It does not always take two to tango: “Syntrophy” via hydrogen cycling in one bacterial cell

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Abstract
Interspecies hydrogen transfer in anoxic ecosystems is essential for the complete microbial breakdown of organic matter to methane. Acetogenic bacteria are key players in anaerobic food webs and have been considered as prime candidates for hydrogen cycling. We have tested this hypothesis by mutational analysis of the hydrogenase in the model acetogen Acetobacterium woodii. Hydrogenase-deletion mutants no longer grew on H₂ + CO₂ or organic substrates such as fructose, lactate, or ethanol. Heterotrophic growth could be restored by addition of molecular hydrogen to the culture, indicating that hydrogen is an intermediate in heterotrophic growth. Indeed, hydrogen production from fructose was detected in a stirred-tank reactor. The mutant grew well on organic substrates plus caffeate, an alternative electron acceptor that does not require molecular hydrogen but NADH as reductant. These data are consistent with the notion that molecular hydrogen is produced from organic substrates and then used as reductant for CO₂ reduction. Surprisingly, hydrogen cycling in A. woodii is different from the known modes of interspecies or intraspecies hydrogen cycling. Our data are consistent with a novel type of hydrogen cycling that connects an oxidative and reductive metabolic module in one bacterial cell, “intracellular syntrophy.”

Introduction
Molecular hydrogen is present only in trace concentrations (550 parts per billion) in the Earth’s atmosphere [1], but plays an important part in the global carbon cycle and is a major constituent of microbial metabolism. In anoxic ecosystems it is rapidly produced and consumed by microorganisms resulting in a large turnover [2]. Hydrogen connects different parts of the anaerobic food web and is usually produced by primary fermenters [3]. Fermentations typically yield between 1 and 4 mol of ATP per mol of sugar, and the maximum is only observed if electrons can be blown away into the environment as molecular hydrogen thus allowing the cells to make acetate according to Eq. (1) [4]:

\[
\text{C}_6\text{H}_{12}\text{O}_6 + 2\text{H}_2\text{O} + 4\text{ADP} + 4\text{Pi} \rightarrow 2\text{CH}_3\text{COOH} + 2\text{CO}_2 + 4\text{H}_2 + 4\text{ATP} \quad \Delta G^\circ = -206.3 \text{ kJ/mol (1)}
\]

However, hydrogen formation from reduced pyridine nucleotides or flavins is energetically unfavourable and growth according to Eq. (1) requires removal of hydrogen by a syntrophic partner such as a sulfate reducing bacterium, a methanogenic archaeon or an acetogenic bacterium [5–8]. The latter produces acetate according to Eq. (2):

\[
4\text{H}_2 + 2\text{CO}_2 + x\text{ADP} + x\text{Pi} \rightarrow \text{CH}_3\text{COOH} + 2\text{H}_2\text{O} + x\text{ATP} \quad \Delta G^\circ = -95 \text{ kJ/mol (2)}
\]

Since acetogens grow by conversion of H₂ + CO₂ to acetate, the reaction has to be coupled to net synthesis of ATP [9]. Detailed studies in the acetogenic model organism Acetobacterium woodii estimated the amount of ATP to 0.3 mol per mol of acetate produced [9].

In contrast to methanogenic archaea, acetogenic bacteria do not only grow lithotrophically according to Eq. (2) but also by fermentation [10]. Acetogenesis is a modular
metabolism with an oxidative and a reductive branch [11] (Fig. 1). In the oxidative branch, hydrogen (during litho-
trophic growth) or an organic carbon and energy source
(during heterotrophic growth) are oxidised. Electrons are
carried over to the reductive branch (the Wood-Ljungdahl
pathway [WLP]) in which 2 mol of CO₂ are reduced to
acetate according to Eq. (2). Overall, fermentation of fruc-
tose to three molecules of acetate by a combination of
Eqs. (1) and (2) gives the highest ATP yield in fermenting
bacteria of 4.3 mol ATP/mol of sugar [11].

