

Growth of the acetogenic bacterium *Acetobacterium woodii* on glycerol and dihydroxyacetone

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Summary

More than 2 million tons of glycerol are produced during industrial processes each year and, therefore, glycerol is an inexpensive feedstock to produce bio-commodities by bacterial fermentation. Acetogenic bacteria are interesting production platforms and there have been few reports in the literature on glycerol utilization by this ecophysiologicaly important group of strictly anaerobic bacteria. Here, we show that the model acetogen *Acetobacterium woodii* DSM1030 is able to grow on glycerol, but contrary to expectations, only for 2–3 transfers. Transcriptome analysis revealed the expression of the *pdu* operon encoding a propanediol dehydratase along with genes encoding bacterial microcompartments. Deletion of *pduAB* led to a stable growth of *A. woodii* on glycerol, consistent with the hypothesis that the propanediol dehydratase also acts on glycerol leading to a toxic end-product. Glycerol is oxidized to acetate and the reducing equivalents are reoxidized by reducing CO₂ in the Wood–Ljungdahl pathway, leading to an additional acetate. The possible oxidation product of glycerol, dihydroxyacetone (DHA), also served as carbon and energy source for *A. woodii* and growth was stably maintained on that compound. DHA oxidation was also coupled to CO₂ reduction. Based on transcriptome data and enzymatic analysis we present the first metabolic and

bioenergetic schemes for glycerol and DHA utilization in *A. woodii*.

Introduction

Acetogenic bacteria are a specialized group of strictly anaerobic bacteria that are facultative autotrophs (Diekert and Wohlfarth, 1994; Drake *et al.*, 2008; Müller and Frerichs, 2013). Carbon dioxide is reduced to acetate with electrons derived from molecular hydrogen or carbon monoxide in the Wood–Ljungdahl pathway (WLP), the only pathway of carbon dioxide fixation that is coupled to net synthesis of ATP thus enabling an autotrophic life style (Ragsdale and Pierce, 2008; Poehlein *et al.*, 2012; Schuchmann and Müller, 2014). The WLP also conveys enormous metabolic flexibility to acetogens (Schuchmann and Müller, 2016). During heterotrophic growth, the WLP serves as an electron sink and many substrates can be oxidized with the electrons shuffled to the WLP. Redox balancing between the oxidation module and the WLP is achieved by the Rnf complex (ferredoxin ↔ NAD) (Westphal *et al.*, 2018), the Ech complex (ferredoxin ↔ protons) (Schölmerich and Müller, 2019), the electron bifurcating hydrogenase (hydrogen ↔ NAD + ferredoxin) (Schuchmann and Müller, 2012) and the transhydrogenases Nfn and Stn (NADPH ↔ NAD + ferredoxin; Mock *et al.*, 2015; Kremp *et al.*, 2020). By transferring the electrons to the WLP, sugar oxidation yields the highest amount of ATP (4.3 mol ATP/mol hexose) in fermenting bacteria and thus makes it possible for acetogens to compete with other bacteria in anoxic ecosystems (Müller, 2008).

In the oxidation module, molecular hydrogen, carbon monoxide, sugars, primary and secondary alcohols, carbonic acids or methyl groups are oxidized (Bertsch and Müller, 2015; Schuchmann *et al.*, 2015; Bertsch *et al.*, 2016; Trifunović *et al.*, 2016; Dönig and Müller, 2018; Kremp *et al.*, 2018; Lechtenfeld *et al.*, 2018). In recent years, many of these pathways have been elucidated on a molecular level. However, little is known on the ability to grow on C3 sugars such as glycerol or dihydroxyacetone (DHA). The latter has not been described as a growth substrate for acetogens. Glycerol has been described to promote growth of

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Acetobacterium and *Eubacterium* species (Eichler and Schink, 1984). We have followed up these observations and analysed glycerol metabolism in *Acetobacterium woodii*. The unexpected result was that cells did grow after the first transfer but could not be subcultured on glycerol. This led us to analyse whether the oxidation product of glycerol, DHA, is a growth substrate for *A. woodii*.

Results

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

In 1984, Eichler & Schink reported the isolation of a new *Acetobacterium* strain, *Acetobacterium carbinolicum*. In the course of the characterization of the strain, the substrates utilized were determined and the data summarized in a table ('+' for growth, '-' for no growth). The same table included *A. woodii* strain NZva16 as a control. Both species were reported to grow on glycerol but growth curves were not presented. Since glycerol is a cheap feedstock and would be interesting for acetogenic conversions in biotechnological applications, we decided to follow up this observation and to study glycerol metabolism in the acetogenic model strain *A. woodii* DSM1030. After transfer of *A. woodii* from a preculture grown on fructose to complex medium containing 5 mM glycerol as carbon and energy source, cells grew with a rate of 0.12 h^{-1} to a final OD of 0.36 (Fig. 1.). The medium contained 0.02% yeast extract that enabled poor growth up to an OD₆₀₀ of only 0.13 (Fig. 1). When an aliquot of the glycerol-grown culture was used to inoculate (20%) a second culture, growth was much slower ($\mu = 0.06 \text{ h}^{-1}$) and the final OD was only 0.21. After the third transfer, there was no growth anymore. We were puzzled by this effect and tried to adapt *A. woodii* on glycerol by decreasing the fructose concentration in the preculture and increasing the glycerol concentration in the main culture (5, 10, 50 mM). We also precultivated cells on fructose + glycerol (5 mM each) or used ethylene glycol or DHA as substrate for the preculture. A change of the medium to minimal medium with and without bicarbonate/CO₂, to phosphate buffered medium with and without bicarbonate/CO₂, to complex medium containing tryptone (2 g L^{-1}), to complex medium with 120, 180 or 240 mM bicarbonate, or to the medium used by Eichler and Schink (1984) did not prevent the effect. Still, *A. woodii* could not be subcultured on glycerol.

