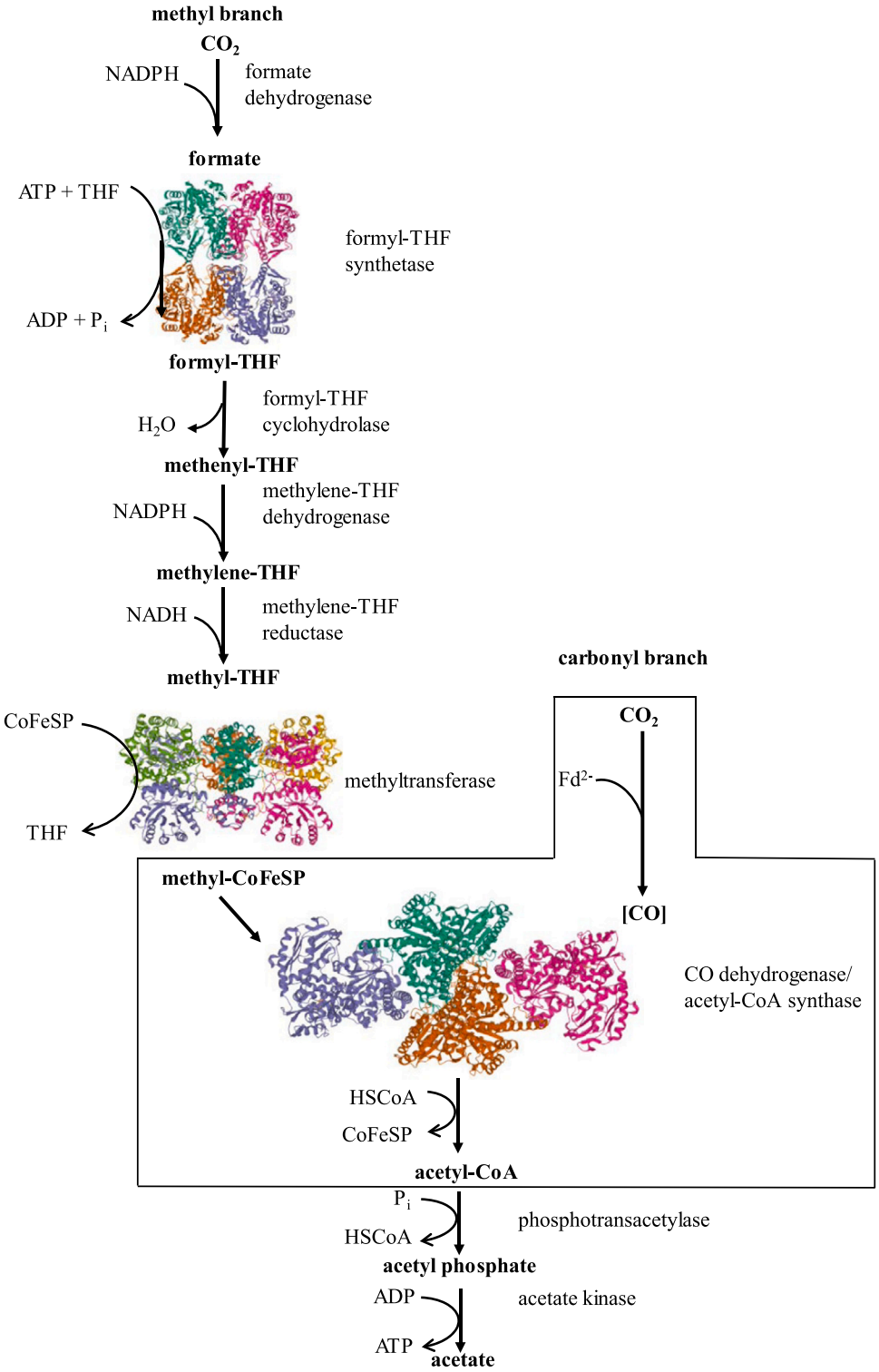


**Fig. 5.** Enzymology of the WLP in *M. thermoacetica*. The structures of the formyl-THF synthase (PBD-ID: 1EG7), folate-free CoFeSP in complex with its methyltransferase (PBD-ID: 4DJD) and the CODH/ACS (PBD-ID: 1MJG) were solved at high resolution. THF, tetrahydrofolate; CoFeSP, corrinoid/iron sulfur protein; [CO], enzyme-bound carbon monoxide; HSCoA, coenzyme A. Reactions catalyzed by CODH/ACS are boxed.
(modified after [12,88,91,96])

