Autotrophy at the thermodynamic limit of life: a model for energy conservation in acetogenic bacteria

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Abstract | Life on earth evolved in the absence of oxygen with inorganic gases as potential sources of carbon and energy. Among the alternative mechanisms for carbon dioxide (CO_2) fixation in the living world, only the reduction of CO_2 by the Wood–Ljungdahl pathway, which is used by acetogenic bacteria, complies with the two requirements to sustain life: conservation of energy and production of biomass. However, how energy is conserved in acetogenic bacteria has been an enigma since their discovery. In this Review, we discuss the latest progress on the biochemistry and genetics of the energy metabolism of model acetogens, elucidating how these bacteria couple CO_2 fixation to energy conservation.

Acetogenic bacteria are a specialized group of bacteria that are able to produce acetate from two molecules of carbon dioxide (CO_2) via the Wood–Ljungdahl pathway (WLP). Acetogens are facultative autotrophs that can grow by the oxidation of a large variety of organic substrates, including hexoses, pentoses, alcohols, methyl groups and formic acid, or by the oxidation of inorganic substrates such as hydrogen (H_2) or carbon monoxide (CO), which is usually coupled to the reduction of CO_2. The metabolic ability of acetogenic bacteria to convert many fermentation substrates to acetate links fermentative bacteria to methanogens, which can use acetate as a substrate, and makes acetogens an essential part of anaerobic food webs. Furthermore, the metabolic flexibility of acetogens gives these bacteria an ecological advantage: they are ubiquitously distributed in nature and are found in alkaline as well as in high-salt and hot environments, in deep subsurface samples as well as in the termite hindgut and the human intestines, and in terrestrial soil as well as in freshwater and marine sediments[1].

Acetogens are present in 23 different bacterial genera, which highlights that acetogenesis is not a phylogenetic trait[1]. Most acetogens are found in one phylum, the Firmicutes (which are Gram-positive bacteria with low GC content). Several genera such as *Clostridium* contain acetogenic as well as non-acetogenic species, whereas other genera such as *Acetobacterium* or *Sporomusa* only contain acetogens. Most known acetogens belong to the genera *Clostridium* and *Acetobacterium*. From the large number of acetogenic bacteria, three model organisms have been studied in detail: *Moorella thermoacetica, Acetobacterium woodii* and *Clostridium ljungdahlii*.

M. thermoacetica is a Gram-positive, motile, endospore-forming thermophile with an optimal growth temperature of 55–60 °C and is a common inhabitant of soils[2]. *M. thermoacetica* was the biochemical model strain for acetogenesis for decades, and all of the enzymes of the WLP have been isolated and characterized in this species. *M. thermoacetica* can grow on CO, H_2 and CO_2, formate, methanol, hexoses, pentoses, methoxylated benzoic acids and several two-carbon compounds (including oxalate, glycolate and glyoxylate)[2–7]. In addition to CO_2, *M. thermoacetica* can use thiosulphate[8,9], nitrate[10] and nitrite[11] as alternative electron acceptors. This organism contains membrane-bound cytochromes and menaquinones. Importantly, growth and acetate formation from H_2 and CO_2 in *M. thermoacetica* does not require sodium ions (Na^+)[12–14].

The genus *Acetobacterium* was established when the first Gram-positive, non-spore-forming acetogen — *A. woodii* — was isolated. *A. woodii* cells are motile and mesophilic, and they grow robustly on H_2 and CO_2 but can also grow on formate, methanol, ethanol, 1,2-propanediol, 2,3-butanediol, ethylene glycol, acetoin, lactate, glycerate, sugars, betaine and several methoxylated aromatic acids[15–18]. *A. woodii* uses CO_2 as an electron acceptor but can also use aromatic acrylates[16,19]. In contrast to *M. thermoacetica, A. woodii* growth, motility and acetate formation from H_2 and CO_2 are strictly dependent on Na^+ (REFS 20,21). *A. woodii* has neither cytochromes nor quinones.

Wood–Ljungdahl pathway (WLP). The pathway was named according to its discoverers, Harland G. Wood and Lars G. Ljungdahl. It is also referred to as the reductive acetyl-CoA pathway. In this pathway, two molecules of CO_2 are reduced and joined to form acetyl-CoA and then acetate.

Autotrophs
Organisms that have the ability to grow in the absence of organic carbon. The organic carbon for biosynthesis is assimilated from inorganic carbon sources such as CO_2. (Heterotrophy is used to describe a dependence on organic carbon sources).

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C. ljungdahlii is a Gram-positive, endospore-forming and motile bacterium that grows on H_2 and CO_2, CO, formate, ethanol, pyruvate, fumarate and sugars (including fructose and xylose)[22]. Similarly to *A. woodii*, *C. ljungdahlii* does not have cytochromes, but its growth, like *M. thermoacetica*, does not require Na^+.

As the synthesis of acetate from 2 moles of CO_2, with H_2 as the reductant, enables the growth of acetogens, this pathway must be coupled to net ATP formation. Indeed, the WLP is the only pathway of CO_2 fixation that is coupled to energy conservation, and it is considered to be one of the oldest biochemical pathways (if not the oldest pathway) that enabled biomass and ATP production in a primordial world[23]. Despite its importance for the evolution of life, the question of how the WLP is coupled to ATP synthesis in acetogens has been an enigma for decades.

ATP is the universal energy carrier in biology and every living cell has to conserve energy in this energy equivalent. Two modes of energy conservation are known: substrate level phosphorylation (SLP) and chemiosmotic ion gradient-driven phosphorylation (see Supplementary information S1 (box)). SLP couples a chemical reaction directly to the phosphorylation of ADP, which generates ATP. Chemiosmotic energy conservation couples an exergonic reaction (in most cases an electron-transfer reaction) to the translocation of ions across a membrane, which results in the generation of an electrical and/or ion gradient (that is, a transmembrane electrochemical ion gradient) across the membrane, which then drives ATP synthesis by a membrane-bound ATP synthase (see Supplementary information S1 (box)). Although a reaction that results in SLP is involved in the WLP, there is no net ATP formation by SLP in this pathway. Therefore, energy conservation in acetogens must rely on the establishment of a chemiosmotic mechanism.

Basic differences in how a transmembrane ion gradient is established in acetogens led to their classification into two bioenergetic classes: the cytochrome-containing and Na^+-independent (proton (H^+)-dependent) acetogens, with *M. thermoacetica* as the model organism; and the cytochrome-free, Na^+-dependent acetogens, with *A. woodii* as the model organism. However, this classification was challenged by the discovery of *C. ljungdahlii* and other species that neither have cytochromes nor depend on Na^+ for growth. In fact, it is now clear that these interpretations were incorrect, and in our view, this classification should no longer be maintained. In this Review, we highlight the latest results on the energy conservation of acetogens, discuss how it is possible to survive using a reaction with a free energy change that results in the synthesis of less than 1 mole of ATP and present a new bioenergetic classification of acetogens on the basis of their putative mechanisms of energy conservation.

Biochemistry and energy profile of the WLP

Hexose fermentation in acetogens proceeds via glycolysis to pyruvate, which is then oxidized to acetyl-CoA and CO_2. The electron acceptor that is used in the oxidation of pyruvate is a low-molecular-weight, iron–sulphur centre-containing protein, ferredoxin, which has a standard redox potential (E_0') that is much more negative than that of the NAD^+/NADH couple, which is usually used as the electron acceptor during pyruvate oxidation in other organisms[24]. Acetyl-CoA is then converted to acetate via acetyl phosphate, in a reaction that is catalysed by the enzymes phosphotransacetylase and acetate kinase (FIG. 1). Hexose oxidation is coupled to the synthesis of 4 moles of ATP by SLP (where [H] is reducing equivalent (= $1e^- + 1H^+$)):

$$C_6H_{12}O_6 + 4ADP + 4P_i \rightarrow 2CH_3COOH + 2CO_2 + 4ATP + 2H_2O + 8[H] \quad (1)$$

The reducing equivalents and the 2 moles of CO_2 that are gained in this reaction are shuttled to the WLP and form the third molecule of acetate:

$$2CO_2 + 8[H] + nADP + nP_i \rightarrow CH_3COOH + nATP + (2+n)H_2O \quad (2)$$

In sum, glucose is oxidized to 3 moles of acetate:

$$C_6H_{12}O_6 + (4+n)ADP + (4+n)P_i \rightarrow 3CH_3COOH + (4+n)ATP + (4+n)H_2O \quad (3)$$

The reducing equivalents from equation 2 derive from the oxidation of sugars, but they can also derive from the oxidation of H_2, making acetogens facultative autotrophs that can convert H_2 and CO_2:

$$2CO_2 + 4H_2 + nADP + nP_i \rightarrow CH_3COOH + nATP + (2+n)H_2O \quad (4)$$

The WLP consists of two separate branches — the methyl branch and the carbonyl branch — one for each CO_2 molecule (FIG. 1). CO_2 is reduced to CO in the carbonyl branch and to a methyl group in the methyl branch. The first reaction in the methyl branch is the reduction of CO_2 to formate. The formyl group is then bound to tetrahydrofolate (THF), which generates formyl-THF in a reaction that requires the hydrolysis of ATP[25,26]. Water is split off to yield methenyl-THF, which is then reduced via methylene-THF to methyl-THF. The methyl group is eventually transferred via a methyltransferase and a corrinoid iron–sulphur protein to a subunit of the CO dehydrogenase/acetyl-CoA synthase (CODH/ACS)[25,26].

