environmental microbiology

Environmental Microbiology (2022) **24**(7), 3124–3133 doi:10.1111/1462-2920.16011

One substrate, many fates: different ways of methanol utilization in the acetogen *Acetobacterium woodii*

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Summary

Acetogenic bacteria such as *Acetobacterium woodii* use the Wood–Ljungdahl pathway (WLP) for fixation of CO_2 and energy conservation. This pathway enables conversion of diverse substrates to the main product of acetogenesis, acetate. Methyl group containing substrates such as methanol or methylated compounds, derived from pectin, are abundant in the environment and a source for CO_2. Methyl groups enter the WLP at the level of methyltetrahydrofolic acid (methyl-THF). For methyl transfer from methanol to THF a substrate-specific methyltransferase system is required. In this study, we used genetic methods to identify *mtaBC2A* (Awo_c22760-Awo_c22740) as the methanol-specific methyltransferase system of *A. woodii*. After methyl transfer, methyl-THF serves as carbon and/or electron source and the respiratory Rnf complex is required for redox homeostasis if methanol + CO_2 is the substrate. Resting cells fed with methanol + CO_2, indeed converted methanol to acetate in a 4:3 stoichiometry. When methanol was fed in combination with other electron sources such as H_2 + CO_2 or CO, methanol was converted Rnf-independently and the methyl group was condensed with CO to build acetate. When fed in combination with alternative electron sinks such as caffeate methanol was oxidized only and resulting electrons were used for non-acetogenic growth. These different pathways for the conversion of methyl-group containing substrates enable acetogens to adapt to various ecological niches and to syntrophic communities.

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Introduction

Acetogenic bacteria are characterized by a special pathway for utilization of C1 compounds for acetogenesis (Drake, 1994; Müller, 2003; Ragsdale and Pierce, 2008). Carbon dioxide is reduced in the carbonyl branch of the two-branched Wood–Ljungdahl pathway (WLP) to formate which is then activated by ATP hydrolysis and bound to the C1 carrier tetrahydrofolic acid (THF). The resulting formyl-THF is dehydrated to methenyl-THF which is subsequently reduced *via* methylene- to methyl-THF. In the carbonyl branch, a second molecule of CO_2 is reduced to CO that is bound to the enzyme CO dehydrogenase/acetyl-CoA synthase (CODH/ACS). The CODH/ACS condenses the carbonyl-group with the methyl-group of methyl-THF and CoA to acetyl-CoA. The latter is converted *via* acetylphosphate to acetate and ATP. The ATP balance of this pathway is zero (Poehlein *et al*., 2012). Additional ATP is synthesized by one of two possible respiratory chains present in acetogens, consisting of an ATP synthase and either the Rnf- or the Ech complex, which both are fueled by oxidation of reduced ferredoxin (Biegel and Müller, 2010; Schuchmann and Müller, 2014; Westphal *et al*., 2018; Schoelmerich and Müller, 2019). Both complexes are hooked up to the WLP and have the general function of energy-coupled transhydrogenases used to build up an ion-motive force when reduced ferredoxin is oxidized but they also work as ion potential-driven reverse electron transport chains to enable reduction of ferredoxin with high potential electron donors such as NADH or H_2.

The WLP is an ideal pathway for the utilization of C1 compounds of different oxidation/reduction levels (Balch *et al*., 1977; Bache and Pfennig, 1981; Schink, 1994; Katsyv and Müller, 2020). CO is oxidized to CO_2 and further converted as described above; electrons required for the process solely derive from CO. If H_2 + CO_2 is the substrate, electrons are derived from the oxidation of hydrogen gas. Formate is a substrate for some acetogens (Tanner *et al*., 1993; Litty and Müller, 2021; Moon *et al*., 2021) as well. It is taken up by a formate transporter and/or diffusion and then disproportionated to CO_2 and acetate (Moon *et al*., 2021). 75% of the substrate is oxidized to CO_2 generating the six electrons required for formate and CO_2 reduction to acetate.

Formaldehyde enters the pathway at the level of methylene-THF and, thereafter, is also disproportionated. 50% of the carbon is oxidized to CO_2 generating the four electrons required to reduce methylene-THF and CO_2 to acetate. Methanol is the most reduced carbon and energy source that is used by some acetogens. The methyl group is transferred by a methyltransferase system to THF (Kremp and Müller, 2021) and methyl-THF is then oxidized to CO_2. Oxidation of one methyl group yields six electrons that are used to reduce three more methyl groups and 3 mol CO_2 to acetate according to:

$$4\,CH_3OH + 2\,CO_2 \rightarrow 3\,CH_3COOH + 2\,H_2O \qquad (1)$$

Methanol metabolism has been studied in the model acetogen *A. woodii* recently (Kremp *et al*., 2018). Its metabolism according to Eq. 1 is depicted in Fig. 1. Methanol oxidation leads to the reduction of NAD^+ and the production of H_2. Since the CO dehydrogenase requires reduced ferredoxin as reductant, the electron-bifurcating hydrogenase and the Rnf complex are essential to convert 1 H_2 and 2 NADH to 3 mol of reduced ferredoxin. Energy is conserved solely by substrate-level phosphorylation.

Acetogenesis from methanol according to Eq. 1 is already textbook knowledge but the methyltransferase involved still remained to be elucidated. Methanol originates in nature from pectin and is always present together with other C1 substrates or electron donors (Siragusa *et al*., 1988). Therefore, the question arose, whether *A. woodii* can adapt its methanol metabolism to the environmental conditions.