Electron carriers involved in transferring electrons
from the oxidative to the reductive branch in acetogens
are NADH, NADPH, or reduced ferredoxin [9]. A third
module, the redox balance module, ensures that the
different reduced electron carriers from the oxidative module
are fed in and are converted to the specific redox carriers
required by the WLP [11]. In the model acetogen
A. woodii, 2 mol of NADH from glycolysis and 2 mol of
reduced ferredoxin from conversion of pyruvate to acetyl-
CoA are converted to 1 mol of hydrogen, 2 mol of NADH
and 1 mol of reduced ferredoxin by the combined action
of the Rnf complex and the electron-bifurcating hydro-
genase [11, 12]. NADH is used as reductant for the
methylenetetrahydrofolate (THF) reductase [13] and the
methylenethf dehydrogenase [14], and reduced ferre-
doxin is used by the CO dehydrogenase/acetyl-CoA
synthetase as electron donor for CO₂ reduction in the car-
bonyl branch of the WLP [15, 16]. In contrast, the first
enzyme used for CO₂ reduction in the methyl branch of A.
woodii (Fig. 1), the hydrogen-dependent CO₂ reductase
(HDCR), uses H₂ as reductant in vitro [17], indicating the
need for electron transfer via hydrogen from the oxidative
module (glycolysis) to the reductive module (CO₂
reduction). On the other hand, the purified HDCR can also
accept electrons from reduced ferredoxin, albeit with
17-fold lower activities [17]. To address a potential
hydrogen transfer from the oxidative to the reductive
branch of the acetogenic metabolism, we have deleted the
only hydrogenase in A. woodii and studied the phenotype
of the mutant.

Materials and methods

Growth of A. woodii

A. woodii DSM1030 was cultivated at 30 °C under anoxic
conditions in complex medium as previously described
[18]. When using the pyrE deletion mutant, 50 mg/l uracil
was added to the medium [12]. Unless otherwise sta-
ted 20 mM fructose or 20 mM fructose together with H₂ +
CO₂ (80:20 [v/v]) were used as carbon source. Gaseous

Fig. 1 The modularity of acetogenesis in A. woodii. Shown are the
oxidation of fructose to acetate in the oxidative branch (left) and the
reduction of CO₂ to acetate (right) in the reductive branch (WLP).
Redox balancing is achieved by a third module, in which the Rnf
complex and the electron-bifurcating hydrogenase produce the
reductants required for the the WLP. Fd, ferredoxin; Fd²⁻, reduced
ferredoxin; THF, tetrahydrofolate; HDCR, hydrogen-dependent CO₂
reductase; CODH/ACS, carbon monoxide dehydrogenase/acetyl-CoA
synthetase; Co-Fe-S-P, corrinoid iron-sulfur protein.
substrates were used at a pressure of 1.0 × 10^5 Pa. For growth experiments, concentrations of the used carbon sources were as follows: lactate, 80 mM; ethanol, 50 mM; formate, 100 mM. Minimal medium used for genetic manipulations was prepared as previously described [19] in which yeast extract was omitted and higher amounts of 0.2 g l^{-1} KH_{2}PO_{4}, 1.35 g l^{-1} NH_{4}Cl, and 1.5 ml l^{-1} of selenite/tungstate solution were used and 10 μg ml^{-1} of νl-panthenolate was added. For mutant selection, 1.7 μg ml^{-1} uracil and 1.5 mg ml^{-1} 5-fluoroorotic acid were added.