The carbonyl group of acetate derives from a second mole of CO_2 that is reduced to enzyme-bound CO in a reaction catalysed by CODH/ACS. The CODH/ACS catalyses the synthesis of acetyl-CoA from the methyl group (from the methyl branch), the carbonyl group (from the carbonyl branch) and CoA. Acetyl-CoA is released from CODH/ACS and converted to acetate by the action of two enzymes: a phosphotransacetylase and an acetate kinase[25,26]. In this process, CoA is recycled and thus has only a catalytic function. 1 mole of ATP is synthesized by SLP during the acetate kinase reaction, but 1 mole of ATP is necessary for the activation of formate, which results in zero net synthesis of ATP by SLP, explaining the need for a chemiosmotic mechanism of ATP synthesis to be present in acetogenic bacteria[6,26–28].

From a thermodynamic perspective, the overall reduction of two molecules of CO_2 to acetate with H_2 as

Cytochromes
Membrane-bound, haem group-containing electron-transfer proteins that are especially involved in chemiosmotic energy conservation in the aerobic and anaerobic respiratory chains or photosynthesis.

Quinones
Lipid-soluble electron carriers that are often associated with chemiosmotic respiratory chains.

Exergonic reaction
A reaction that releases energy (the change in the free energy is negative) and thus takes place without external energy input.

Tetrahydrofolate
(THF). A cofactor that is involved in transfer reactions of single carbon groups. It is important not only in acetogenesis but also in the metabolism of amino acids or nucleic acids.

Corrinoid
A cofactor based on the corrin skeleton that is, in acetogenesis, involved in methyl-transfer reactions. Cobalamines (as vitamin B_{12}) are the most prominent example of this group of cofactors that contain a cobalt ion bound in the centre of the corrin system.

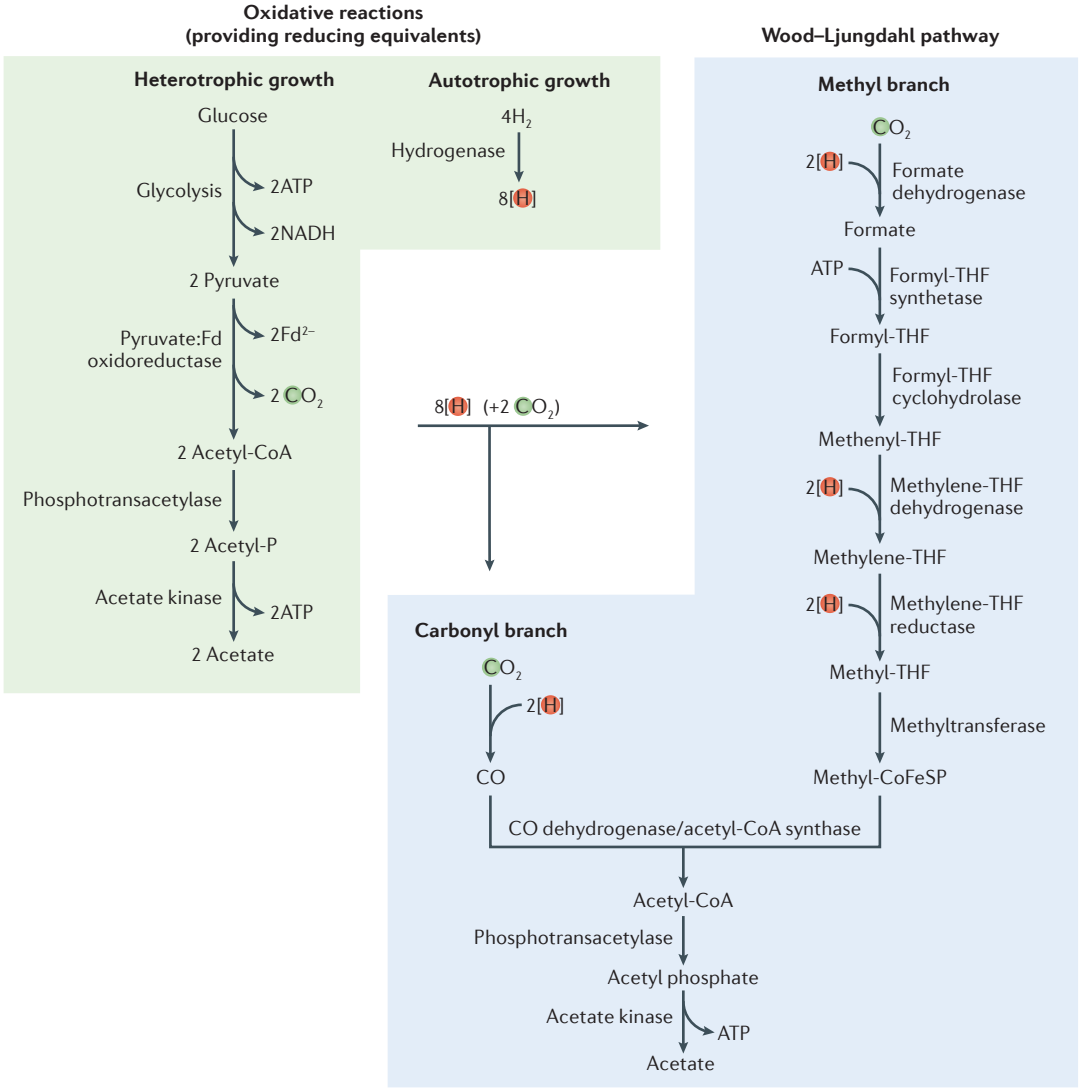


Figure 1 | **The Wood–Ljungdahl pathway of acetogenesis.** Acetogenic bacteria produce acetate from two molecules of carbon dioxide (CO_2) via the Wood–Ljungdahl pathway (WLP). During heterotrophic acetogenesis, glucose is oxidized to two molecules of acetate and two molecules of CO_2 via a combination of glycolysis, pyruvate:ferredoxin oxidoreductase, phosphotransacetylase and acetate kinase. The reducing equivalents are reoxidized by the reduction of the two molecules of CO_2 to acetate in the WLP. During autotrophic acetogenesis, acetate is formed from $4H_2$ and $2CO_2$. In the carbonyl branch of the WLP, one molecule of CO_2 is reduced to CO via the carbon monoxide (CO) dehydrogenase/acetyl-CoA synthase (CODH/ACS). In the methyl branch of the WLP, one molecule of CO_2 is reduced to formate via a formate dehydrogenase and bound to the cofactor tetrahydrofolate (THF), yielding formyl-THF via the formyl-THF synthetase. Formyl-THF is used by the formyl-THF cyclohydrolase to generate methenyl-THF, which in turn is reduced to methylene-THF via the methylene-THF dehydrogenase. Methylene-THF is reduced to methyl-THF via the methylene-THF reductase. Finally, a methyltransferase transfers the methyl group from methyl-THF via a corrinoid iron-sulphur protein (CoFeSP) to the CODH/ACS. This bifunctional enzyme reduces CO_2 to CO in the carbonyl branch and fuses it with the methyl group from the methyl branch and with CoA to form acetyl-CoA, which is then used by a phosphotransacetylase to generate acetyl phosphate, and then turned into acetate via an acetate kinase. Fd, ferredoxin; Fd^{2-}, reduced ferredoxin; [H], reducing equivalent (= 1e– + $1H^+$).

the electron donor is an exergonic reaction with a total free energy change of around $\Delta G' = -40$ kJ per mole at physiological conditions ($\Delta G_0' = -95$ kJ per mole; see BOX 1 for details of the calculations of $\Delta G_0'$, $\Delta G'$, $\Delta E_0'$ and $\Delta E'$). However, this is only the sum of the free energy changes of all intermediate reactions: some individual reactions can be more exergonic, whereas others can be endergonic. Indeed, some reactions involve high energy barriers, and only few individual reactions are thought to be exergonic enough to enable energy conservation in the form of the generation of a chemiosmotic ion gradient.