has a specific activity of 7.7 U/mg and its structure was solved at a high resolution [18,89]. The CoFeSP is a αβ dimer with 55 and 33 kDa, respectively, which contains 5′-methoxybenzimidazolylcobamide and a $[4Fe\text{-}4S]^{2+/1+}$ cluster as cofactors [90]. The structure of the methyltransferase was solved at a resolution of 2.38 Å [91].

The carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS) is the key enzyme of the WLP since it catalyses C-C bond formation [92]. The enzyme from *M. thermoacetica* is a Ni-containing metalloenzyme that consists of two subunits, α and β, with a molecular mass of 78 and 71 kDa, respectively [93]. The CODH/ACS catalyses two reactions, the reversible reduction of $CO_2$ to CO with $Fd^{2-}$ as reductant, which is the only reaction of the carbonyl branch [23,93]. Thereby, the CO remains bound at the $Ni^{2+}$ centre of the ß-subunit [23,90,94]. In the second reaction the methyl group of the methyl-CoFeSP is

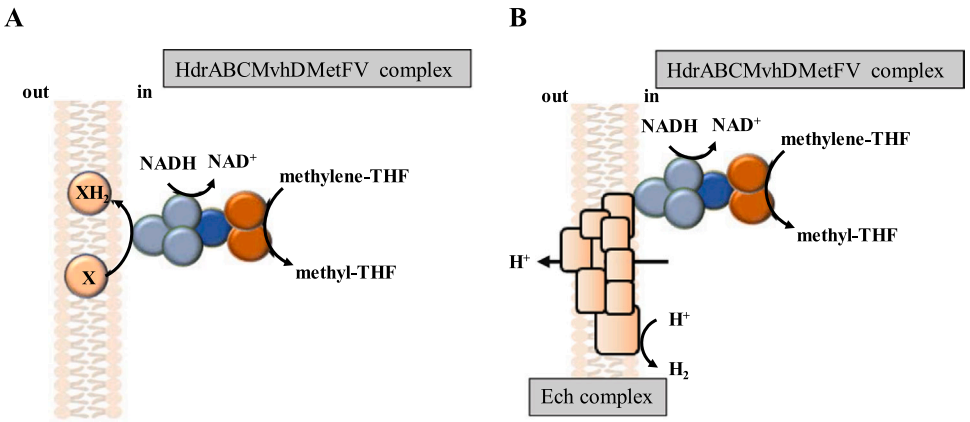


**Fig. 6.** Hypothetical coupling of NADH oxidation to the reduction of methylene-THF. The oxidation of NADH and methylene reduction by the putative electron-bifurcating HdrABCMvhDMetFV maybe coupled to a membrane-bound electron carrier such as menaquinone or cytochrome (A) or to the Ech complex (B). X, cytochrome- or menaquinone oxidized; $XH_2$, cytochrome- or menaquinone reduced. For further explanations, see text.

transferred to the α subunit of the CODH/ACS, which is subsequently used to form acetyl-CoA together with the enzyme-bound CO and coenzyme A [20,95]. The purified CODH/ACS is a homotrimer with a molecular mass of 440 kDa and contains three zinc, six nickel, 33 iron and 42 acid-labile sulfur [93]. The CODH/ACS was capable of oxidizing $Fd^{2-}$ from *M. thermoacetica* as well as rubredoxin, flavodoxin and Fd from *Clostridium formicoaceticum* [93]. In addition, artificial electron acceptors such as methyl- and benzyl viologen and methylene blue could also serve as electron acceptor. Using methyl viologen as electron acceptor, the maximum velocity ($V_{max}$) and the, $K_m$ were determined, which were found to be 750 U/mg at pH 8.4 and 50 °C with a $K_m$ of 3 mM [93]. The structure of the CODH/ACS was solved first by Doukov and colleagues in 2002 with a resolution of 2.2 Å [96].