**Genetic modifications**

For deletion of the hydBA genes, an uracil auxotroph pyrE deletion mutant was generated using the suicide plasmid pMTL\_AW\_KO1. The background of the plasmid pMTL\_AW\_KO1 is pMTL84151 [20] out of which the Gram+ origin of replication was partially deleted by digestion of the vector with XmnI and FspI following a blunt-end ligation. The pyrE deletion cassette was cloned into the multiple cloning site, consisting of a 393 bp upstream flanking region including the first 52 bp of pyrE and a 399 bp downstream flanking region including the last 68 bp of pyrE. Both flanking regions were amplified via PCR, joined by splice-by-overlap-PCR (SOE-PCR) and cloned into the plasmid using BamHI and EcoRI. Transformation and integration of the plasmid into the A. woodii wild type as well as recombination of the plasmid at its homologous regions toward the loss of the pyrE gene has already been described elsewhere [12]. The deletion of 456 bp of the pyrE gene was verified by DNA sequencing analysis [21]. The suicide plasmid pMTL\_AW\_KO2 for the in-frame deletion of hydBA genes in A. woodii was built in the pMTL\_AW\_KO1 background. First, the pyrE cassette from Eubacterium limosum KIST612, consisting of the pyrE gene (ELI\_0961) and 66 bp of its promoter region, was placed behind the catP resistance marker cassette to be used as a counter selectable marker as described previously [12], generating pMTL\_AW\_KO1\_pyrE\_Elim. Second, both fragments of the hydBA deletion cassette, consisting of a 486 bp upstream flanking region including the first 18 bp of hydB and a 462 bp downstream flanking region including the last 15 bp of hydBA, where amplified via PCR, ligated via SOE-PCR and were cloned into pMTL\_AW\_KO1\_pyrE\_Elim by using EcoRI and XbaI, which replaced the original pyrE deletion cassette with the hydBA deletion cassette. For plasmid transformation into the pyrE mutant and further integration and recombination of the hydBA deletion cassette, the same protocol as for the pyrE gene deletion, as described above, was followed. The deletion of 3538 bp of the hydBA region was verified by DNA sequencing analysis [21].

**Preparation of cell-free extracts**

Cells were harvested in the late exponential growth phase and resuspended in lysis buffer, containing 25 mM Tris-HCl buffer, pH 7.8, 420 mM sucrose, 2 mM DTE, 4 μM resazurin, for a 1 h treatment with 2.8 mg ml^{-1} lysozyme. After washing the protoplasts in analytical buffer, containing 25 mM Tris-HCl, pH 7.6, 20 mM MgSO_{4}, 20 % [v/v] glycerol, 2 mM DTE, 4 μM resazurine, 0.5 mM PMSF, 0.1 mg ml^{-1} DNaseI, protoplasts were passed twice through a french pressure cell at 110 MPa (Thermo, Needham Heights, MA, USA). The cell-free extract was separated from cell debris by centrifugation at 12,000 × g for 15 min.

**Hydrogenase activity assays**

The activity of the electron-bifurcating hydrogenase was determined as described previously [22].

**Western blot analysis**

For detection of HydB and HydA subunits in cell-free extracts, Western blot analysis was performed as described before [23].

**Stirred-tank reactor cultivations**

Bioreactor cultivations were conducted in a 21 working volume Biostat Aplus fermenter (Sartorius, Melsungen, Germany). The vessel was equipped with temperature probe, sparger, baffles, two Rushton-impeller, pH-probe (Hamilton, Bonaduz, Switzerland) and a redox potential probe (Hamilton, Bonaduz, Switzerland). The gas stream into the reactor was maintained at a constant rate by a digital mass-flow controller (Bronkhorst High-Tech, Ruurlo, Netherlands).

