Adh4, an alcohol dehydrogenase controls alcohol formation within bacterial microcompartments in the acetogenic bacterium Acetobacterium woodii

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Summary
Acetobacterium woodii utilizes the Wood-Ljungdahl pathway for reductive synthesis of acetate from carbon dioxide. However, A. woodii can also perform non-acetogenic growth on 1,2-propanediol (1,2-PD) where instead of acetate, equal amounts of propionate and propanol are produced as metabolic end products. Metabolism of 1,2-PD occurs via encapsulated metabolic enzymes within large proteinaceous bodies called bacterial microcompartments. While the genome of A. woodii harbours 11 genes encoding putative alcohol dehydrogenases, the BMC-encapsulated propanol-generating alcohol dehydrogenase remains unidentified. Here, we show that Adh4 of A. woodii is the alcohol dehydrogenase required for propanol/ethanol formation within these microcompartments. It catalyses the NADH-dependent reduction of propionaldehyde or acetaldehyde to propanol or ethanol and primarily functions to recycle NADH within the BMC. Removal of adh4 gene from the A. woodii genome resulted in slow growth on 1,2-PD and the mutant displayed reduced propanol and enhanced propionate formation as a metabolic end product. In sum, the data suggest that Adh4 is responsible for propanol formation within the BMC and is involved in redox balancing in the acetogen, A. woodii.

Introduction
Acetogens are characterized by their ability to synthesize acetyl-CoA by the reduction of CO₂ by H₂ using the Wood-Ljungdahl pathway (Wood and Ljungdahl, 1991). While they can sustain their carbon demand solely from gaseous carbon (autotrophic growth), they can also utilize several other organic substrates for growth as sole carbon source like hexoses, pentoses, diols, alcohols, lactate, formate, glyoxylate and acetate (Drake et al., 1997; Müller, 2003; Schuchmann and Müller, 2016). Growth on either of these substrates leads to the formation of the common product, acetate (Ljungdahl, 1969; Ragsdale and Wood, 1985; Drake, 1994). However, some acetogens like A. woodii can also grow non-acetogenically, as for example, by dismutation of 1,2-propanediol (1,2-PD) to propionate and propanol (Schuchmann et al., 2015). Metabolism of 1,2-PD by A. woodii is thought to occur within specialized proteinaceous bacterial microcompartments encasing multiple metabolic enzymes responsible for catabolism of 1,2-PD. Bacterial microcompartments (BMCs) are a functionally unique group of proteinaceous ‘organelles’ that are used to compartmentalize specific metabolic module(s), which lead to the formation of toxic or volatile intermediates. While these BMCs are solely composed of selectively permeable shell proteins, the movement of substrates or intermediates in and out of the metabolic core is selectively controlled by restricting the outward diffusion of toxic intermediates from BMCs (Kerfeld et al., 2005; Sampson and Bobik, 2008; Chowdhury et al., 2014).

BMCs are assembled by mainly three type of shell proteins, BMC-H (hexamers), BMC-T (pseudohexamer) and BMC-P (pentamer) (Kerfeld et al., 2005; Klein et al., 2009), while the function of a particular BMC is designated by the specific signature enzymes encapsulated within the BMC core. Depending on the encapsulated enzymes, BMCs are classified in two main categories, anabolic (carboxysome) and catabolic (metabolosome) (Kerfeld et al., 2005; Axen et al., 2014). While carboxysomes are characterized by their signature enzymes like carbonic anhydrase and RubisCO (Shively et al., 1973; Shively et al., 2001), metabolosomes involved in catabolism of 1,2-PD or ethanolamine are characterized by the presence of a B₁₂-dependent diol...
zyme B12-dependent diol dehydratase (PduCDE) (Toraya et al., 1979; Bobik et al., 1997) or B12-dependent ethanolamine lyase (EutBC) (Blackwell and Turner, 1978; Kofoid et al., 1999) respectively. Metabolosomes functions to retain the toxic propionaldehyde or acetaldehyde generated by the respective signature enzymes, which is further disproportionated to corresponding alcohol and acyl-CoA by an alcohol dehydrogenase and an aldehyde dehydrogenase respectively. The generated acyl-CoA is then activated by an incoming phosphate to an acyl-phosphate by a phosphotransacetylase (PTAC) which is further dephosphorylated to generate ATP by substrate level phosphorylation (SLP) (Rondon and Escalante-Semerena, 1992; Havemann et al., 2002; Penrod and Roth, 2006).