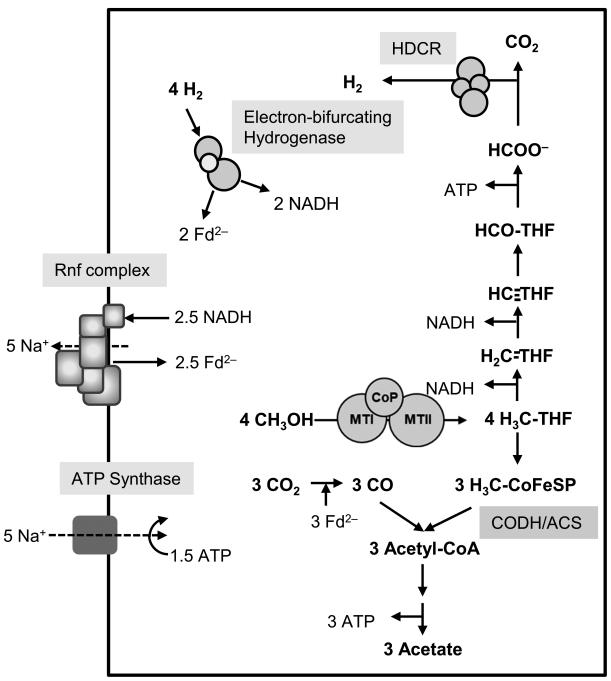

Fig. 1. Biochemistry and bioenergetics of acetogenesis from methanol + CO_2 in *A. woodii*. A stoichiometry of 2 H^+/2 e^- is assumed for the Rnf complex. The stoichiometry of the ATP synthase is 3.3 Na^+/ATP (Matthies *et al*., 2014). Fd, ferredoxin; THF, tetrahydrofolate; CODH/ACS, CO dehydrogenase/acetyl coenzyme A synthase; CoP, corrinoid protein; HDCR, hydrogen-dependent CO_2 reductase, MTI, methyltransferase I; MTII, methyltransferase II.

Results

Identification of the methanol-specific methyltransferase system

Transfer of the methyl group of methanol to THF requires three proteins: the substrate-specific methyltransferase I (MT I) that abstracts the methyl group and transfers it to the corrinoid cofactor of the second protein, the corrinoid protein (CoP), which changes from Co(I) to methyl Co(III) (Kaufmann *et al*., 1998b; Kreher *et al*., 2010). The methyl group is then transferred to THF by the third protein, methyltransferase II (MT II) yielding Co(I) back and methyl-THF (Kreft and Schink, 1993; Kaufmann *et al*., 1998a; Kremp and Müller, 2021). *Acetobacterium woodii* has 23 different MT I-enzyme encoding genes, highlighting the tremendous importance of different pectin-derived methyl groups as carbon and energy source for *A. woodii* (Kremp and Müller, 2021). A methanol-specific methyltransferase had been identified in the acetogen *Eubacterium callanderi* (Chen *et al*., 2016) and *A. woodii* has a homologue with 71% identical amino acids. This homologue is encoded by *mtaB* (Awo_c22760). *mtaB* has a length of 1395 bp and is translated into a protein with a predicted molecular mass of 50.5 kDa. MtaB has conserved cysteine and glutamine residues responsible for Zn^{2+} binding (E170, C226 and C272) (Hagemeier *et al*., 2006). Upstream of *mtaB* is *mtaY* with a length of 393 bp (Fig. S1A). *mtaY* encodes for a protein of 14.4 kDa that is similar to the cap domain of methionine synthases (Bandarian *et al*., 2002). Downstream of *mtaB* is *mtaC2* that encodes a CoP (Awo_c22750). *mtaC2* has a length of 639 bp and is translated into a protein with a predicted molecular mass of 22.7 kDa. The derived amino acid sequence is 69% identical to MtaC of *E. callanderi* and shows the characteristic DxHxxG-motif of cobamide-binding proteins (Drennan *et al*., 1994; Matthews *et al*., 2008). *mtaC2* is followed by *mtaA* (Awo_c22740), a gene with a length of 801 bp that encodes a protein with a predicted molecular mass of 29.4 kDa. MtaA shows 77% identity to MTII (Eli_2005) of *E. callanderi* and has a conserved pterin-binding domain. Downstream of *mtaA* is an exopolyphosphatase encoded by *ppx2* (Fig. S1A). To address the function of this cluster in methanol metabolism in *A. woodii*, *mtaBC2A* was deleted using the procedure described before (Wiechmann *et al*., 2020). After