**Analytical methods**

Protein concentrations were determined by the method described by Bradford [24]. Fructose, formate and acetate concentrations were measured enzymatically (Hoffmann-La Roche, Basel, Switzerland). Hydrogen concentrations were determined by gas chromatography (GC) as described previously [17, 25]. Fermentation off-gas analysis was conducted by a Micro-GC (Inficon, Bad Ragaz, Switzerland) equipped with two measurement modules. Module one had argon as carrier gas for determination of hydrogen, oxygen and nitrogen, module two had helium as carrier gas for determination of carbon dioxide and water. Sampling time of the GC was 25 s. The analytical conditions for module one were: injector temperature, 90 °C; column pressure, 2.07 × 105 Pa; column temperature, 80 °C, column, Rt-Molsive 5 Å, 0.25 mm × 10 m with a Rt-Q-Bond, 3 m precolumn and for module two:
injector temperature, 90 °C; column pressure, 1.72 x 10^5 Pa; column temperature, 60 °C; column, Rt-Q-Bond, 0.25 mm x 8 m. Each module was equipped with a thermal conductivity detector and the sampling rate was set to 100 Hz. Analysis time was 90 s for both modules. Off-line samples were taken in 2 ml volume. OD_{600} was measured in a spectrophotometer, Genesys 10 s UV-Vis (Thermo Scientific, Madison, USA) at a wavelength of 600 nm. Samples were diluted with 0.9% NaCl solution, if necessary. Samples were centrifuged at 16,363 x g for 10 min and the supernatant was used for analysis of acetic acid, formic acid and fructose using enzymatic assays as described above.

Results

Deletion of the hydBA genes of the hydrogenase operon (hydCEDBA)

Apart from the hydrogenase module in the HDCR, A. woodii has only one hydrogenase (HydABCD), a soluble, electron-bifurcating enzyme [22]. To delete the genes coding for the two major subunits HydB (Awo_c26980) and HydA (Awo_c26970) of the electron-bifurcating hydrogenase encoded by the hydCEDBA operon (Awo_c27010-Awo_c26970), the suicide plasmid pMTL_AW_KO2 was generated. The plasmid contains homologous flanking regions of ~480 bp upstream and downstream of the hydBA genes each leaving 15 bp plus the start codon of hydB and 12 bp plus the stop codon of hydA intact after deletion. The suicide plasmid was integrated into the chromosome at one of the flanking regions under antibiotic pressure. The following desintegration was forced by the presence of 5-fluoroorotate since the plasmid contains a pyrE gene together with its promoter for production of a functioning orotate phosphoribosyltransferase. However, isolation of mutants using fructose as only carbon source failed. Since this may have been caused by the lack of molecular hydrogen produced by HydABCD, hydrogen was added to the culture. Indeed, colonies were obtained, isolated and characterised. PCR (Fig. 2a) and DNA sequencing analyses confirmed the absence of hydBA in the genome. HydB and HydA could not be detected with antibodies against the two subunits (Fig. 2b) and cell-free extract of these cells did not catalyse hydrogen-dependent NAD$^+$ and ferredoxin reduction. These experiments demonstrate that hydBA were deleted.

Hydrogen restores growth of the hydBA mutant on organic substrates

That growth of the hydBA mutant on fructose is dependent on the addition of hydrogen is in line with the hypothesis that hydrogen is an electron carrier between the oxidative and reductive branch of acetogenesis (Fig. 1). This is also supported by the observation that the mutant did not grow on lactate or ethanol without addition of hydrogen. Formate is an intermediate of the WLP, and is dismutated to CO$_2$ and acetate. As expected, the hydBA mutant did not grow on formate and addition of hydrogen also did not restore growth of the mutant (Table 1). Formate oxidation to CO$_2$ yields hydrogen and obviously, the hydrogenase is essential to reduce NAD and ferredoxin, two electron carriers essential for CO$_2$ reduction, with H$_2$ as reductant.

Hydrogen transfer in A. woodii

Next, we tested for hydrogen consumption and production during growth of the mutant and the wild type in bicarbonate-supplied medium supplemented with 20 mM

![Image](https://example.com/image.png)

Table 1 Growth of the A. woodii wild type and the hydBA mutant in bicarbonate-supplied medium supplemented with different carbon sources.

|                | Without H$_2$ | Addition of H$_2$ |
|----------------|--------------|------------------|
| **Wild type**  |              |                  |
| HydBA mutant   |              |                  |
| **Wild type**  |              |                  |
| HydBA mutant   |              |                  |

| Carbon source   | Without H$_2$ | Addition of H$_2$ |
|-----------------|---------------|-------------------|
| Fructose        | ±             | +                 |
| Lactate         | ±             | +                 |
| Ethanol         | ±             | +                 |
| 2,3-Butanediol  | ±             | +                 |
| Formate         | ±             | +                 |