To determine whether glycerol was actually consumed in the first transfer, we determined the glycerol concentration over time in the culture. After transfer from a preculture containing only 2 mM fructose to a culture containing 5 mM glycerol, glycerol consumption started

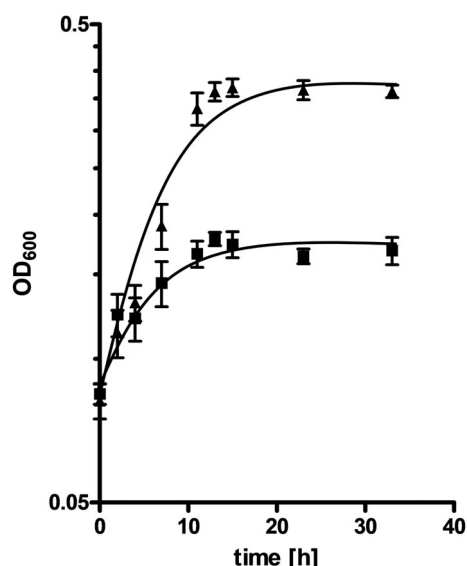


Fig 1. Growth of *Acetobacterium woodii* after the first transfer to medium containing glycerol. 5% of a preculture grown on 2 mM fructose was used to inoculate 5 ml CO₂/bicarbonate-buffered medium containing yeast extract [0.2% (w/v)] under a N₂/CO₂ [80/20% (v/v)] atmosphere. The cultures were cultivated at 30°C in the absence (■) or presence of 5 mM glycerol (▲). Growth was followed by measuring the optical density (OD) at 600 nm. All data points are mean ± SEM; N = 3 independent experiments.

immediately (Fig. 2) and acetate was produced simultaneously. When glycerol was exhausted, growth ceased. Glycerol (4.5 mM) was utilized with a rate of $0.61 \pm 0.05 \text{ mM h}^{-1}$, 5.1 mM acetate were produced and the pH in the medium stayed constant over time. This experiment clearly demonstrates that glycerol is used as carbon and energy source by *A. woodii*.

A pduAB deletion mutant grows stably on glycerol

The genome of *A. woodii* encodes a propanediol-dehydratase (PduCDE) that is encapsulated by a bacterial microcompartment (Schuchmann *et al.*, 2015). This gene cluster is apparently also induced during growth on other substrates (Schuchmann *et al.*, 2015). Transcriptome analysis with cells grown on glycerol (see below) revealed a twofold (log₂) overexpression of the *pdu* genes. We speculated that the propanediol dehydratase may also act on glycerol, leading to the dead end-product 3-hydroxypropionaldehyde, a broad-spectrum antibiotic also known as Reuterin (Talarico *et al.*, 1988). To check this possibility, we took advantage of a $\Delta pduAB$ stain in which not only the genes *pduAB* but also the upstream promoter region was deleted; this mutant will be described elsewhere. Indeed, the $\Delta pduAB$ mutant could be subcultured on glycerol with rates and yields not decreasing over time (Fig. 3A). Using this strain, we determined the concentration dependence for

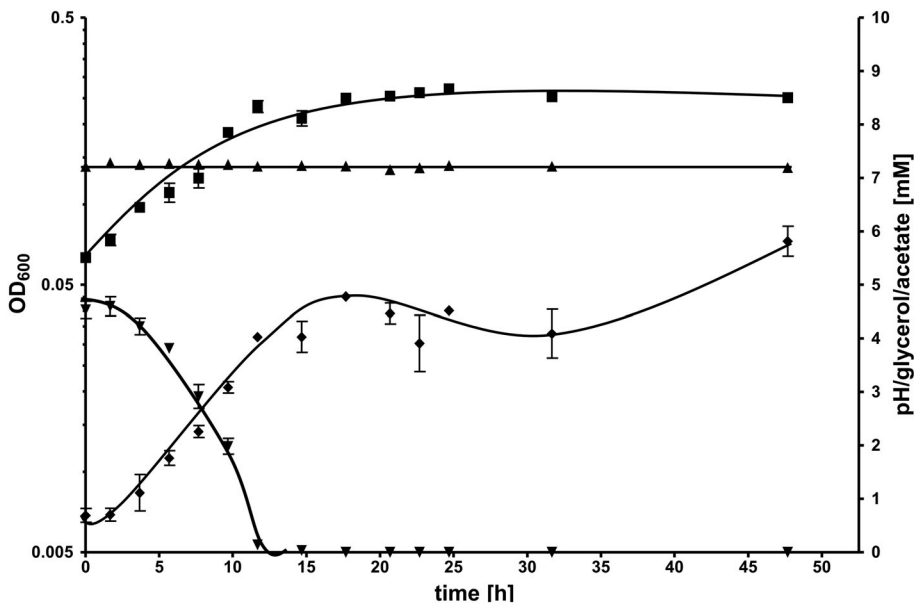


Fig 2. Glycerol utilization by *Acetobacterium woodii* after the first transfer to glycerol-containing media. 5% of a preculture grown on 2 mM fructose was used to inoculate 250 ml CO₂/bicarbonate-buffered medium under a N₂/CO₂ [80/20% (v/v)] atmosphere and 4.5 mM glycerol as a sole carbon and energy source. The cultures were cultivated at 30°C and at the time points indicated 2 ml samples were taken. Concentrations of glycerol (▼), acetate (◆) and pH (▲) were measured as described in Material and Methods. OD₆₀₀ (■) was determined photometrically. All data points are mean ± SEM; N = 3 independent experiments.