Acetobacterium woodii also produce BMCs during growth on 1,2-PD (Schuchmann et al., 2015) where the substrate is first dehydrated to propionaldehyde by coenzyme B12-dependent diol dehydratase (PduCDE). The generated propionaldehyde is then converted to propionate by a coenzyme A-dependent propionaldehyde dehydrogenase (PduP), phosphotransacetylase and propionate kinase. In the parallel branch, another molecule of propionaldehyde is reduced to propanol by a putative alcohol dehydrogenase by the NADH generated by PduP (see Fig. 1B). The internal recycling of NAD+ within the BMC lumen is achieved by two separate encapsulated enzymes working in tandem. In A. woodii, the genes responsible for BMC formation and also its internal enzymes are encoded in a 20-gene contiguous pdu gene cluster (Chowdhury et al., 2020). The pdu cluster consists of two genes for a two-component system (histidine kinase and response regulator), six genes encoding shell proteins (pduABB’KNT), five genes for 1,2-PD degradation (pduCDELP) and four accessory genes for enzyme reactivation (pduÖSgh) (Schuchmann et al., 2015; Chowdhury et al., 2020). Interestingly, a comparison of the pdu gene cluster of A. woodii to that of Salmonella enterica (Bobik et al., 1999) revealed that several genes are missing in A. woodii (Fig. 1A). Among them, three genes for shell formation (pduJMj), one accessory gene for vitamin B12 synthesis (pduX), propionate kinase (pduw) and most importantly propanol dehydrogenase (pduQ). While A. woodii codes for an acetate kinase (ackA) that is located elsewhere in the genome, it harbours 11 genes encoding putative alcohol dehydrogenases (Poehelein et al., 2012). One of them, adhE (bifunctional alcohol dehydrogenase) is the key enzyme involved in ethanol and acetaldehyde metabolism and not produced during metabolism of 1,2-PD (Bertsch et al., 2016). Two genes are annotated as putative butanol dehydrogenase (bdh1 and bdh2) and another as putative 1,2-propanediol dehydrogenase (dhaT), the remaining seven genes as alcohol dehydrogenase (adh1-adh7) (Bertsch et al., 2016). As the current model of 1,2-PD metabolism (Fig. 1B) describes NAD+ recycling to occur within the BMC, it is imperative that at least one of the 10 alcohol dehydrogenases co-localizes with the PduP within the BMC and recycles the reducing cofactor NAD+.

In our prior study, with enriched BMCs we have shown that the CoA-dependent propionaldehyde dehydrogenase enzyme, PduP, localizes within the BMC of A. woodii (Chowdhury et al., 2020). In this study, we show that Adh4 is the missing alcohol (propanol) dehydrogenase involved in 1,2-PD degradation by A. woodii. With heterologously over-produced enzyme, we characterize the enzymatic properties of Adh4 and with the help of in vivo pull-down experiments with histidine-tagged PduB we show that both PduP and Adh4 interacts with PduB and co-localizes within the BMC. To address the potential function of the propanol forming alcohol dehydrogenase (Adh4) in the reductive branch of 1,2-PD metabolism, we deleted the adh4 gene in A. woodii and studied the phenotype of the mutant strain. The results presented here identified the missing propanol dehydrogenase to localize within the BMC and on the other hand, revealed a complex electron balancing within two metabolic modules of non-acetogenic and acetogenic growth of A. woodii on 1,2-PD.

**Results**

**Expression analysis of putative alcohol dehydrogenase genes of A. woodii during BMC forming conditions**

Our earlier studies have shown that BMCs are not only produced during growth on 1,2-PD but also during heterotrophic growth of A. woodii on ethanol (Bertsch et al., 2016), acetaldehyde (Trifunovic et al., 2020), 2,3-butanediol (2,3-BD) (Hess et al., 2015), ethylene glycol (Trifunovic et al., 2016) or fructose (Schuchmann et al., 2015). During growth on many of these substrates, alcohol dehydrogenases apparently play an important role. While the A. woodii genome encodes 11 putative Adhs, only the bifunctional AdhE has been purified and characterized to be the key enzyme involved in ethanol and acetaldehyde metabolism of A. woodii (Bertsch et al., 2016; Trifunovic et al., 2020). However, during growth on either 1,2-PD or 2,3-BD, AdhE was not produced by A. woodii. This led us to question and investigate which of the other 10 Adhs localize within the BMC during metabolism of 1,2-PD. From our earlier study, semi-quantitative measurement of expression levels of the putative adh genes during growth on ethanol or fructose revealed apart from adhE elevated transcript abundance of adh4, adh6 and adh1 genes (Bertsch et al., 2016). Further, a transcriptomic analysis of...
A. woodii cells grown on 1,2-PD also revealed that among all the adhs present in the genome, only these three adhs (adh1, adh6 and adh4) were by far the most expressed (Chowdhury et al., 2020). To address which of these three putative alcohol dehydrogenases were differentially expressed during growth of A. woodii on 1,2-PD, 2,3-BD or ethylene glycol (BMC-forming conditions), we analysed mRNA levels of the representative adh genes during growth on these substrates. qRT-PCR analysis revealed very low levels of adh1 transcripts during growth under BMC-forming conditions whereas up to 20 times higher transcript levels of adh6 or adh4 were obtained during growth on 2,3-BD and ethylene glycol or 1,2-PD. Expression of both adh4 and adh6 was highest during growth on 2,3-BD followed by 1,2-PD and ethylene glycol (Fig. 2B).

Adh4 and Adh6 shared only 33% identity to each other and around 35% identity to propanol dehydrogenase (PduQ) from Salmonella enterica. Both contains a putative N-terminal iron binding domain (Reid and Fewson, 1994), typical of bacterial oxygen-sensitive alcohol dehydrogenases. Interestingly, a closer look at the genetic organization of these two genes within the A. woodii genome revealed that adh6 (Awo_c25410) is localized between two MFS_1 (major facilitator family) sugar transporter (Awo_c25420 and Awo_c25400) genes.
genes, whereas \textit{adh4} (Awo\textunderscore c06220) localizes as a single gene with a downstream LysR family transcriptional regulator (Awo\textunderscore c06230) (Fig. 2A).