The largest thermodynamic barrier in the WLP is the reduction of CO_2 to CO in the carbonyl branch of the pathway. This redox couple has a very low standard redox potential ($E_0' = -520$ mV (REF. 29)), which is more

Endergonic
A term used to describe a reaction that consumes energy (the change in the free energy is positive) and thus can only take place with external energy input.

Box 1 | **Changes in free energy of the reactions involved in the WLP**

The standard Gibbs free energy changes ($\Delta G_0'$) of reactions that are used in this work reflect the change of free energy that accompanies a reaction with an activity of 1 for substrates and products at 25 °C and pH 7.0. They were calculated from reported equilibrium constants or from standard redox potentials (E_0')[29,38,78]. Similarly, the E_0' refers to a concentration of 1 M for all reactants at pH 7. The actual Gibbs free energy change of a reaction ($\Delta G'$) and the actual cellular redox potential (E') are dependent on the concentrations of substrates and products in the cell, the pH and the temperature[29]. As cellular concentrations for most reactants are unknown, we mostly use the $\Delta G_0'$ and $\Delta E_0'$ values for our considerations. However, a tenfold change in the concentrations of the reactants changes the redox potential by ±60 mV, which corresponds to approximately 12 kJ per mole (according to $\Delta G' = -n \times F \times \Delta E'$, where F is the Faraday constant and n is the number of electrons). Therefore, for the evaluation of whether a given reaction may be feasible, we consider a $\Delta G_0'$ ±12 kJ per mole. These values reflect the range of cellular concentrations expected to be found within a cell, based on experimental analyses and the K_m values for the metabolic enzymes around the low mM range[78,79]. The G_0' values are based on the following E_0' values: [NAD/NADH] and [NADP/NADPH] = −320 mV, [ferredoxin (Fd)/reduced ferredoxin (Fd^{2-})] = −450 mV. The $\Delta G'$ values are based on the following E' values: [NAD/NADH] = −280 mV (REF. 79), [NADP/NADPH] = −370 mV (REF. 79), [H^+/H_2] ≈ −340 mV (based on the determined minimal hydrogen partial pressure of *Acetobacterium woodii*)[33]. The actual standard redox potentials of all of the ferredoxins in acetogens are not known, but characterized ferredoxins from various organisms typically had redox potentials of around −400 mV to −450 mV. There are also ferredoxins that are known to have redox potentials of −500 mV and even lower[30,80]. Ferredoxin II from *Moorella thermoacetica* has two [4Fe–4S] clusters with different midpoint potentials of −454 mV and −487 mV[81]. We therefore used −450 mV as E_0' for the [Fd/Fd^{2-}], but this value might vary between different organisms and ferredoxins in the range of −400 mV to −500 mV. The E' of ferredoxin might also be different, but there are no data in acetogens. THF, tetrahydrofolate; P_i, inorganic phosphate.

Reaction	$\Delta G_0'$ [kJ per mole]
$2H_2 + Fd + NAD^+ \rightleftharpoons 3H^+ + Fd^{2-} + NADH$	−11
$H_2 + NADP^+ \rightleftharpoons H^+ + NADPH$	−18
$H_2 + Fd \rightleftharpoons 2H^+ + Fd^{2-}$	+7
$CO_2 + NADH \rightleftharpoons Formate + NAD^+$	+22
$CO_2 + H_2 \rightleftharpoons Formate + H^+$	+4
$CO_2 + NADPH \rightleftharpoons Formate + NADP^+$	+22
$CO_2 + Fd^{2-} + H^+ \rightleftharpoons Formate + Fd$	−4
$Formate + THF + ATP \rightleftharpoons Formyl\text{-}THF + ADP + P_i$	−10
$Formyl\text{-}THF + H^+ \rightleftharpoons Methenyl\text{-}THF + H_2O$	+6
$Methenyl\text{-}THF + NADH \rightleftharpoons Methylene\text{-}THF + NAD^+$	−5
$Methenyl\text{-}THF + NADPH \rightleftharpoons Methylene\text{-}THF + NADP^+$	−5
$Methylene\text{-}THF + NADH \rightleftharpoons Methyl\text{-}THF + NAD^+$	−23 to −37
$Methylene\text{-}THF + 2NADH + Fd \rightleftharpoons Methyl\text{-}THF + 2NAD^+ + H^+ + 2Fd^{2-}$	−12 to +2
$CO_2 + Fd^{2-} + 2H^+ \rightleftharpoons CO + Fd + H_2O$	+14
$ADP + P_i \rightleftharpoons ATP + H_2O$	+32
$Fd^{2-} + NAD^+ + H^+ \rightleftharpoons Fd + NADH$	−25

negative than the H^+/H_2 couple (E_0' = −414 mV (REF. 29)). The known electron carriers in the WLP are NADH, NADPH and ferredoxin, and of these, only reduced ferredoxin (Fd^{2-}) can provide electrons for this reduction (BOX 1). Most ferredoxins have standard redox potentials of −400 mV to −450 mV, but the physiological potential can be as low as −500 mV (REFS 30–32). The physiological redox potentials of the Fd/Fd^{2-} and the CO_2/CO couple are not known in acetogens, but the E' of the H^+/H_2 couple is closer to −340 mV (REF. 33). Nevertheless, there is a large energetic barrier associated with the electron flow from hydrogen to ferredoxin, but all acetogens that have been examined so far solve this problem in a similar way, using enzymes that are capable of flavin-based electron bifurcation during this electron transport (BOX 2).

The reactions that are involved in the flow of carbon in the methyl branch of the WLP are highly conserved in all acetogenic bacteria. However, the electron flow in the redox reactions that are involved in this branch differs strongly between acetogens[25]. For example, in the first step of this pathway, CO_2 is reduced to formate; the standard redox potential (E_0') of the CO_2/formate pair is −432 mV[29], and thus, oxidation of NADH is not sufficient to drive this reaction. However, ferredoxin, NADPH and H_2 all have physiological redox potentials that can possibly be used for the reduction of CO_2 to formate (BOX 1). Indeed, different acetogens evolved to use different electron donors: *A. woodii* uses H_2; *M. thermoacetica* uses NADPH; and the details of this reaction in *C. ljungdahlii* are unknown, but it probably involves the use of ferredoxin and NADPH as electron donors[34].

In the next step of the methyl branch of the WLP, methenyl-THF is reduced to methylene-THF (E_0' = −295 mV (REF. 35)) in a slightly exergonic reaction that uses either NADH or NADPH as the reductant[36,37] (BOX 1). Methylene-THF is then reduced to methyl-THF. The methylene-THF/methyl-THF redox couple has the most positive redox potential of the WLP (E_0' = −200 mV to −130 mV (REF. 38)), and this reaction is highly exergonic even with NADH as the electron donor ($\Delta G_0'$ = −23 kJ to −37 kJ per mole) (BOX 1). Therefore, it was speculated for decades that the reduction of methylene-THF is coupled to energy conservation, probably as the electron acceptor of a cytochrome-dependent, ion-translocating electron-transfer chain that receives electrons from NADH, NADPH or reduced ferredoxin[18,28,32]. However, none of the examined acetogens contains a methylene-THF reductase that is either membrane-integral or membrane-attached[33,39–41], which suggests that the methylene-THF reductase is not involved in the generation of a chemiosmotic gradient. Therefore, none of the reactions that are involved in the flow of carbon in the WLP is directly responsible for energy conservation in acetogenic bacteria. Instead, chemiosmosis is coupled to the reactions of the WLP via the accumulation of reduced ferredoxin, which is then oxidized by membrane-integral protein complexes to generate a chemiosmotic gradient that is used for ATP generation. This strategy enables acetogens to use different modules for energy conservation without the constraint of changing any reaction of the carbon assimilation pathway.

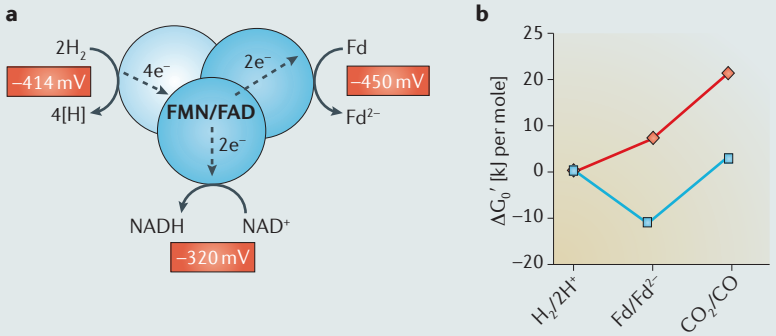
Energy conservation in *A. woodii*

The mesophilic acetogen *A. woodii* was the first acetogen in which the question of how energy is conserved during acetogenesis was answered. Analysis of the genome sequence of *A. woodii* revealed that all enzymes that are

Flavin
A cofactor for redox reactions based on pteridine. Depending on the attached moiety, flavins are found as flavine adenine dinucleotide or flavine adenine mononucleotide.

