2,3-Butanediol Metabolism in the Acetogen *Acetobacterium woodii*

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The acetogenic bacterium *Acetobacterium woodii* is able to reduce CO₂ to acetate via the Wood-Ljungdahl pathway. Only recently we demonstrated that degradation of 1,2-propanediol by *A. woodii* was not dependent on acetogenesis, but that it is disproportionated to propanol and propionate. Here, we analyzed the metabolism of *A. woodii* on another diol, 2,3-butanediol. Experiments with growing and resting cells, metabolite analysis and enzymatic measurements revealed that 2,3-butanediol is oxidized in an NAD⁺-dependent manner to acetate via the intermediates acetoin, acetaldehyde, and acetyl coenzyme A. Ethanol was not detected as an end product, either in growing cultures or in cell suspensions. Apparently, all reducing equivalents originating from the oxidation of 2,3-butanediol were funneled into the Wood-Ljungdahl pathway to reduce CO₂ to another acetate. Thus, the metabolism of 2,3-butanediol requires the Wood-Ljungdahl pathway.

The ability to use molecular hydrogen as reductant for the fixation of two molecules of CO₂ to acetate is characteristic for the phylogenetically diverse group of acetogenic bacteria (1–3). The pathway involved, the Wood-Ljungdahl pathway (WLP), not only allows the bacteria to grow autotrophically but also provides energy for growth: electron transfer from molecular hydrogen to CO₂ is coupled to the generation of an electrochemical ion gradient across the cytoplasmic membrane (4–7). In the acetogenic model organism *Acetobacterium woodii*, the coupling ion was shown to be Na⁺ (5, 8) and the electrochemical Na⁺ potential is established by a membrane-bound Fd²⁺:NAD⁺ oxidoreductase, most likely encoded by the *rnf* genes (9, 10). During autotrophic growth, reduced ferredoxin is provided by an electron bifurcating hydrogenase that transfers electrons from molecular hydrogen not only to NAD⁺ but also, in equal amounts, to ferredoxin (11). The Na⁺ gradient established by the Rnf complex is then used to drive phosphorylation of ADP by an Na⁺-dependent F₁F_o ATP synthase (5, 12–14).

However, acetogenic bacteria are not restricted to molecular hydrogen as reductant for carbon dioxide. Electrons may also derive from sugars (hexoses, as well as pentoses), acids (e.g., pyruvate and lactate), methyl groups, or alcohols (2, 15–17). Thus, the products of these fermentations are the oxidation products of the electron donor as well as acetate derived from CO₂ reduction. Acetogens are metabolically very diverse and can, for example, not only reduce CO₂ but other electron acceptors such as phenylacrylates (15), fumarate (18–20), and nitrate (17, 21–23). Recently, we discovered another nonacetogenic metabolism carried out by the model acetogen *A. woodii*: 1,2-propanediol (1,2-PD) is dehydrated to propionaldehyde that is then disproportionated to propionate and propanol. This fermentation does not involve the WLP and ATP is synthesized by substrate level phosphorylation in the course of the propionate kinase reaction (24). Degradation of 1,2-PD involves the formation of bacterial microcompartments, and these compartments were also present when another diol, 2,3-butanediol (2,3-BD), was the growth substrate (24). Since 2,3-BD metabolism in acetogens had not been studied in much detail and to test whether nonacetogenic growth on diols is a general rule, we analyzed the fermentation of 2,3-BD by *A. woodii*.

MATERIALS AND METHODS

Growth conditions. *A. woodii* DSM 1030 was grown under anaerobic conditions at 30°C in complex medium that was prepared as described previously (8, 25). 2,3-BD, acetoin, or fructose at 20 mM was used as a substrate. Growth was monitored by measuring the optical density at 600 nm (OD₆₀₀).

