Energy Conservation Associated with Ethanol Formation from H₂ and CO₂ in Clostridium autoethanogenum Involving Electron Bifurcation

Johanna Mock,a Yanning Zheng,a Alexander P. Mueller,b San Ly,b Loan Tran,b Simon Segovia,b Shilpa Nagaraju,b Michael Köpke,b Peter Dürre,a Rudolf K. Thauera

Max Planck Institute for Terrestrial Microbiology, Marburg, Germany; aLanzaTech Inc., Skokie, Illinois, USA; bInstitute of Microbiology and Biotechnology, University of Ulm, Ulm, Germany

ABSTRACT

Most acetogens can reduce CO₂ with H₂ to acetic acid via the Wood-Ljungdahl pathway, in which the ATP required for formate activation is regenerated in the acetate kinase reaction. However, a few acetogens, such as Clostridium autoethanogenum, Clostridium ljungdahlii, and Clostridium ragsdalei, also form large amounts of ethanol from CO₂ and H₂. How these anaerobes with a growth pH optimum near 5 conserve energy has remained elusive. We investigated this question by determining the specific activities and cofactor specificities of all relevant oxidoreductases in cell extracts of H₂/CO₂-grown C. autoethanogenum. The activity studies were backed up by transcriptional and mutational analyses. Most notably, despite the presence of six hydrogenase systems of various types encoded in the genome, the cells appear to contain only one active hydrogenase. The active [FeFe]-hydrogenase is electron bifurcating, with ferredoxin and NADP as the two electron acceptors. Consistently, most of the other active oxidoreductases rely on either reduced ferredoxin and/or NADPH as the electron donor. An exception is ethanol dehydrogenase, which was found to be NAD specific. Methylenetetrahydrofolate reductase activity could only be demonstrated with artificial electron donors. Key to the understanding of this energy metabolism is the presence of membrane-associated reduced ferredoxin:NAD⁺ oxidoreductase (Rnf), of electron-bifurcating and ferredoxin-dependent transhydrogenase (Nfn), and of acetaldehyde:ferredoxin oxidoreductase, which is present with very high specific activities in H₂/CO₂-grown cells. Based on these findings and on thermodynamic considerations, we propose metabolic schemes that allow, depending on the H₂ partial pressure, the chemiosmotic synthesis of 0.14 to 1.5 mol ATP per mol ethanol synthesized from CO₂ and H₂.

IMPORTANCE

Ethanol formation from syngas (H₂, CO, and CO₂) and from H₂ and CO₂ that is catalyzed by bacteria is presently a much-discussed process for sustainable production of biofuels. Although the process is already in use, its biochemistry is only incompletely understood. The most pertinent question is how the bacteria conserve energy for growth during ethanol formation from H₂ and CO₂; considering that acetyl coenzyme A (acetyl-CoA), is an intermediate. Can reduction of the activated acetic acid to ethanol with H₂ be coupled with the phosphorylation of ADP? Evidence is presented that this is indeed possible, via both substrate-level phosphorylation and electron transport phosphorylation. In the case of substrate-level phosphorylation, acetyl-CoA reduction to ethanol proceeds via free acetic acid involving acetaldehyde:ferredoxin oxidoreductase (carboxylate reductase).