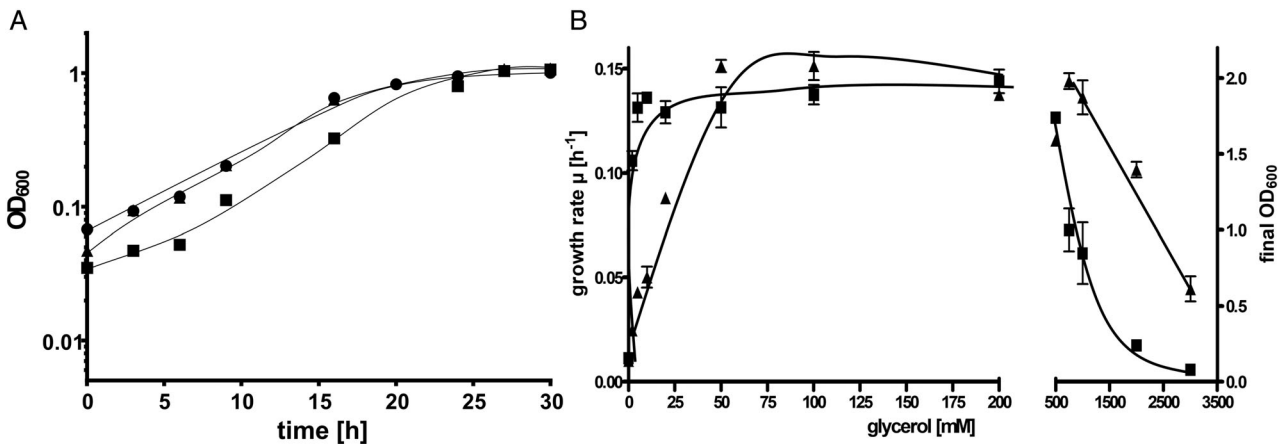


Fig 3. Growth of *Acetobacterium woodii* $\Delta pduAB$ during the first three transfers (A) and dependence of growth on the glycerol concentration (B). A. 5% of a preculture grown on 20 mM fructose was used to inoculate 5 ml CO₂/bicarbonate-buffered medium containing yeast extract [0.2% (w/v)] and 20 mM glycerol under a N₂/CO₂ [80/20% (v/v)] atmosphere. The first (●), second (■) and third transfer (▲) were cultivated at 30°C. B. 5% of a fully adapted preculture on 20 mM glycerol was used to inoculate 5 ml CO₂/bicarbonate-buffered medium containing different amounts of glycerol and yeast extract [0.2% (w/v)] under a N₂/CO₂ [80/20% (v/v)] atmosphere. Growth was followed by measuring the optical density (OD) at 600 nm. Growth yield and final OD₆₀₀ were plotted against the glycerol concentration. These data points represent three different biological replicates.

growth on glycerol. As seen in Fig. 3B, the final yield and growth rate were dependent on the glycerol concentration. By adding more glycerol, the growth rate increased and reached a maximum at 5 mM glycerol with $0.13 \pm 0.01 \text{ h}^{-1}$. Higher concentrations led a slight decrease, until 500 mM glycerol. By using more than 500 mM the growth rate was decreased drastically until only slight growth was observed at 3 M glycerol (Fig. 3B). The final yield was maximal at 50–100 mM and declined slightly thereafter. Surprisingly, even with 500 mM glycerol the $\Delta pduAB$ strain was able to grow fine with slightly reduced growth rate of $0.127 \pm 0.004 \text{ h}^{-1}$ and a final OD₆₀₀ of 1.6 ± 0.05 . Deletion of *pduAB* did not only lead

to stable growth on glycerol but also to a higher tolerance towards glycerol.

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

Dihydroxyacetone phosphate (DHAP) is a likely intermediate of glycerol metabolism. To address whether *A. woodii* is able to grow on DHA, cells were precultured on complex medium with 2 mM fructose and then transferred into complex medium with 10 mM DHA as carbon and energy source. As can be seen in Fig. 4, cells started to grow without an apparent lag phase and a growth rate

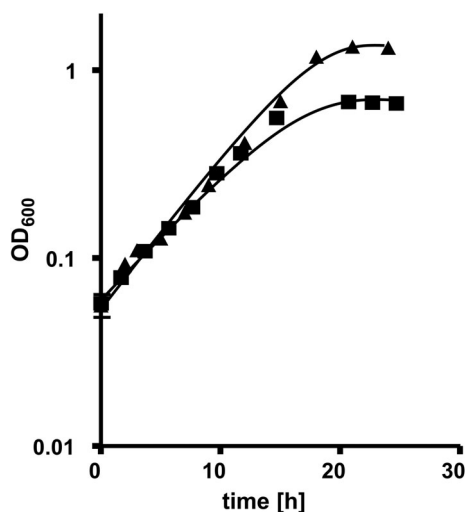
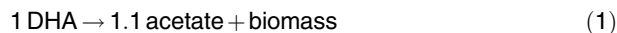


Fig 4. Adaptation of *Acetobacterium woodii* to grow on dihydroxyacetone. 5% of a preculture grown on 2 mM fructose was used to inoculate 5 ml CO₂/bicarbonate-buffered medium under a N₂/CO₂ [80/20% (v/v)] atmosphere and 10 mM dihydroxyacetone as carbon and energy source. The first (■) and third adaptation step (▲) were cultivated at 30°C and growth was followed by measuring the optical density (OD) at 600 nm. All data points are mean ± SEM; N = 3 independent experiments.

of 0.15 h⁻¹ to a final optical density of 0.68. At the third transfer, the growth rate was slightly increased and the final OD was increased to 1.07.

To determine the optimal substrate concentration, cells were grown at different DHA concentrations. At 10 mM DHA, the growth rate was 0.16 and the final OD₆₀₀ only 1.1 (Fig. 5A). Increasing DHA concentrations led to a decrease in growth rates but to an increase in final yield; the growth rate was optimal at 5 mM DHA whereas the highest yield was obtained at 50 mM DHA (Fig. 5B). With 10 mM DHA as substrate, DHA consumption and acetate formation had a lag phase of about 5 h. Then, DHA decreased with a rate of 0.41 ± 0.03 mM h⁻¹ whereas acetate was produced with a rate of 0.42 ± 0.04 mM h⁻¹ (Fig. 6). Ethanol was not produced. At the end of growth, 7.8 mM of DHA was converted to 8.6 mM acetate plus biomass, corresponding to.



Resting cells also converted DHA to acetate (Fig. 7). In the presence of NaCl in the buffer, 8.5 mM DHA were oxidized with a rate of 4.4 mM h⁻¹ to finally 10.4 mM acetate, giving a acetate to DHA ratio of 1.2.

The WLP is involved in DHA utilization

The data presented so far are in line with the hypothesis that electrons coming from DHA oxidation are transferred

to the WLP for disposal. The WLP in *A. woodii* requires Na⁺ (Heise *et al.*, 1989) and indeed, in the absence of additional Na⁺, acetate formation as well as DHA utilization was drastically reduced (Fig. 7). A hydrogenase mutant of *A. woodii* was not able to grow on fructose anymore because the reducing equivalents from glycolysis can no longer be disposed by the WLP (Wiechmann *et al.*, 2020). Same is true for growth on DHA which is impaired in the $\Delta hydBA$ mutant (data not shown). Moreover, a Δrnf mutant (Westphal *et al.*, 2018) lacking the energy conserving Rnf complex did also not grow on DHA. These experiments underline the notion that the WLP is essential as electron disposal module for growth on DHA.