Preparation of cell suspensions. *A. woodii* was grown as described in 5 liters of complex medium to an OD₆₀₀ of 0.4 to 0.5 when 2,3-BD was the carbon source or to an OD₆₀₀ of 1.0 to 1.2 when acetoin was the carbon source. The culture was centrifuged anaerobically at 11,500 × g and 4°C for 10 min. The cells were washed twice in imidazole buffer (50 mM imidazole, 20 mM MgSO₄, 20 mM KCl, 2 mM dithioerythritol [DTE], 4 μM resazurin; pH 7.0) and resuspended in 2 to 3 ml of imidazole buffer. The protein concentration was determined as described previously (26). In order to investigate the conversion of 2,3-BD or acetoin to acetate, 100-ml serum flasks (Glasgerätebau Ochs GmbH, Bovenden-Lengeln, Germany) were filled with 10 ml of imidazole buffer containing either 10 mM 2,3-BD or 10 mM acetoin. If indicated, KHCO₃ and NaCl were added to a concentration of 20 mM each. If applied, the ionophore ETH2120 was added to a concentration of 80 μM. If the buffer contained bicarbonate, the gas phase of the serum flasks was changed to 10⁵ Pa of N₂ + CO₂ (80:20 [vol/vol]), if there was no bicarbonate present the gas phase was 100% N₂. The vessels were preheated to a temperature of 30°C in a water bath. The reaction was started by addition of resting cells to a final concentration of 1 mg/ml. Henceforth, 500-μl samples were taken at time points as indicated. Samples were centrifuged immediately at 18,000 × g for 1 min, and the supernatant was stored at –20°C.

Preparation of cell extract and membranes. *A. woodii* was grown as described in 5 liters of complex medium to an OD₆₀₀ of 0.4 to 0.5 when 2,3-BD was the carbon source or to an OD₆₀₀ of 1.0 to 1.2 when acetoin

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FIG 1 Growth of *A. woodii* on 2,3-BD and acetoin. *A. woodii* was grown on either 20 mM 2,3-BD (■) or 20 mM acetoin (▲) in phosphate-carbonate-buffered complex medium. The OD₆₀₀ was determined. The curves shown are representative for three independent growth experiments.

was the carbon source. The culture was centrifuged anaerobically at $11,500 \times g$ and 4°C for 10 min. Cells were washed twice in washing buffer (25 mM Tris, 420 mM sucrose, 2 mM DTE, 4 μM resazurin; pH 7). Afterward, the cells were resuspended in 25 ml of washing buffer containing 200 mg of lysozyme and incubated at 37°C for 1 h. After sedimentation of the cells at $11,500 \times g$ and 4°C for 10 min, the cells were resuspended in 15 ml of storage buffer (50 mM KPi, 2 mM DTE, 4 μM resazurin; pH 7) and disrupted by two passages through a French press (110 MPa). Cell debris and whole cells were removed by a centrifugation step for 30 min at $23,700 \times g$. The cell extract was stored at 4°C . For preparation of membranes, the cell extract was centrifuged at $150,000 \times g$ at 4°C for 2 h and washed once in storage buffer. After the second centrifugation step, membranes were resuspended in 3 to 4 ml of storage buffer.

Measurement of NAD⁺-dependent decrease of acetoin. Glass vessels (10 ml) were filled with 6.3 ml of assay buffer (100 mM Tris, 0.5 mM

MgCl₂, 2 mM DTE, 4 μM resazurin; pH 7.5), and cell extract was added to a concentration of 1 mg/ml. Subsequently, acetoin, coenzyme A (CoA) and thiamine pyrophosphate (TPP) were added to concentrations of 8 mM, 100 μM , and 0.8 mM, respectively. The assay was started by the addition of 10 mM NAD⁺. Henceforth, 500- μl samples were taken at the indicated time points, and 50 μl of trichloroacetic acid was added immediately. Samples were centrifuged at $18,000 \times g$ for 1 min, and the supernatant was stored at -20°C .

Determination of acetate, ethanol, and acetoin. Remaining suspended particles were removed from the supernatant of all samples by a centrifugation step ($15,000 \times g$; 5 min). A total of 400 μl of the resulting supernatant was mixed with 500 μl of 13.6 M acetone and 50 μl of 2 M phosphoric acid. The samples were analyzed by gas chromatography on a Clarus 580 GC (Perkin-Elmer, Waltham, MA) with an ELITE-FFAP capillary column (30 m by 0.32 mm; Perkin-Elmer) with the following temperature profile: 60°C for 3 min, followed by a temperature gradient to 150°C with $10^\circ\text{C min}^{-1}$. Helium was used as the carrier gas with a flow rate of 40 cm min^{-1} . The sample volume was 0.5 μl , injected with an autosampler (injection temperature of 250°C , split of 1:50). Acetate, acetoin, and ethanol were analyzed with a flame ionization detector at 250°C . The peak areas were proportional to the concentration of each substance and calibrated with standard curves. A total of 10 mM 1-propanol was used as the internal standard in all cases.

