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BRIEF REPORT

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No acetogen is equal: Strongly different H_2 thresholds reflect diverse bioenergetics in acetogenic bacteria

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Abstract

Acetogens share the capacity to convert H_2 and CO_2 into acetate for energy conservation (ATP synthesis). This reaction is attractive for applications, such as gas fermentation and microbial electrosynthesis. Different H_2 partial pressures prevail in these distinctive applications (low concentrations during microbial electrosynthesis [<40 Pa] vs. high concentrations with gas fermentation [>9%]). Strain selection thus requires understanding of how different acetogens perform under different H_2 partial pressures. Here, we determined the H_2 threshold (H_2 partial pressure at which acetogenesis halts) for eight different acetogenic strains under comparable conditions. We found a three orders of magnitude difference between the lowest and highest H_2 threshold (6 ± 2 Pa for *Sporomusa ovata* vs. 1990 ± 67 Pa for *Clostridium autoethanogenum*), while *Acetobacterium* strains had intermediate H_2 thresholds. We used these H_2 thresholds to estimate ATP gains, which ranged from 0.16 to 1.01 mol ATP per mol acetate (*S. ovata* vs. *C. autoethanogenum*). The experimental H_2 thresholds thus suggest strong differences in the bioenergetics of acetogenic strains and possibly also in their growth yields and kinetics. We conclude that no acetogen is equal and that a good understanding of their differences is essential to select the most optimal strain for different biotechnological applications.

INTRODUCTION

Acetogenic bacteria (or acetogens) are a group of phylogenetically diverse anaerobes (Drake et al., 2008). They share their ability to convert carbon dioxide (CO_2) and molecular hydrogen (H_2) into mainly acetate by the Wood–Ljungdahl pathway (WLP) as given by Equation (1) (Schiel-Bengelsdorf & Dürre, 2012):

$$2\,CO_2 + 4\,H_2 \rightarrow CH_3COO^- + H^+ + 2\,H_2O \qquad (1)$$

Acetogens are suitable candidates for different biotechnological applications. In synthesis gas (syngas) fermentation, acetogens convert a gas mixture consisting of mainly H_2, CO_2 and/or CO into organic compounds (Bertsch & Müller, 2015). The percentage of H_2 in the gas mixture varies depending on the origin of the syngas, but is usually relatively high, for example 9%–32% (Munasinghe & Khanal, 2010). Typical acetogens used for syngas fermentation are *Clostridium ljungdahlii*, *Clostridium autoethanogenum*, and *Acetobacterium woodii* (Bengelsdorf et al., 2018; Kantzow et al., 2015; Köpke et al., 2010).

Microbial electrosynthesis (MES) is another biotechnological application using acetogens, in which cathodes deliver electrons for CO_2 reduction (Nevin et al., 2010). In MES, H_2 evolves in situ on the cathode surface at sufficiently negative potentials (Rabaey & Rozendal, 2010). The H_2 partial pressure at the cathode surface results from the balance between electrochemical H_2 evolution and microbial H_2 consumption

and is often much lower than with syngas fermentation (e.g., <40 Pa; Deutzmann et al., 2015; Nevin et al., 2010). Acetogenic enrichments and single acetogenic strains have been tested in bioelectrochemical systems. *Sporomusa ovata* so far leads to the highest conversion rates (51.1 g of acetate m^{-2} d^{-1}) in pure culture (Das et al., 2020; Madjarov et al., 2022), while enrichments are usually dominated by *Acetobacterium* (Philips, 2020).

Similarly as on a cathode, anoxic oxidation of metallic iron (Fe^0) generates H_2. Acetogens are able to consume this H_2 and thereby cause microbial induced corrosion (Philips et al., 2019). Acetogenic strains related to *Acetobacterium malicum*, *Acetobacterium wieringae* and *Sporomusa sphaeroides* have been isolated from Fe^0 (Kato et al., 2015; Philips et al., 2019), while the H_2 partial pressure during this process was below 0.15% (Philips et al., 2019).

Interestingly, Kato et al. (2020) also observed that different acetogens prevailed in microbial communities enriched at low versus high H_2 partial pressures. This difference is likely related to different H_2 consumption characteristics of acetogens, which results in competitive benefits depending on the H_2 partial pressure.