Identification of genes involved in DHA and glycerol utilization

Glycerol could be taken up by a transporter and then oxidized to DHA by glycerol dehydrogenases followed by phosphorylation of DHA by a kinase. Alternatively, glycerol could be phosphorylated by a glycerol kinase to glycerol-3-phosphate followed by oxidation to DHAP. Inspection of the genome sequence revealed a gene possibly encoding a glycerol facilitator (Awo_c29540), two genes encoding potential glycerol dehydrogenases (Awo_c24450, Awo_c06880), five genes encoding glycerol-3-phosphate dehydrogenases (Awo_c01050, Awo_c09230, Awo_c12570, Awo_c12750, Awo_c17750), six genes encoding potential glycerol kinases (Awo_c09050, Awo_c12560, Awo_c12770, Awo_c16650, Awo_c23340, Awo_c23380) and three genes encoding potential DHA kinase (Awo_c20330 - Awo_c20310). Thus, the pathways for entry of glycerol and DHA could not be proposed unambiguously from the genome sequence. Therefore, we studied gene expression in DHA- and glycerol-cultivated cells on a genome-wide level using transcriptomics. Interestingly, genes with the highest degree of differential regulation are apparently not involved in glycerol metabolism but in ion homeostasis (Table S1). Nitrogenase was upregulated ≈5 fold as well as nickel, iron and molybdate transporter. As mentioned above, also *pdu* genes were highly upregulated. Among the gene clusters potentially related to glycerol metabolism was only Awo_c12730 - c12770 that encodes for five different proteins (Fig. 8). Awo_c12770 encodes a glycerol kinase with 68.8% identity to the GlpK from *Escherichia coli*, Awo_c12760 is a glycerol-3-phosphate responsive antiterminator, Awo_c12750 a glycerol-3-phosphate dehydrogenase with 23.4% similarity to GlpA from *E. coli*, Awo_c12740 is a FAD-dependent and Awo_c12730 is a Fe-containing oxidoreductase that are 20.2% and 23.5% similar to GlpB and GlpC from *E. coli* (Fig. 8). This cluster was not only upregulated

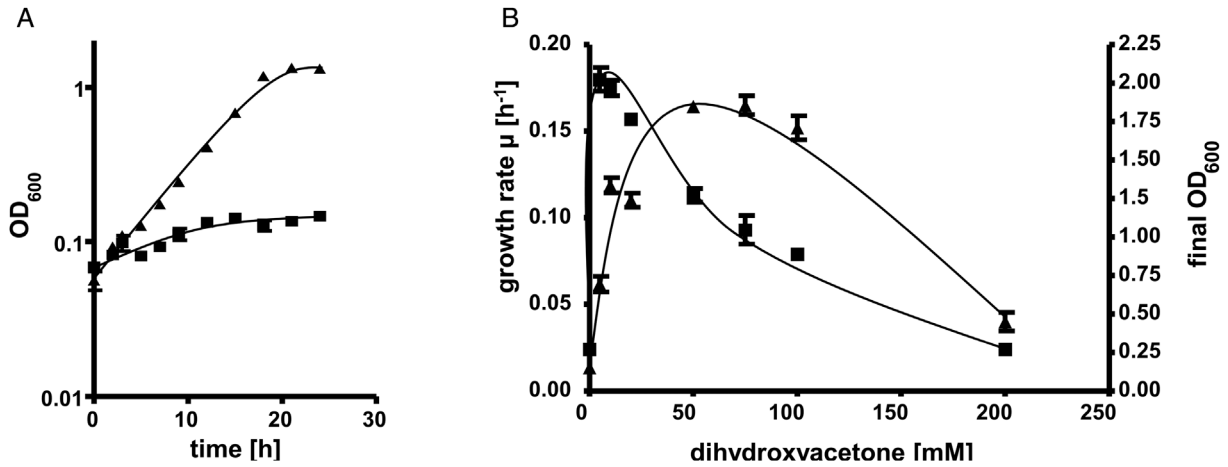


Fig 5. Determination of the dihydroxyacetone concentration optimal for growth for *A. woodii*.

A. 5% of a preculture grown on 10 mM dihydroxyacetone was used to inoculate 5 ml CO₂/bicarbonate-buffered medium under a N₂/CO₂ [80/20% (v/v)] atmosphere. The cultures were cultivated at 30 °C in absence (■) or presence of 10 mM dihydroxyacetone (▲). Growth was followed by measuring the optical density (OD) at 600 nm.

B. The growth rate μ (■) and final optical density at 600 nm (OD₆₀₀, ▲) were plotted against the dihydroxyacetone concentration. All data points are mean \pm SEM; *N* = 3 independent experiments.