Measurement of enzymatic activities. All enzyme assays were carried out at 30°C in glass cuvettes sealed with rubber stoppers containing a 100% N₂ atmosphere.

2,3-BD:NAD⁺ oxidoreductase activity was measured in 1 ml of buffer containing 200 mM glycine, 2 mM DTE, 4 μM resazurin (pH 9), and 150 μg of cell-free extract. 2,3-BD was added to a concentration of 300 mM and the reaction was started by addition of 1 mM NAD⁺. Formation of NADH was measured photometrically at 340 nm.

Acetoin:NADH oxidoreductase activity was measured in 1 ml of buffer containing 200 mM glycine, 2 mM DTE, 4 μM resazurin (pH 9), and 150 μg of cell extract. Acetoin was added to a concentration of 10 mM, and the reaction was started by addition of 300 μM NADH. Oxidation of NADH was measured photometrically at 340 nm.

Acetoin:NAD⁺ oxidoreductase activity was measured in 1 ml of buffer containing 100 mM Tris, 0.5 mM MgCl₂, 2 mM DTE, 4 μM resazurin (pH 7.5), and 150 μg of cell extract. NAD⁺, TPP, and CoA were added to concentrations of 2 mM, 0.8 mM, and 130 μM , respectively. The reaction was started by the addition of 10 mM acetoin. The formation of NADH was measured photometrically at 340 nm.

Acetoin:DCPIP oxidoreductase activity was measured in 1 ml of buffer containing 100 mM Tris, 0.5 mM MgCl₂, 2 mM DTE, and 4 μM resazurin

FIG 2 Acetate formation from 2,3-BD (A) and acetoin (B) by resting cells of *A. woodii*. *A. woodii* was grown on 2,3-BD, and resting cells were prepared. The reaction was started by the addition of either 2,3-BD (A) or acetoin (B) to a final concentration of 10 mM each. Concentrations of acetoin (■) and acetate (▲) were determined by gas chromatography. The data shown are representative for three independent experiments (A), or the values are means from four replicates (B).

(pH 7.5). TPP and DCPIP were added to concentrations of 0.8 mM and 150 μ M, respectively, cell extract was added to a concentration of 150 μ g/ml. The reaction was started by the addition of 10 mM acetoin. Reduction of DCPIP was measured photometrically at 604 nm.

NADH:lipoamide oxidoreductase activity was measured in 1 ml of buffer containing 100 mM Tris, 0.5 mM MgCl_2 , 2 mM DTE, 4 μ M resazurin (pH 7.5), and 150 μ g of cell extract. NADH was added to a concentration of 200 μ M. The reaction was started by addition of 2 mM lipoamide. Oxidation of NADH was measured photometrically at 340 nm.

Acetaldehyde:NAD⁺ oxidoreductase activity was measured in 1 ml of buffer containing 35 mM KPi (pH 8), 2 mM DTE, and 4 μ M resazurin. NAD⁺, cell extract, and acetaldehyde were added to concentrations of 1.8 mM, 150 μ g/ml, and 10 mM, respectively. The reaction was started by the addition of 200 μ M CoA. The formation of NADH was measured photometrically at 340 nm.

Measurement of Fd^{2+} :NAD⁺ oxidoreductase (Rnf) activity at membranes was performed as described previously (27).

Analytical methods. Protein concentrations were measured according to the method of Bradford (28).

Calculations. Curve fitting and determination of the kinetic parameters K_m (Michaelis constant) and $V_{\max}$ (maximal enzyme velocity) were performed with GraphPad Prism version 4.03 and the Michaelis-Menten equation [$Y = (V_{\max} \times X)/(K_m + X)$] using a nonlinear fit based on least-squares analysis. The standard Gibbs free energy of a reaction ($\Delta G^{\circ'}$) was calculated from the standard Gibbs free energy of formation (ΔG_f°) of the products and reactants (29, 30).