\textbf{Overproduction and purification of histidine tagged Adh4 and Adh6}

Further, to analyse and identify the specific alcohol dehydrogenase involved in NAD\textsuperscript{+} recycling within the BMC and generation of propanol from propionaldehyde, both \textit{adh4} and \textit{adh6} genes were cloned separately in the plasmid pET21a(+). A DNA sequence encoding a His-tag was inserted at the 3'-end of each gene and the plasmid was transformed into \textit{Escherichia coli} BL21 (DE3). His\textsubscript{6}-tagged Adh4 and Adh6 were produced in large amounts via a T7 expression system. Cell-free extract prepared from these recombinant strains was loaded on to a Ni\textsubscript{NTA} column and histidine-tagged proteins were purified by an affinity chromatography. Both Adh4 (Fig. 3A) and Adh6 (Fig. 3B) with an expected size of \( \approx 40 \) kDa (as deduced from the respective amino acid sequence) were purified to a 95% homogeneity.

\textbf{Enzymatic analysis of purified Adh4 and Adh6}

To analyse the function of Adh4 and Adh6, NADH-dependent propionaldehyde reduction and NAD\textsuperscript{+}-dependent 1-propanol oxidation activities for both of these His\textsubscript{6}-tagged enzymes (affinity purified) were tested under strict anoxic conditions. While Adh6 exhibited very low NADH oxidation with propionaldehyde, Adh4 rapidly oxidized NADH with propionaldehyde. Adh4 had about 150-fold higher propionaldehyde reductase activity than Adh6. The experimentally obtained propionaldehyde-reductase specific activity (\( V_{\text{max}} \)) for Adh4 was about \( 140.5 \pm 6.5 \) \( \mu \text{mol min}^{-1} \text{mg}^{-1} \) with a \( K_m \) value of \( 50 \pm 6 \) mM for propionaldehyde (Fig. 4). When propionaldehyde was replaced by butyraldehyde or acetaldehyde, the \( V_{\text{max}} \) was \( 153.4 \pm 2 \) \( \mu \text{mol min}^{-1} \text{mg}^{-1} \) and \( 170.6 \pm 1.7 \) \( \mu \text{mol min}^{-1} \text{mg}^{-1} \) respectively (Fig. S1). When butyraldehyde was replaced by isobutyraldehyde, \( V_{\text{max}} \) was only 10\% of the optimum, 12.7 \( \pm 0.5 \) \( \mu \text{mol min}^{-1} \text{mg}^{-1} \). Oxidation of NADH was strictly propionaldehyde dependent and followed allosteric sigmoidal kinetics, the apparent \( K_m \) value for NADH was determined to be 0.27 mM. The pH and the temperature optima for Adh4 were at pH 7.0 and 30°C respectively. The activity of the enzyme rapidly decreased with either increasing pH of the assay buffer or temperature (Fig. S2), which is consistent with the pH and temperature preference of several other alcohol dehydrogenases (Reid and Fewson, 1994).

Adh4 also catalysed the reverse reaction, the oxidation of 1-propanol to propionaldehyde, which was strictly NAD\textsuperscript{+} dependent. NAD\textsuperscript{+} was reduced with 1-propanol with an activity of \( 0.63 \pm 0.02 \) \( \mu \text{mol min}^{-1} \text{mg}^{-1} \) and the apparent \( K_m \) value for NAD\textsuperscript{+} was determined to be 0.25 mM (Fig. S3). Surprisingly, while Adh4 exhibited bidirectional activity, Adh6 exhibited an extremely low propionaldehyde-reductase activity (\( V_{\text{max}} = 0.3 \) \( \mu \text{mol min}^{-1} \text{mg}^{-1} \)). While both the enzymes were purified under similar conditions, the activities of Adh4 and Adh6 are apparently different.

\textbf{Adh4 is an integral component of intact BMCs}

To determine the localization of Adh4, BMCs were enriched from \textit{A. woodii} cells grown on 1,2-PD. The enriched BMCs were separated on a 12% SDS polyacrylamide gel and a protein fractionation pattern similar to earlier studies (Chowdhury \textit{et al.}, 2020) was observed on staining the gel with Coomassie brilliant blue (Fig. 5). Since several proteins with expected molecular mass of...
Adh4 (~41 kDa) were observed on the polyacrylamide gel, antibodies were raised against Adh4 (see Experimental procedures) for the purpose of immunoblotting experiments.

The presence of Adh4 was determined immunologically with anti-Adh4 antibodies. Indeed, the anti-Adh4 antibody reacted with only one protein that also fitted in size to Adh4. These results clearly confirmed the presence of Adh4 within the BMCs. Further, the presence of the internal protein (PduP) within the same protein enrichment was also analysed immunologically. As seen in Fig. 5, the enriched preparation contained both the encapsulated signature enzymes Adh4 and PduP together within the same BMC preparation. In our earlier report, we proved that BMCs purified in this method were intact and enclosed (Chowdhury et al., 2020). Hence, the results presented here show that indeed Adh4 localizes within the BMCs and is likely involved in NAD⁺ recycling.