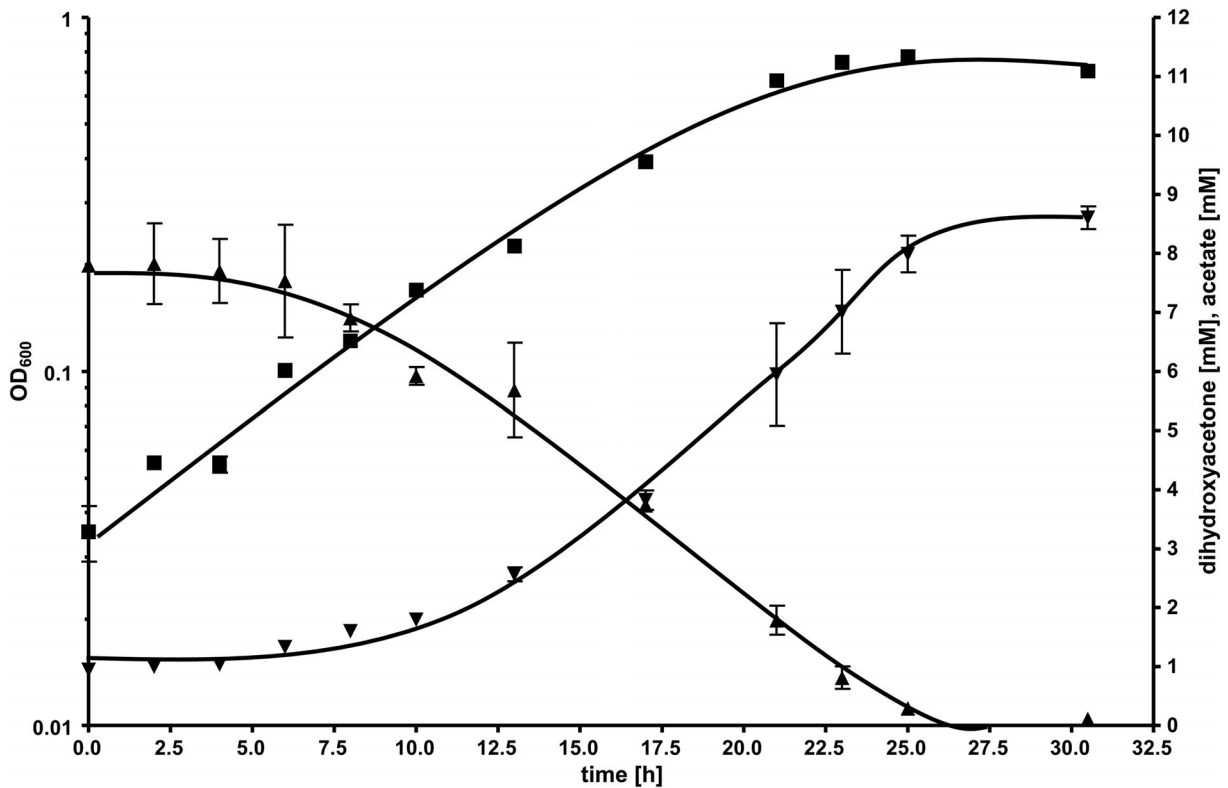


Fig 6. Dihydroxyacetone utilization by *Acetobacterium woodii*. 5% of a preculture grown on 10 mM dihydroxyacetone was used to inoculate 250 ml CO₂/bicarbonate-buffered medium under a N₂/CO₂ ([80/20% (v/v)]) atmosphere and 10 mM dihydroxyacetone as carbon and energy source. The cultures were cultivated at 30 °C and at the time points indicated 2 ml samples were taken. Concentrations of dihydroxyacetone (▲) and acetate (▼) were measured as described in Experimental procedures, OD₆₀₀ (■) was determined photometrically. All data points are mean \pm SEM; *N* = 3 independent experiments.

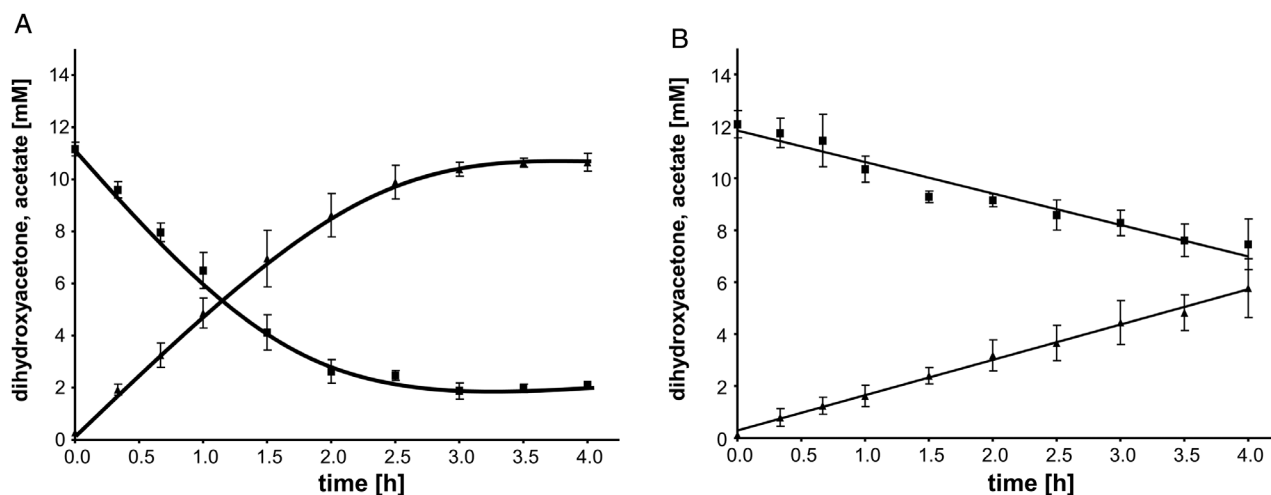


Fig 7. Dihydroxyacetone utilization by resting cells of *Acetobacterium woodii* in the presence (A) or absence (B) of NaCl. Resting cells (1 mg ml^{-1}) of *A. woodii* were incubated in 20 ml imidazole buffer (50 mM imidazole, 20 mM KCl, 50 mM MgSO_4 , 60 mM KHCO_3 , 2 mM DTE and $4 \mu\text{M}$ resazurin, pH 7.0; the contaminating Na^+ was $70 \mu\text{M}$) containing 10 mM dihydroxyacetone in presence (A) or absence (B) of 20 mM NaCl at 30°C . Concentrations of dihydroxyacetone (\blacktriangledown) and acetate (\blacktriangle) were determined as described in Experimental procedures. All data points are mean \pm SEM; $N = 3$ independent experiments.

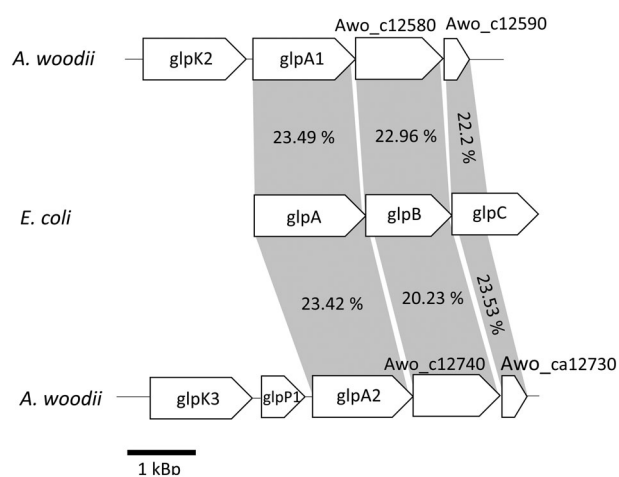


Fig 8. Potential genes involved in glycerol utilization in *Acetobacterium woodii*. *glpK*, glycerol kinase; *glpP1*, glycerol-3-phosphate responsive antiterminator, *glpA*, glycerol-3-phosphate dehydrogenase, *glpB*, glycerol-3-phosphate dehydrogenase subunit B, *glpC*, glycerol-3-phosphate dehydrogenase subunit C.

during growth on glycerol, but had very high transcript levels (Table S3). There was another potential glycerol kinase gene upregulated (Awo_c16650) but the transcript levels were ~ 50 fold lower. Awo_c12730–c12770 is annotated as a *glpK3* operon, interestingly, there is a similar operon, *glpK2* (Awo_c12560–12,590) that is not upregulated (Table S3). Same holds for the potential glycerol facilitator (Awo_c29540), solitary glycerol-3-phosphate dehydrogenases (Awo_c01050, Awo_c09230, Awo_c17750), glycerol kinases (Awo_c09050, Awo_c23340, Awo_c23380) and glycerol dehydrogenases (Awo_c06880). In sum, these data

suggest phosphorylation of glycerol followed by oxidation to DHAP, catalysed by the *glpK3* operon gene products as most likely pathway.