RESULTS

Growth of *A. woodii* on 2,3-butanediol and acetoin. 2,3-BD allowed growth of *A. woodii* up to a final OD_{600} of 0.68 ± 0.11 with an optimal substrate concentration of 20 mM (Fig. 1) and a doubling time of 8.3 h. The oxidation product of 2,3-BD, acetoin, also allowed growth of *A. woodii* as described previously for *Pelobacter carbinolicus* and some strains of *A. woodii* (30, 31). The optimal acetoin concentration was determined to be 20 mM. A final OD_{600} of 1.87 ± 0.17 was reached within 36 h, and the doubling time was 4.6 h (Fig. 1). Analysis of the product pool after growth on 2,3-BD or acetoin showed that both substrates were converted exclusively to acetate. Other end products, such as ethanol, could not be detected (data not shown).

Acetate is the only end product of 2,3-BD and acetoin degradation by resting cells of *A. woodii*. To get a first insight into the pathway of 2,3-BD degradation in *A. woodii*, we analyzed the product pool in cell suspensions oxidizing 2,3-BD. As evident from Fig. 2A, 10 mM 2,3-BD was converted to 22 mM acetate within 30 h with an initial rate of $1.6 \mu\text{mol of acetate mg}^{-1} \text{ h}^{-1}$. The formation of ethanol could not be detected. A quantification of 2,3-BD was not possible due to the presence of several stereoisomers with unknown identity. Therefore, we performed a stoichiometric analysis for the conversion of acetoin. As evident from Fig. 2B, resting cells of *A. woodii* grown on 2,3-BD degraded 10 mM acetoin within 90 min. Again, only acetate could be detected as final end product with a concentration of 23 ± 2 mM. One mole of acetoin was converted to 2.3 mol of acetate. Since the ratio exceeds 1:2, additional acetate must have been produced via a second pathway. Interestingly, as seen before with cells grown on 1,2-PD (24), acetoin consumption was faster than acetate production: 10 mM acetoin was consumed completely within 90 min with an initial rate of $14 \mu\text{mol of acetoin mg}^{-1} \text{ h}^{-1}$, whereas 23 mM acetate was produced only after 4 h with an initial rate of $11 \mu\text{mol of acetate mg}^{-1} \text{ h}^{-1}$. Based on equation 1 (see below), the carbon recovery from acetoin was calculated to be 89%.

FIG 3 Enzymatic characterization of the 2,3-BD:NAD⁺ oxidoreductase activity in cell extract of *A. woodii* grown on 2,3-BD. Electron transfer from 2,3-BD to NAD⁺ in cell extract of *A. woodii* was measured at 30°C anaerobically under an N₂ atmosphere in buffer containing 200 mM glycine (pH 9), 4 μ M resazurin, and 2 mM DTE. 2,3-BD and NAD⁺ were added to concentrations of 300 and 1 mM, respectively (A). Michaelis-Menten kinetics for 2,3-BD (B) and NAD⁺ (C) are shown. All values are means from three replicates.

Characterization of an NAD⁺-dependent 2,3-BD dehydrogenase. In order to unravel the enzymatic reactions mediating the conversion of 2,3-BD to acetate, the enzymatic activities were determined. Cell extract of *A. woodii* grown on 2,3-BD catalyzed the

FIG 4 NAD^+ -dependent decrease of acetoin in cell extract of 2,3-BD-grown cells. The assay was performed anaerobically (N_2 atmosphere) in buffer (100 mM Tris-HCl, 0.5 mM MgCl_2 , 2 mM DTE, 4 μM resazurin; pH 7.5) containing 8 mM acetoin, 100 μM CoA, and 0.8 mM TPP. At time point zero, NAD^+ was added to a final concentration of 10 mM (■), or water was added as a control (▲). The decrease in acetoin was measured by gas chromatography. The curves shown are representative for three experiments using independent preparations of cell extract.

endergonic electron transfer from 2,3-BD to NAD^+ ($\Delta G^{0'} = +23.96$ kJ/mol) (Fig. 3A). This activity was not observed in fructose-grown cells. Electron transfer was strictly dependent on the addition of both substrates and followed Michaelis-Menten kinetics (Fig. 3B and C). The K_m value for NAD^+ was determined to be 413 ± 3 μM , the K_m value for the substrate 2,3-BD was comparatively high (99 ± 14 mM), and the $V_{\max}$ was 222 ± 7 mU/mg. The optimal pH for NAD^+ reduction was 9. The cell extract not only oxidized 2,3-BD but also 1,3-BD, 1,2-BD, and 1,2-PD with 24, 30, and 73%, respectively, of the activity obtained with 2,3-BD (data not shown). Ethanol was not used as electron donor for NAD^+ reduction under the conditions tested. Cell extract also catalyzed the reverse reaction, reduction of acetoin with NADH as reductant. This was observed in cell extract from both, acetoin and

2,3-BD-grown cells with specific activities of 240 ± 24 mU/mg and 170 ± 7 mU/mg, respectively.