Acetyl-CoA is further metabolized to acetyl phosphate *via* a phosphotransacetylase. The genome of *M. thermoacetica* encodes for two putative phosphotransacetylases PduL1 and PduL2 [67]. Drake et al. (1981) purified and characterized a tetrameric phosphotransacetylase with a molecular mass of 88.1 kDa. The purified enzyme was stimulated by $Mn^{2+}$ and had a specific activity of 24.8 U/mg. The pH optimum as well as the temperature optimum were 7.6 and 75 °C, respectively but no amino acid sequence was published [18]. In a recent study, both putative phosphotransacetylases were heterologously produced and purified from *E. coli*. Both proteins were described as homotetrameric proteins with a molecular mass of about 90 kDa. Both enzymes had phosphotransacetylase activity under aerobic conditions. The activity of PduL1 was 9.0 U/mg and that of PduL2 was 2.0 U/mg at 55 °C and a pH of 7.6 [97]. Furthermore, the $K_m$ of PduL1 and PduL2 for acetyl-CoA was determined to be 0.49 and 0.04 mM, respectively. In contrast to the study by Drake et al. (1981) the authors observed an inhibitory effect of $Mn^{2+}$ [97].

In the final step, acetyl phosphate is further metabolized to acetate and ATP is synthesized by substrate level phosphorylation. The acetate kinase has a molecular mass of 60 kDa and a specific activity of 282 U/mg with a pH optimum of 9 and a temperature optimum at 60 °C. Furthermore, the $K_m$ for acetate and ATP were determined to be 0.14 M and 1.64 mM, respectively [24].

## 5. The genetic toolbox for *M. thermoacetica*

*M. thermoacetica* is not naturally competent for DNA uptake. Therefore, a protocol for plasmid integration based on electroporation was established. In order to avoid degradation of plasmids by restriction enzymes of *M. thermoacetica*, plasmids were first methylated and then transformed into *M. thermoacetica*. To ensure the correct methylation, two methylases were cloned into plasmid pBAD33 and transformed into *E. coli* Top 10 [98]. The plasmid used to transform *M. thermoacetica* was first transformed into the *E. coli* strain that contains the plasmid encoding the *M. thermoacetica*-specific methyltransferases to ensure *M. thermoacetica*-specific methylation. The correctly methylated plasmid was then isolated from *E. coli* and used to transform *M. thermoacetica* [98].

The plasmid used for genetic modification of *M. thermoacetica* is based on the backbone of the plasmid pK18mob. To select for plasmid integration and disintegration, the *pyrF*-method was used. Deletion of *pyrF* leads to an uracil auxotrophic mutant that was used for markerless mutagenesis. To generate the uracil auxotrophic mutant, plasmid pK18 carrying up- and downstream regions of 1 kb of the *pyrF* gene present in *M. thermoacetica* were introduced into *M. thermoacetica.* By homologous recombination the plasmid was integrated into the genome. In a second recombination event forced by 5-fluoroorotic acid the plasmid was deleted from the genome resulting in either the wild type or the *pyrF* deletion mutant. 5-fluoroorotic acid is metabolized by the wild type to 5-fluorouracil, which is toxic to the cell and leads to cell death [99,100]. In contrast to the wild type, the Δ*pyrF* mutant is not able to metabolize 5-fluoroorotic acid due to the missing enzyme orotate monophosphate decarboxylase and thus, grow is not affected by 5-fluorouracil [100]. The Δ*pyrF* mutant strain was then used for further mutagenesis.