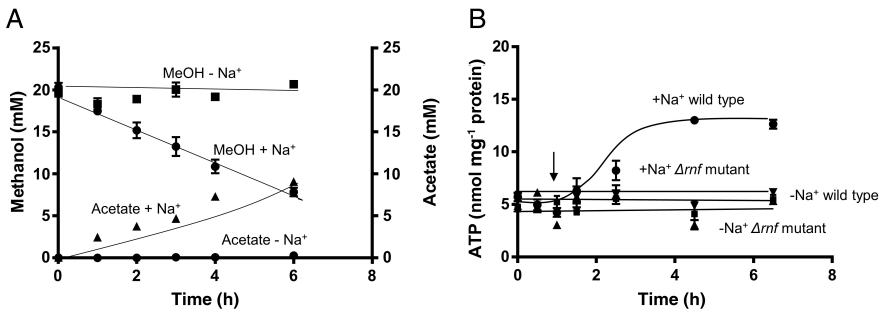

Fig. 2. Acetogenesis and ATP synthesis of *A. woodii* from methanol + CO_2 is Na^+-dependent. Resting cells of *A. woodii* wild type or the *Δrnf* mutant (protein concentration: 1 mg ml^{-1}) in 40 mM imidazole buffer containing 20 mM KCl, 20 mM NaCl, 20 mM $MgSO_4$, 2 mM DTE and 4 μM resazurin (pH 7.0) were incubated at 30°C under a N_2/CO_2 atmosphere [80:20% (vol./vol.)] in the presence or absence of Na^+ with 20 mM methanol as substrate. The contaminating amount of Na^+ in absence of Na^+ was 171 μM.
A. Methanol consumption (●) and acetate formation (▲) of the wild type in the presence of Na^+; Methanol consumption (■) and acetate formation (▼) of the wild type in the absence of Na^+.
B. ATP concentration of the wild type in the presence (●) or absence (■) of Na^+; ATP concentration of the *Δrnf* mutant in the presence (▲) or absence (▼) of Na^+. The data represent the result of three independent experiments ($n = 3$).

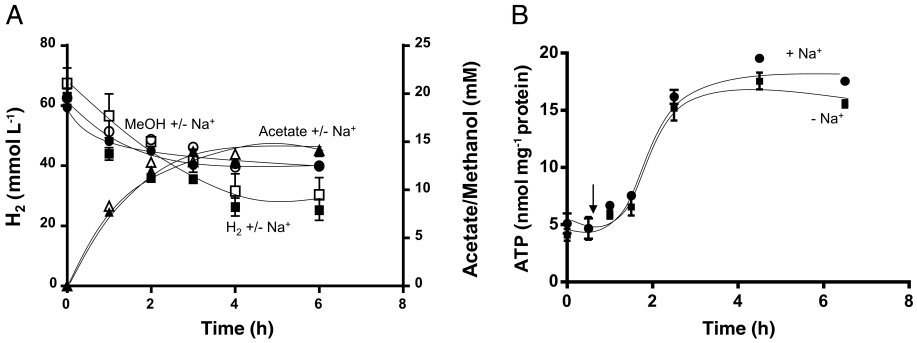

Fig. 3. Acetogenesis and ATP synthesis of *A. woodii* from methanol + H_2 + CO_2 is Na^+-independent. Resting cells of *A. woodii* wild type (protein concentration, 1 mg ml^{-1}) in 40 mM imidazole buffer containing 20 mM KCl, 20 mM $MgSO_4$, 2 mM DTE and 4 μM resazurin (pH 7.0) were incubated at 30°C in the presence or absence of Na^+ with 20 mM methanol + 101 kPa H_2 + CO_2 [80:20% (vol.:vol.)] as substrates. The contaminating amount of Na^+ was 171 μM.
A. The concentrations of methanol (●), H_2 (■) and acetate (▲) in the presence of Na^+. The concentrations of methanol (○), H_2 (□) and acetate (△) in the absence of Na^+.
B. Cellular ATP pools in the presence (20 mM) (●) or absence (■) of Na^+. The addition of methanol + H_2 + CO_2 is indicated by an arrow. The data represent the result of three independent experiments ($n = 3$).

selection on 5-fluoroorotic acid, 11 out of 12 colonies screened showed the genotype of the revertant and one colony (colony 9) showed the genotype of the deletion mutant, which was verified by PCR analysis (Table S1; Figs S1B and S1C) and DNA sequencing. The *mtaBC2A* mutant grew on fructose, H_2 + CO_2, formate and glycine betaine (not shown), but no longer on methanol (Fig. S2), demonstrating first that *mtaBC2A* codes for the methanol-specific methyltransferase system of *A. woodii* and second, there is no redundancy since none of the other methyltransferases could apparently substitute for *mtaBC2A*.

Growth of A. woodii *on methanol*

As discussed above, oxidation of the methyl group of methanol proceeds *via* a reversal of the WLP, this should require the Rnf complex to provide reduced ferredoxin for the CODH reaction. To experimentally verify this assumption, cells were pregrown on methanol in the presence of CO_2/HCO_3^- and resting cells were prepared. These cell suspensions consumed methanol and produced acetate at a ratio of 1:0.73 (Fig. 2A) which fits the Eq. 1 very nicely. In the absence of Na^+ (contaminating Na^+ concentration 171 μM), methanol was not consumed and acetate was not produced. Also, the *Δrnf* mutant (Westphal *et al*., 2018) did neither consume methanol nor produce acetate (data not shown), fitting the model that Na^+ and the Rnf complex are essential for methyl group oxidation and acetogenesis according to Eq. 1 and Fig. 1. Along with acetate, the cells produced ATP but only in the presence of Na^+ (Fig. 2B) with a theoretical yield of 0.83 ATP/acetate and 0.63 ATP/methanol (based on Fig. 1); the *Δrnf* mutant was not able to synthesize ATP.