Box 2 | **Flavin-based electron bifurcation**

The mechanism of soluble, flavin-based electron bifurcation (FBEB) was first shown for the butyryl-CoA dehydrogenase complex of *Clostridium kluyveri*[82]. This enzyme couples the endergonic reduction of ferredoxin (Fd) with NADH to the exergonic reduction of butyryl-CoA with NADH. Since then, other enzyme complexes have been discovered that use the mechanism of FBEB to couple endergonic redox reactions to exergonic redox reactions. All of these enzymes are soluble and consist of multiple subunits, at least one of which binds a flavin cofactor (FMN or FAD)[32,83]. All FBEB enzymes oxidize one electron donor and deliver the electrons simultaneously and tightly coupled to two different electron acceptors. The reduction of one acceptor is always exergonic, which drives the endergonic reduction of the second acceptor. In the case of the acetogenic bifurcating hydrogenases, the reduction of NAD drives the reduction of ferredoxin (see the figure, part **a**). FBEB is essential to drive the overall electron transfer from H_2 ($[H^+/H_2]$ $E_0' = -414$ mV) to the CO_2/CO couple (standard redox potential (E_0') = −520 mV). Under physiological conditions, the redox potential of the CO_2/CO couple might be more positive[80]. As the exact redox potential of the ferredoxin is not known, we use the E_0' value ($[Fd/Fd^{2-}]$ $E_0' = -450$ mV) for simplicity in our discussion (BOX 1), as the redox potential of the electron donor H_2 is shifted under physiological conditions to more positive values at comparable magnitudes, thus leaving the same energetic problems. In both cases, it is obvious that the cells must have coupling mechanisms to catalyse the endergonic reaction of ferredoxin reduction with H_2 as the electron donor. Part **b** of the figure shows the energy profile of the electron transfer first from hydrogen to ferredoxin, which is catalysed by the hydrogenase ($\Delta G_0' = +6.9$ kJ per mole), followed by the electron transfer from ferredoxin to CO_2, which is reduced to CO, catalysed by the CO dehydrogenase/acetyl-CoA synthase (CODH/ACS) ($\Delta G_0' = +13.5$ kJ per mole). Without FBEB, the sum of both electron-transfer reactions is highly endergonic (red line, $\Delta G_0' = +20.4$ kJ per mole). By coupling the hydrogen oxidation to NAD reduction with FBEB, the reaction sequence is close to the equilibrium (blue line, $\Delta G_0' = +2.3$ kJ per mole; $\Delta G_0'$ of electron transfer from H_2 to ferredoxin and NAD = −11.2 kJ per mole and $\Delta G_0'$ of electron transfer from ferredoxin to CO_2/CO = +13.5 kJ per mole).



involved in the WLP and the hydrogenases are soluble[33]. However, *A. woodii* has a membrane-integral F_0F_1 ATP synthase, which is specific for Na^+ (REFS 42–44). The ATP synthase has three ATP synthesizing centres and ten ion-binding sites, which results in the use of 3.3 moles of Na^+ to generate 1 mole of ATP; but what is the driving force for the generation of the Na^+ gradient?

***Membrane-bound reactions in* A. woodii.** Early work found that corrinoids were present in the membrane fraction of protein extracts from *A. woodii*, which suggested that *A. woodii* could express a corrinoid-containing, Na^+-translocating methyltransferase, similar to the one that has been described in methanogenic archaea[14,45]. However, a membrane-bound corrinoid-containing protein complex has never been described, and recent genome analysis suggests that such a complex does not exist, which indicates that the corrinoids found in the membrane preparations most probably resulted from contaminations with cytoplasmic methyltransferases. By contrast, *A. woodii* was found to have a membrane-integral, multisubunit ferredoxin–NAD^+ oxidoreductase, called the Rnf complex, which catalyses electron transfer from reduced ferredoxin to NAD^+ (REFS 1,46,47) (FIG. 2). This reaction should provide sufficient energy ($\Delta G_0' = -25$ kJ per mole; E_0' $[Fd/Fd^{2-}] = -450$ mV, E_0' $[NAD^+/NADH] = -320$ mV; $\Delta G'$ might be more exergonic as E' $[NAD^+/NADH] = -280$ mV) for the translocation of 2 moles of Na^+ across the cytoplasmic membrane (assuming a transmembrane electrochemical ion potential of around −180 mV)[48]. The Rnf complex is the only coupling site that is found in *A. woodii*, and thus the entire acetogenic metabolism must be designed to have a high Fd^{2-}/NADH ratio — that is, more reactions that produce reduced ferredoxin lead to higher levels of ATP synthesis. Reduced ferredoxin is thus a high-energy intermediate, and the amount of ATP that can be produced by *A. woodii* depends on which reactions generate or consume reduced ferredoxin.

***The oxidative part of acetogenesis in* A. woodii.** Every electron for autotrophic acetogenesis from H_2 and CO_2 must be derived from the oxidation of hydrogen. *A. woodii* has a soluble hydrogenase, HydABCD, which is a tetramer that contains iron–sulphur centres and flavin[49] (FIG. 2). HydABCD catalyses the endergonic reduction of ferredoxin with hydrogen ($\Delta G_0' = +7$ kJ per mole) (BOX 1). The driving force for this reaction is neither ATP nor a chemiosmotic ion gradient across the cytoplasmic membrane, but rather flavin-based electron bifurcation[32,50] (BOX 2). Briefly, the endergonic reduction of ferredoxin is coupled to the exergonic reduction of NAD^+; ferredoxin and NAD^+ are reduced simultaneously in a 1/1 stoichiometry in an overall exergonic reaction ($\Delta G_0' = -11$ kJ per mole)[49] (BOX 2). Therefore, the oxidative part of acetogenesis yields NADH and reduced ferredoxin in equal amounts. Part of the ferredoxin is used in the reductive reactions of acetogenesis and the remaining ferredoxin can be used by the Rnf complex to generate a chemiosmotic gradient.

***The reductive part of acetogenesis in* A. woodii.** In the reductive part of acetogenesis, one molecule of CO_2 is reduced to CO by a ferredoxin-dependent CODH/ACS, which has been purified and characterized[51,52]. Another molecule of CO_2 is reduced to formate. Interestingly, *A. woodii* uses an enzyme complex for this reaction that has not previously been observed: a hydrogen-dependent CO_2 reductase (HDCR) that contains a hydrogenase module (HydA2) and a formate dehydrogenase module (FdhF1 or FdhF2), which are connected by two electron-transferring subunits (HycB2 and HycB3)[53]. The HDCR uses H_2 directly as an electron donor for CO_2 reduction[53], and coupling of the reaction to H_2 is energetically advantageous for the cell; the indirect way would be at the expense of a high-energy intermediate — such as reduced ferredoxin — which would be lost as a substrate for the Rnf complex to generate the chemiosmotic gradient. The minimal H_2 concentration