Clostridium autoethanogenum (1) is industrially used in fermentations of syngas (H₂/CO/CO₂) or industrial off-gases, like basic oxygen furnace (BOF) gas from steel manufacturing (2, 3). It is a close relative of Clostridium ljungdahlii (4) and Clostridium ragsdalei (5). The 16S RNA sequences between the three strains are 99% similar; also, the gene arrangement and sequence in the Wood-Ljungdahl operon are almost identical (6). For C. autoethanogenum (7, 8) and C. ljungdahlii (9), complete genome sequences are available, and a comparative genomic analysis suggested some key differences (7). C. autoethanogenum can grow on CO as the sole carbon and energy source, forming mainly ethanol and acetic acid but also 2,3-butanediol, lactic acid, and some H₂ as fermentation products (6, 10). This organism can also grow on H₂ and CO₂, albeit at lower rates and to lower cell concentrations than on CO. A characteristic of C. autoethanogenum, C. ljungdahlii, and C. ragsdalei is that they grow optimally between pH 5 and pH 5.5 and that, besides acetic acid, they are able to form ethanol during growth on H₂ and CO₂ (see reactions 1 and 2, below) (11). The three clostridia differ in this respect from the well-studied Acetobacterium woodii, which has a pH growth optimum near 7 and reduces CO₂ with H₂ to acetic acid (reaction 1) but not to ethanol (reaction 2) (12). The three Clostridium species also differ from A. woodii in that A. woodii is dependent on sodium ions, whereas the three clostralid species are not, and in that A. woodii is unable to grow on CO in minimal medium (13) unless cultures are provided with formate (14). A common feature, however, is the lack of cytochromes and of menaquinone, which distinguish these acetogens from Moorella thermoacetica, which contains cytochromes and menaquinone (12, 15). M. thermoacetica, a ther-
mophile, can also grow on H₂ and CO₂ and form mainly acetic acid (16–18).

\[ 2 \text{CO}_2 + 4 \text{H}_2 \rightarrow \text{CH}_3\text{COO}^- + \text{H}^+ + 2 \text{H}_2\text{O} \]
\[ \Delta G^\circ = -95 \text{kJ/mol acetate} \]  
\[ 2 \text{CO}_2 + 6 \text{H}_2 \rightarrow \text{CH}_3\text{CH}_2\text{OH} + 3 \text{H}_2\text{O} \]
\[ \Delta G^\circ = -105 \text{kJ/mol ethanol} \]  

Under standard conditions (pH₂ and pCO₂ each at 10⁵ Pa, [acetate] at 1 M, pH 7), ethanol formation from H₂ and CO₂ is more exergonic than acetic acid formation from H₂ and CO₂. However, the opposite is true under physiological conditions, where the pH₂ is lower than 10⁵ Pa. This is because the free energy change of reaction 2, with six H₂ molecules, decreases with the H₂ concentration more than the free energy change of reaction 1, with four H₂ molecules. The advantage of forming ethanol is therefore the avoidance of acidification and not based in thermodynamics.

It has recently been elucidated how A. woodii growing on H₂ and CO₂ conserves energy (12). The scheme shown in Fig. 1A illustrates that in A. woodii, CO₂ is reduced with H₂ to acetic acid via the Wood-Ljungdahl pathway, which involves formyltetrahydrofolate (formyl-H₄F), acetyl coenzyme A (acetyl-CoA), and acetyl-phosphate as “energy-rich” intermediates. The ATP required for formate activation to formyl-H₄F is quantitatively regenerated in the acetate kinase reaction. Net ATP formation is achieved via electron transport phosphorylation involving the membrane-associated complexes RnfA to -G and F₁F₀ ATP synthase. The exergonic reduction of NAD⁺ with reduced ferredoxin catalyzed by the RnfA to -G complex is associated with the buildup of an electrochemical sodium ion potential (1 Na⁺ translocated per electron) (19), which in turn drives the phosphorylation of ADP via the F₁F₀ ATP synthase complex (3.3 Na⁺ translocated per ATP) (20). The reduced ferredoxin is regenerated via reduction of ferredoxin and NAD⁺ with 2 H₂ catalyzed by the electron-bifurcating and NAD-dependent [FeFe]-hydrogenase HydABCD (21). NADH is reoxidized via reduction of methyl-H₄F to methylene-H₄F and of methylene-H₄F to methyl-H₄F, reactions which are catalyzed by NAD-specific methylene-H₄F dehydrogenase (22) and NAD-specific methylene-H₄F reductase (23), respectively. CO₂ reduction to formate is catalyzed by a cytoplasmic formate-hydrogen-lyase complex (24). Via this scheme (Fig. 1A), a net 0.3 mol ATP is generated per mol of acetic acid formed from H₂ and CO₂ (12). The low ATP gain of 0.3 mol ATP/mol of acetate is consistent with an H₂ threshold concentration near 0.2% in the gas phase reported for the acetogen (25–27). However, growth yields corrected for maintenance (Yₚ) of nearly 7 g of cells/mol of acetate (26, 28) indicate that the ATP gain (μ) could be somewhat higher: Yₚ = k·μ·Yₚₐₜₕ, with k representing the yield coefficient and Yₚₐₜₕ the theoretical amount of cells per mole of ATP (29). Yₚₐₜₕ has been calculated to be about 15 g of cells/mol of ATP when starting with acetate and CO₂ as carbon sources and H₂ as electron donor (30–33). The yield coefficient k is generally less than 1 and usually between 0.4 and 0.8 (29).