Growth of A. woodii *on methanol + H_2 + CO_2*

Methanogenic archaea disproportionate the methyl group *via* the WLP to CO_2 and CH_4, but under an H_2 atmosphere, methanol is exclusively reduced to methane (Blaut and Gottschalk, 1984; Müller *et al*., 1986). Therefore, we tested whether this 'reductive only' mode of methanol conversion is also possible for *A. woodii*. To this end, cells were incubated under H_2 + CO_2, the latter because CO_2 is required as precursor for the carboxyl group of acetate, and in the presence of methanol. As can be seen in Fig. 3A, acetate was produced and both, methanol and H_2 were consumed simultaneously. In contrast to acetate formation from methanol + CO_2 (Fig. 2A), acetate formation from methanol + CO_2 + H_2 was Na^+-independent (Fig. 3A). The ratio of H_2:methanol was 5.3:1 and the ratio of acetate:methanol was 2.2:1 resulting in a fermentation balance of:

$$5\,H_2 + 3\,CO_2 + 1\,CH_3OH \rightarrow 2\,CH_3COOH + 3\,H_2O \quad (2)$$

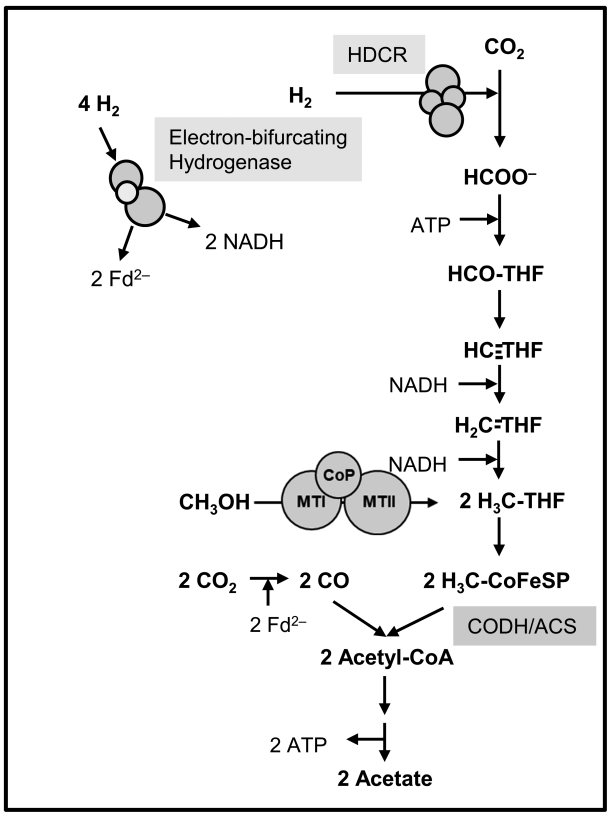

Fig. 4. Biochemistry and bioenergetics of acetogenesis from methanol + H_2 + CO_2 in *A. woodii*. A stoichiometry of 2 H^+/2 e^- is assumed for the Rnf complex. The stoichiometry of the ATP synthase is 3.3 Na^+/ATP (Matthies *et al*., 2014). Fd, ferredoxin; THF, tetrahydrofolate; CODH/ACS, CO dehydrogenase/acetyl coenzyme A synthase; CoP, corrinoid protein; HDCR, hydrogen-dependent CO_2 reductase MTI, methyltransferase I; MTII, methyltransferase II.

Acetogenesis from methanol + H_2 + CO_2 was not only independent of Na^+ but also did not require the Rnf complex; the fermentation balance in the *Δrnf* mutant was identical to the wild type (Fig. S3). Moreover, as expected, acetogenesis from methanol + H_2 + CO_2 was coupled to ATP synthesis (Fig. 3B) that was independent of Na^+ and did not require the Rnf complex. The *Δrnf* mutant grew even faster and to higher optical densities with methanol +-H_2 + CO_2 (in the presence of Na^+) than the wild type (Fig. S4). These data are in line with the hypothesis that the methyl group is not oxidized to CO_2 under these conditions. Instead, H_2 is the electron donor and 4 mol of H_2 are oxidized by the electron-bifurcating hydrogenase to yield 2 NADH and 2 Fd^{2-} (Fig. 4). The only way to reoxidize NADH is in the WLP and 1 mol H_2 is required in addition for the reduction of CO_2 to formate, as catalysed by the hydrogen-dependent CO_2 reductase, HDCR. In sum, the two methyl groups derived from one methanol and one CO_2 in the methyl branch and 2 mol CO_2 from the carbonyl branch give 2 mol of acetate. The theoretical ATP yield is 0.5 ATP/acetate and 1 ATP/methanol. It would also be possible to draw a Na^+-dependent scenario, but this was not observed experimentally; a Na^+-dependent conversion has a theoretical ATP yield of 0.7 ATP/acetate but only 0.7 ATP/methanol.

Growth of A. woodii *on methanol + CO*

The unexpected finding that the reducing equivalents derived from H_2 oxidation were disposed Na^+-

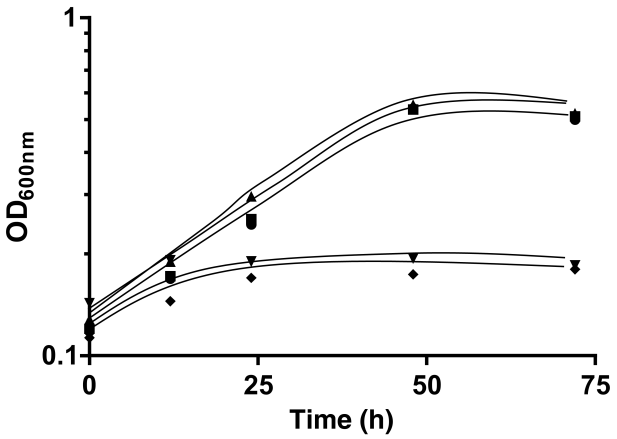

Fig. 5. Growth of *A. woodii* on methanol + CO. *A. woodii* wild type was grown at 30°C in phosphate-buffered medium (Braun and Gottschalk, 1981) with 60 mM methanol and 10% CO (+ 90% N_2). Growth was followed by measuring the optical density (OD) at 600 nm. (●) Growth of *A. woodii* on methanol + CO in presence of Na^+ after the first transfer from fructose + methanol-pregrown cells; (■) Second transfer of *A. woodii* on methanol + CO; (▲) Third transfer of *A. woodii* on methanol + CO; (▼) Growth of *A. woodii* on phosphate-buffered medium without any substrate; (♦) Growth of *A. woodii* on 60 mM methanol as sole carbon source under an N_2-atmosphere.