One of the parameters reflecting the H_2 consumption characteristics of acetogens is the H_2 threshold (Philips, 2020). The H_2 threshold is defined as the H_2 partial pressure at which acetogenesis halts, because it is no longer thermodynamically favourable (Cord-Ruwisch et al., 1988; Kotsyurbenko et al., 2001). So far, H_2 thresholds are only available for just a few acetogens. Philips (2020) reviewed the experimental H_2 thresholds previously reported for acetogens. Most of the available data refers to *A. woodii*, since this is a well-studied model strain. However, the H_2 thresholds values reported for *A. woodii* show high variability and range between 14 and 250 Pa (Conrad & Wetter, 1990; Cord-Ruwisch et al., 1988; Le Van et al., 1998; Leclerc et al., 1997; Peters et al., 1998; Poehlein et al., 2012). Different factors influence the H_2 threshold, including the partial pressure of CO_2, total pressure, the pH, and temperature (Hoehler et al., 1998; Philips, 2020), which were not consistent among the different studies reporting experimental H_2 threshold values. Therefore, comparable conditions are needed to compare the H_2 threshold of different acetogens (Philips, 2020).

The H_2 threshold also depends on the bioenergetics of the acetogen (Poehlein et al., 2012). Hence, the measurement of H_2 thresholds could be a useful tool to evaluate the energy metabolism of acetogens. Acetogens share the WLP pathway, but the WLP pathway does not yield ATP itself. For energy conservation, acetogens use chemiosmotic ion gradient-driven phosphorylation coupled to the WLP (Schuchmann & Müller, 2014). Acetogens differ in the cation involved in the chemiosmotic gradient (Na^+ or H^+), the module of energy conservation (Rnf or Ech complex), and other enzyme complexes involved in their energy conservation strategy (Katsyv & Müller, 2020; Rosenbaum & Müller, 2021).

The energy conservation mechanism has only been fully unravelled for few acetogens, as it requires extensive biochemical and enzymatic characterizations. When the mechanism is completely known, the ATP gain (mole ATP per mol acetate) can be determined from the balanced stoichiometry of the electron carriers that generate the chemiosmotic gradient (Schuchmann & Müller, 2014). For *A. woodii*, an ATP gain of 0.3 mol ATP per mol acetate from H_2 and CO_2 has been determined (Katsyv & Müller, 2020). So far, however, the ATP gain is still uncertain or unknown for other acetogens. Nevertheless, insights into the ATP gain would be useful for comparing the bioenergetics of different acetogens.

Here, we measured the H_2 thresholds in comparable conditions of eight acetogenic strains, which are of biotechnological interest and are known to prevail under different H_2 levels. In addition, we estimated the ATP gain from the experimental H_2 threshold and the experimental conditions. Insights into H_2 consumption characteristics and ATP gains of acetogens are relevant to allow the selection of the most optimal acetogen strains for different biotechnological applications.

EXPERIMENTAL PROCEDURES

Microorganisms and culture conditions

The H_2 threshold of eight different acetogenic strains was determined. The acetogenic strains included in this study were *S. ovata* (DSM 2662 and DSM 2663), *S. sphaeroides* (DSM 2875), *A. malicum* (DSM 4132), *A. woodii* (DSM 1030), *A. wieringae* (DSM 1911), *C. autoethanogenum* (DSM 10061) and *C. ljungdahlii* (DSM 13528). All the strains were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). Strains were revived in the DSMZ recommended medium with betaine or fructose as substrate and stored frozen at −80°C in 10% dimethyl sulfoxide.