In vivo pull-down assay using His6-tagged PduB

To further confirm the observed internalization of Adh4 within the BMCs, potential binding interaction studies between the shell protein PduB and Adh4 were
performed. For this purpose, a strategy was developed where a DNA sequence encoding a His-tag was inserted at the 3'-end of the pduB and cloned into the pMTL82254 plasmid (Heap et al., 2007) containing the pdu promoter (see Materials and methods) and transformed into the wild type A. woodii. In our earlier study, we observed strong activation of the pdu promoter by 20 mM 2,3-BD (Chowdhury et al., 2020). Hence, transformed A. woodii cells were grown on 20 mM 2,3-BD as a sole carbon source and production of the His6-PduB was auto-induced during growth on 2,3-BD. Cell-free extracts containing His6-tagged PduB were prepared from the 2,3-BD grown cells and passed over a Ni-NTA affinity column. Low affinity binding proteins were washed out with buffers containing low imidazole concentrations (20 mM). His6-PduB was then eluted from the column with 250 mM imidazole in the elution buffer. SDS-PAGE of the eluted His6-PduB protein demonstrated that multiple proteins co-eluted with the major protein, PduB (Fig. 6A). Western blot analysis of the eluted protein with antibodies against PduB, PduT, PduP, PduC and Adh4 revealed that indeed PduB interacts with the outer shell protein PduT and also with the internal proteins PduC/PduP and Adh4 (Fig. 6B). Binding of PduB to Adh4 is consistent with the finding that Adh4 localizes within the BMCs and also further proves that Adh4 is an encapsulated protein that also interacts with the major shell protein, PduB.

Deletion of Adh4 causes delayed growth phenotype on 1,2-propanediol

As Adh4 was found associated with the BMCs and likely fulfills the essential function of NAD+ recycling within the BMCs during growth on 1,2-PD, we aimed to delete the gene (Awo_06220) encoding Adh4 from the A. woodii genome, and study the phenotype of the mutant. The immediate downstream gene of adh4 (Awo_06220) is a LysR-type regulator (Awo_06230) that was not modified to avoid hindrance to any other regulated processes (Fig. 2A). In order to create the adh4 deletion in A. woodii, a suicide plasmid pMTL84151_JM_dadh4 was generated, carrying approximately 1000 bp region (both upstream and downstream) flanking the adh4 gene. The plasmid was integrated into the chromosome at one flanking region under antibiotic pressure (thiamphenicol) and subsequently, disintegration was forced by the presence of 5-fluoroorotate since the plasmid contained the pyrE cassette together with its promoter for production of a functional orotate phosphoribosyltransferase (Westphal et al., 2018). The mutants were isolated using fructose as a sole carbon source. The potential adh4 mutants were screened and verified using PCR and sequence analysis. The adh4 mutant failed to grow on media containing 15 mM 1,2-PD as sole carbon source even after several attempts. Since A. woodii needs a longer adaptation for growth on 15 mM 1,2-PD, this may have caused the growth phenotype. In our earlier study, we reported when A. woodii cells grown on 100 mM formate to a stationary phase and then 15 mM 1,2-PD was added, a rapid growth on 1,2 PD was observed (Chowdhury et al., 2020). Both ΔpyrE and Δadh4 strains were grown on 100 mM formate as a sole carbon and energy source and on reaching the stationary phase (OD600 = 0.35), 15 mM 1,2-PD was added to the culture. Growth was monitored after addition of 1,2-PD and growth of Δadh4 strain was at least 54% slower than the ΔpyrE strain (doubling time of 9.6 ± 0.2 h versus 6.3 ± 0.3 h). The Δadh4 strain also grew to a much lower cell density compared to the ΔpyrE strain (OD600 of 0.60 versus 0.75) (Fig. 7). A similar observation of delayed growth phenotype and lower final optical density of a pduQ (propanol

![Fig. 6. His6-PduB interacts with the internal core enzymes of BMC. SDS-PAGE monitoring of the purification process of the His6-PduB from A. woodii. (A) After each purification step, 20 μg protein fractions was applied on to 12% polyacrylamide gel (Laemmli, 1970). Lane 1, cell-free extract of A. woodii producing His6-PduB. Lane 2-4, wash fractions. Lane 5-6, elution fractions. (B) The presence of PduB and PduT (i), PduP and Adh4 (ii) and PduC and His6-PduB (iii) was determined immunologically in the cell extracts, wash (W1, W6 and W7) and elution fractions (E1 and E2).]

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...dehydrogenase) deletion mutant in *S. enterica* was reported earlier (Cheng et al., 2012).

Further, we tested whether the Δadh4 mutant also displayed similar growth defects during growth on BMC-forming substrates like ethanol (50 mM) or 2,3-BD (20 mM). Therefore, the Δadh4 mutant and ΔpyrE strain were adapted and grown on the respective substrates. Growth was monitored and doubling time of both these strains were recorded. Compared to ΔpyrE, the Δadh4 mutant displayed only 13% and 10% prolonged doubling time when grown on ethanol or 2,3-BD respectively (data not shown). Apparently, deletion of *adh4* in *A. woodii* did not severely affect growth on ethanol or 2,3-BD as was observed on 1,2-PD.

**Δadh4 mutant produced less propanol during growth on 1,2-propanediol**

Further, to show that Adh4 is involved in the metabolism of 1,2-PD, we analysed the product pool of the mutant strains during growth on 1,2-PD. As evident from Fig. 8, 1,2-PD was completely consumed within 12 h by both, the ΔpyrE and Δadh4 mutant and propionate and propanol were formed as end products. As expected, ΔpyrE cells produced equimolar amounts of propanol and propionate (7.3 mM versus 8 mM) from 1,2-PD (15 mM). Strikingly, the Δadh4 mutant produced much less propanol compared to propionate (3.5 mM versus 13.6 mM). Though the consumption rate of 1,2-PD was similar for the ΔpyrE and Δadh4 mutant (3.9 mM/h versus 4 mM/h), the rate of propionate formation was markedly lower in the Δadh4 mutant (1.1 mM/h versus 0.3 mM/h). While the results for the ΔpyrE are in agreement with the stoichiometry of the fermentation balance: 1,2-propanediol → 0.5 propanol +0.5 propionate, the ratio of propionate to propanol formation was found to be 4:1 in the Δadh4 mutant. Apparently, deletion of *adh4* dis-balances the propionaldehyde to propanol pathway with a shift towards the formation of propionate.