Characterization of acetoin oxidation. Growth of cells on acetoin as well as NAD^+ -dependent oxidation of 2,3-BD implies acetoin as an intermediate of the pathway. Therefore, we next analyzed the fate of acetoin. However, acetoin-dependent NAD^+ reduction was not observed in cell extracts of 2,3-BD-grown cells. Since this was surprising and may result from other activities re-oxidizing NADH, we did not analyze for NAD^+ reduction but for acetoin utilization by gas chromatography. As can be seen in Fig. 4, acetoin disappeared from cell extract with a specific activity of 93 ± 21 mU/mg. This activity was NAD^+ dependent.

To further analyze the kinetic parameters of the acetoin dehydrogenase, we took advantage of the fact that this is a multisubunit enzyme complex catalyzing individual reactions (32). The E1 subunit catalyzes electron transfer from acetoin to the artificial electron carrier DCPIP (32, 33) and indeed this activity was catalyzed by cell extract of 2,3-BD-grown cells of *A. woodii* (Fig. 5A). The specific activity was determined to be 423 ± 3 mU/mg. A similar value (470 ± 10 mU/mg) was obtained for the same reaction in cell extract from cells grown on acetoin. Furthermore, the activity of subunit E3 of the acetoin dehydrogenase could also be measured in cell extract from 2,3-BD-grown cells (Fig. 5B), albeit the rate of the reaction was slow. Electron transfer from NADH to lipoamide occurred with a specific activity of 12 ± 1 mU/mg. The same activity was also present in acetoin-grown cells with an electron transfer rate of 22 ± 2 mU/mg. None of these oxidoreductase activities was detectable in cell extracts of fructose-grown cells (data not shown). In order to characterize the kinetic parameters of NAD^+ -dependent oxidation of acetoin, we tested whether electron transfer from acetoin to NAD^+ could be measured in the cell extracts of acetoin-grown cells. Indeed, NAD^+ was reduced after the addition of acetoin to the assay, and the activity was strictly dependent on the presence of acetoin, CoA, and cell extract (Fig. 6A and B). The specific activity of electron transfer from acetoin to NAD^+ was determined to be 140 ± 5 mU/mg. The

FIG 5 Enzymatic assay measuring the acetoin:DCPIP (A) or NADH:lipoamide (B) activity in cell extract of 2,3-BD-grown cells of *A. woodii*. Measurements were performed at 30°C in 1-ml-glass cuvettes under a N_2 atmosphere containing 1 ml of buffer (100 mM Tris-HCl [pH 7.5], 0.5 mM MgCl_2 , 4 μM resazurin, and 2 mM DTE). (A) To measure the acetoin:DCPIP oxidoreductase activity, TPP and DCPIP were added to concentrations of 0.8 and 0.15 mM, respectively. The reaction was started by addition of 10 mM acetoin. Reduction of DCPIP was measured at 604 nm, either in the presence (solid line) or in the absence (dotted line) of 150 μg of cell extract. (B) For measurement of NADH:lipoamide oxidoreductase activity, NADH was added to a concentration of 200 μM . The reaction was started by the addition of 2 mM lipoamide. Oxidation of NADH was measured at 340 nm. The curves shown are representative for three experiments from independent preparations of cell extract.

FIG 6 Enzymatic characterization of the acetoin:NAD⁺ oxidoreductase activity in cell extract of *A. woodii* grown on acetoin. Electron transfer from acetoin to NAD⁺ in cell extract of *A. woodii* was measured at 30°C anaerobically under an N₂ atmosphere in buffer containing 100 mM Tris (pH 7.5), 0.5 mM MgCl₂, 4 μM resazurin, and 2 mM DTE. Acetoin, NAD⁺, TPP, and CoA were added to concentrations of 10, 2, 0.8, and 0.13 mM, respectively (A). Michaelis-Menten kinetics for NAD⁺ (C), acetoin (D), and CoA (E) are shown. All values are mean from three replicates.

dependence on NAD⁺, acetoin, and CoA, followed Michaelis-Menten kinetics (Fig. 6C to E) and the apparent K_m values were determined to be 1 ± 0.3 mM for NAD⁺, 0.9 ± 0.17 mM for acetoin, and 44 ± 8 μM for CoA.