Before the experiment, frozen stocks were revived in their respective medium with organic substrate and transferred once. When the late exponential growth phase was reached, the culture was filtered through a 0.2 μm cellulose microcrystalline filter and washed two times with autotrophic growth medium inside an anaerobic chamber (80% N2, 20% CO2) (Jacomex, France). The washed cells were resuspended in autotrophic growth medium till a final optical density (OD_{600nm}) of 0.2 was obtained and 60 mL of this cell suspension was brought inside serum bottles (160 mL). The autotrophic growth medium used for all the H_2 threshold determinations consisted of 1 g NH_4Cl, 0.1 g KCl, 0.8 g NaCl, 0.1 g KH_2PO_4, 0.077 g $MgCl_2$, 0.02 g

$CaCl_2.2H_2O$, 0.3 g L-Cysteine-HCl.H_2O, 20 mM $NaHCO_3$, 50 mM 3-(*N*-morpholino)propane sulfonic acid brought to pH 7.0 with NaOH, 0.1 g yeast extract, 10 mL vitamin solution DSMZ 141, 1 mL trace element solution (Patil et al., 2015) and 0.1 mL tungstate–selenite solution per litre (Patil et al., 2015).

H_2 threshold measurement

The experiment was initiated by flushing the headspace of the bottles with a gas mix of 2.1% (21,000 ppm) H_2 in CO_2. The total pressure in the bottles was brought to 1.4 bar with the same gas. Three to five replicates were included for each strain. The bottles were incubated upside-down at 30°C with shaking at 100 rpm. The gas phase was frequently sampled with a syringe for H_2 analysis (1.5 mL), until a stable H_2 concentration was measured in at least three consecutive samples. The total pressure was measured with a digital manometer (OMEGA DPG108, UK) before each sampling event. Samples of 1 mL of the liquid phase were taken with a syringe for measurement of the OD, which was measured with a spectrophotometer Thermo Scientific™ SPECTRONIC 20. At the end of the experiment, the liquid phase was also sampled (1 mL) to measure the acetate concentration.

Analytical methods

Headspace samples were analysed using a CompactGC 4.0 (Interscience, Netherlands). This GC was especially configured to obtain a low H_2 detection limit (0.5 Pa or 5 ppm) and perform fast measurements. This GC has a Thermal Conductivity Detector (TCD) operated at 90°C and two columns: Molsieve 5A 30 m × 0.32 mm and Rt-QBond 3 m × 0.32 mm at 70°C. The CompactGC was calibrated with H_2 containing gas mixtures prepared by gas dilutions with Brooks SLA5800 mass flow controllers. Three calibration curves for different ranges were used (5%–100%, 50 ppm to 5% and 5–50 ppm).

A Peak performer 1 (PP1) (Peak Laboratories, USA) GC unit based on HgO–Hg conversion with a reducing compound photometer (RCP) was used to measure trace H_2 levels (0.1–50 ppm of H_2) below the quantification limit of the CompactGC.

VFAs and alcohols were analysed by GC-flame ionization detection (FID) (system 7890, column: HP-INNOWAX 19091N-133, 30 m, 0.25 µm, 0.25 ID, Agilent Technologies, USA) with helium as the carrier gas and H_2 and air as FID detector gases. The detector temperature was 250°C. Pivalic acid (175 mg L^{-1} in 0.3 M oxalic acid) was used as internal standard. The detection limit for acetate was 0.3 mM.

The values of the measured H_2 concentrations in the headspace (obtained in ppm) were converted to H_2 partial pressures (Pa) using the total pressure. The dissolved concentrations of H_2 in the liquid phase were calculated using the Henry's constant (7.8×10^{-4} M atm^{-1}, at 30°C) (Sander, 2015).

Critical Gibbs free energy calculation and ATP gain estimation

The Gibbs free energy change at the conditions of the H_2 threshold is usually not zero (Conrad, 1999; Thauer et al., 2008), as would be expected for a reaction in thermodynamic equilibrium. This Gibbs free energy change is called the critical Gibbs free energy ΔG_c (kJ·mol^{-1}) (Conrad, 1999; Seitz et al., 1990) and can be calculated for acetogens as:

$$\Delta G_c = \Delta G^0_{acetogenesis} + R \cdot T \cdot ln\left(\frac{[CH_3COO^-] \cdot [H^+]}{{p_{CO_2}}^2 \cdot {\theta_{H_2}}^4}\right) \quad (2)$$

with $\Delta G^0{}_{acetogenesis}$ the Gibbs free energy change of acetogenesis in standard conditions (pH 0) (− 55.8 kJ.mol^{-1}) (Haynes, 2017), θ_{H_2} the experimental H_2 threshold (atm), p_{CO2} the CO_2 partial pressures (atm), $[H^+]$ the proton concentration (M) and $[CH_3COO^-]$ the acetate concentration (M).