**Resting cell experiments with Δadh4 mutant**

The stoichiometry of propanol and propionate formation from 1,2-PD was analysed in resting cells since carbon is not utilized for biomass production. Therefore the ΔpyrE and Δadh4 mutant were grown on 1,2-PD as described above and harvested until no further change in OD<sub>600</sub> was observed. The cells were washed two times, resuspended in 10 ml of reaction buffer with a total protein concentration adjusted to 1 mg ml<sup>−1</sup>. 1,2-PD was added to the cell suspension at time point zero (<i>t<sub>0</sub></i>) to a concentration of 15 mM. As shown in Fig. 9, 1,2-PD (15 mM) was rapidly degraded and completely consumed within 15 min by both the ΔpyrE and Δadh4 mutant. At the same time, at least 10 mM propionaldehyde appeared in the medium, which was then completely consumed in the next 135 min. In the ΔpyrE cells, the products propanol and propionate were formed in equimolar amounts (7.1 mM versus 7.5 mM) with a rate of 0.34 μmol min<sup>−1</sup> mg<sup>−1</sup> each. Interestingly, the same was not true for the Δadh4 mutant. Though propionaldehyde was consumed at a similar rate (0.10 μmol min<sup>−1</sup> mg<sup>−1</sup>) as in the ΔpyrE strain (0.11 μmol min<sup>−1</sup> mg<sup>−1</sup>), the rate of formation of propionate was much faster (0.5 μmol min<sup>−1</sup> mg<sup>−1</sup> but propanol formation was much slower (0.20 μmol min<sup>−1</sup> mg<sup>−1</sup>). As is evident from Fig. 9, the final concentration of propionate formed (10.7 mM) was more than the double of propanol formed (4.4 mM). Apparently, the Δadh4 mutant produced 0.9 mM of acetate compared to 0.1 mM by the ΔpyrE strain indicating that electrons are re-directed into the WLP.

**Discussion**

Bacterial microcompartments are essential for optimizing segments of metabolic pathways and also to encapsulate toxic intermediates (acetalddehyde or propionaldehyde) to reduce cytotoxicity (Cheng et al., 2008; Kerfeld et al., 2010; Jorda et al., 2013; Chowdhury et al., 2014). While BMCS are produced by various classes of bacteria in form of carboxysomes or metabolosomes, the acetogen *A. woodii* has recently been described to utilize BMCS for a non-acetogenic mode of life (Schuchmann et al., 2015). *Acetobacterium woodii* grows on 1,2-PD as a sole carbon and energy source to produce propionate.
and propanol as metabolic end products and it is speculated that \textit{A. woodii} produces BMCs during growth on diols and ethanol to encase toxic aldehyde intermediates generated during metabolism, as suggested for \textit{Salmonella} (Sampson and Bobik, 2008). In \textit{A. woodii}, aldehyde detoxification occurs in a bifurcated manner by two concomitant parallel enzymatic steps; in one direction by coenzyme A-dependent oxidation of aldehyde to its corresponding CoA thioester (propionyl-CoA) by CoA-dependent propanediol dehydrogenase (PduP) and on the other by reduction of aldehyde to its corresponding alcohol (propanol) by an uncharacterized alcohol dehydrogenase.

Existing knowledge of 1,2-PD metabolism by other organisms like \textit{Salmonella enterica} established that the 1-propanol dehydrogenase (\textit{pduQ}) is solely responsible for reduction of propionaldehyde to propanol and also for internal recycling of NAD⁺ (Cheng et al., 2012). In this study, we demonstrated that during growth of \textit{A. woodii} on 1,2-PD and 2,3-BD as sole carbon source, among 11 alcohol dehydrogenases encoded in the genome, two putative alcohol dehydrogenase genes, \textit{adh4} and \textit{adh6} were highly transcribed. While both gene products (Adh4 and Adh6) reduced propionaldehyde, Adh4 was by far much more active than Adh6. The finding that Adh4 co-purified with the intact \textit{A. woodii} BMC and also during \textit{in vivo} pull-down assays using His₆-PduB as a bait, indicated that Adh4 is indeed an integral component of \textit{A. woodii} BMCs. Adh4 exhibited high propionaldehyde reduction activity (~150 μmol min⁻¹ mg⁻¹) with low propanol oxidation activity (0.63 μmol min⁻¹ mg⁻¹), clearly indicating that the forward reaction towards propanol formation is favoured by the enzyme. While it is absolutely essential that at least one alcohol

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**Fig. 8.** Products formed from 1,2-PD by the \(\Delta\text{adh4}\) and \(\Delta\text{pyrE}\) mutant. \textit{Acetobacterium woodii} \(\Delta\text{pyrE}\) (A) and \(\Delta\text{adh4}\) (B) were grown as described in Fig. 7. Samples were drawn at different time points during growth and concentration of 1,2-PD (■), propionaldehyde (♦), propanol (●) and propionate (▲) was analysed by gas chromatography.