In the presence of glycerol, genes for uptake and phosphorylation of fructose (Awo_c03330, Awo_c03340) were highly downregulated as well as other genes involved in sugar metabolism (Table S2). As expected, genes encoding enzymes of the WLP and the respiratory chain were hardly altered, if at all, the trend was a slight down-regulation during growth on glycerol (Table S3).

Cells grown on DHA did not upregulate genes involved in ion acquisition but otherwise the response was very similar to cells grown on glycerol (Tables S4–S6). Genes possibly involved in uptake and phosphorylation of DHA are Awo_c09000 – c09020 and Awo_c20310 – c20330 that each encode similar set of genes, but they were not differentially transcribed during growth on glycerol or DHA. However, transcript levels for the second cluster were much higher (≈ 10 -fold, Table S6).

Identification of enzymes potentially involved in glycerol activation and oxidation

Cell-free extracts of cells grown on glycerol did not catalyse glycerol-dependent NAD^+ reduction but the reverse reaction, DHA-dependent oxidation of NADH_2 . The activity was $46 \pm 2 \text{ mU mg}^{-1}$ but only 12 ± 1 or $14 \pm 1 \text{ mU mg}^{-1}$ in fructose- or DHA-grown cells. Glycerol-3-phosphate dehydrogenase activity with NAD^+ as electron acceptor was present with 32 mU mg^{-1} in glycerol grown cells but only 25% and 44% in fructose and DHA-grown cells, respectively. Glycerol kinase

activity was also upregulated in glycerol grown cells ($21 \pm 1 \text{ mU mg}^{-1}$), compared to 11.5 ± 0.5 and $11.9 \pm 1 \text{ mU mg}^{-1}$ in DHA- and fructose grown cells.

Discussion

Genes possibly involved in glycerol and DHA oxidation

The utilization of DHA is well described in bacteria (Magasanik *et al.*, 1953; Gutnick *et al.*, 1969; Lin, 1976; Forage and Lin, 1982) and there are different ways to phosphorylate DHA to DHAP, an intermediate of glycolysis. At least to our knowledge, DHA has not been described as carbon and energy source for acetogenic bacteria. Here we describe that the model organism *A. woodii* grows on DHA. The growth rate ($\mu = 0.16 \text{ h}^{-1}$) is comparable to growth on 10 mM fructose ($\mu = 0.17 \text{ h}^{-1}$). DHAP is a central intermediate of glycolysis and the unique feature of DHA metabolism is its uptake and phosphorylation. The genome sequence of *A. woodii* encodes proteins with high similarity to a DHA kinase that consists of three soluble subunits. They are encoded in one potential operon comprising three genes (Awo_c20330 - Awo_c20310). Awo_c20330 is 996 bp long and encodes for DhaK that is only separated 46 bp from Awo_c20320, that is 639 bp long and encoding DhaL. Of note, 4 bp upstream is Awo_c20310, which has a length of 348 bp and encodes for DhaM. DhaK has a molecular mass of 34.98 kDa and DhaL of 22.55. They are similar to the N- and C-terminal halves of the ATP-dependent DHA kinase ubiquitous in bacteria, plants and animals (Daniel *et al.*, 1995; Gutknecht *et al.*, 2001), respectively. Like in *A. woodii*, the kinase is encoded in *E. coli* by two genes, *dhaK* and *dhaL*. DhaK and DhaL of *E. coli* and *A. woodii* share a 50.6% and 45.4% similarity. The phosphoryl group donor for DhaKL in *E. coli* is phosphoenolpyruvate, not ATP, with DhaM acting as donor. DhaM of *E. coli* is 51.5 kDa and consists of three domains, EIIA, Hpr and EI (Gutknecht *et al.*, 2001). Each of the domains has an active site histidine that is phosphorylated by the general PTS proteins EI and Hpr; the EII-domain of DhaM then transfers the phosphoryl group to DhaKL (Gutknecht *et al.*, 2001). DhaM of *A. woodii* (Awo_c20310) is only 13.1 kDa and similar to the EII-domain of DhaM_{Ec}. Therefore, it is likely that the phosphoryl group of PEP is transferred *via* the general PTS proteins EI and Hrp to DhaM; DhaM is at the end of the phosphoprotein relay and phosphorylates DHA by DhaKL. General PTS proteins are encoded in the genome of *A. woodii* but no DHA-PTS. There are only three phosphotransferase systems annotated in the genome of *A. woodii*: PtsG of the glucose family, FruA/B of the Fru family and SrlA/E, SrlB and SrlE of the sorbitol family. We could not find data in the literature that any of

these are known to transport DHA as well. Therefore, DHA transport in *A. woodii* remains an open question. One potential candidate for DHA uptake could be the glycerol uptake facilitator GlpF (Awo_c29540). Unlike in *E. coli*, the up- and downstream genes are apparently not involved in DHA or glycerol metabolism.

From the data presented here, it seems that phosphorylation of glycerol to glycerol-3-phosphate followed by an oxidation to DHAP is the most likely pathway for activating and channelling glycerol into glycolysis mediated by the products of the *glpK3* gene cluster, the kinase is a three-component (glpABC) glycerol-3-phosphate dehydrogenase. Since other pathways may also be present as indicated by the genome analysis, transcriptomics and enzyme measurements, the pathway postulated is not unambiguously. It also may be possible that different feeding pathways for glycerol are active simultaneously. The role of different pathways in glycerol metabolism requires future mutational analysis.