To insert genes into the genome of *M. thermoacetica,* plasmid pK18 was prepared carrying the *pyrF* gene with its up- and downstream regions as well as the sequence of the gene of interest. This plasmid was integrated into the genome of *M. thermoacetica via* homologous recombination. Transformants did not require uracil supplementation due to the presence of the *pyrF* gene. Using this methodology, genes encoding for a lactate dehydrogenase from *Thermoanaerobacter pseudethanolicus* or the acetone biosynthetic pathway consisting of the 3-oxoacid CoA-transferase (Tmel_1135/6) from *Thermosipho melanesiensis*, thiolase (TTE0549) from *Caldanaerobacter subterraneus* subsp. *tengcongensis*, and acetoacetate decarboxylase (CA_P0165) from *C. acetobutylicum* or the aldehyde dehydrogenase from *M. thermoacetica* for ethanol production were integrated into the genome of *M. thermoacetica* [98,101–104]. In addition, genes encoding for the phosphotransacetylase PduL1 and PduL2 were deleted by the *pyrF* method [99]. These approaches were successful and led to strains that produced lactate, acetone or ethanol from sugars [91–104].

Lactate is produced by the wild type in minor amounts, whereas the mutant Δ*pduL1*Δ*pduL2*:*ldh* produces up to 3.56 g/l, but approximately 20 of 45 mM of the substrate is not metabolized, giving a lactate/fructose ratio of 1.5:1. Unfortunately, $H_2$ + $CO_2$ was not metabolized to lactate [98,102]. For acetone production a Δ*pduL2*::acetone mutant was created, which metabolized fructose to acetone in a ratio of 1:1 [104]. In addition to fructose, $H_2$ + $CO_2$ and CO were also tested as feedstocks for acetone production. Cells grew poorly on $H_2$ + $CO_2$ and produced only 0.10 g/l acetone and 0.20 g/l acetate after 254 h of incubation. Similar results were obtained with CO as feedstock. The best results were obtained by using CO and $H_2$ in a ratio of 1:1, the amount of acetone produced rose to 0.17 g/l [104].

Ethanol is produced by the Δ*pduL1*Δ*pduL2*:*aldh* mutant with a yield of approximately 9 g/l using 21.6 g/l glucose. In this study as well, the

substrate was not consumed completely. Nevertheless, a ratio of ethanol produced and glucose consumed of 2:1 was determined [101]. The Δ*pduL1*Δ*pduL2*:*aldh* mutant did not grow on $H_2$ + $CO_2$ or CO and only traces of ethanol and acetate were formed, demonstrating that the production of ethanol from $H_2$ + $CO_2$ or CO requires a different approach [101,103]. It can be speculated that using an aldehyde ferredoxin oxidoreductase instead of an aldehyde dehydrogenase would be more appealing [105].

Recently, the genetic toolbox for *M. thermoacetica* was extended by constructing a replicating plasmid, pMTLK-Tp that was used to produce proteins from a plasmid. To create the shuttle vector for *M. thermoacetica* the plasmid pMTL84141 was used as a backbone. The plasmid pMTLK-Tp contains a thermostable kanamycin resistance gene under the control of the glyceraldehyde-3-phosphate dehydrogenase promotor from *M. thermoacetica* and the replicon from *Thermotoga petrophila* pRKU1. After successful transformation of pMTLK-Tp into *M. thermoacetica,* transformants were checked *via* qPCR for the presence of the genes encoding for the kanamycin resistance and the replicon. To show that the plasmid-based genetic tool can be used to produce valuable products by *M. thermoacetica,* plasmids were constructed encoding for the native aldehyde dehydrogenase Aldh from *M. thermoacetica* or the bifunctional aldehyde-alcohol dehydrogenase, AdhE1 from *Clostridium autoethanogenum.* Transformants carrying the plasmids with *aldh* or *adhE1* were able to produce ethanol with a yield of 63 or 99 mg/l, respectively [106].

## 6. Future perspectives for the biotechnological platform *M. thermoacetica*

Currently no thermophilic acetogen, neither *M. thermoacetica* nor *T. kivui* is used in industrial applications although thermophilies have several advantages. Higher temperature reduces the risk of contaminations, volatile compounds are easier to recover, and intensive cooling of the fermenter is not required [38]. *T. kivui* would be preferred over *M. thermoacetica* as future platform for industrial application simply because its physiology is understood better. To overcome the lack of physiological data, a bioinformatical metabolic model was constructed to characterize the metabolic profile of *M. thermoacetica* using various substrates such as $H_2$ + $CO_2$, CO or methanol [107].