The addition of hydrogen (H$_2$ + CO$_2$, 80:20 [v/v], at 1.0 x 10$^5$ Pa) is indicated. Growth was observed, whereby a plus symbol (+) indicates growth and a minus symbol (−) indicates no growth.
fructose under an H2 + CO2 (80:20 [v/v]) atmosphere. In theory, 1 mol of hydrogen should be produced by the electron-bifurcating hydrogenase per mol of fructose consumed, which then should be used by the HDCR for CO2 reduction (Fig. 1). The growth rate of wild type and mutant (Fig. 3a) was similar (each ~0.16 h⁻¹) as well as the acetate:fructose ratio of 3:1 in the stationary phase (Fig. 3b), but the mutant cells used only ~20 mM of hydrogen (and 20 mM fructose), in contrast to the wild type which consumed all the hydrogen (Fig. 3a). This result confirms that the HDCR requires hydrogen and that hydrogen is produced by the electron-bifurcating hydrogenase when grown on substrates such as fructose, lactate or ethanol.

**Initial stirred-tank reactor (STR) cultivation of the A. woodii wild type revealed in vivo hydrogen formation**

If hydrogen is produced in vivo to connect the oxidative and reductive parts of acetogenesis, then, due to its volatile nature, it should be detectable in the gaseous phase. To determine a possible hydrogen production, a bench-scale anaerobic bioreactor for cell cultivation was set up together with off-gas analysis via GC. Initial cultivations of the A. woodii wild type in bicarbonate-supplied medium with 20 mM fructose as carbon and energy source showed exponentially rising hydrogen flow rates in the off-gas of up to 0.24 µmol min⁻¹ (Fig. 4). Hydrogen levels in the off-gas dropped to 0.1 µmol min⁻¹ after fructose was completely consumed. The cultures reached a maximum OD₆₀₀ of 2.28 after 24 h of cultivation, the growth rate was 0.19 h⁻¹. From the available 20 mM fructose, 50 mM of acetate was produced after 24 h. Overall 2.4 mol of acetic acid and 8.4 ± 0.2 mmol of hydrogen were produced per mol of fructose consumed. A. woodii requires Na⁺ [18] and a functional Rnf complex for energy conservation [12]. When grown with fructose in the absence of Na⁺, the acetate:fructose ratio of the wild type decreased to 2 and 2.1-times more hydrogen was released. Similar results were observed for the rnf deletion mutant. Maximum values for OD₆₀₀ and growth rate were 1.85 and 0.13 h⁻¹ for the rnf mutant and 1.68 and 0.11 h⁻¹ for the wild type (data not shown). Table 2 summarises the above mentioned fermentation parameters.
Replacing the WLP with other, non-hydrogen consuming reductive pathways restores growth of the hydBA mutant on fructose

H₂ + CO₂ is converted to formate by the HDCR [17]. Therefore, we speculated that the hydBA mutant should be able to grow on fructose + formate (+ CO₂) instead of fructose + CO₂, since formate reduction only requires NADH and reduced ferredoxin (Fig. 5). Indeed, the hydBA mutant grew with a doubling time of ~5 h on 20 mM fructose and 100 mM formate, which is slightly slower than the growth of the wild type (~3.5 h−4 h−1). The final OD₆₀₀ of the hydBA mutant reached 80–85 % of the final OD₆₀₀ of the wild type.