The critical Gibbs free energy is not zero, likely because acetogenesis should be considered coupled to ATP generation (Conrad, 1999; Thauer et al., 2008). Including ATP generation from ADP and P_i with *n* the ATP gain (mol ATP per mol acetate) in Equation (1) gives:

$$\begin{aligned} &2\,CO_2 + 4\,H_2 + n\,ADP + n\,P_i \\ &\rightarrow CH_3COO^- + H^+ + n\,ATP + 2\,H_2O \end{aligned} \quad (3)$$

When the H_2 threshold is reached, the Gibbs free energy change of Equation (3) assumingly equals zero (overall reaction in equilibrium) (Poehlein et al., 2012; Thauer et al., 2008), which gives:

$$\Delta G_c + n \cdot \Delta G_{ATP} = 0 \quad (4)$$

With ΔG_{ATP} the Gibbs free energy change for the phosphorylation of ADP to ATP (also called the phosphorylation potential), which we assume here to be 75 kJ.mol^{-1} (Schink, 1997). The ATP gain *n* can thus be estimated as:

$$n = -\frac{\Delta G_c}{\Delta G_{ATP}} \quad (5)$$

RESULTS

We measured the H_2 thresholds of eight biotechnologically relevant acetogenic strains. Abiotic controls showed that the H_2 partial pressure slightly decreased over time (the initial headspace concentration of 3000 Pa (2.1%) dropped till 2500 Pa over 25 days) (Figure 1), likely because of the loss of H_2 due to sampling. For all tested strains, the H_2 partial pressure dropped more than in abiotic conditions, demonstrating that all evaluated acetogens were able to use H_2 as electron donor at the initial headspace concentration (Figure 1). The OD remained stable during the experiment for all strains, indicating no significant growth (Figure S1). Acetogenesis by the different strains halted at different H_2 levels. The H_2 thresholds were determined as the average of at least three H_2 partial pressures, measured after H_2 consumption had stopped (Table 1). H_2 was re-injected to the same initial level after reaching the threshold for the *Acetobacterium* strains, to assess whether the halt in H_2 consumption was only due to the H_2 threshold. All three strains