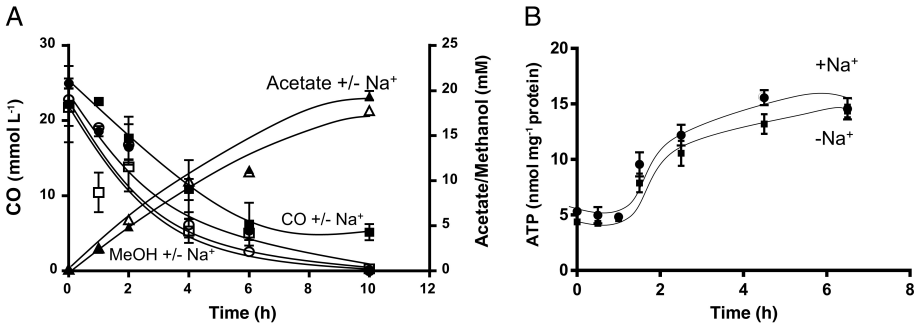

Fig. 6. Acetogenesis and ATP synthesis from methanol + CO of *A. woodii* is Na^+-independent. Resting cells of *A. woodii* wild type (protein concentration, 1 mg ml^{-1}) in 40 mM imidazole buffer containing 20 mM KCl, 20 mM NaCl, 20 mM $MgSO_4$, 2 mM DTE and 4 μM resazurin (pH 7.0) were incubated at 30°C in the presence or absence of Na^+ with 20 mM methanol + CO (10%) as substrates. N_2 was used as make-up gas. The contaminating amount of Na^+ was 121 μM.
A. The concentrations of methanol (●), CO (■) and acetate (▲) in the presence of Na^+. The concentrations of methanol (○), CO (□) and acetate (△) in the absence of Na^+.
B. The cellular ATP pools in the presence (20 mM) (●) or absence (■) of Na^+. The addition of methanol + CO is indicated by an arrow. The data represent the result of three independent experiments ($n = 3$).

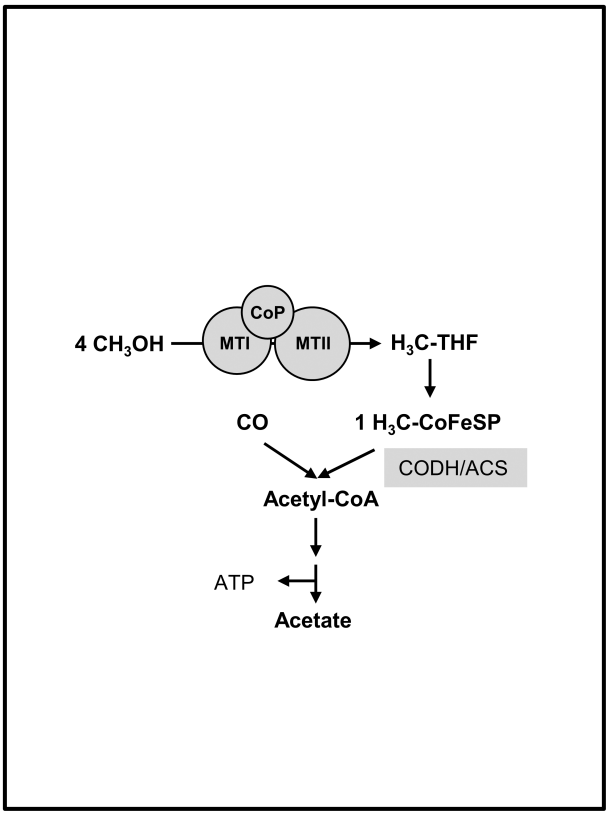

Fig. 7. Biochemistry and bioenergetics of acetogenesis from methanol + CO in *A. woodii*. THF, tetrahydrofolate; CODH/ACS, CO dehydrogenase/acetyl coenzyme A synthase; CoP, corrinoid protein; MTI, methyltransferase I; MTII, methyltransferase II.