A. woodii can use caffeate as electron acceptor alternative to CO₂ [26, 27]. Reduction of caffeate to hydrocaffeate does not require hydrogen but only NADH as reductant [28] (Fig. 6a). Therefore, the hydBA mutant should be able to grow on, for example, fructose + caffeate. After addition of fructose + caffeate to the wild type and the mutant, growth of the two strains could be detected (Fig. 6b). Doubling times of the mutant and the wild type were similar (~6 h), but growth of the mutant culture stopped much earlier, with a final OD₆₀₀ of 0.68 compared with 3.2 of the wild type. The mutant also grew on lactate + caffeate (Fig. 6c) or ethanol + caffeate (Fig. 6d), but the growth rate of the hydBA mutant was one fifth and one half of the growth rate of the wild type, respectively. Like for fructose + caffeate, the final OD₆₀₀ was 0.47 or 0.25 for the mutant and 1.5 or 0.78 for the wild type when grown on lactate + caffeate or ethanol + caffeate, respectively. The lower final OD₆₀₀ may be due to the inability of the strains to use the HDCR reaction for recapturing CO₂ which is produced during the oxidation of the respective substrate.

Table 2 Selected fermentation parameters from STR-cultivations of the A. woodii wild type (+ Na⁺) and the rnf mutant.

| Parameter        | WT + Na⁺ | WT − Na⁺ | rnf mutant |
|------------------|----------|----------|------------|
| OD₆₀₀, max/−     | 2.28 ± 0.06 | 1.66 ± 0.01 | 1.85 ± 0.01 |
| μ/h⁻            | 0.19     | 0.11     | 0.13       |
| Y₆₅₀/mmol mol⁻¹  | 2.44 ± 0.02 | 2.01 ± 0.05 | 2.06 ± 0.03 |
| Y₇₅₂/mmol mmol⁻¹ | 8.39 ± 0.25 | 18.74 ± 0.62 | 17.81 ± 1.84 |

OD₆₀₀, max, maximum OD; μ, growth rate; Y₆₅₀, yield of acetic acid per fructose; Y₇₅₂, yield of hydrogen per fructose; WT, wild type.

Discussion

Sulfate reducing bacteria grow by oxidation of lactate or other organic substrates coupled to energy conservation by the reduction of sulfate [29, 30]. They also grow lithotrophically and, indeed, hydrogen is the most effective hydrogen donor for sulfate reduction [4, 29]. This led Odom and Peck to postulate hydrogen cycling as a general mechanism for energy coupling in sulfate reducing bacteria [31] and they postulated this mechanism to be present in methanogens and acetogens as well [5]. According to their model, hydrogen is produced inside the cell and diffuses across the membrane to the periplasm. The authors speculated that the same organism oxidises hydrogen to 2 H⁺ + 2e⁻ at the periplasmic side of the membrane, thus, producing scalar protons that create a proton motive force (pmf) across the cytoplasmic membrane that drives ATP synthesis. This mechanism requires a soluble, cytoplasmic hydrogenase and a periplasmic, membrane-bound hydrogenase. Biochemical and physiological experiments performed are in line with this hypothesis [32]. More than 35 years later, hydrogen cycling as a model of pmf generation was directly demonstrated by mutational analyses for the archaeon Methanosarcina barkeri [33]. When grown on methanol, 25 % of the substrate is oxidised to CO₂ to generate the electrons needed to reduce the other 75 % to methane. The oxidation of methanol is coupled to the reduction of protons to H₂, as catalysed by the F₄₂₀-reducing hydrogenase. Hydrogen diffuses out of the cell and is harnessed by the membrane-bound Vht hydrogenase that reduces the membrane-integral electron-carrier methanophenazine, the electron donor for the respiratory enzyme, the heterodisulfide reductase [33]. The proton potential established by the heterodisulfide reductase drives the synthesis of ATP in this archaeon [34]. Deletion of the soluble hydrogenase abolished hydrogen formation and deletion of the Vht hydrogenase was lethal, demonstrating nicely that there is hydrogen cycling in one cell, important for energy conservation [33].

The second type of hydrogen transfer in anoxic ecosystems is observed between different species and is called interspecies hydrogen cycling [5]. There, one partner oxidises a substrate linked to the production of hydrogen. Since hydrogen formation is thermodynamically unfavourable, the hydrogen producer can only survive if the hydrogen concentration in the environment is kept low by hydrogen oxidising microorganisms such as methanogenic archaea [6, 7].