**Fig. 9.** Conversion of 1,2-PD by resting cells of \textit{A. woodii} \(\Delta\text{pyrE}\) and \(\Delta\text{adh4}\). \textit{Acetobacterium woodii} \(\Delta\text{pyrE}\) (A) and \(\Delta\text{adh4}\) (B) mutant strains were grown on 1,2-PD as described in Fig. 7, harvested, washed twice and resuspended to a final protein concentration of 1 mg ml⁻¹ in 10 ml buffer in 115-ml serum bottles under a N₂/CO₂ atmosphere (80:20 [vol/vol]). Samples were drawn at different time points for quantification of 1,2-PD (■), propionaldehyde (♦), propanol (●) propionate (▲) and acetate (×) by gas chromatography. All experiments were performed in duplicates.
dehydrogenase is present along with the PduP enzyme within the BMC for NAD\(^+\) recycling, this does not rule out the presence of a second alcohol dehydrogenase within the BMCs. *Acetobacterium woodii* grows on a variety of substrates like 2,3-BD, ethanol, ethylene glycol or 1,2-PD, which are individually metabolized utilizing different pathways and also involving multiple different alcohol dehydrogenases. Despite the difference of the substrates, BMCs are always produced. Therefore, it is also possible that a second alcohol dehydrogenase is present within the BMCs and plays an equally important role in reduction of aldehyde to its corresponding alcohol. While it has been reported that trafficking of internal enzymes into the BMCs is accomplished by encapsulation peptides generally located at the N- or C-terminus of each enzyme, such an encapsulation peptide could not be identified at the C-terminal end of BMC-associated alcohol dehydrogenase (Aussignargues et al., 2015). However, on closer inspection of the N- and the C-termini of both Adh4 and Adh6, a short peptide (\(~\sim 12\) aa) at the C-terminus, predicted to fold as an alpha-helix could be identified (See Fig. S4) using the JPred server (Cole et al., 2008). A similar short peptide sequence was also identified at the C-terminal end of PduQ, but the predicted alpha-helix fold does not fit the definition of an encapsulation peptide (no apparent amphipathicity of the helix) (Kinney et al., 2012). Moreover, it has been shown that PduQ forms a complex with PduP suggesting it may be encapsulated by piggy-banking (Cheng et al., 2012). In *A. woodii*, it is likely that the alcohol dehydrogenase Adh4 co-localizes within the BMCs by protein–protein interaction with PduB as revealed by pull-down experiments.

Though not being a part of the *pdu* cluster, deletion of the *adh4* gene indeed resulted in a slow growth phenotype compared to the \(\Delta pyrE\) strain when grown on 1,2-PD as carbon source. Theoretically, at the end of 1,2-PD metabolism by *A. woodii*, equimolar amounts of propionate and propanol should be formed as metabolic end products from metabolism of 15 mM 1,2-PD. Indeed, this was true in case of the \(\Delta pyrE\) strain but not in the \(\Delta adh4\) mutant that produced a much higher amount of propionate compared to propanol. In absence of Adh4, propionaldehyde reduction was severely impaired whereby the extra propionaldehyde (carbon) flux was pushed towards the formation of propionate. One would expect that on impairment of the NAD\(^+\) regeneration pathway, the mutant cells would exhibit a severe growth phenotype. In this case, our experimental results suggested that *A. woodii* has another alcohol dehydrogenase, which allowed NAD\(^+\) recycling with propanol formation albeit with much lower activity. Low concentrations of propanol could also be formed by Adh6, which was found to be highly expressed during growth on 1,2-PD or any other alcohol dehydrogenase that might be activated by deletion of *adh4*. It is possible that another alcohol dehydrogenase is either imported within the BMC or propionaldehyde traverses outside the BMC to be reduced to propanol for NAD\(^+\) regeneration. In either of the case, in the \(\Delta adh4\) mutant, this turns out to be less efficient. It is clear that Adh4 is required for a complete functional BMC and that its removal causes imbalance in 1,2-PD metabolism.

Furthermore, one question arises: how is the excess NADH reoxidized in the *adh4* mutant? It is likely that NADH is reoxidized in the Wood-Ljungdahl pathway, which is consistent with the observed production of acetate. How this is achieved remains open but it shows a complex electron-balancing between acetogenic and non-acetogenic lifestyle. In summary, in this study we could identify, characterize and illustrate the localization of the missing BMC-associated-alcohol dehydrogenase in *A. woodii*. We have successfully assigned function to one of the 11 alcohol dehydrogenases involved in propanol formation. This study also broadens our knowledge and understanding how metabolic modules involving BMCs are controlled in an ancient organism along with other energy conserving processes like chemiosmosis and ATP hydrolysis.

**Experimental procedures**

**Growth of A. woodii**

*Acetobacterium woodii* DSMZ 1030 and mutants were cultivated under strictly anoxic conditions at 30°C in carbonate buffered complex media as described previously (Heise et al., 1989). Fructose (20 mM), 1,2-propanediol (15 mM), 2,3-butanediol (20 mM) or ethylene-glycol (20 mM) served as carbon and energy source unless otherwise mentioned. For growth studies on 1,2-propanediol (1,2-PD), *A. woodii* strains (\(\Delta pyrE\) and \(\Delta adh4\)) were first grown on 100 mM sodium formate. At stationary phase when all carbon (formate) was consumed, 1,2-PD was given to a final concentration of 15 mM as described previously (Chowdhury et al., 2020). Growth was monitored by measuring the optical density at 600 nm. The growth experiments were performed in 115-ml serum flasks containing 50 ml of media.