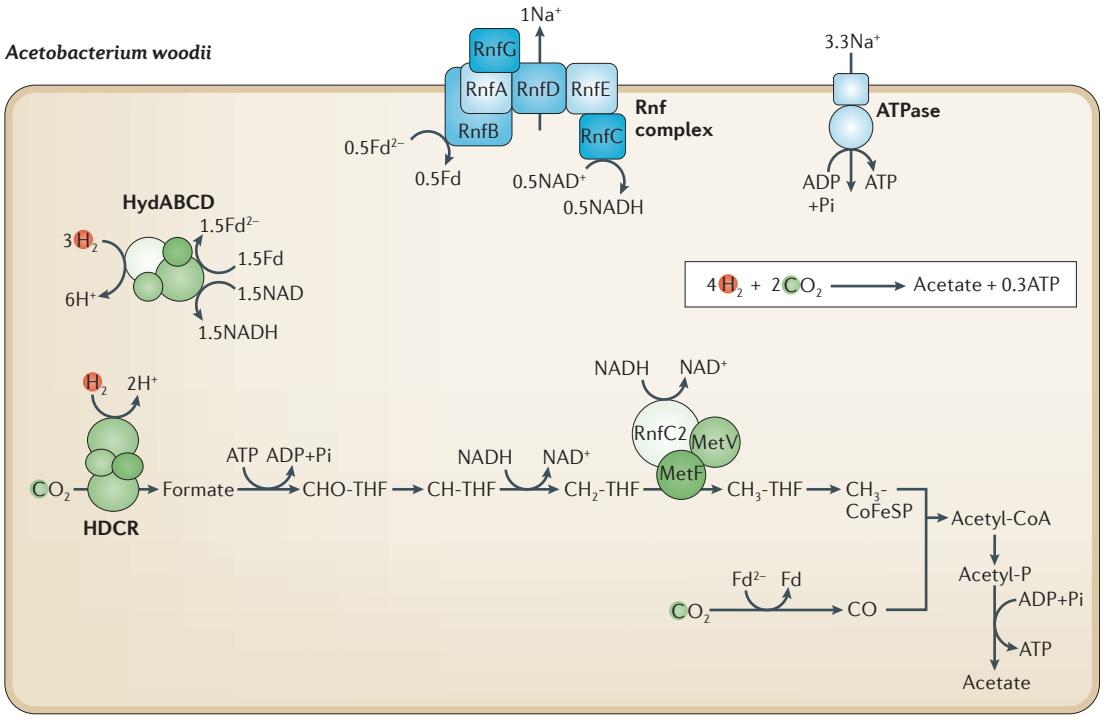


Figure 2 | **Model for acetogenesis in *Acetobacterium woodii*.** In *A. woodii*, hydrogen (H_2) is oxidized by an electron-bifurcating hydrogenase (HydABCD) that couples the endergonic reduction of ferredoxin (Fd) with the exergonic reduction of NAD. In the Wood–Ljungdahl pathway (WLP), CO_2 is reduced to formate by a hydrogen-dependent CO_2 reductase (HDCR) and is then further converted to a methyl group (CH_3). The reduction of methylene-tetrahydrofolate (CH_2-THF) is catalysed by a trimeric enzyme that consists of RnfC2, MetF and MetV. The membrane-bound Rnf complex is proposed to couple the electron transfer from reduced ferredoxin (Fd^{2-}) to NAD with the translocation of sodium ions (Na^+) across the cytoplasmic membrane. The Na^+ gradient is then used by a membrane-bound ATP synthase (ATPase) to generate ATP. To obtain enough NADH for the WLP, 0.5 moles of Fd are oxidized at the Rnf complex, producing 0.5 moles of NADH and translocating 1 mole of Na^+, which is enough for the phosphorylation of 0.3 moles of ADP by the ATP synthase. CH-THF, methenyl-THF; CH_3-THF, methyl-THF; CHO-THF, formyl-THF; CoFeSP, corrinoid iron–sulphur protein, Pi, inorganic phosphate.

that enables acetogenesis in *A. woodii* is 250 Pa[33] and it was calculated that, under such conditions, CO_2 reduction to formic acid with H_2 as the electron donor is still possible[33].

The next redox enzymes in this pathway are a methylene-THF dehydrogenase that uses NADH as electron donor[36], followed by a methylene-THF reductase. The genomic data of *A. woodii* are consistent with the presence of a soluble methylene-THF reductase that is composed of MetF, MetV and RnfC2. This complex uses NADH and is not capable of electron bifurcation (V.M., unpublished observations).

Collectively, these data show that all bioenergetically relevant enzymes of the WLP from *A. woodii* have been characterized and that the reductive part of the WLP uses 1 mole of H_2, 2 moles of NADH and 1 mole of reduced ferredoxin as electron donors for the reduction of 2 moles of CO_2 to 1 mole of acetate (FIG. 2).

***The complete energy metabolism of* A. woodii.** In the reductive branch of acetogenesis, 1 mole of H_2 is directly used as the donor for the CO_2 reductase, HDCR. The remaining 3 moles of hydrogen are oxidized by the electron-bifurcating hydrogenase HydABCD, which yields 1.5 moles each of NADH and reduced ferredoxin. 1 mole of reduced ferredoxin is required to reduce 1 mole of CO_2 to CO in the course of the CODH/ACS reaction, which leaves 0.5 moles of reduced ferredoxin to be used by the Rnf complex for the establishment of a chemiosmotic gradient. The Rnf complex generates 0.5 moles of NADH. The total 2 moles of NADH that remain are used to reduce 1 mole of methenyl-THF and 1 mole of methylene-THF. The oxidation of 0.5 moles of reduced ferredoxin by the Rnf complex leads to the export of up to 1 mole of Na^+. Considering the stoichiometry of 3.3 Na^+ per mole of ATP of the ATP synthase, this results in the generation of 0.3 moles of ATP per mole of acetate (FIG. 2).

Energy conservation in *C. ljungdahlii*

Like *A. woodii*, *C. ljungdahlii* does not contain cytochromes, but it contains an Rnf complex, which suggests that both organisms use the same mode of energy conservation[41]. However, growth and chemiosmotic ATP synthesis in *C. ljungdahlii* do not depend on Na^+ (REF. 41). The ion specificity of the Rnf complex in *C. ljungdahlii* is unknown[54], but it is likely that the bioenergetics of *C. ljungdahlii* are based on an electrochemical H^+ potential[41,55]. This is also supported by the lack of an Na^+-binding motif in the *atpE* gene of the ATP synthase. It

is not unusual for a complex membrane protein to have different ion specificities in different organisms; for example, the flagellar motor and ATP synthase use H^+ in most species but use Na^+ in others[56,57].

Finally, there is no genetic or experimental evidence to support the existence of another membrane-bound energy-conserving enzyme complex in *C. ljungdahlii*, which suggests that reduced ferredoxin is the primary energy source that is used by this bacterium, similar to *A. woodii*. However, the establishment of a high ferredoxin/NAD^+ ratio is achieved in a different way in *C. ljungdahlii*.

***Enzymatic variations in the WLP of* C. ljungdahlii.** The basic chemistry of acetogenesis from H_2 and CO_2 is the same in *C. ljungdahlii* and *A. woodii*. However, the enzymes that catalyse hydrogen oxidation, the reduction of CO_2 to formate and the reduction of methylene-THF to methyl-THF are different.

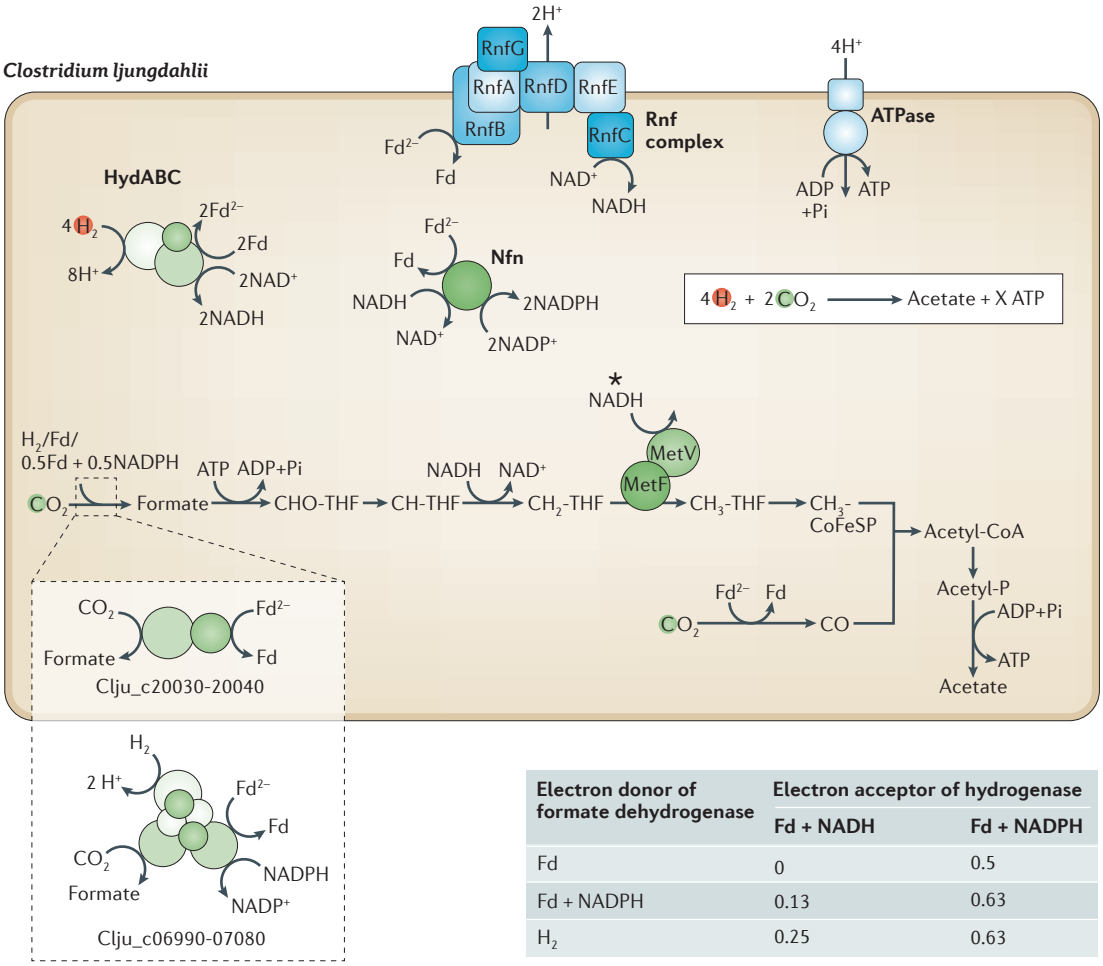
C. ljungdahlii has the genetic potential to encode four [FeFe] hydrogenases and one [NiFe] hydrogenase[41,58]. One of the [FeFe] hydrogenases (Clju_c14720–14700) is very similar to the electron-bifurcating hydrogenase of *A. woodii* but has only one, not two, small electron-transfer proteins, which is similar to the electron-bifurcating hydrogenase of *Thermotoga maritima* or *M. thermoacetica*[59,60]. A gene that encodes another electron-bifurcating [FeFe] hydrogenase in complex with a formate dehydrogenase is also present (Clju_c06990-07080), and a similar enzyme complex was recently identified in *Clostridium autoethanogenum*, which simultaneously uses ferredoxin and $NADP^+$ as the electron acceptors[34] (FIG. 3). The electron acceptors of the remaining three hydrogenases are unknown, but available transcriptomic data suggest that these enzymes are not involved in acetogenesis[58].