Whether or not hydrogen cycling occurs in the ecophysiology relevant group of acetogenic bacteria remained to be established. In this report, we show clear evidence that A. woodii releases hydrogen into the atmosphere but only ~8.4 mmol hydrogen per mol of fructose was liberated, which is less than 10 % of the acetic acid generated, but similar to the result of Braun et al. [35]. This result indicates that the HDCR efficiently captures the hydrogen produced by the electron-bifurcating hydrogenase. If the electron-bifurcating hydrogenase is missing, cells growing on fructose, ethanol or lactate are unable to produce hydrogen needed for CO₂ reduction by the HDRC.
and therefore are unable to grow, except when they grow mixotrophically on fructose + hydrogen or fructose + formate. This is also clear evidence that hydrogen evolution takes place inside the cell and hydrogen is directly used within the cell before it can diffuse through the cell membrane. Since A. woodii lacks a membrane-bound hydrogenase, hydrogen oxidation is not linked to energy conservation and, therefore, does not fall into the category “intraspecies hydrogen cycling linked to energy conservation”, as postulated by Odom and Peck [5].

In contrast, A. woodii combines the metabolic features of two syntrophic partners in one bacterial cell. Depending on the environmental conditions A. woodii can play the part of the fermenting partner as in coculture with a methanogen [36] or the hydrogen consuming partner in syntrophic interactions. When grown together with methanogens on H₂ + CO₂, acetogens usually would be outcompeted by methanogens since methanogenesis from H₂ + CO₂ delivers much more energy than acetogenesis [37]. However, under certain conditions acetogens dominate as H₂ + CO₂-consuming partner in syntrophic interactions. For example, hydrogen-utilising acetogens compete successfully with hydrogen-utilising methanogens in wood-feeding termites [38]. Further studies showed that acetogens can outcompete methanogens for hydrogen at a pH of 6.2 and also at more acidic pH values [39] and at low temperature, for example at an in situ temperature of 4 °C in sediments of Lake Constance [40]. The partial pressure of hydrogen measured in pore water is too low to allow growth of pure cultures and it is speculated that the in situ partial pressure of hydrogen might be higher for acetogens living in close proximity to the hydrogen-producing organism [38]. Here, we demonstrate that, in addition, A. woodii can also play both parts—the fermenting and the hydrogen consuming part—in one cell. This is the closest proximity one can get. We propose to call this novel type of hydrogen cycling that connects an oxidative and reductive metabolic module in one bacterial cell “intracellular syntrophy.”

Acetogenic microorganisms are phylogenetically very divers [41]. Acetogenesis has been found in different phylogenetic clades of the Bacteria and has been studied there for the last hundred years, but rather recently acetogenesis was also found in different phyla of the Archaea. This is not only based on genomic but also on physiological analyses [42–44]. These archaea are supposed to grow autotrophically on H₂ + CO₂ to produce acetate but also on organic substrates. It is postulated that they ferment the organic substrates such as short chain fatty acids to acetate, alcohols and molecular hydrogen [43]. At the same time the WLP can act as electron sink to make the fermentation energetically possible, which makes fermentation independent from a syntrophic partner [44]. Our mutational analyses in A. woodii fully support this model.

The coupling of hydrogen-dependent CO₂ reduction to hydrogen-producing fermentations allows acetogens to grow, for example, in the deep biosphere on substrates that are
otherwise inaccessible for energetic reasons. Hydrogen production from fermentation is common in aquatic sediments and hydrogen production and consumption is essential for anaerobic food webs [45]. Hydrogen production from organic substrates also puts a new perspective on the origin of the eukaryotic cell [43]. Many acetogens have just one soluble hydrogenase like \textit{A. woodii} [46], others such as \textit{Moorella thermoacetica} [47], \textit{Thermoanaerobacter kivui} [48] or \textit{Heimdallarchaeota} and \textit{Odinarchaeota} [43] have membrane-bound, ion-translocating hydrogenase activities as hydrogen consuming respiratory enzymes. Their role in hydrogen transfer is still an open question.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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