However, *M. thermoacetica* offers several advantages over *T. kivui*. In contrast to *T. kivui*, *M. thermoacetica* can grow on methanol, a promising feedstock for acetogens that can be easily produced from $CO_2$ by chemical processes and does not require laborious gas fermentation with its technical limitations and the limitations of gas-mass transfer [108]. In addition, *M. thermoacetica* but not *T. kivui* can use alternative electron acceptors [109]. For the latter, nitrate, DMSO and thiosulfate have been described [49,74,110]. Reduction of alternative electron acceptors by *M. thermoacetica* reduces the amount of acetate formed, indicating that in most cases $CO_2$ is reduced simultaneously to the alternative electron acceptors [77,111]. However, reduction of alternative electron acceptors can be growth stimulating, supplementation of cultures growing on glucose or $H_2$ + $CO_2$ with DMSO, or cultures growing on methylated compounds with nitrate may enhance ATP yields, product formation and biomass production [49,75]. *M. thermoacetica* does not naturally produce a compound other than acetate when growing on $H_2$ + $CO_2$ [52]. In an effort to enhance the ethanol yield from fructose, Kobayashi et al. [110] let *M. thermoacetica* grow mixotrophically on fructose and $H_2$ + $CO_2$. The wild type consumed fructose and formed acetate as the sole end product. Compared to the wild type, the mutant produced mainly ethanol and small amounts of acetate. In addition, $H_2$ was formed to dispose excess electrons. Whereas the wild type grew better mixotrophically and showed increased acetate production, the ethanol producing mutant strain Δ*pduL1*Δ*pduL2*:*aldh* grew poorly under mixotrophic condition. Despite the inhibition of growth and small amount of ethanol produced, the ethanol:fructose ratio was increased. The authors speculated that the apparent inhibition of metabolism by $H_2$ is due to an imbalance in the ratio of reduced to oxidized pyridine nucleotides with an increase in the reduced form. If that is true, the addition of an external electron acceptor should overcome growth inhibition and initiate ethanol production. Indeed, this was observed by addition of DMSO. This elegant study underlines the importance of metabolic flexibility of the organism used to establish the acetogenic production platform. Mixotrophic growth of acetogens is certainly of advantage in some applications, for example to recover carbon dioxide [110].

In addition to *M. thermoacetica*, several acetogenic bacteria can use alternative electron acceptors, for example nitrate reduction has also been found in *Sporomusa ovata* and *Clostridium ljungdahlii* [112,113]. *Sporomusa ovata* presumably uses a membrane-bound cytochrome *c*-dependent nitrate reductase for nitrate reduction [112]. The published proteome analysis supports this hypothesis, but further physiological and biochemical analyzes are lacking. *C. ljungdahlii* growing on $H_2$ + $CO_2$ reduces nitrate in an energy conserving manner. Growth was stimulated and the production of acetate was reduced [113]. As in *M. thermoacetica*, the genes coding for the WLP enzymes were downregulated. Furthermore, it was shown that the supplementation with nitrate (in form of nitric acid) reduces the production costs for the formation of biomass from $H_2$ + $CO_2$ and the formation of desired products such as lactate, ethanol or isoprene [113].

In contrast to *T. kivui M. thermoacetica* has quinones and cytochromes [46,114]. The role of these membrane integral electron carrier is neither understood nor well studied. Since scientists found cytochromes and menaquinone in *M. thermoacetica* first and the Ech complex was identified much later, it was hypothesized that a cytochrome- and quinone-dependent respiratory chain that is involved in energy conservation are responsible for energy conservation, similar to the methanogenic archaeon *Methanosarcina barkeri* but so far experimental evidence for this is missing [115–118]. The menaquinone is postulated as a reductant for several enzymes such as the DMSO reductase, Fix complex and NDH, whereas the role of the cytochromes remains enigmatic.