Conversion of acetaldehyde to acetyl-CoA by cell extract of cells grown on 2,3-BD. The experiments described thus far are consistent with an oxidative cleavage of acetoin to acetyl-CoA and acetaldehyde. To analyze the fate of acetaldehyde, we searched for

acetaldehyde dehydrogenase activity. Indeed, the cell extract of 2,3-BD-grown cells catalyzed the exergonic acetaldehyde-dependent reduction of NAD⁺ ($\Delta G^{0'} = -17.5$ kJ/mol [29]) (Fig. 7) with a specific activity of 284 ± 12 mU/mg. This activity was not observed in fructose-grown cells and the reaction was strictly CoA dependent (Fig. 7), indicating acetyl-CoA as product of the reaction.

In sum, the data are consistent with the oxidation of 2,3-BD to

FIG 7 CoA- and acetaldehyde-dependent reduction of NAD^+ in cell extract of 2,3-BD-grown cells. Electron transfer from acetaldehyde to NAD^+ was measured at 30°C under N_2 atmosphere in 1 ml of KP_i buffer (35 mM KP_i , 2 mM DTE, 4 μM resazurin; pH 8) containing 150 μg of cell extract, 1.8 mM NAD^+ , and 10 mM acetaldehyde. The reaction was started by the addition of 200 μM CoA. The formation of NADH was measured photometrically at 340 nm. The curve shown is representative for three experiments.

2 mol of acetyl-CoA and the concomitant reduction of 3 mol of NAD^+ . Since all of the acetyl-CoA is converted to acetate, the question arose how NADH is reoxidized. For *A. woodii* as an acetogenic bacterium, CO_2 is the obvious electron acceptor. The involvement of the WLP in 2,3-BD conversion was tested next with cell suspensions.

Acetate formation from 2,3-BD is Na^+ and CO_2 dependent.

In the presence of CO_2 , bicarbonate, and NaCl, resting cells of *A. woodii* converted 10 mM 2,3-BD to 22 mM acetate within 30 h (Fig. 8). Acetate formation was strictly dependent on CO_2 /carbonate. If no additional Na^+ was added or in the presence of a sodium ionophore, acetate formation from 2,3-BD was largely inhibited. A similar pattern of acetate formation was observed when acetoin was the substrate (data not shown). The dependence of acetate formation on Na^+ or a transmembrane Na^+ gradient, as evident from the inhibition of acetate formation by a sodium ionophore, also indicates the involvement of the WLP in 2,3-BD metabolism.

In order to further prove the involvement of the WLP in 2,3-BD degradation, we measured the enzymatic activity of the coupling site of the WLP, the Rnf complex. Indeed, electron flow from reduced ferredoxin to NAD^+ at membranes of 2,3-BD-grown cells of *A. woodii* occurred with a rate of 53 ± 3 mU/mg. This is in the same range as measured before for fructose-grown cells (50 ± 5 mU/mg [27]).

DISCUSSION

Acetogenic bacteria are able to grow chemolithoautotrophically with H_2 as an electron source and CO_2 as a carbon source, thereby forming acetate as the end product (6). Furthermore, they are also able to grow chemoorganoheterotrophically on sugars, acids, or alcohols (17). The oxidation of these organic substrates leads to the formation of reducing equivalents, which are usually disposed of in the WLP, resulting in the reduction of CO_2 to acetate. Only recently, we found that growth of the model acetogen *A. woodii* on the diol 1,2-PD is nonacetogenic, since the substrate is disproportionated into propanol and propionate (24). In the present study, we show that degradation of the diol 2,3-BD by *A. woodii* is not independent of acetogenesis but that oxidation of 2,3-BD is coupled to the reduction of CO_2 via the WLP.

FIG 8 Acetate formation from 2,3-BD by resting cells of *A. woodii* is CO_2 and Na^+ dependent. Whole cells of *A. woodii* were incubated with 10 mM 2,3-BD in buffer containing 50 mM imidazole, 20 mM KCl, 5 mM MgSO_4 , 2 mM DTE, and 4 μM resazurin (pH 7). Supplements and the gaseous atmosphere were as indicated. Symbols: ■, 20 mM NaCl, 20 mM KHCO_3 , N_2/CO_2 atmosphere; ◆, 20 mM NaCl, 20 mM KHCO_3 , 80 μM ETH2120, N_2/CO_2 atmosphere; ▲, 20 mM KHCO_3 , N_2/CO_2 atmosphere; ▼, 20 mM NaCl, N_2 atmosphere. The data shown are representative for three independent experiments.