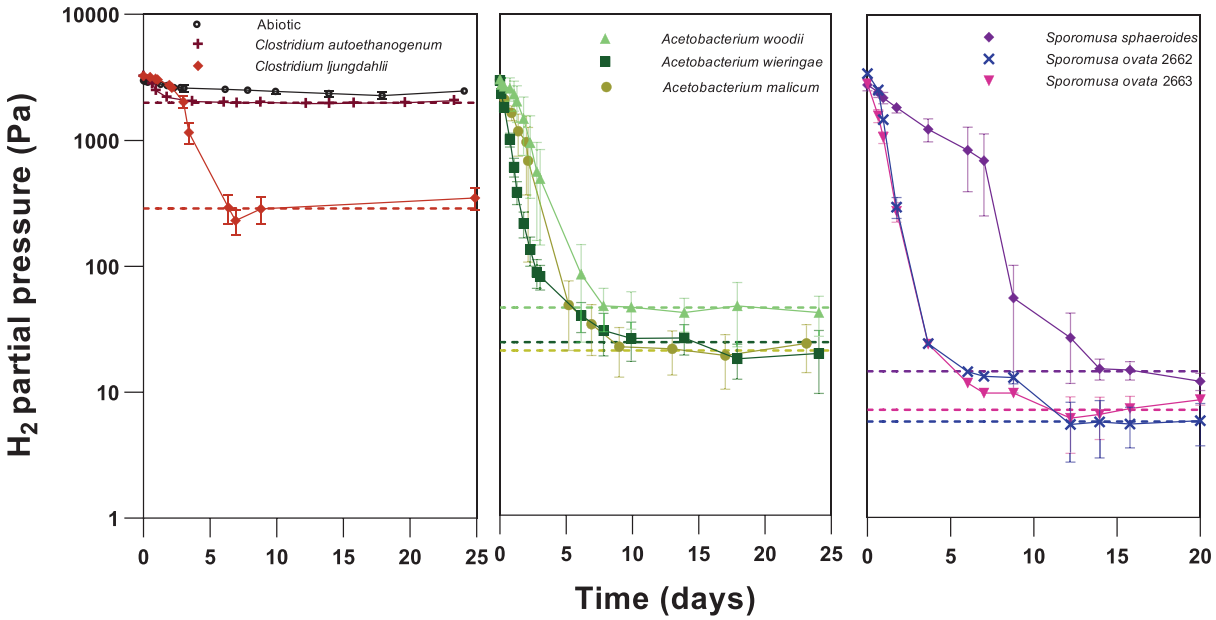

FIGURE 1 Change of the H_2 partial pressure (Pa) over time (days) in abiotic controls and with *Clostridium*, *Acetobacterium* and *Sporomusa* strains. The data points represent the average of three to five replicates, the error bars show the standard deviation. The dotted lines indicate the calculated H_2 thresholds for the different strains.

TABLE 1 H_2 thresholds measured for eight different acetogenic strains, expressed as partial pressures in the headspace (both in ppm and Pa) and as concentration dissolved in the liquid phase (nM).

	H2 threshold			Finale acetate concentration	ΔG_c	Estimated ATP gain *n*
Strain	ppm	Pa	nM	mM	kJ·mol^{-1}	mol ATP per mol acetate
Clostridium autoethanogenum	15,787 ± 832	1990 ± 67	15,325 ± 518	1.03 ± 0.38	−75.8	1.01
Clostridium ljungdahlii	2273 ± 590	289 ± 75	2226 ± 574	6.82 ± 0.87	−51.4	0.69
Acetobacterium woodii	389 ± 143	46 ± 17	346 ± 127	0.94 ± 0.09	−37.4	0.50
Acetobacterium wieringae	214 ± 84	24 ± 10	183 ± 73	0.99 ± 0.46	−30.9	0.41
Acetobacterium malicum	205 ± 75	21 ± 10	165 ± 58	0.90 ± 0.03	−29.4	0.39
Sporomusa sphaeroides	115 ± 29	15 ± 4	113 ± 27	4.09 ± 0.48	−22.6	0.30
Sporomusa ovata 2663	66 ± 20	7 ± 2	56 ± 17	10.65 ± 0.70	−12.8	0.17
Sporomusa ovata 2662	48 ± 16	6 ± 2	44 ± 15	6.44 ± 0.64	−11.8	0.16

Note: The acetate concentration corresponds to the timepoint when H_2 consumption had halted. Both the values of the H_2 thresholds and the acetate concentrations represent the average of three to five replicates and their standard deviation. The critical Gibbs free energy change was calculated by Equation 2 using the H_2 threshold and final acetate concentration. The ATP gain was estimated from critical Gibbs free energy change using Equation 5.

consumed H_2 again until the same level (Figure S2), confirming the cells were still active and there were no other limiting factors, besides H_2.

The highest H_2 thresholds were found for the two *Clostridium* strains (>200 Pa). The H_2 threshold of *C. autoethanogenum* was close to the initial H_2 partial pressure, while a seven times lower H_2 threshold was measured for *C. ljungdahlii*. The three *Acetobacterium* strains had comparable H_2 thresholds (around 30 Pa), which were at least one order of magnitude lower than for the *Clostridium* strains. For *A. woodii*, the H_2 threshold was 46 Pa, which is close to those previously determined by Cord-Ruwisch et al. (53 Pa) and Le Van et al. (37 Pa). The lowest H_2 thresholds were observed for the three *Sporomusa* strains (<20 Pa).

All H_2 thresholds were still above the detection limit of our GC-TCD method, nevertheless the lowest H_2 thresholds were also measured using GC-RCP. The H_2 thresholds for the *S. ovata* strains, measured with GC-RCP, were slightly lower (3.0 ± 0.2 Pa), but in the same low range, as those in Table 1. This confirms the low H_2 thresholds for the *Sporomusa* strains, which were at least one and two order of magnitude lower than for the *Acetobacterium* and *Clostridium* strains, respectively.

