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Brief Report

Growth of the acetogenic bacterium *Acetobacterium woodii* by dismutation of acetaldehyde to acetate and ethanol

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Summary

Acetogenic bacteria are a group of strictly anaerobic bacteria that may have been first life forms on Earth since they employ an ancient pathway for CO_2 fixation into acetyl-CoA that is coupled to the synthesis of ATP, the Wood–Ljungdahl pathway. Electrons for CO_2 reduction are derived from oxidation of H_2 or CO and thus, these bacteria can grow lithotrophically on gases present on early Earth. Among the organic molecules present on early Earth is acetaldehyde, a highly volatile C2 compound. Here, we demonstrate that the acetogenic model bacterium *Acetobacterium woodii* grows on acetaldehyde. Acetaldehyde is dismutated to ethanol and acetyl-CoA, most likely by the bifunctional alcohol dehydrogenase AdhE. Acetyl-CoA is converted to acetate by two subsequent enzymes, phosphotransacetylase and acetate kinase, accompanied by the synthesis of ATP by substrate-level phosphorylation. Apparently, growth on acetaldehyde does not employ the Wood–Ljungdahl pathway. Our finding opens the possibility of a simple and ancient metabolic pathway with only three enzymes that allows for biomass (acetyl-CoA) and ATP formation on early Earth.

Introduction

The metabolic reactions leading to the synthesis of the cellular energy currency adenosine triphosphate (ATP) are central to the origin of life (Lipmann, 1941). The 'last universal common ancestor' (LUCA) is discussed as being anaerobic, N_2-fixing, H_2-dependent with an ancient pathway for CO_2 fixation that is coupled to the synthesis of ATP, the Wood–Ljungdahl pathway (Lane *et al*., 2010; Weiss *et al*., 2016). Today's acetogenic bacteria have these characteristics of LUCA and are model systems for metabolic reactions in the early history of life (Schuchmann and Müller, 2014; Weiss *et al*., 2018). One such organism, *Acetobacterium woodii*, has been used to unravel the enzymatic reactions leading to the synthesis of ATP during autotrophic growth (Schuchmann and Müller, 2014). Interestingly, this bacterium can also grow heterotrophically as, for example, on ethanol (Eichler and Schink, 1984; Bertsch *et al*., 2016). Ethanol is oxidized by the bifunctional alcohol dehydrogenase AdhE via the intermediate acetaldehyde to acetate (Bertsch *et al*., 2016). This led to the question whether acetaldehyde can also serve as sole carbon and energy source for this bacterium. Here, we will report that the acetogenic model bacterium *A. woodii* grows on acetaldehyde.

Results and discussion

Acetobacterium woodii *is able to use acetaldehyde as sole carbon and energy source*

To address whether *A. woodii* is able to grow on the toxic compound acetaldehyde, cells were precultured on a complex medium with 50 mM ethylene glycol as carbon and energy source. Upon transfer into the same medium without carbon and energy source, the optical density increased to only 0.079 $\pm$ 0.011 (Fig. 1A) due to trace amounts of growth supportive substrates in yeast extract (Dönig and Müller, 2018; Lechtenfeld *et al*., 2018). However, the addition of acetaldehyde to a final concentration of 5 mM increased the optical density of the culture to 0.26 $\pm$ 0.01. Growth rate as well as final cell yield was dependent on the acetaldehyde concentration (Fig. 1A). After four transfers, the growth rate increased from 0.042 $\pm$ 0.003 h^{-1} to 0.1 $\pm$ 0.01 h^{-1}, indicating that the cells had adapted to acetaldehyde. Concentrations

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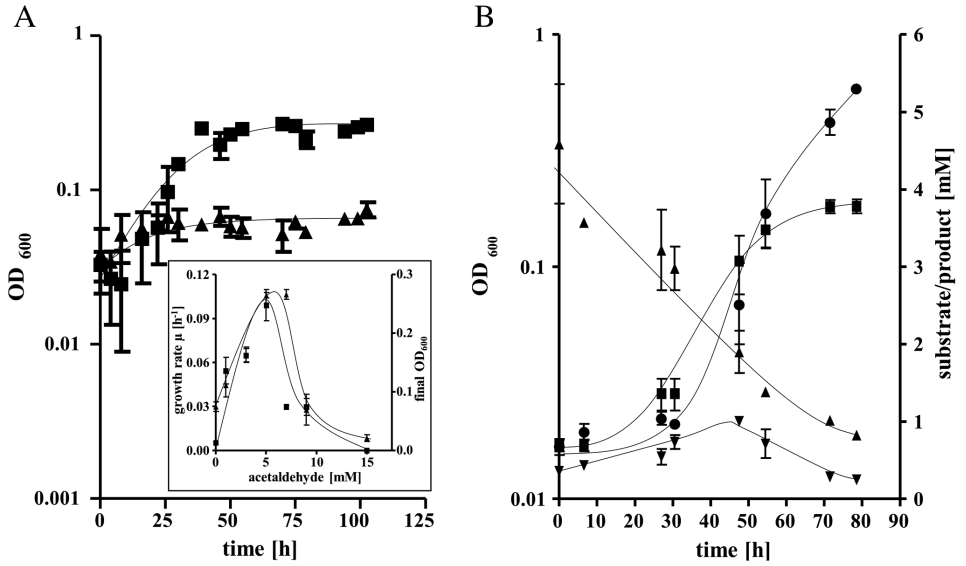