independently by apparently reducing CO_2 led us to determine the effect of CO on methanol metabolism. CO is an effective inhibitor of the first enzyme of the methyl branch of the WLP, the HDCR (Schuchmann and Müller, 2013; Ceccaldi *et al*., 2017), and should inhibit CO_2 reduction by the WLP. Moreover, CO is an intermediate of the carbonyl branch of the WLP and some acetogens are known to grow on CO (Daniel *et al*., 1990; Tanner *et al*., 1993; Chang *et al*., 1997; Weghoff and Müller, 2016). Therefore, we tested methanol conversion by *A. woodii* under a CO atmosphere. Indeed, *A. woodii* did grow on methanol + CO in the absence of CO_2/bicarbonate (Fig. 5). Therefore, we analysed acetogenesis from methanol + CO in the cell suspensions. CO was consumed alongside methanol and only traces of H_2 were produced (0.2 mM), but acetate was formed with high rates up to 19.4 mM, which is comparable to acetate formation from methanol alone or methanol + H_2 + CO_2 (Fig. 6A). The acetate:methanol ratio was 0.92:1 and the CO:methanol ratio was 1:1. The metabolism was not dependent on Na^+ (Fig. 6A). Conversion of methanol + CO led to synthesis of ATP that was neither dependent on Na^+ (Fig. 6B) nor on the Rnf complex (data not shown). These stoichiometries are in line with the following equation:

$$1\,CO + 1\,CH_3OH \rightarrow 1\,CH_3COOH \qquad (3)$$

which is in accordance with the metabolic scheme presented in Fig. 7. As expected, acetogenesis from methanol + CO was not only Na^+-independent but also not affected by a deletion of the *rnf* genes (data not shown). Apparently, under these conditions methanol was converted only by the 'condensing mode', in which CO condenses with the methyl group and CoA to acetyl-CoA (Fig. 7). The theoretical ATP yield is 1 ATP/acetate or 1 ATP/methanol.

Growth of A. woodii *on methanol + caffeate*

So far, we have addressed metabolic variants that included disproportionation of methanol or simply the

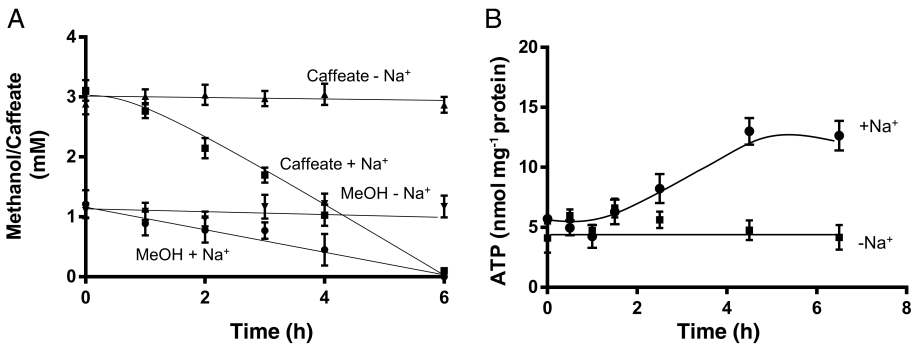

Fig. 8. Acetogenesis and ATP synthesis from methanol + caffeate of *A. woodii* is Na^+-dependent. Resting cells of *A. woodii* wild type (protein concentration, 1 mg ml^{-1}) in 40 mM imidazole buffer containing 20 mM KCl, 20 mM NaCl, 20 mM $MgSO_4$, 2 mM DTE and 4 μM resazurin (pH 7.0) were incubated at 30°C in the presence or absence of Na^+ with 1 mM methanol and 3 mM caffeate under an N_2 atmosphere. The contaminating amount of Na^+ was 97 μM.
A. Methanol (●) and caffeate consumption (▲) in the presence of Na^+; Methanol (■) and caffeate (▼) consumption in the absence of Na^+.
B. The cellular ATP pools in the presence (●) or absence (■) of Na^+ (20 mM). The addition of methanol + caffeate is indicated by an arrow. The data represent the result of three independent experiments ($n = 3$).

condensation of the methyl group with carbon monoxide. However, data in the literature suggest that *A. woodii* can grow non-acetogenically by complete oxidation of methanol to CO_2 ('oxidation mode') when the electrons are consumed by a methanogenic partner (Winter and Wolfe, 1980). Also, it has been demonstrated that *A. woodii* can grow on methyl group-containing phenylacrylates non-acetogenically (Bache and Pfennig, 1981). Caffeate is used by *A. woodii* as alternative electron acceptor (Dilling *et al*., 2007) and the biochemistry and bioenergetics of caffeate reduction are well established (Hess *et al*., 2013). Cell suspensions pregrown on methanol + caffeate consumed methanol and caffeate but acetate was not produced (Fig. 8A). The ratio of methanol:caffeate consumed was 1:3, as expected from the equation:

$$1\,CH_3OH + 3\,caffeate + H_2O \rightarrow 3\,hydrocaffeate + CO_2 \quad (4)$$

Along with the consumption of methanol and caffeate, the cells produced ATP but only in the presence of Na^+ (Fig. 8B). Conversion of methanol + caffeate required Na^+ and was abolished in the *Δrnf* mutant. These data are in line with the hypothetical model presented in Fig. 9. The theoretical ATP yield of this pathway is 1.0 ATP/caffeate and 3.1 ATP/methanol.