We quantified acetate as the main end product (Table 1), since no significant production of ethanol or other end products was observed. Final acetate concentrations were higher than expected, since the complete conversion of 3000 Pa H_2 could maximally yield 0.5 mM acetate. Limited to no increase of the acetate concentration was measured in biological controls without H_2 (results not shown), suggesting that the relatively high acetate concentrations measured for several of our strains (Table 1), cannot be due to acetate formation from the small amount of yeast extract and cysteine in the medium. In related experiments, we measured acetate already at the start of the experiment (Table S1), suggesting that acetate resulted from the carry-over from the inoculum, even though cells were washed.

The finale acetate concentrations were used together with the experimental H_2 thresholds, the remaining CO_2 partial pressure (total pressure in the bottle) and the pH, to calculate the critical Gibbs free energy change ΔG_c for each strain using Equation 2 (Table 1). The most negative ΔG_c was calculated for *C. autoethanogenum* (-75.8 kJ.mol^{-1}), while the least negative ΔG_c was obtained for the *S. ovata* strains (-11.8 kJ.mol^{-1}). The *Acetobacterium* strains had an intermediate ΔG_c of about -30 kJ.mol^{-1}. The wide range of obtained ΔG_c (Table 1) is only for a minimal extent due to the varying final acetate concentrations, since with a constant acetate concentration of 2.5 mM, the experimental H_2 thresholds still result in ΔG_c values ranging from to -73.5 to -14.3 kJ.mol^{-1}(Table S2).

Next, ΔG_c values were used to estimated ATP gains n using Equation 5. ATP gains were highest for *C. autoethanogenum* (1.01 mol ATP per mol acetate) and lowest for *S. ovata* strains (0.16 mol ATP per mol acetate; Table 1).

DISCUSSION

Acetogens have different H_2 Thresholds

Acetogens have different H_2 thresholds under comparable conditions (pH, temperature and CO_2 partial pressure). We observed a difference of three orders of magnitude between the highest and lowest H_2 threshold (Table 1). Members of the same genus have H_2 thresholds in similar ranges (Figure 1). In initial studies, the H_2 thresholds were described to be reaction-specific and only dependent on the terminal electron acceptor (Cord-Ruwisch et al., 1988; Seitz et al., 1990). Here, we demonstrate that H_2 thresholds are also strain-dependent, as different acetogens performing the exact same reaction diverged in their H_2 thresholds (Table 1). Significant differences in H_2 thresholds have also been observed between methanogenic strains (Kaster et al., 2011; Thauer et al., 2008).

Interestingly, these H_2 thresholds correlate well with the H_2 level of the application in which the respective acetogens are used. *Clostridium* strains are often used for syngas fermentation (>9% H_2; Liew et al., 2016) and were found to have the highest H_2 thresholds (Table 1). In contrast, the lowest H_2 thresholds were found for *Sporomusa* strains, which are known for their high current consumption during MES and their prevalence on Fe^0 (low H_2 levels) (< 40 Pa; Aryal et al., 2017; Bian et al., 2018; Kato et al., 2015; Philips et al., 2019). Intermediate H_2 thresholds were found for *Acetobacterium* strains, which need more negative potentials (higher H_2 supply rates) to grow on cathodes than *S. ovata* (Philips, 2020). In addition, the measured H_2 thresholds correlate with the H_2 level used to enrich these different acetogens. Kato et al. (2020) found that an acetogenic community enriched on high H_2 concentrations was dominated by *Clostridium* spp., while *Sporomusa* and *Acetobacterium* spp. dominated the enrichment on low H_2 concentrations.

Intriguingly, the H_2 threshold measured for the *S. ovata* strains was lower than all those previously reported for acetogens (overview given by Philips [2020]) and in the range of the lowest H_2 thresholds reported for methanogens (1–10 Pa) (Kaster et al., 2011; Lovley, 1985; Thauer et al., 2008). It has always been thought that acetogens cannot compete with methanogens at low H_2 levels, due to the lower H_2 threshold of methanogens (Le Van et al., 1998; Leclerc et al., 1997; Schuchmann & Müller, 2016). Our results, however, suggest that there possibly exist acetogens that can win the competition for H_2 from methanogens. Future studies should characterize the H_2 consumption kinetics of *Sporomusa* strains to further investigate this possibility.