A. woodii does not form ethanol when growing on H₂ and CO₂ (12, 34). However, when growing on glucose, this bacterium can form ethanol in addition to acetic acid, indicating that the organism has the potential to reduce acetyl-CoA to ethanol. Both the acetaldehyde dehydrogenase (CoA acetylator) and the ethanol dehydrogenase were found to be NAD specific (35). The CoA-linked acetaldehyde dehydrogenase has been characterized (36). The free energy change associated with reaction 2 at H₂ concentrations above 0.1% would, in principle, allow ATP formation. The inability to form ethanol during growth on H₂/CO₂ must

FIG 1 Energy metabolism of Acetobacterium woodii growing on H₂ and CO₂ at pH 7. (A) Metabolic scheme showing how acetic acid is formed from H₂ and CO₂ and which steps are associated with energy conservation (12). (B) Metabolic scheme assuming ethanol is formed from H₂ and CO₂, for which the overall reaction is thermodynamically feasible. However, ethanol formation from CO₂/H₂ does not occur, although enzymes required for ethanol formation are present, but with these the ATP gain is negative as predicted with the scheme. For explanations, see the text. Hyd, electron-bifurcating and NAD-dependent [FeFe]-hydrogenase; Rnf, membrane-associated and energy-conserving reduced ferredoxin:NAD oxidoreductase; ADH, alcohol dehydrogenase; ALD, coenzyme A-dependent acetaldehyde dehydrogenase.
therefore be due to factors other than thermodynamics. A possible explanation is given in Fig. 1B, in which the scheme shown in Fig. 1A has been expanded to include ethanol formation from acetyl-CoA. Via these reactions, the ATP gain (moles of ATP per mole of ethanol) in A. woodii would be negative (−0.1 mol ATP/mol ethanol).

In contrast to A. woodii, C. autoethanogenum forms ethanol in addition to acetic acid when growing on H₂ and CO₂ (1). Our study addresses the questions of how and why ethanol can be formed. We identified five enzymes involved in ethanol formation from H₂ and CO₂ that are present in H₂/CO₂-grown C. autoethanogenum cells but, based on the genome sequence (27), are not present in A. woodii: an electron-bifurcating and NADP-dependent [FeFe]-hydrogenase (Hyt) (catalyzing reaction 3, below), an electron-bifurcating and ferredoxin-dependent transhydrogenase (Nfn) (reaction 4), an NADP-specific methylene-H₄F dehydrogenase (FoD) (reaction 5), a methylene-H₄F reductase (MetFV) for which the physiological electron donor has not yet been found (reaction 6), and an acetaldehyde:ferredoxin oxidoreductase (AOR) (reaction 7).

The finding that H₂/CO₂-grown cells of C. autoethanogenum contain only an electron-bifurcating and NADP-dependent [FeFe]-hydrogenase (Hyt) (reaction 3) was unexpected, because in vivo, NADP generally has a lower redox potential (NADP⁺/ NADPH, <1) than NAD (NAD⁺/NADH, >1) (37), and therefore NAD rather than NADP should be the thermodynamically favored electron acceptor of electron-bifurcating hydrogenases (38). In vivo, the redox potential of ferredoxin in acetonogens is probably near −400 mV (39), while the in vivo redox potential of NAD is near −300 mV (40) and that of NADP is near −360 mV (37).

Nagarajan and colleagues stated that “a thorough understanding of various factors governing the metabolism, in particular energy conservation mechanisms, is critical for metabolic engineering of acetonogens for targeted production of desired chemicals” (41), and this increased understanding was the motivation for our performed work.