The genome of *C. ljungdahlii* also contains three gene clusters that code for potential formate dehydrogenases (FDHs). The first gene cluster (*Clju_c06990–07080*), already mentioned above, encodes an FDH and an electron-bifurcating hydrogenase, and this complex might use either H_2 or ferredoxin and NADPH as electron donors[58]. The second gene cluster (*Clju_c20030–20040*) encodes an FDH and a small ferredoxin-like protein, and this complex could potentially receive electrons from ferredoxin (FIG. 3). The third FDH gene (*Clju_c08930*) encodes a selenocysteine-containing enzyme and seems to be an isogene of *Clju_c20040*, which encodes a selenocysteine-free FDH.

The methylene-THF reductase from *C. ljungdahlii* (Clju_c37610–37620) has never been purified, and there are no data available on the reductant that is used and whether the enzyme uses electron bifurcation to drive ferredoxin reduction. The genome of *C. ljungdahlii* codes for two subunits that are similar to the subunits MetF and MetV of the *A. woodii* enzyme, but the gene for the third subunit of the *A. woodii* enzyme, RnfC2, is missing. Notably, NAD(P)H-dependent reduction of methylene-THF has not been observed in cell-free extracts of *C. authoethanogenum*, which is an acetogen that also contains genes coding for MetF and MetV but that lacks RnfC2. However, for the following calculations, we assume that the methylene-THF reductase from *C. ljungdahlii* can use an electron donor that is energetically equivalent to NADH, such as flavodoxin, thioredoxin or a direct coupling of enzymes. Therefore, the open questions in the bioenergetics of *C. ljungdahlii* concern the electron acceptor of the hydrogenase (either ferredoxin and NAD or ferredoxin and NADP), the electron donor for the FDH (ferredoxin, hydrogen, or ferredoxin and NADPH) and whether or not the methylene-THF reductase is electron bifurcating and reduces ferredoxin. These are important questions that have direct effects on the energy-conserving mechanism.

***Models for the complete energy metabolism of* C. ljungdahlii.** The only coupling site for energy conservation in *C. ljungdahlii* is the Rnf complex. Thus, the only energy-conserving reaction is the electron transfer from reduced ferredoxin to NAD, which is coupled to H^+ translocation and the generation of a chemiosmotic gradient. Depending on the electron acceptors of the hydrogenase and the electron donors of the WLP, the ratio between reduced ferredoxin and NAD is different (FIG. 3). By calculating how much NAD must be reduced with electrons from reduced ferredoxin to get a balanced electron stoichiometry, it is possible to predict the amount of energy that can be conserved in the form of ATP. For example, assuming that *C. ljungdahlii* uses the bifurcating [FeFe] hydrogenase (Clju_c14720–14700) — which uses ferredoxin and NADH as the electron acceptors — and the FDH (Clju_c06990–07080) — which is capable of electron bifurcation with ferredoxin and NADPH as the electron donors, then the electrons from 4 moles of H_2 are provided by 2 moles of NADH and 2 moles of reduced ferredoxin. The reduction of 2 moles of CO_2 to acetate via the WLP needs 2 moles of NADH, 1.5 moles of reduced ferredoxin and 0.5 moles of NADPH. The genome of *C. ljungdahlii* codes for a putative electron-bifurcating transhydrogenase, Nfn[58]. This protein, which is encoded by *Clju_c37240*, is similar to the NfnAB complex of *Clostridium kluyveri*, which reduces 2 moles of NADP with 1 mole of reduced ferredoxin and 1 mole of NADH, which thus connects the electron pool from NAD and ferredoxin with NADP[61]. However, in contrast to *C. kluyveri*, the two subunits of NfnAB in *C. ljungdahlii* seem to be encoded on a single polypeptide. In this scenario, 0.25 moles of NAD must be reduced via the Rnf complex with reduced ferredoxin, which leads to the translocation of 0.5 moles of H^+ and enables the generation of 0.13 moles of ATP.

Similarly, the theoretical ATP gain assuming all possible combinations of the enzymes can be calculated. The variables are the use of ferredoxin, or of ferredoxin and NADPH, or of hydrogen as the electron donors for the formate dehydrogenase and the use of ferredoxin and NADH or of ferredoxin and NADPH as the electron acceptors for the hydrogenase (FIG. 3). Only one combination without net energy conservation is observed and can thus be excluded: a hydrogenase with ferredoxin and NADH as electron acceptor and a



Electron donor of formate dehydrogenase	Electron acceptor of hydrogenase	
	Fd + NADH	Fd + NADPH
Fd	0	0.5
Fd + NADPH	0.13	0.63
H_2	0.25	0.63

Figure 3 | **Model for acetogenesis in *Clostridium ljungdahlii*.** *C. ljungdahlii* oxidizes hydrogen either by an electron-bifurcating hydrogenase (HydABC), as described for *Acetobacterium woodii*, or by an enzyme complex of hydrogenase and formate dehydrogenase (Clju_c06990–07080), resulting in the reduction of ferredoxin (Fd) and NAD or NADP (the model of the enzyme represents all possible electron donors and acceptors, but the physiological electron transfer is unknown). The electron-bifurcating enzyme Nfn catalyses the interconversion of Fd, NADH and NADPH. In the Wood–Ljungdahl pathway (WLP), CO_2 is reduced to formate, with electrons provided either by Fd (Clju_c20030–20040) or either by hydrogen or by Fd and NADPH (Clju_c06990–07080). Reduction of methylene-THF (CH_2-THF) is catalysed by a dimeric enzyme (MetF and MetV). The membrane-bound Rnf complex in *C. ljungdahlii* is proposed to couple the electron transfer from reduced ferredoxin (Fd^{2-}) to NAD with the translocation of protons (H^+) across the cytoplasmic membrane. The H^+ gradient is then used by a membrane-bound ATP synthase (ATPase) to generate ATP (assuming four H^+ to phosphorylate one ADP). In the different proposed models for acetogenesis in *C. ljungdahlii*, the total amount of conserved energy in the form of ATP is variable owing to differences in the enzymes that are proposed to be involved. The table provides the ATP gain per mole of acetate, depending on the different enzyme combinations (mole ATP/mole acetate). *The electron donor for MetFV is not known but is assumed to be equivalent to the redox potential range of NADH. CH-THF, methenyl-THF; CH_3-THF, methyl-THF; CHO-THF, formyl-THF; CoFeSP, corrinoid iron–sulphur protein, Pi, inorganic phosphate.

ferredoxin-dependent FDH. In all other scenarios, the ATP gain ranges from 0.13 to 0.63 moles of ATP per mole of acetate (FIG. 3). The highest ATP gain is predicted when a hydrogenase uses ferredoxin and NADPH and when a formate dehydrogenase uses hydrogen or ferredoxin and NADPH as electron donors. However, an NADP-dependent bifurcating hydrogenase would depend on high H_2 partial pressures. If *C. ljungdahlii* has an electron-bifurcating methylene-THF reductase, this would result in the additional production of 0.5 moles of ATP per mole of acetate in all scenarios. Therefore, these calculations show that energy conservation is possible with the predicted models in *C. ljungdahlii*, and the theoretical values calculated for ATP generation are in agreement with the existing experimental data.

In summary, *C. ljungdahlii* is another example of an acetogen that uses the Rnf complex as the only coupling site for energy conservation. Despite the variations in some soluble enzymes that are used in the WLP by *C. ljungdahlii* and *A. woodii*, which influence the overall ATP yield, the basics of the energy metabolism during acetogenesis are the same in these two organisms. The major difference is that *A. woodii* uses an Na^+ gradient for chemiosmotic ATP synthesis, whereas *C. ljungdahlii* uses an H^+ gradient.