Different H_2 thresholds reflect different bioenergetics in acetogens

The different H_2 thresholds measured for the acetogenic strains entail that these acetogens also differ in their critical Gibbs free energy (ΔG_c; Table 1). For the *Acetobacterium* strains and *S. sphaeroides*, the ΔG_c is in the range of previously reported critical Gibbs free energies for acetogens (−20 to −40 kJ.mol^{-1}, expressed per mol acetate and determined using *Acetobacterium* strains) (Conrad & Wetter, 1990; Kotsyurbenko et al., 2001; Seitz et al., 1990). Interestingly, ΔG_c for the *Clostridium* and the *S. ovata* strains (Table 1) are outside of this range. This finding thus demonstrates that acetogens differ stronger in their bioenergetics than originally expected.

Several studies have previously related the ATP gain *n* to the critical Gibbs free energy (and thus the H_2 threshold) using Equation (5) (Kaster et al., 2011; Mock et al., 2015; Poehlein et al., 2012; Thauer et al., 2008). It should be noted though that Equation (5) only gives an estimate of the ATP gain, mainly because Equation (5) strongly depends on the phosphorylation potential. Here, we used a ΔG_{ATP} of 75 kJ per mol ATP, since this is considered as the minimum amount of energy required to synthesize ATP in a living cell losing some energy as heat (Schink, 1997). However, this ΔG_{ATP} can vary between different organisms, as it depends on the intracellular ATP, ADP and P_i concentrations, but has only been accurately measured for few species. Moreover, anaerobes, living under strong energy limitations, such as acetogens, possibly need less energy to synthesize ATP (Schink, 1997). For *A. woodii*, a ΔG_{ATP} of 32.1 kJ per mol ATP has been determined (Spahn et al., 2015), which is lower than measured for any other microbe, but this value does not include heat loss. Another reason why Equation (5) only gives an estimate of the ATP gain is that acetogenesis could proceed uncoupled from energy conservation (Poehlein et al., 2012; Thauer et al., 2008). This would entail that experimental H_2 thresholds (and thus ATP gains) are lower than theoretically expected.

Despite these limitations, the estimated ATP gains (Table 1) are in the expected range, since the lowest ATP gain proposed for an acetogen in literature is 0.14 mol ATP per mol acetate (for *C. autoethanogenum*, assuming methylene-tetrahydrofolate reductase [MTHFR] non-electron bifurcating and NADP dependent; Mock et al., 2015), while the theoretical maximal ATP gain is 1.36 ($\Delta G^{0'}_{acetogenesis}/\Delta G_{ATP}$ [Philips, 2020] with $\Delta G^{0'}_{acetogenesis}$ the Gibbs free energy change of acetogenesis in physiological standard conditions [pH 7; −95.6 kJ.mol^{-1}; Haynes, 2017]).

Our estimated ATP gains range from 0.16 mol ATP per mol acetate for *S. ovata* 2662 to 1.01 mol ATP per mol acetate for *C. autoethanogenum* (Table 1). Similarly, as the critical Gibbs free energies, the estimated ATP gains thus suggest strong differences in the bioenergetics between acetogens. These differences are most likely related to the energy conservation mechanism coupled to the WLP (Poehlein et al., 2012), as the enzymes and electron carriers involved in the energy conservation of acetogens are different (Bengelsdorf et al., 2018; Katsyv & Müller, 2020; Kremp et al., 2020; Poehlein et al., 2015). So far, only the energy conservation mechanism of *A. woodii* is completely unravelled, and those biochemical characterizations allowed to calculate an ATP gain of 0.3 mol ATP per mol acetate from H_2:CO_2 (Schuchmann & Müller, 2014). This value is slightly lower than the ATP gain calculated from our experimental H_2 thresholds (0.50) (Table 1). Curiously, also an estimate of the ATP gain for *A. woodii* based on growth yield measurements was higher than 0.3 mol ATP per mol acetate (Mock et al., 2015).