Fig. 1. A. Growth of *A. woodii* on acetaldehyde. The media were prepared as described previously (Hungate, 1969; Bryant, 1972; Heise *et al*., 1989). *Acetobacterium woodii* DSM 1030 was cultivated at 30°C in 5 ml of CO_2/bicarbonate-buffered medium in the absence (▲) or presence of 5 mM acetaldehyde (■) under a N_2/CO_2 (80/20% [v/v]) atmosphere and growth was followed by measuring the optical density (OD) at 600 nm. The inset shows the dependence on growth rate (▲) and final OD_{600} (■) on the acetaldehyde concentration. B. Concentrations of acetaldehyde (▲), acetate (●) and ethanol (▼) were measured by gas chromatography as described previously (Trifunović *et al*., 2016), OD_{600} (■) was determined photometrically. All data points are mean ± SEM; *N* = 3 independent experiments.

higher than 5 mM acetaldehyde led to growth inhibition. Therefore, cells were routinely grown on 5 mM acetaldehyde; to achieve higher cells densities, substrate was added back after its consumption. Eight successive additions of 5 mM acetaldehyde led to an optical density of 1 ± 0.02 (data not shown). During growth on acetaldehyde *A. woodii* produced acetate and ethanol but product formation was diauxic (Fig. 1B). In the first phase, up to 47.5 h, acetaldehyde was converted to acetate and ethanol. From 4.6 ± 0.8 mM acetaldehyde, 1 ± 0.04 mM ethanol and 2.5 ± 0.5 mM acetate were formed. At around 47.5 h, ethanol disappeared although acetaldehyde was still present and acetate was formed up to 5.3 ± 0.02 mM. This indicates that *A. woodii* oxidizes the ethanol produced to acetate in a second stage of growth, as observed before during growth on ethylene glycol (Trifunović *et al*., 2016). Ethanol oxidation is coupled to the reduction of carbon dioxide in the wood–ljungdahl pathway (WLP) (Bertsch *et al*., 2016).

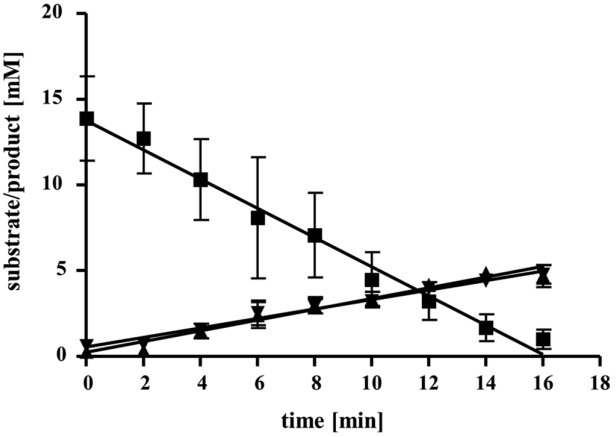

Fig. 2. Dismutation of acetaldehyde to ethanol and acetate by resting cells of *A. woodii* in the absence of bicarbonate/CO_2. Resting cells (1 mg ml^{-1}) of *A. woodii* were prepared (Trifunović *et al*., 2016) from cells grown on 5 mM acetaldehyde in 3 l complex media (Heise *et al*., 1989) and were incubated in 10 ml imidazole buffer (50 mM imidazole, 20 mM KCl, 20 mM $MgSO_4$, 2 mM DTE and 4 μM resazurin, pH 7.0) containing 12.8 mM acetaldehyde in a 100% N_2 atmosphere at 30°C. At the time points indicated 1 ml samples were taken, cells were removed by centrifugation (14.000×*g*, 1 min) and concentrations of acetaldehyde (■), acetate (▲) and ethanol (▼) in the supernatant were determined by gas chromatography as described previously (Trifunović *et al*., 2016). All data points are mean ± SEM; *N* = 3 independent experiments.