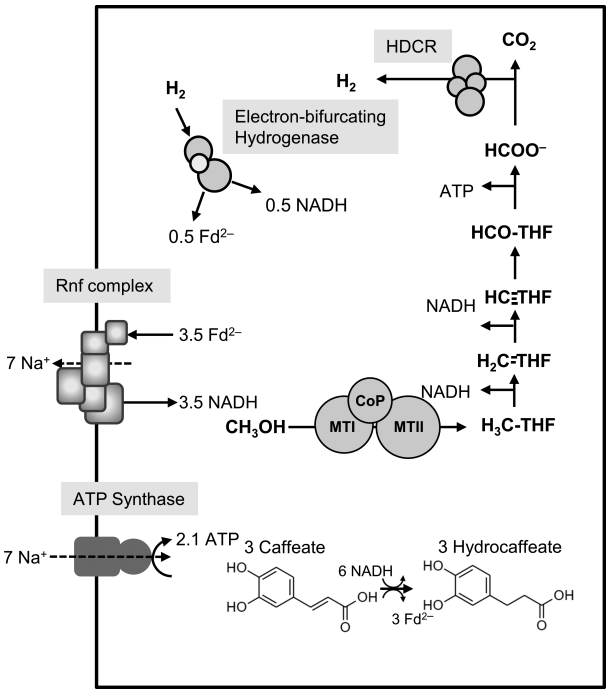

Fig. 9. Biochemistry and bioenergetics of acetogenesis from methanol + caffeate in *A. woodii*. A stoichiometry of 2 H^+/2 e^- is assumed for the Rnf complex. The stoichiometry of the ATP synthase is 3.3 Na^+/ATP (Matthies *et al*., 2014). Fd, ferredoxin; THF, tetrahydrofolate; CODH/ACS, CO dehydrogenase/acetyl coenzyme A synthase; CoP, corrinoid protein; HDCR, hydrogen-dependent CO_2 reductase MTI, methyltransferase I; MTII, methyltransferase II.

Discussion

Channelling of the methyl group of methanol and other methyl group-containing substrates into the WLP requires a methyltransferase system that consists of a methyltransferase I, a CoP and a methyltransferase II; their action leads to the production of methyl-THF (Kreft and Schink, 1993, 1994; Das *et al*., 2007; Visser *et al*., 2016; Kremp *et al*., 2018). A transcriptomic approach was used to possibly identify the methanol-dependent methyltransferase system of *A. woodii* (Kremp *et al*., 2018). Although transcription of *mtaBC2A* was found to be highly upregulated when grown on methanol (compared to fructose-grown cells), two more

potential methyltransferase systems were upregulated significantly but to a lower extent and the question arose if one of them (or both) might also act on methanol as a substrate. From our genetic analysis it is evident that *mtaBC2A* (Awo_c22760-Awo_c22740) encodes the only methanol-specific methyltransferase system in the acetogenic model organism *A. woodii* and none of the other methyltransferase systems is able to complement the deletion of *mtaBC2A*.

Here, we demonstrated different fates of methanol in *A. woodii*. Under an N_2/CO_2 atmosphere, methyl-THF was disproportionated to CO_2 and acetate; this is the lifestyle as observed in pure laboratory cultures grown under N_2/CO_2 and this lifestyle yields 0.83 ATP/acetate or 0.63 ATP/methanol. However, in their natural environment also other acetogenic substrates are present in addition to methanol or methyl groups in general (Drake *et al*., 2008). In the presence of $H_2 + CO_2$, mixotrophic acetogenesis occurred from $H_2 + CO_2$ + methanol. Since the only way for H_2-dependent reduction of ferredoxin (which is required to reduce CO_2 to CO) is by way of the electron-bifurcating hydrogenase, NADH is the obligatory second product. NADH can only be oxidized in the WLP and thus, reduction of CO_2 to methyl-THF is required in addition. Although electron transfer from NADH to ferredoxin could also have been catalysed by the Rnf complex, this apparently did not happen since acetogenesis from methanol + H_2 + CO_2 was Na^+- and Rnf-independent. The Rnf-dependent route would yield 0.7 ATP/acetate or 0.7 ATP/methanol, whereas the Rnf-independent way with CO_2 as an additional electron sink yields 0.5 ATP/acetate or 1 ATP/methanol. The better energy balance per mol methanol consumed may be the reason for employing the Rnf-independent way.

In the presence of hydrogen, NADH is produced that needs to be re-oxidized by the WLP thus making CO_2 reduction essential for conversion of H_2 + methanol. Although *A. woodii* does not grow on CO alone, it can grow on CO + formate, circumventing the CO-inhibited HDCR reaction (Bertsch and Müller, 2015). As shown here *A. woodii* can also grow on methanol + CO, without using the pathways leading from CO_2 to CO or methyl-THF. Moreover, hydrogen was not produced due to the inhibition of the electron-bifurcating hydrogenase. The 'condensation' mode was also observed in resting cells.

Another type of methanol metabolism is the non-acetogenic way in which methyl-THF is only oxidized to CO_2. This was observed during growth of *A. woodii* on phenylmethyl ethers and also in the coculture with a methanogen that apparently used the electrons from methanol oxidation for methanogenic CO_2 reduction (Winter and Wolfe, 1980; Bache and Pfennig, 1981). The 'oxidation only' mode was also observed in the presence of the alternative electron acceptor caffeate. Methanol-dependent caffeate reduction yields 3.1 ATP/methanol and 1.03 ATP/caffeate.

In sum, the route of methanol conversion in *A. woodii* is strongly dependent on the environmental conditions and the availability of other substrates. When only methanol is present, it has to be disproportionated to yield electrons for acetate production. In the presence of $H_2 + CO_2$ and methanol, mixotrophic growth occurred and when CO is present, the HDCR is inhibited and cells switch to the 'condensation' mode in which the methyl group, CO and CoA are condensed to acetyl-CoA. This mode of acetogenesis maybe the oldest one since it requires only the CODH/ACS to form an acyl thioester from a methylated compound, i.e. methyl sulphide and carbon monoxide (Wächtershäuser, 1992; Martin, 2012). In coculture with H_2-removing microbes such as methanogens, the 'oxidation only' mode is likely the preferred mode. Apparently, the different ways to consume methyl groups give a tremendous ecological advantage to acetogens such as *A. woodii*.