*T. kivui* and *M. thermoacetica* are both able to grow on CO as feedstock but in contrast to *T. kivui M. thermoacetica* that requires nicotinic acid as supplement for growth on CO [119]. *M. thermoacetica* can not utilize $H_2$ + $CO_2$ or CO in mineral medium but in the presence of nicotinic acid growth was obtained in minimal medium with doubling times of 25 and 10 h and final optical densities (OD) of 0.1 and 0.2, forming 21.0 and 8.4 mM acetate, respectively [119].

In contrast, *T. kivui* is able to utilize CO in mineral medium with doubling times of 40 h and a final OD of 0.21 with a 50% CO atmosphere, this is an advantage since it reduces the production cost since no additional vitamins are required [119,120]. CO metabolism in *T. kivui* was also observed using complex medium, thereby cultures reached an OD of 0.41 with a doubling time of 10 h and produced 82.7 mM acetate under a 70% CO atmosphere [120]. Nevertheless, the production of high value products like acetone with CO as feedstock can be economically feasible using *M. thermoacetica* as production platform [121]. Notwithstanding the differences between *M. thermoacetica* and *T. kivui*, both microorganisms can be used to produce acetate from syngas, which can serve as a feedstock for other microorganisms. Applying this principle, a second microbiological platform is used to produce a high-quality product such as amino acids from the acetate produced by the acetogens. Thereby, biotechnological processes can be made environmentally friendly, and a previously sugar-based biotechnology can be changed to a process based on a waste product.

Some acetogens can use electrons shared by an electrode to reduce $CO_2$ to acetate with high efficiency [122]. *M. thermoacetica* and *T. kivui* are far less studied in microbial electrosynthesis as model organisms but both organisms are in general capable to be electrosynthesized [122,123]. In this approach, sustainable produced electricity is used as electron source for the microbial metabolism.

An economically favourable approach for electronsynthesis or electroacetogenesis is the use of solar energy. A promising attempt to

harness this energy has been made by scientists at UC Berkeley. Growing cultures of *M. thermoacetica* formed cadmium sulfide (CdS) nanoparticles in the presence of cadmium and a sulfur source [124]. These nanoparticles covered the cell surface and allowed the cell to capture solar energy and converted this energy into reducing equivalents, which allowed the microorganism to achieve higher acetate yields [124]. However, a recently published study showed that the increased amount of acetate formed was not dependent on light, but on the reducing agent cysteine and in combination with a response to the toxic cadmium ions [125]. Similar studies were performed using other acetogens such as *C. autoethanogenum* or *S. ovata* [126,127]. In general, this approach needs further studies and improvements in order to be understood and demonstrate its feasability for industrial applications.

## 7. Conclusions

Due to its metabolic flexibility and bioenergetics *M. thermoacetica* is a suitable candidate for future biotechnological applications. It is capable to use waste (gases) in a sustainable way to produce valuable products. *M. thermoacetica* can grow and produce acetate from sugar but also mixotrophically from sugars + $H_2$ + $CO_2$ or CO. It can also use methanol, a promising feedstock derived from $CO_2$ by chemical processes. Currently, the potential of *M. thermoacetica* to gain broader interest in biotechnology is limited due to the lack of knowledge about its physiology and bioenergetics. A further current drawback is the toolbox for genetic modification, which needs to be improved. In contrast to most acetogens *M. thermoacetica* is not restricted to one energy conserving system. In addition to the Ech complex, cytochromes and quinones may be involved in energy conservation by, for example, DMSO respiration. The extra energy conserved by multiple ways either using electricity or reduction of alternative electron acceptors can be used to form highly valuable but energy-demanding products. A basic system to genetically modify *M. thermoacetica* has been established allowing a closer look into the physiology, bioenergetics and afterwards redirect the carbon and energy flux to produce highly valuable products.

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## Data Availability

Data presented in this study are available on request from the corresponding author.

## Authors contributions

F.P.R. and V.M. wrote the manuscript; literature search, analysis and study: F.P.R. and V. M; Drafting of review F.P.R. and V.M; Editing: F.P.R. and V.M.

## Declaration of Competing Interest

Volker Müller is an editorial board member for Green Carbon and was not involved in the editorial review or the decision to publish this article. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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