The Wood–Ljungdahl pathway is not involved in acetaldehyde utilization

To exclude ethanol oxidation coupled to CO_2 reduction and to further analyse the fate of acetaldehyde, experiments with resting cells in the absence of bicarbonate/CO_2 were performed. Cells were pregrown on acetaldehyde, harvested by centrifugation and resuspended in buffer under a N_2 atmosphere. Upon addition, acetaldehyde was consumed immediately and acetate and ethanol were produced in equal amounts (Fig. 2); 12.9 ± 3.3 mM acetaldehyde were converted to

4.5 ± 0.6 mM ethanol and 4.3 ± 0.4 mM acetate. From 1 mol of acetaldehyde, only 0.37 mol of ethanol and 0.35 mol of acetate were produced with a carbon recovery of 68.2%. These data are similar to data observed studying acetaldehyde utilization in resting cells of lactic acid bacteria. Acetaldehyde was dismuted to ethanol and acetate and the carbon recovery varied between 40% and 75% (Osborne *et al*., 2000).

To trace whether acetaldehyde reacts spontaneously with any buffer components, the same suspension experiments were performed, but without any addition of cells. Over 20 min, 3.8 mM acetaldehyde disappeared without the formation of acetate, ethanol or any other compound (data not shown). About 1.8 mM was found in the gas phase, the rest could have reacted with buffer components such as DTE. Anyway, when the loss of acetaldehyde is accounted for, the corrected fermentation balance is:

$$1\,\text{acetaldehyde} \rightarrow 0.49\,\text{ethanol} + 0.47\,\text{acetate} \quad (1)$$

with a carbon recovery of 96.7%. Ethanol is apparently not oxidized to acetate in the absence of CO_2. Ethanol oxidation requires carbonate as oxidant, but the effect of bicarbonate/CO_2 on utilization of ethanol produced from acetaldehyde could not be tested in growing cultures because bicarbonate/CO_2 is essential for growth of *A. woodii* on this C_2 substrate. Biomass from a two-carbon substrate requires carboxylation reactions. However, the hydrogenase activity (measured as H_2-dependent methylviologen reduction) was downregulated by 68.4% during growth on acetaldehyde (from 325 U/mg during growth on fructose versus 102 U mg^{-1} during growth on acetaldehyde). Since hydrogen produced by the electron bifurcating hydrogenase is the electron carrier between organic substrates and the WLP, this experiment underlines the notion that the WLP is not involved in acetaldehyde conversion.

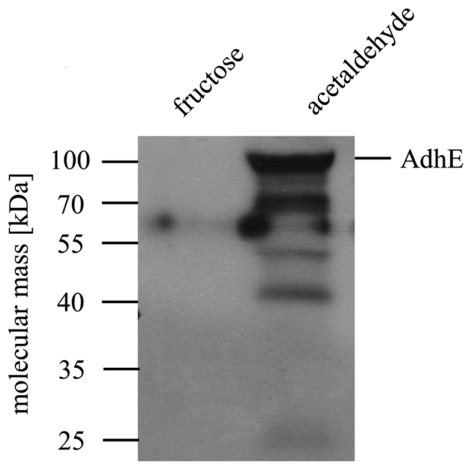

Fig. 3. Immunological detection of the bifunctional alcohol dehydrogenase AdhE in cells grown on acetaldehyde. *Acetobacterium woodii* was grown on either acetaldehyde or fructose and harvested in the mid-exponential growth phase. About 20 μg of the whole cell extracts were separated on a 12% SDS-PAGE according to Laemmli (1970) and the presence of AdhE was determined immunologically as described previously (Hess *et al*., 2011) with an antiserum against AdhE (Bertsch *et al*., 2016).

The key reactions of acetaldehyde utilization are catalyzed by AdhE

To determine the activities of enzymes involved in acetaldehyde metabolism, cells were grown on acetaldehyde, harvested and disrupted in a 'french press'. The cell free extract was incubated in the presence of NADH. Upon the addition of acetaldehyde, NADH was oxidized (Supporting Information Fig. S1). NADH-dependent acetaldehyde reduction was dependent on the acetaldehyde concentration and activity followed a Michaelis–Menten kinetic with an apparent K_m of 3.8 ± 0.3 mM. The dependence of the NADH concentration also followed a Michaelis–Menten kinetic with an apparent K_m of 60.8 ± 14 μM. Both apparent K_m correlate with both K_m measured in cell free extracts of ethylene glycol grown cells (52.1 ± 7.1 μM for NADH and 2.6 ± 0.2 mM for acetaldehyde; Trifunović *et al*., 2016).