**Quantitative PCR (qRTPCR) analysis.** RNA was prepared from *A. woodii* cells grown on 1,2-PD, 2,3-BD or ethylene glycol to mid-exponential growth phase as described earlier (Chowdhury et al., 2020). 1 \(\mu\)g of RNA from each sample was converted into cDNA by using M-MLV Reverse Transcriptase according to the manufacturer’s protocol (Promega, Mannheim, Germany). All quantitative PCRs were performed in triplicate, using...
Ssolfast Evagreen Supermix (Bio-Rad, CA, USA) with 1 ng cDNA as template and 500 nM of gene specific primers in a final reaction volume of 25 μl. Transcript levels of adh1, adh4 and adh6 were analysed using gene specific primers (Table S1). Transcript levels of all the genes tested were normalized to transcript levels of the gene gyrA as described previously (Chowdhury et al., 2020).

Cloning and overproduction of Adh4 and Adh6

The genes encoding Adh4 (Awo_c06220) and Adh6 (Awo_c25410) were amplified from chromosomal DNA of A. woodii with gene specific primer (Table S1). The PCR products were cloned into pET-21a (+) vector and the resulting plasmids were transformed into E. coli BL-21 (DE3) for overproduction of the His6 tagged Adh4 or Adh6 proteins. The transformants were grown at 37°C in LB medium to an OD600 of 0.6–0.8 and gene expression was induced by addition of isopropyl-β-D-galactopyranoside (IPTG) to a final concentration of 1 mM. After incubation at 16°C for 16 h, cells were harvested by centrifugation and disrupted by passage through a French press (SLM Instruments, United States) at 100 MPa. Proteins were purified using 6X-histidine tag from the cell lysates under strictly anoxic conditions using buffer A (50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl) containing 250 mM imidazole. The protein purity was analysed on a 12% denaturing SDS-polyacrylamide gel (Laemmli, 1970) and further staining with Coomassie brilliant blue G250 (Meyer and Lambert, 1965).

Measurement of enzymatic activities

All enzymatic assays were performed with His6-tagged Adh4 or Adh6. Measurements were carried out in 1 ml of anoxic buffer in 1.8 ml sealed cuvettes with a gas phase of 100% N2. The activities of Adh4 and Adh6 were measured by propionaldehyde-dependent oxidation of NADH at 340 nm with 10 μg of purified proteins in a 50 mM Tris–HCl buffer (pH 7.5) containing 50 mM NaCl, 2 mM DTE, 250 μM NADH and 50 mM propionaldehyde. The reverse reaction (1-propanol oxidation) was measured in a 25 mM CHES buffer (pH 9.0), 0.6 mM NAD+ and 400 mM 1-propanol. The increase of NADH concentration (ε = 6.3 mM⁻¹ cm⁻¹) (Ziegenhorn et al., 1976) for the determination of Vmax and Km values, the data were fitted by non-linear curve fitting using GraphPad Prism 5.01 Software (GraphPad Software, San Diego, CA).

Antibody generation and analytical method

Concentration of purified proteins was measured according to Bradford (Bradford, 1976). For generation of Adh4 antibody, Adh4-His6 protein (see above) was separated and analysed by SDS-PAGE and visualized by staining with Coomassie brilliant blue G250 (Sigma-Aldrich Chemie GmbH). Adh4 was cut from a Coomassie-stained SDS gel and used to immunize rabbits. Antisera prepared from immunized rabbits were used for blotting purpose. Other antibodies against PduB, PduT, PduP and PduC proteins were used as described earlier (Schuchmann et al., 2015). For immunological detection of BMC associated proteins, purified BMC fractions (10–20 μg) were separated on 12% polyacrylamide gels. Proteins were transferred on to a nitrocellulose membrane (Protran BA 83; GE Healthcare, United Kingdom) followed by immunoblotting with a 1:10,000 dilution of the rabbit antisera antibodies as earlier described (Hess et al., 2011).

Pull down assays

BMCs were routinely prepared from A. woodii cells grown on 1,2-PD or 2,3-BD as earlier described (Chowdhury et al., 2020). For homologous production of PduB and pull-down assay in A. woodii, the plasmid (291bpUp_pMTL82254_CAT) was used as a backbone (Chowdhury et al., 2020). The pduB gene including a DNA sequence at the 3’-end that encodes a 6X-histidine-tag was amplified using A. woodii genomic DNA as a template and cloned in to the 291bpUp_pMTL82254_CAT plasmid between the Ndel and NcoI restriction sites. In the resulting plasmid, the catP gene was replaced by pduB, allowing the pduB gene to be directly under the control of pdu promoter (291 bp). The plasmid was named 291bpUP_pMTL82254_PduB_His. Transformation was performed according to earlier described procedure (Westphal et al., 2018). The transformants were grown in volume of 2 l of carbonate buffered media (Heise et al., 1989) containing 20 mM 2,3-BD and 15 μg ml⁻¹ erythromycin, as 2,3-BD strongly induced the pdu promoter resulting in native-production of His6-PduB (Chowdhury et al., 2020). On reaching the stationary growth phase, cells were harvested by centrifugation and disrupted by passage through a French press (SLM Instruments, United States) at 100 MPa. Cell debris was removed by centrifugation at 17,000 × g for 20 min at 4°C. The supernatant was applied on a 2 ml of nickel-nitritolactric acid (Ni-NTA) column, which was equilibrated with buffer A. The histidine tagged PduB was eluted with two column volumes of 250 mM imidazole in buffer A. The purified His6-PduB and its interacting proteins were analysed using PAGE and further by western blotting.