Energy conservation in *M. thermoacetica*

The third model organism of acetogenesis, *M. thermoacetica*, uses H^+ as the chemiosmotic coupling ion (as in *C. ljungdahlii*), but it lacks the Rnf complex that is essential for energy conservation in both organisms, as discussed previously[6,14,29,62,63]. *M. thermoacetica* has cytochromes and quinones, which have been predicted to mediate electron transfer from a donor, such as H_2, to methylene-THF[12–14]. However, there is no experimental evidence to support the existence of such a membrane-based electron-transport chain, and inspection of the genome of *M. thermoacetica* and experimental data did not reveal that the methylene-THF reductase is membrane integral[39,40,62]. These observations raise the question of how energy is conserved in the absence of an Rnf complex.

The only membrane-bound, potentially ion-translocating enzyme that can be linked to the WLP in *M. thermoacetica* is an energy-converting hydrogenase (Ech) (FIG. 4). Ech complexes are widespread in anaerobic or facultative anaerobic bacteria and archaea and couple the exergonic electron transfer from reduced ferredoxin to H^+, thereby producing H_2 (REF. 64). Examples of such complexes are: the EchA–EchF complex of *Methanosarcina barkeri,* which catalyses electron transfer from reduced ferredoxin to H^+ (REF. 65); the CO-induced hydrogenase Coo from *Rhodospirillum rubrum,* which reduces H^+ with electrons that result from oxidation of CO[66]; and the formate hydrogen lyase from *Escherichia coli*, which is capable of coupling H^+ reduction to formate oxidation[67]. All Ech hydrogenases share a core module of two membrane-integral subunits, two [NiFe] hydrogenase subunits and two additional hydrophilic subunits.

The gene cluster *Moth_2184–2193* of *M. thermoacetica* codes for an Ech complex and is preceded by a gene for a putative formate dehydrogenase. Another cluster (*Moth_0977–0987*) also codes for an Ech complex, but the hydrogenase subunit seems to lack the nickel-binding site that is necessary for its activity. Therefore, the first cluster could encode an Ech complex that is a possible candidate for the energy-coupling site in the autotrophic metabolism of *M. thermoacetica.* This is similar to what has been reported in the methanogenic archaeon *Methanosarcina mazei,* in which Ech activity is coupled to the generation of a transmembrane electrochemical H^+ gradient[68]. The translocated H^+/produced H_2 stoichiometry of the Ech complex is unknown, but it has been proposed to be 1/1. Importantly, the reaction that is catalysed by the Ech complex releases less than one-third of the energy of the reaction that is catalysed by the Rnf complex ($\Delta G_0' = -7$ kJ per mol; the $\Delta G'$ value depends directly on the hydrogen partial pressure)[68,69]. Similarly to the Rnf complex, there is no reason to think that H^+ is the exclusive coupling ion in the Ech complexes in different species, and some may use Na^+ as the coupling ion.

***A model for energy conservation in* M. thermoacetica.** *M. thermoacetica* oxidizes hydrogen with a soluble electron-bifurcating hydrogenase (HydABC) that reduces ferredoxin and NAD^+, similar to *A. woodii* and *C. ljungdahlii*. In addition, *M. thermoacetica* has a soluble $NADP^+$-reducing hydrogenase (Moth_1883–1888)[40,60] (FIG. 4). Furthermore, an electron-bifurcating transhydrogenase (NfnAB) enables the further interconversion of 1 mole of ferredoxin and 1 mole of NADH to 2 moles of NADPH[40,61]. This combination of the electron-bifurcating hydrogenase and the transhydrogenase leads to the same result of 2 moles of NADPH produced with the NADP-dependent hydrogenase. NADPH is the electron donor for the reduction of CO_2 to formate, which is catalysed by FDH[70,71]. The *M. thermoacetica* genome codes for two additional formate dehydrogenases (FIG. 4): *Moth_2193* is in the Ech gene cluster, and the corresponding enzyme may be bound to the membrane-integral Ech complex; the gene cluster *Moth_0450–0452* codes for a periplasmic formate dehydrogenase that transfers the electrons to cytochrome *b* and may be involved in nitrate reduction rather than in acetogenesis[72,73].

The cyclization of formyl-THF and the reduction of methenyl-THF are catalysed by one enzyme: a bifunctional formyl-THF cyclohydrolase–methenyl-THF dehydrogenase that uses NADPH as the reductant[37]. In addition, the CODH/ACS uses ferredoxin for the reduction of CO_2 to CO[74]. The enzyme that catalyses the reduction of methylene-THF — the methylene-THF reductase — was recently partially purified and characterized[39]. Interestingly, the genes that encode the methylene-THF reductase are preceded by genes that encode homologues of a soluble electron-bifurcating heterodisulphide reductase (HdrABC), and the partial purification of the methylene-THF reductase of *M. thermoacetica* provided evidence that this is a heterohexameric complex that consists of MetFV, HdrABC and MvhD and that uses NADH as the electron donor. However, this complex does not catalyse NADH-dependent methylene-THF reduction and might require a second electron acceptor for this reaction. Notably, ferredoxin is not reduced in this process, and whether or not HdrABC uses electron bifurcation with a second electron acceptor remains to be established[39] (FIG. 4).

Thus, all enzymatic steps and their respective electron flow in the WLP of *M. thermoacetica* can be tracked, except for the second electron acceptor of the methylene-THF reductase. For simplicity, we assume that ferredoxin is the second acceptor of the methylene-THF reductase when testing the hypothesis of Ech-based energy conservation, but an energetic equivalent electron carrier or a direct coupling of enzymes (for example, methylene-THF reductase and CO-dehydrogenase or Ech) could be used as a substitute without influencing the bioenergetic consideration. In this model, the reactions of the WLP require 2 moles of NADPH and 2 moles of NADH for the reduction of 2 moles CO_2 (FIG. 4). The hydrogenases can provide the 2 moles of NADPH from the oxidation of 2 moles of H_2. 1 mole of NADH can be generated by the electron-bifurcating hydrogenase, which results in the simultaneous electron transfer from the last mole of H_2 to ferredoxin. Therefore, how is the second mole of NADH generated from reduced ferredoxin? In