Glycerol utilization in the pduAB mutant

The *pdu* gene cluster of *A. woodii* encodes a diol dehydratase along with proteins forming a bacterial microcompartment (Schuchmann *et al.*, 2015; Chowdhury *et al.*, 2020). In contrast to *Salmonella*, in which the system is only involved in propanediol metabolism, the *pdu*- encoded BMS's are produced in *A. woodii* during growth on several substrates such as fructose, several diols, $\text{H}_2 + \text{CO}_2$ and ethanol (Schuchmann *et al.*, 2015) which raises the question about the nature of the inducer/repressor for gene activation that is not answered yet. Obviously, the *pdu* expression was induced during growth on glycerol. We hypothesize that the diol dehydratase dehydratases glycerol to 3-hydroxypropionaldehyde, a toxic compound with antimicrobial activities (Talarico *et al.*, 1988). This hypothesis is in line with the observation that deletion of *pduAB* led to stable growth and high tolerance of *A. woodii* on glycerol.

The DHA and potential glycerol metabolism in A. woodii

From the data presented here, we propose the following metabolic pathway of acetogenesis from DHA and glycerol. Two molecules of DHA are taken up and phosphorylated to DHAP *via* the dihydroxyacetone kinase pathway. DHAP is then converted to glyceraldehyde-3-phosphate by the triosephosphate isomerase which is further metabolized to PEP and pyruvate. Pyruvate is then decarboxylated to acetyl-CoA and further converted to acetate. The generated 2 CO_2 , 2 Fd_{red} and 2 NADH are used in the WLP to generate one additional acetate. In addition, 0.3 ATP are produced by the respiratory chain (Schuchmann and Müller 2014). This metabolic scenario

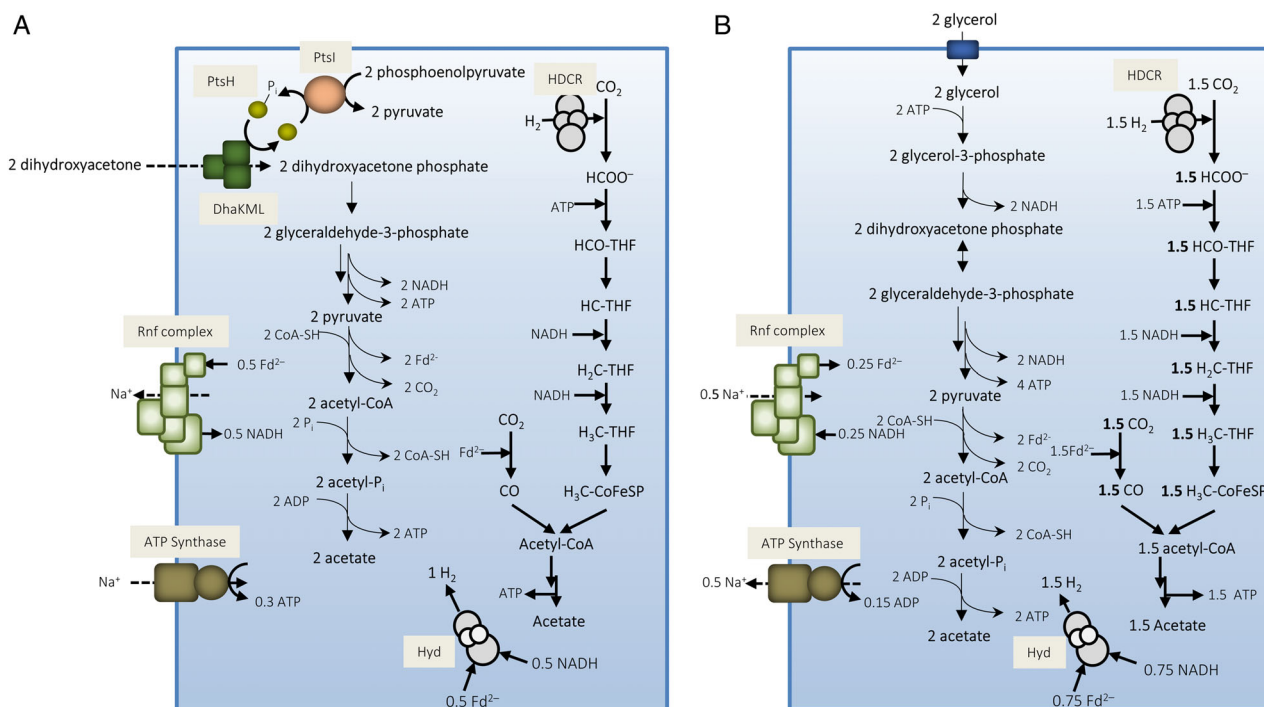


Fig 9. Hypothetical dihydroxyacetone (A) and glycerol metabolism (B) in *Acetobacterium woodii*. For explanations, see text. DHA, dihydroxyacetone; Hyd, bifurcating hydrogenase; HDCR, hydrogen dependent CO₂ reductase; PtsI, phosphoenolpyruvate phosphotransferase; PtsH, phosphocarrier protein; THF, tetrahydrofolate. [Color figure can be viewed at wileyonlinelibrary.com]

is consistent with the observation that Na⁺ is required for DHA oxidation and that DHA oxidation is impaired in mutants with defects in WLP genes. Oxidation of 1 mol DHA yields 2.15 mol of ATP corresponding to the following equation (Fig. 9A):



This is the highest C3 substrate:ATP ratio for acetogenic bacteria. On the other hand, with glycerol as a substrate the energy yield is different. The initial phosphorylation of glycerol leads to an investment of ATP. The further oxidation of glycerol-3-phosphate to DHAP leads to additional 2 NADH, and to reoxidize the excess NADH, additional CO₂ has to be taken up and reduced to acetate *via* the WLP. In this scenario 0.25 mol ferredoxin have to be reduced with NADH as reductant to get the reduced ferredoxin required for CO₂ reduction to CO. To drive ferredoxin reduction with NADH as reductant, reversed electron transport is required, energized by ATP hydrolysis (Fig. 9B). In sum, glycerol is converted to acetate according to:



Experimental procedures

Cultivation of *A. woodii*

A. woodii DSMZ 1030 (WT or $\Delta pduAB$) was cultivated at 30 °C in complex medium (Heise *et al.*, 1989). Minimal media did not contain yeast extract, and the amount of KH₂PO₄ and NH₄Cl was increased to 0.2 and 1.35 g L⁻¹ respectively, selenite tungsten solution to 1.5 ml L⁻¹ and 10 µg ml⁻¹ of D/L-pantothenic acid was added. For some experiments, the medium described by Eichler and Schink (1984) was used. Fructose (2–20 mM), ethylene glycol (50 mM), DHA (10 mM) or glycerol (5–50 mM) was used as growth substrate. Since the deletion strain is uracil auxotrophic, uracil was supplemented to the media with a final concentration of 50 mg L⁻¹. Growth was monitored by measuring the optical density at 600 nm (OD₆₀₀).