MATERIALS AND METHODS

Chemicals. NAD⁺, NADP⁺, NADH, NADPH, FAD, FMN, thiamine pyrophosphate, coenzyme A, pyruvate, glyceraldehyde-3-phosphate (GAP), acetaldehyde, H₂F, benzyl viologen (BV), dithiothreitol (DTT), and phosphotransacetylase from Bacillus steatorrhophilus were obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Methylene-H₄F was prepared from H₂F and formaldehyde by spontaneous reaction. H₂, N₂, and O₂ with highest purity (99.996%) were sourced from Messer (Düsseldorf, Germany) and Air Liquide Speciality Gases USA LLC (Hous-

September 2015 Volume 197 Number 18 Journal of Bacteriology 2967

Downloaded from http://jb.asm.org on November 3, 2017 by guest
then washed three times with anoxic buffer. Unbroken cells and cell debris were removed by centrifugation at 20,000 × g and 4°C for 30 min. The supernatant was used for enzyme assays. The membrane fraction was obtained by centrifugation at 150,000 × g and 4°C for 60 min (46).

**Specific activity measurements.** Enzyme activities were measured under strictly anoxic conditions at 37°C in 1.5-ml anaerobic cuvettes [NAD(P), ferredoxin, and viologen dye reduction] or 6.5-ml anaerobic serum bottles (H₂ formation and CO₂ reduction) sealed with rubber stoppers and filled with 0.8 ml assay mixture and N₂ (100%), H₂ (100%), or CO (100%) at 1.2 × 10⁵ Pa as the gas phase. After the start of the reaction with enzyme, the reduction of NADP⁺ or NAD⁺ and the oxidation of NADPH or NADH were monitored spectrophotometrically at 340 nm (ε = 6.2 mM⁻¹ cm⁻¹) or at 380 nm (ε = 1.2 mM⁻¹ cm⁻¹). *C. pasteurianum* ferredoxin reduction was monitored at 430 nm (Δε₉₉₉₉₉ red ≈ 13.1 mM⁻¹ cm⁻¹), and benzyl viologen reduction was monitored at 578 nm (ε = 8.6 mM⁻¹ cm⁻¹). The reduction of ¹⁴CO₂ to [¹⁴C]formate was followed by quantifying ¹⁴C radioactivity in a Beckman LS6500 liquid scintillation counter (Fullerton, CA). One unit was defined as the transfer of 2 micromoles of electrons per minute. Protein levels were determined using the Bio-Rad protein assay (Munich, Germany) with bovine serum albumin as the standard (46).

**Hydrogenase (reaction 3).** For determination of hydrogenase specific activity, the assay mixtures contained 100 mM Tris-HCl (pH 7.5), 2 mM DTT and, where indicated, 25 μM ferredoxin, 1 mM NAD⁺, or 1 mM NADP⁺. The gas phase was 100% H₂.

**Ferrodoxin-dependent transhydrogenase (reaction 4).** For ferredoxin-dependent transhydrogenase specific activity measurements, the assay mixtures contained 100 mM morpholinopropanesulfonic acid (MOPS)-KOH (pH 7.0), 10 mM 2-mercaptoethanol, 10 mM ascorbate, 10 mM ferredoxin, 1 mM NAD⁺ or 1 mM NADH, and 10 μM acetic acid to stop the reaction by acidification. The 200-μl assay mixture contained 100 μM NADPH or 10 μM FAD, 20 mM benzyl viologen, and 1 mM methyl-H₂-F. Before the start of the reaction with enzyme, benzyl viologen was reduced to an A₅₇₈ of 0.3 with sodium dithionite. The gas phase was 100% N₂.

**Reduced ferredoxin:NAD(P)⁺ oxidoreductase.** For determination of reduced ferredoxin:NAD(P)⁺ oxidoreductase activity, the assay mixtures contained 100 mM potassium phosphate (pH 7.0), 2 mM DTT, 15 μM ferredoxin, and 1 mM NAD⁺ or NADP⁺. The gas phase was 100% CO for the reduction of ferredoxin via the ferredoxin-dependent CO dehydrogenase in the cell extract (19).