It was previously shown that *A. woodii* can grow on 2,3-BD and acetoin, respectively; however, not all strains grew on these substrates (31). Therefore, we started by analyzing whether *A. woodii* DSM 1030 can grow on 2,3-BD and acetoin. Indeed, this was the case and *A. woodii* only produced acetate as end product, ethanol could not be detected. It was striking that the final OD_{600} when growing on acetoin was 150% higher than growth on 2,3-BD, and also the doubling time on acetoin was only half as long as with 2,3-BD. The higher optical density might indicate a higher ATP yield from acetoin as carbon and energy source compared to 2,3-BD. According to equation 1, the metabolism of acetoin by *A. woodii* might allow the phosphorylation of 1.55 mol ADP per mol of acetoin, whereas the degradation of 1 mol 2,3-BD (equation 2) only allows the phosphorylation of 1.3 mol ADP per mol of 2,3-BD (see Fig. 10):

where $\Delta G^{0'} = -117.1$ kJ/mol, and

where $\Delta G^{0'} = -93.6$ kJ/mol.

The increased doubling time on acetoin might be due to a low affinity of the butanediol-oxidizing enzyme for its substrate. Indeed, a following enzymatic characterization of the 2,3-BD dehydrogenase of *A. woodii* showed a high K_m of 99 mM 2,3-BD and V_{max} was only achieved in the presence of 300 mM 2,3-BD. The K_m of the acetoin dehydrogenase for acetoin was determined to be only 1 mM, giving a reason for the faster growth of *A. woodii* on this substrate. Of course, this calculation does not include the intracellular substrate concentration due to active import of 2,3-BD and acetoin, but a 2,3-BD concentration of 300 mM in *A. woodii* is very unlikely. A similar observation was seen with cell suspensions: again, only acetate was the end product on either 10 mM 2,3-BD or 10 mM acetoin and, again, acetate production from acetoin was 5 times faster than from 2,3-BD. This supports the hypothesis made above that the low affinity of the butanediol dehydrogenase for the diol is the bottleneck in 2,3-BD conversion.

FIG 9 Genetic organization of the *aco* gene cluster of *A. woodii*. *acoR* is annotated as a putative transcriptional activator, *acoA* and *acoB* encode the subunits α and β , respectively, of subunit E1 (TPP-dependent acetoin dehydrogenase), *acoC* encodes subunit E2 (dihydrolipoamide acetyltransferase), and *acoL* codes for subunit E3 (dihydrolipoamide dehydrogenase) of the acetoin dehydrogenase complex.

Another interesting observation was the slow formation of acetate compared to the rate of acetoin consumption. This was seen before for the conversion of 1,2-PD and might indicate that an intermediate of the pathway is stored within the cells. Since the formation of bacterial microcompartments in *A. woodii* on 2,3-BD-grown cells was already shown (24), the assumption is likely that the toxic product of the acetoin dehydrogenase reaction, acetaldehyde, is stored within those compartments and that it is only slowly further converted to acetate.

In this study we could show that in *A. woodii*, 2,3-BD is first oxidized to acetoin in an NAD^+ -dependent reaction and then further oxidized to acetyl-CoA and acetaldehyde. The first reaction is mediated by an alcohol dehydrogenase, which we could characterize here, but an assignment of this enzyme to 1 of the 11 alcohol dehydrogenases encoded in the genome of *A. woodii* (34) is not possible. However, the situation was different for the second step of this metabolism: the oxidative cleavage of acetoin. In *P. carbinolicus*, acetoin is known to be oxidized NAD^+ and CoA dependent by a heterotetrameric enzyme complex, giving the end products acetyl-CoA and acetaldehyde (32, 35). Indeed, the genome of *A. woodii* revealed two gene clusters (Awoc_01720-01690 and Awoc_29430-29400) that encode all subunits of a putative acetoin dehydrogenase (36). The first of the two clusters is preceded by a gene annotated as putative transcriptional activator (*acoR*/Awoc_01730, Fig. 9). Since the oxidation of α -oxoglutarate to succinyl-CoA in the tricarboxylic acid cycle is a “housekeeping”

activity and catalyzed by a highly similar complex, we would assume that the second gene cluster without the upstream regulator codes for an α -ketoglutarate dehydrogenase. The genes *acoA* and *acoB* encode the subunits α and β , respectively, of the subunit E1 of the acetoin dehydrogenase, catalyzing the oxidation of acetoin. *acoB* codes for the subunit E2, a dihydrolipoamide dehydrogenase and *acoL* encodes subunit E3, catalyzing electron transfer from dihydrolipoamide to NAD^+ (32).