Preparation of cell suspensions

All buffers and media were prepared using the anaerobic techniques described previously (Hungate, 1969; Bryant, 1972) and all preparation steps were performed at room temperature in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI; filled with 95%–98% N₂ and 2%–5% H₂) under strictly anoxic conditions.

A. woodii was grown with DHA as substrate to an OD₆₀₀ of 0.6–0.7, harvested by centrifugation (8.000×g, 10 min) and washed and resuspended in imidazole buffer (50 mM imidazole-HCl, 20 mM MgSO₄, 20 mM KCl, 2 mM DTE, 4 μM resazurin, pH 7.0). The protein concentration of the cell suspension was measured as described (Schmidt *et al.*, 1963). Resting cell experiments were done in 20 ml imidazole buffer (50 mM imidazole-HCl, 20 mM MgSO₄, 20 mM KCl, 2 mM DTE, 4 μM resazurin, 60 mM KHCO₃, pH 7.0) under a N₂/CO₂ atmosphere. Cells were added to a final protein concentration of 1 mg ml⁻¹. These cell suspensions were incubated at 30 °C for 5 min and the experiment was started by the addition of 200 μl DHA (1 M). For the determination of the substrate and product concentrations, 1 ml samples were taken at the indicated time points, cells were removed by centrifugation (14.000×g, 1 min) and the supernatant was stored at -20 °C for further analysis.

Preparation of cell-free extracts

Cells grown on fructose, glycerol or DHA were cultivated till mid exponential growth phase (OD₆₀₀ = 1.25 for fructose, 0.71 for DHA and 0.2 for Glycerol) and were harvested aerobically by centrifugation (8.000×g, 10 min) and washed and resuspended in 3 ml buffer A (50 mM Tris, 20 mM MgSO₄, pH 7.5). Cells were disrupted by passing two times through a 'French Press' cell (SLM Aminco, SLM instruments, USA) at 100 MPa. Cells and cell debris were removed by centrifugation (13.000 rpm 10 min, 4 °C). The protein concentration was measured according to Bradford (1976). The cell-free extract was stored at 4 °C until further use.

Determination of enzyme activities

All measurements were performed at 30 °C under oxic conditions. To measure the electron transfer from glycerol to NAD⁺ in cell-free extracts from *A. woodii* grown on different substrates, different amounts of cell-free extracts and 3 mM NAD⁺ were added to the buffer (0.1 M Na₄P₂O₇, pH 8.0). The reaction was started by addition of 30 mM glycerol and monitored by following the absorption at 340 nm. NADH-dependent DHA reduction was measured under identical conditions, with 30 mM DHA as electron acceptor.

For electron transfer from NADH to DHAP 0.45 mM NADH were added to the buffer (0.3 M triethanolamine, pH 7.6). The reaction was started by addition of 0.5 mM DHAP.

To determine glycerol kinase activity 30 mM glycerol and 3 mM ATP were added to the buffer (0.2 M glycine, 20.9 g ml⁻¹ hydrazine, 1.8 mM MgCl₂, pH 9.8). ADP produced was determined by a coupled optical-enzymatic

assay by adding 160 μl premixed 'Reaction Start solution' ('Glycerol Assay Kit' (Megazyme, Bray, Ireland) following the instructions of the manufacturer, but suspension 4 (Glycerol kinase) was omitted. The ADP produced was used by phosphoenolpyruvate kinase and to generate pyruvate from phosphoenolpyruvate. Pyruvate was then reduced to lactate by a lactate dehydrogenase and NADH oxidation was monitored at 340 nm.

The change in absorption during the electron transfer and the extinction coefficient of NADH/NAD⁺ ($\epsilon = 6.2 \text{ mM}^{-1} \text{ cm}^{-1}$) were used to calculate the apparent enzymatic activities in the cell-free extracts. 1 Unit (U) is defined as 1 μmol substrate utilized per minute.

Determination of acetate, glycerol and DHA

For acetate determination 400 μl of the samples were mixed with 50 μl phosphoric acid (2 M), 50 μl 1-propanol (200 mM) and 500 μl acetone (13.6 M). The samples were analysed by gas chromatography on a Gas chromatograph 7890B GC Systems (Agilent Technologies, Santa Clara, California, USA) with an DB-Wax column (30 m × 0.25 mm, Agilent Technologies, Santa Clara, California, USA). The following temperature profile was used: 60 °C 3 min followed by a temperature gradient to 180 °C with 10 °C min⁻¹ followed by a hold at 180 °C for 5 min. Acetate was analysed with a flame ionization detector at 250 °C. The peak areas were proportional to the concentration of each substance and calibrated with standard curve.

Glycerol concentration was measured using a 'Glycerol Assay Kit' (Megazyme, Bray, Ireland) following the instructions of the manufacturer. The DHA concentrations were measured as described (Sponholz and Wünsch, 1980).

Transcriptome analyses

For transcriptome analyses, wild type cells were grown on 10 mM DHA or during the first adaptation step with 5 mM glycerol and were harvested in the exponential growth phase with OD₆₀₀ of 0.31 and 0.15 respectively. All the following steps were carried out as described previously (Kremp *et al.*, 2018). Genes with a log₂-fold change (FC) of +2/-2 and a *P*-adjust value < 0.05 were considered differentially expressed. The sequence data have been submitted to the SRA database.

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Conflict of Interest

The authors declare no potential conflict of interest.

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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:

Table S1. The most upregulated genes of *A. woodii* during the first transfer on glycerol.

Table S2. The most downregulated genes of *A. woodii* during the first transfer on glycerol.

Table S3. Transcript abundance of potential genes involved in glycerol metabolism of *A. woodii*.

Table S4. The most upregulated genes of *A. woodii* during growth on dihydroxyacetone.

Table S5. The most downregulated genes of *A. woodii* during growth on dihydroxyacetone.

Table S6. Transcript abundance of potential genes involved in dihydroxyacetone metabolism of *A. woodii*.