For *S. ovata*, the energy conservation mechanism is not yet fully unravelled, but an ATP gain of 0.25 mol ATP per mol acetate was proposed from on a genome-based model and biochemical enzymes characterizations (Kremp et al., 2020). This value is slightly higher than the one estimated from our experimental H_2 thresholds (0.16). For *C. autoethanogenum*, biochemical characterizations of the energy conservation mechanism have led to two possible ATP gain values: 0.4 or 1 mol ATP per mol acetate, depending on whether or not the key enzyme MTHFR is bifurcative (Katsyv & Müller, 2020). For *C. ljungdahlii*, a metabolic scheme with an ATP gain of 0.75 mol ATP per mol acetate was proposed for growth on H_2 and CO_2 (Zhu et al., 2020), which is highly comparable with our estimate (0.69). Also for *C. ljungdahlii*, the MTHFR enzyme was recently characterized to be not bifurcative (Öppinger et al., 2021; Yi et al., 2021), but these studies could not reach a final conclusion on the ATP gain, since the reaction of MTHFR with ferredoxin, determined as the reductant in vitro, seemed to be unfeasible in vivo. This example demonstrates the extensive biochemical characterizations, required for each single strain to accurately determine its ATP gain. The simple estimation of the ATP gain from the experimental H_2 threshold, as used here, could thus present a useful tool for the relative comparison of the ATP gain of different acetogens.

It should be noted that our analysis assumes that the energy conservation mechanism, and thus ATP gain, is a fixed trait for an acetogenic strain when growing on H_2 and CO_2. Nevertheless, some acetogens can form other end products than acetate from H_2 and CO_2, for example ethanol, which alters the ATP gain (Mock et al., 2015). However, as in our experiment, acetate is usually the main or only end product of acetogenesis on H_2 and CO_2 (Groher & Weuster-Botz, 2016; Liew et al., 2016). Acetogens will of course also have a higher ATP gain when growing on more favourable substrates, such as organic compounds and CO

(Schuchmann & Müller, 2016; Zhu et al., 2020). For the methanogen *Methanococcus maripaludis*, a fixed maximum methanogenesis rate was found, independent of whether formate was provided at a low or high rate (Müller et al., 2021). Similarly, there exists no experimental evidence that acetogens could adjust their energy conservation mechanism to the substrate supply rate, when growing on one specific substrate.

Implications for the ecological role and the industrial applications of acetogens

This work demonstrates a wide diversity in H_2 thresholds, critical Gibbs free energies and ATP gain estimates among acetogenic bacteria (Table 1). This diversity entails that acetogens likely also differ in their H_2 consumption kinetics (affinity for H_2 [Monod half saturation constant K_m] and the maximum cell specific H_2 consumption rate [V_{max}]) and their growth yields. A higher ATP gain most likely corresponds to a higher growth rate and biomass yield (Thauer et al., 2008). This implies that acetogens are confronted with a trade-off between the capacity to grow fast at high H_2 partial pressures (high ATP gain) and the capacity to efficiently use H_2 down to low H_2 levels (low H_2 threshold). This resembles the rate-yield trade-off, which is well known for microbial metabolism and ecology (Frank, 2010; Pfeiffer et al., 2001). So far, only little information is available on the kinetic parameters and growth yields of acetogenic bacteria. Philips (2020) reviewed the literature and found kinetic parameter values for only few acetogenic strains. Nevertheless, a good understanding of the diversity in the H_2 thresholds, ATP gains, growth rates and biomass yields of acetogens is required to comprehend the prevalence of certain strains in specific environments, as well as their ecological interactions with other microorganisms. Moreover, insights in all those parameters will also contribute to a better understanding of which acetogenic bacteria play a role in microbial corrosion and the development of tools to diagnose microbial corrosion. Finally, such insights are required to enable the selection of the most optimal strain for the diverse biotechnological applications of acetogens, as well as to estimate the expected carbon and energy flow in these applications (Bertsch & Müller, 2015).

AUTHOR CONTRIBUTIONS

Laura Munoz: Conceptualization (equal); investigation (lead); methodology (lead); writing – original draft (lead); writing – review and editing (equal). **Jo Philips:** Conceptualization (equal); funding acquisition (lead); investigation (supporting); methodology (supporting); supervision (lead); writing – original draft (supporting); writing – review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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