Experimental procedures

Cultivation of A. woodii

Acetobacterium woodii wild type (DSM 1030), the *Δrnf* or the *ΔmtaBC2A* mutant were grown on complex medium at 30°C as described previously (Heise *et al*., 1989; Heise *et al*., 1992). For growth of the mutants 50 mg L^{-1} uracil was added to the medium before inoculation. As growth substrate 60 mM methanol, 60 mM methanol + 101 kPa H_2 + CO_2 [80/20% (vol./vol.)], 60 mM methanol + CO (10%) or 60 mM methanol + 2 mM caffeate were used.

Generation of the mtaBC2A mutant

The upstream flanking region (UFR) of *mtaBC2A* (including the first 78 bp of *mtaB*) and the downstream flanking region (DFR) of *mtaBC2A* (including the last 99 bp of *mtaA*) were amplified with the Q5® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, USA) using the oligonucleotide pairs mtaBCA_up F/R and mtaBCA_dn F/R (Table S1). The UFR and DFR were fused by 'overlap extension PCR' using the oligonucleotides mtaBCA_up_F and mtaBCA_dn_R (Table S1). The PCR-product was inserted between the restriction sites of EcoRI and XbaI in vector pMTL_AW_KO1_pyrE_Elim (Wiechmann *et al*., 2020). The integrity of the resulting plasmid pMTL8_151_ΔmtaBC2A was confirmed by sequencing (Microsynth, Seqlab, Goettingen, Germany). The plasmid was transformed into *A. woodii ΔpyrE* (Wiechmann *et al*., 2020) and further integration and recombination of the *mtaBC2A* deletion cassette were

performed as described before (Westphal *et al*., 2018; Wiechmann *et al*., 2020). The deletion of the *mtaBC2A* region was verified by PCR analysis using oligonucleotide pairs mtaBCA_del.area_for/rev and mtaBCA_out_for/rev (Table S1) and by DNA sequencing (Microsynth, Seqlab).

Preparation of resting cells

For the experiments with resting cells, *A. woodii* wild type or the *Δrnf* mutant were grown on 60 mM methanol, 60 mM methanol + 101 kPa H_2 + CO_2 [80/20% (vol./vol.)] or 60 mM methanol + 2 mM caffeate. The cells were harvested in late exponential growth phase by centrifugation (11.300*g*, 10 min, 4°C; Beckman Avanti J25, JA10 rotor, Beckmann Coulter, Krefeld, Germany) and washed twice with suspension buffer (40 mM imidazole, 20 mM KCl, 20 mM $MgSO_4$, 2 mM DTE, 4 μM resazurin, pH 7). The cells were re-suspended in 5 ml suspension buffer and kept in gas-tight Hungate tubes. All steps were carried out under strictly anoxic conditions in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI, USA). The protein concentration of the cell suspension was determined according to Schmidt *et al*. (1963) and the cells were directly used for the subsequent cell suspension experiments.

Experiments with resting cells

For experiments with resting cells, cell suspensions (final protein concentration: 1 mg ml^{-1}) were filled into 58 ml serum flasks to a final volume of 10 ml suspension buffer under an N_2 atmosphere and incubated for 10 min at 30°C. Subsequently, bicarbonate (40 mM $KHCO_3$ or $NaHCO_3$) was added. As substrates, methanol (20 mM) + N_2/CO_2, methanol (20 mM) + CO (10%), methanol (20 mM) and H_2 + CO_2 {101 kPa, [80/20% (vol./vol.)]} or methanol (1 mM) + caffeate (3 mM) was used. When CO was used as substrate, the gas phase was changed to 10% CO and 90% N_2. When H_2 + CO_2 was used as substrate, the gas phase was changed to 100% H_2 + CO_2 [80/20% (vol./vol.)]. The cell suspension experiments were started by addition of the substrate. When Na^+-free or bicarbonate-free conditions were required, NaCl or $KHCO_3$ were omitted. All resting cell experiments were performed at 30°C in a shaking water bath (200 rpm). To determine metabolites or ATP, 0.5 ml samples were taken at different time points during the experiments. All measurements were performed in triplicates from at least two independent experiments. The amount of Na^+ in the suspension buffer was determined with an Orion 84-111 ROSS sodium electrode (Thermo Electron Corp. Witchford, UK) following the manufacturer's instructions.

Determination of ATP, acetate, CO, H_2 or methanol

The ATP concentration was determined as described in Heise *et al*. (1993) and Kimmich *et al*. (1975). The concentration of acetate, CO, H_2 or methanol was determined by gas chromatography as described previously (Bertsch and Müller, 2015). Caffeate was determined photometrically at 312 nm using an extinction coefficient of 13.72 mM^{-1} cm^{-1} as described previously (Dilling *et al*., 2007).

Acknowledgements

We are indebted to the European Research Council (ERC) for financial support under the European Union's Horizon 2020 research and innovation program (ACETOGENS, grant agreement no. 741791).

Author Contributions

F.K. generated the *ΔmtaBC2A* mutant in *A. woodii* and performed growth experiments with the mutant. D.L. performed growth experiments and cell suspension experiments with *A. woodii* DSM1030 and the *Δrnf* mutant. D.L., F.K. and V.M. analysed the data and wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1 Supporting Information.