Based on the data gained in the present study, the following pathway of 2,3-BD oxidation is proposed. 2,3-BD is initially oxidized to acetoin in an NAD^+ -dependent reaction. Subsequently, acetoin is further oxidized in a second NAD^+ -dependent reaction. Since this reaction is also dependent on the presence of CoA, we presume that the oxidation of acetoin is catalyzed by an acetoin dehydrogenase, resulting in acetyl-CoA and acetaldehyde as intermediates. Product analysis in growing and resting cells by gas chromatography showed that the only end product of 2,3-BD metabolism is acetate, indicating that both acetyl-CoA and acetaldehyde are converted to this product. Acetyl-CoA is converted by the enzymes phosphotransacetylase and acetate kinase, two enzymes whose encoding genes are known to be present in *A. woodii* (34). The presence of a CoA-dependent acetaldehyde dehydrogenase was supported by the measurement of an NAD^+ - and CoA-dependent oxidation of acetaldehyde in cell extract of 2,3-BD-grown cells. The stoichiometries obtained by measuring the substrate and product pool in the present study, as well the carbonate and Na^+ dependence of acetate formation from both 2,3-BD and acetoin, indicate that NADH produced from the stepwise oxidation of 2,3-BD is shuttled into the WLP to produce another acetate via the reduction of CO_2 (equation 2). A model for the conversion of 2,3-BD by *A. woodii* is shown in Fig. 10. In order to provide molecular hydrogen and reduced ferredoxin for the initial reduction of the two molecules CO_2 in the WLP, the membrane-bound Rnf complex has to reduce ferredoxin with electrons coming from NADH, using a transmembrane Na^+ gradient as driving force for this endergonic reduction. This activity is the basis for the observed dependence on Na^+ and a transmembrane Na^+ gradient. Interestingly, apart from lactate metabolism (37), 2,3-BD is the

FIG 10 Model of 2,3-BD metabolism in *A. woodii*. 2,3-BD is initially oxidized to acetoin in an NAD^+ -dependent reaction. Acetoin is further converted to acetyl-CoA and acetaldehyde, producing another NADH. Both intermediates are converted to acetate, thereby conserving energy via substrate level phosphorylation in the acetate kinase reaction. Reducing equivalents formed during oxidation of 2,3-BD are channeled into the Wood-Ljungdahl pathway. Fd^{2-} , reduced ferredoxin.

second known substrate that requires reverse electron transport catalyzed by Rnf to drive ferredoxin reduction. The Na^+ gradient required to drive reverse electron flow is established by the F_1F_0 ATP synthase from ATP hydrolysis (38). Molecular hydrogen for reduction of CO_2 to formate is provided from both reduced ferredoxin and NADH by an electron bifurcating hydrogenase (HydABCD) (11). Energy is conserved only by the activity of the acetate kinase via substrate level phosphorylation. Apart from lactate and 1,2-PD metabolism, this is the third pathway described in *A. woodii* that only relies on substrate level phosphorylation for ATP synthesis. In sum, the oxidation of 2,3-BD results in the production of 2.75 mol of acetate and 1.3 mol of ATP from 1 mol of 2,3-BD and 1.5 mol of CO_2 (Fig. 10). When growing on acetoin as the sole carbon and energy source, less NADH is produced, since the initial NAD^+ -dependent oxidation of 2,3-BD is omitted, and the resulting stoichiometries differ slightly: 1 mol of acetoin and CO_2 is converted to 2.5 mol of acetate (equation 1), resulting in the phosphorylation of 1.55 mol of ADP. This stoichiometry correlates with the values determined experimentally in this study (the acetoin/acetate ratio was determined to be 1:2.3 in cell suspensions), and the higher theoretical ATP yield from acetoin compared to 2,3-BD might be a reason for the higher ODs obtained for acetoin-grown cells.

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