The acetylating activity was measured in the direction of acetaldehyde oxidation. Upon the addition of acetaldehyde to cell free extract of *A. woodii*, NAD^+ was not reduced. Only upon addition of coenzyme A, a rapid reduction of NAD^+ was observed (Supporting Information Fig. S2). Activity followed a Michaelis–Menten kinetic with an apparent K_m of 0.161 ± 0.017 mM for CoA. The dependence on the NADH and acetaldehyde concentration also followed a Michaelis–Menten kinetics with an apparent K_m of 0.4 ± 0.1 mM for NAD^+ and 7.4 ± 0.7 mM for acetaldehyde. Also, these K_m values correlate with the ones measured in cell free extracts of ethylene glycol-grown cells (6 ± 1.1 mM for acetaldehyde, 77.1 ± 13.5 μM for CoA and 160 ± 3 μM for NAD^+; Trifunović *et al*., 2016).

The bifunctional alcohol dehydrogenase AdhE catalyses both reduction of acetaldehyde to ethanol and oxidation of acetaldehyde to acetyl-CoA (Bertsch *et al*., 2016). Indeed, the abundance of AdhE was much higher in acetaldehyde-grown cells compared to fructose-grown cells (Fig. 3).

Conclusion

From the data presented here, it is obvious that acetaldehyde is a carbon and energy source for the acetogen *A. woodii*. The same was observed for the fermenting bacteria *Pelobacter carbinolicus* and *P. acetylenicus*

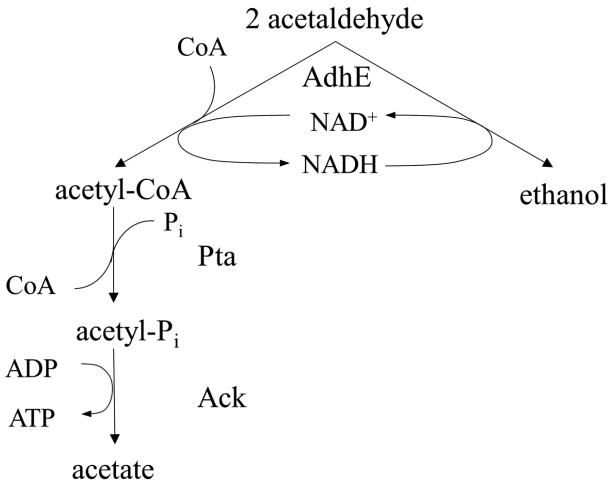

Fig. 4. Acetaldehyde metabolism in *A. woodii*. Acetaldehyde is dismutated by a bifunctional alcohol dehydrogenase (AdhE) to ethanol and acetyl-CoA, which is further converted to acetyl-phosphate and acetate by phosphotransacetylase (Pta) and acetate kinase (Ack).

(Schmidt *et al*., 2014). However, growth of *Pelobacter* species was much slower. In *P. carbinolicus* grown axenically on acetaldehyde acetate and ethanol were produced in equal amounts. There was only little activity for the acetylating aldehyde dehydrogenase, whereas the activity for non-acetylating aldehyde dehydrogenase was much higher. The pathway used was not identified. Acetaldehyde is a toxic compound and, at least to our knowledge, microbial growth of acetaldehyde is not common. Aerobic acetaldehyde oxidating bacteria have recently been isolated from the deep sea (Gao *et al*., 2018), and anaerobic lactic acid bacteria involved in wine making were shown to metabolize acetaldehyde but do not grow on it (Osborne *et al*., 2000). In *A. woodii*, the bifunctional alcohol dehydrogenase AdhE catalyses the disproportion of acetaldehyde to ethanol and acetyl-CoA, the key reaction of this pathway (Fig. 4). The enzyme has been purified from *A. woodii* and its properties described (Bertsch *et al*., 2016). Acetyl-CoA is further converted to acetyl-phosphate and acetate plus ATP. For growth on this C_2 substrate, the WLP is not required, but biosynthetic carboxylation reactions such as pyruvate synthesis from acetyl-CoA are needed. This and other biosynthetic reactions depend on reduced ferredoxin which, in *A. woodii*, can only be generated from NADH by the Rnf complex, a membrane bound transhydrogenase that uses the electrochemical sodium ion gradient to drive the endergonic electron transfer from NADH to ferredoxin (Biegel and Müller, 2010; Hess *et al*., 2013; Westphal *et al*., 2018). In agreement with this hypothesis is the observation that a Δ*rnf* mutant is not able to grow on acetaldehyde. Our discovery of growth of *A. woodii* on acetaldehyde opens the possibility of a heterotrophic origin of life.

Acknowledgements

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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:

Fig. **S1**. Characterization of the NADH:acetaldehyde oxidoreductase activity in cell free extract of *A. woodii* grown on acetaldehyde.

Fig. **S2**. Characterization of the CoA-dependent acetaldehyde:NAD^+ oxidoreductase activity in cell free extract of *A. woodii* grown on acetaldehyde.