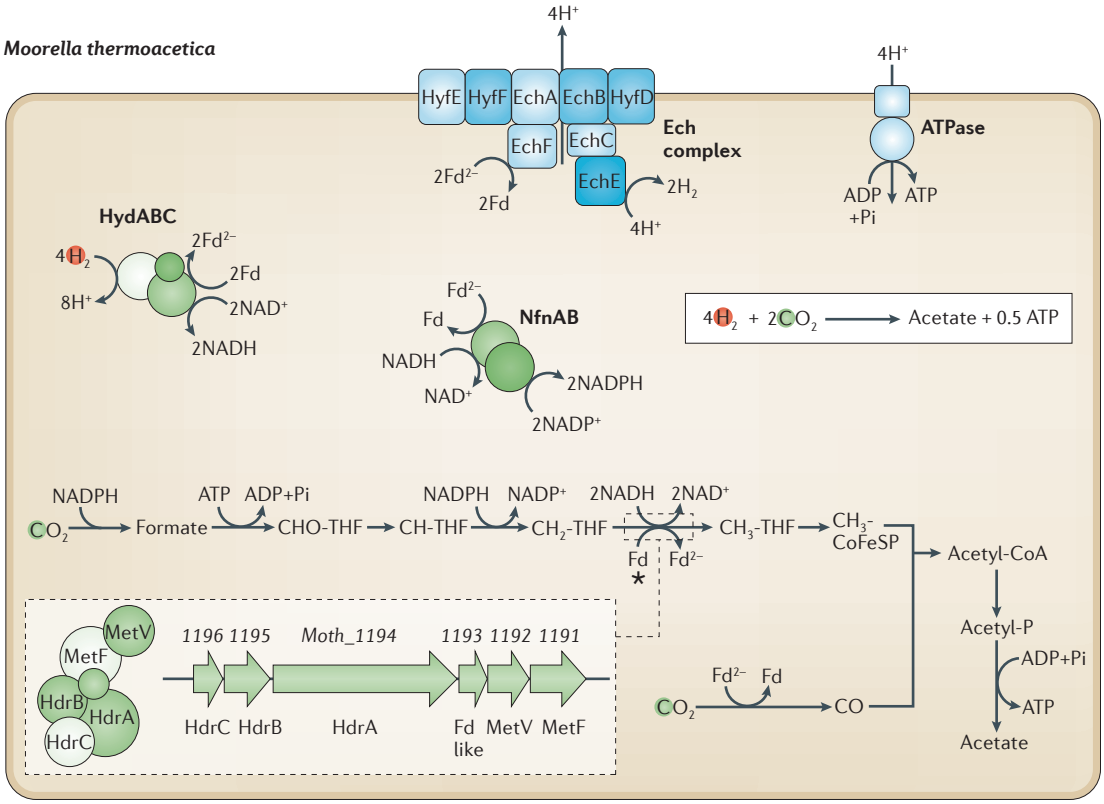


Figure 4 | **Model for acetogenesis in *Moorella thermoacetica*.** In *M. thermoacetica*, hydrogen (H_2) is oxidized by an electron-bifurcating hydrogenase, HydABC, which reduces ferredoxin (Fd) and NAD. NADP is reduced by an electron-bifurcating transhydrogenase, NfnAB, with Fd and NADH as the electron donor. These two enzymes in combination catalyse the same reaction as the NADP-dependent hydrogenase alone (Moth_1883–1885) (not shown). In the Wood–Ljungdahl pathway (WLP), methylene-THF (CH_2-THF) is reduced by an electron-bifurcating enzyme that couples the exergonic electron transfer from NADH to CH_2-THF with the reduction of an energetic equivalent of ferredoxin (Fd). The genetic organization for this enzyme is shown in the inset. The proposed enzyme complex is composed of the subunits MetF and MetV of CH_2-THF reductases and subunits that are similar to the electron-bifurcating Hdr complex of *Methanothermobacter marburgensis*. To balance the electron flow, two molecules of Fd need to be oxidized at the Ech complex that translocates protons (H^+) across the cytoplasmic membrane. The H^+ gradient is then used by a membrane-bound ATP synthase (ATPase) to generate ATP. *The second electron acceptor for the CH_2-THF reductase is not known but for simplicity is assumed to be an energetic equivalent of Fd. CH-THF, methenyl-THF; CH_3-THF, methyl-THF; CHO-THF, formyl-THF; CoFeSP, corrinoid iron–sulphur protein; Fd^{2-}, reduced ferredoxin; Pi, inorganic phosphate.

A. woodii and *C. ljungdahlii*, the answer is the Rnf complex, which is missing in *M. thermoacetica*. A balanced electron stoichiometry is only possible if the Ech complex is involved (FIG. 4). In this scenario, acetogenesis provides the cell with 0.5 moles of ATP per mole of acetate and is thus energetically feasible. The unanswered questions in the proposed models are whether the putative formate dehydrogenase Moth_2193 is indeed part of the Ech complex, whether Moth_2193 has any role in energy conservation and what are the exact mechanism and electron acceptors of the methylene-THF reductase.

The hypothesis of an Ech-based energy-conserving mechanism in *M. thermoacetica* is further supported by the recently published genome sequence of the thermophile *Thermoacetogenium phaeum*[75]. This organism catalyses reverse acetogenesis — that is, the oxidation of acetate to CO_2 by the WLP. There are no genes for an Rnf complex in *T. phaeum*, and this organism also has no cytochromes. The putative coupling sites in *T. phaeum* are an Ech complex that is encoded by the genes *Tph_c21310–Tph_c21360* and/or an Ech complex, together with a formate dehydrogenase that is encoded by the genes *Tph_c26250–Tph_c26370*.

Ecological effect of the different bioenergetics

Although the three model acetogens that are examined here catalyse the same overall chemical conversion of H_2 and CO_2, there are differences in their energetics and in the individual enzymes that are used for the single reactions. A major reflection of the different thermodynamics used by different acetogens could be the threshold concentration of H_2 that enables bacterial growth. If a pathway is mechanistically coupled to an endergonic reaction, such as ATP synthesis or the reduction of a low-potential electron carrier (such as ferredoxin), the H_2 partial pressure has to be high enough to provide the energy that is necessary for this endergonic

reaction. However, if these reactions are uncoupled, the pathway can proceed at lower H_2 partial pressures. Thus the different enzyme combinations that are used by the different acetogens could be a reflection of the substrate concentrations that are observed in the different ecosystems. A prominent example is the Ech complex, which has thermodynamics that are directly coupled to the H_2 partial pressure as the product of the Ech-catalysed reaction. Another explanation for the variations in the acetogenic metabolism could be the various substrates that are used by acetogenic bacteria: most acetogens can use different substrates, but the substrate spectrum varies greatly between different genera. Therefore, the observed enzymatic variations in the different acetogens could be a reflection of a specialization to specific substrates; for example, *C. ljungdahlii* and *M. thermoacetica* are capable of growing with CO as a substrate, but *A. woodii* is not. An example is the HDCR of *A. woodii* that is inhibited by CO, which could explain why the *C. ljungdahlii* and *M. thermoacetica* use different enzymes to reduce CO_2 to formate.

Finally, the ATP yields of the proposed energetic models are not in total agreement with the growth behaviour of the organisms; for example, *A. woodii* grows better on H_2 and CO_2 than *M. thermoacetica*, although its proposed energy yield is lower[3,20,40]. However, in addition to the thermodynamics of acetogenesis, other factors — such as the properties of the enzymes that are involved in acetogenesis and the capacity of the acetogen to transport substances, to deal with pH regulation or to synthesize enzymes that are involved in these reactions — influence the growth rate of different bacteria.

A new bioenergetic classification for acetogens

From the three model organisms that we have presented, it is evident that the old bioenergetics classification of 'corrinoid-containing, Na^+-dependent' and 'cytochrome containing, H^+-dependent' acetogens is no longer meaningful. A cytochrome-dependent methylene-THF reductase or corrinoid-containing membrane proteins, such as a methyltransferase, were speculated to be present in acetogens on the basis of analogies to other bacteria and/or archaea, but recent genome analysis revealed that they do not exist in acetogens. Instead, acetogens seem to have either the Rnf or the Ech complex to couple the WLP to the generation of a transmembrane ion gradient that drives ATP synthesis. Therefore, we propose to classify acetogens bioenergetically as 'Rnf-acetogens' or 'Ech-acetogens'. The ion that is used by these complexes (and the ATP synthase) may be either H^+ or Na^+, which results in two subclasses for each class: Na^+-dependent organisms and H^+-dependent organisms. *A. woodii* is the prototype for the subclass of 'Rnf-containing, Na^+-dependent' acetogens and *C. ljungdahlii* is the model organism for the subclass of 'Rnf-containing, H^+-dependent' acetogens. So far, only members of the subclass 'Ech-containing, H^+-dependent' acetogens are known, and *M. thermoacetica* is the model organism; however, there is no reason to think that an 'Ech-containing, Na^+-dependent' acetogen does not exist.

Conclusions

The WLP seems to be the oldest pathway for generating organic material from CO_2, and acetogenic bacteria use this pathway for both carbon assimilation and energy conservation. The WLP itself is not directly involved in energy conservation and it can be regarded as an isolated module that functions as a CO_2-reducing electron sink. A mechanism of energy conservation is added to the WLP by combining it with an energy-conserving module — the Rnf complex or the Ech complex. The end products of the reactions catalysed by these complexes are either NADH or H_2, which are used as electron carriers to combine both modules.

Although acetogenic bacteria show a large phylogenetic diversity, which could imply that these organisms use diverse metabolic strategies, this is only partially true. The basic chemistry of CO_2 reduction is very similar in all acetogens and their differences can be attributed mainly to the electron donors and acceptors that are used for the redox reactions, rather than the reactions themselves. The diversity among acetogens is present in the energy-conservation module and in how acetogens establish a transmembrane ion gradient as a driving force for ATP synthesis, either by using the Rnf or Ech complex or by using Na^+ or H^+ as the basis for the establishment of a chemiosmotic gradient.

Despite this diversity in the electron-transfer pathway modules, acetogenic bacteria share one important similarity: the actual 'energy equivalent' is the reduced iron–sulphur cluster of the protein ferredoxin, which energizes the ion-translocating membrane complex. This is in sharp contrast to the evolutionarily more 'advanced' respiration chains, such as in aerobic organisms, which all depend on NADH.

The model acetogens share another important similarity: they are strictly dependent on electron bifurcation. This coupling between redox reactions enables the catalysis of otherwise endergonic reactions without using ATP hydrolysis or a transmembrane ion gradient as the driving force. As electron bifurcation has often been observed in organisms that are coping with strong energy limitation, this might be an important adaptation for life at the thermodynamic limit. Acetogenesis is an exciting example of how evolution combined different modules to generate strategies to couple carbon assimilation to ATP synthesis.

Biochemical analysis of acetogenesis is now well advanced. Major parts of the enzymatic reactions are proven by experimental data. The coupling site for the Rnf-acetogens is clearly identified, but the model that has been proposed for the Ech-acetogens needs experimental validation. In recent years, we have seen a slow development of genetic tools for acetogenic bacteria[41,76,77], and this toolbox will certainly expand and help us to address many more exciting questions: why do some acetogens use H^+ and others use Na^+? What are the implications of the enzyme variations that are observed in the WLP in different acetogens? Is there acetogenesis beyond the Rnf and Ech types? Nearly 100 years after their discovery, acetogens are still a treasure trove for microbial physiologists, biochemists, ecologists and bioenergeticists.

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