Generation of A. woodii Δadh4 strain. The deletion of adh4 was made in A. woodii pyrE mutant for counter selective purpose (Wiechmann et al., 2020). The resulting strain is isogenic to the pyrE mutant except for
the adh4 deletion. For deletion of adh4, a plasmid pMTL84151_JM_dadh4 was constructed. Therefore, the adh4 deletion cassette was cloned in the multiple cloning site (MCS) of the background plasmid pMTL84151 (Heap et al., 2007), consisting of 1000 bp of upstream- and downstream flanking regions of adh4 (Awo_c06220), which enables the deletion of adh4 via homologous recombination. The constructed plasmid lacks a replicon for Gram positive bacteria and possesses a gene encoding for thiamphenicol resistance from Clostridium perfringens (Werner et al., 1977) and a heterologous pyrE gene cassette from Clostridium acetobutylicum as a counter selectable marker. Plasmid transformation into the A. woodii pyrE mutant and further integration and recombination of the adh4 deletion cassette was performed as described (Westphal et al., 2018). The deletion the adh4 gene was verified by amplifying the flanking regions and further by DNA sequencing analysis.

Preparation of cell suspensions and analysis
For cell suspension analysis, ΔpyrE and Δadh4 strains were grown as described earlier until no further change in OD600 was observed, harvested by centrifugation (10,000 × g; 10 min) and washed two times with imidazole buffer A (50 mM imidazole-HCl, 20 mM MgSO4, 20 mM KCl, 2 mM dithioerythritol (DTE), 1 mg litre⁻¹ resazurin, pH 7.0) under strictly anoxic conditions in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) filled with 95% to 98% N2 and 2% to 5% H2 as described previously (Heise et al., 1992). The protein concentration of the cell suspension was determined as described previously (Schmidt et al., 1963). Cells were resuspended in 115-ml glass bottles in resuspension buffer (imidazole buffer supplemented with 20 mM NaCl and 60 mM KHCO₃, pH 7.2) under a N₂/CO₂ atmosphere (80:20 [vol/vol]) such that the total protein concentration was 1 mg/ml in a volume of 10 ml. The suspensions were incubated at 30°C in a shaking water bath. Substrate/product analysis were done from 500 μl samples withdrawn with a syringe at different time points. The concentrations of 1,2-PD, 1-propanol, propionate and acetate were determined by gas chromatography as described previously (Schuchmann et al., 2015). The peak areas were proportional to the concentration of each substance and calibrated with standard curves. A total of 5 mM butanol was used as the internal standard for all measurements.

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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.** Dependence of Adh4 activity on the concentration of butyraldehyde (A) or acetaldehyde (B). Assays were performed with 10μg of purified proteins in a 50 mM Tris-HCl buffer (pH 7.5) containing 50 mM NaCl, 2 mM DTE, 250 μM NADH and the indicated concentrations of butyraldehyde or acetaldehyde. Activity was measured by respective aldehyde-dependent oxidation of NADH at 340 nm. Curve fitting and determination of the kinetic parameters $K_m$ and $V_{max}$ were performed using the GraphPad Prism program (version 5).

**Fig. S2.** pH (A) and temperature (B) dependence of propionaldehyde reduction catalyzed by Adh4. Measurement was carried out in 1 ml of anoxic buffer in 1.8 ml sealed cuvettes with gas phase of 100% N$_2$. Adh4 activity was measured by propionaldehyde-dependent oxidation of NADH at 340 nm. For pH optima, combined buffer mix of 25 mM each MES, MOPS, Tris and CHES with different pH values as indicated was used. For temperature optima measurement, assays were performed in Tris-HCl buffer (pH 7.5) at variable temperature as depicted. Each assay contained 10 μg of Adh4 enzyme, 2 mM DTE, 250 μM NADH and 50 mM propionaldehyde.

**Fig. S3.** 1-propanol-dependent reduction of NAD$^+$ catalyzed by Adh4. Measurement was carried out in 1 ml of anoxic buffer in 1.8 ml sealed cuvettes with gas phase of 100% N$_2$. 1-propanol oxidation was measured in a buffer mix of 25 mM each MES, MOPS, Tris, CHES (pH 9.0). Each assay contained 40 μg of enzyme, 400 mM 1-propanol and different concentrations of NAD$^+$. The increase in NADH concentration was monitored at 340 nm ($ε = 6.3$ mM$^{-1}$cm$^{-1}$). Curve fitting and determination of the kinetic parameters $K_m$ and $V_{max}$ were performed using the GraphPad Prism program (version 5).

**Fig. S4.** Encapsulation peptide prediction of Adh4 and Adh6. Encapsulation peptides (EPs) are characterized by a primary structure predicted to form an amphipathic $α$-helix. Secondary structure of the N- and C-terminal ends of Adh4, Adh6 of *A. woodii* and PduQ of *S. enterica* was predicted using the JPred server. For secondary structure prediction, 30 aa from either N- or C-terminal end of each protein were analysed. Helix region is highlighted in grey.

**Table S1.** Primers used in this study.