**CO dehydrogenase.** The assay mixtures for CO dehydrogenase activity measurements contained 100 mM Tris-HCl (pH 7.5), 2 mM DTT, and about 30 μM ferredoxin, 1 mM NAD⁺, or 1 mM NADP⁺. The gas phase was 100% CO.

**Formate-hydrogen lyase.** When the formation of H₂ from formate (formate hydrogen lyase activity) was followed, the assay mixtures contained 100 mM Tris-HCl (pH 7.5), 2 mM DTT, and 20 mM sodium formate. The gas phase was 100% N₂. The serum bottles were continuously shaken at 200 rpm to ensure H₂ transfer from the liquid phase into the gas phase. After start of the reaction with cell extract, gas samples (0.2 ml) were withdrawn every 1 min, and H₂ was quantified by gas chromatography (46).

When the reduction of CO₂ with H₂ to formate was measured, the assay mixtures contained 100 mM potassium phosphate (pH 7.0), 2 mM DTT, and 30 mM [¹³C]K₂CO₃ (24,000 dpm/μmol). The gas phase was 100% H₂. The serum bottles were continuously shaken at 200 rpm to ensure equilibration of the gas phase with the liquid phase. After start of the reaction with cell extract, 100-μl liquid samples were withdrawn every 1.5 min and added to a 1.5-ml safe-seal microtube containing 100 μl of 150 mM acetic acid to stop the reaction by acidification. The 200-μl mixture was then incubated at 40°C for 10 min with shaking at 1,400 rpm in a Thermomixer (type 5436; Eppendorf, Germany) to remove all [¹³C]CO₂, leaving behind the [¹³C]formate formed. Subsequently, 100 μl of the mixture was added to 5 ml of Quicksave A scintillation fluid (Zinsser Analytic, Frankfurt, Germany) and analyzed for [¹³C]radioactivity in a Beckman LS6500 liquid scintillation counter (Fullerton, CA).

**Formate dehydrogenase.** The assay mixtures for determination of formate dehydrogenase activity contained 100 mM Tris-HCl (pH 7.5), 2 mM DTT, 20 mM sodium formate and, where indicated, 25 μM ferredoxin, 1 mM NADP⁺, or 1 mM NAD⁺. The gas phase was 100% N₂.

**Methylene-H₂-F dehydrogenase (reaction 5).** For methylene-H₂-F activity determinations, the assay mixtures contained 100 mM MOPS-KOH (pH 6.5), 50 mM 2-mercaptoethanol, 0.4 mM H₂-F, 10 mM formaldehyde, and 0.5 mM NADP⁺ or 0.5 mM NAD⁺. The gas phase was 100% N₂. After the start of the reaction with the enzyme, the formation of NAD(P)H and methenyl-H₂-F was followed at 350 nm using an ε of 30.5 mM⁻¹ cm⁻¹ [5.6 mM⁻¹ cm⁻¹ for NAD(P)H plus 24.9 mM⁻¹ cm⁻¹ for methenyl-H₂-F].

**Methylene-H₂-F reductase (reaction 6).** For measurement of methylene-H₂-F reductase activity, the assay mixtures contained 100 mM Tris-HCl (pH 7.5), 20 mM ascorbate, 10 μM FAD, 20 mM benzyl viologen, and 1 mM methyl-H₂-F. Before the start of the reaction with enzyme, benzyl viologen was reduced to an A₅₇₈ of 0.3 with sodium dithionite. The gas phase was 100% N₂.

**Acetaldehyde:ferredoxin oxidoreductase (reaction 7).** The assay mixtures for determination of acetaldehyde:ferredoxin oxidoreductase activity contained 100 mM Tris-HCl (pH 7.5), 2 mM DTT, 1.1 mM acetaldehyde, and about 25 μM ferredoxin. The gas phase was 100% N₂.

**Alcohol dehydrogenase (reaction 8).** For measurement of the specific activity of alcohol dehydrogenase, the assay mixtures contained 100 mM potassium phosphate (pH 6), 2 mM DTT, 1.1 mM acetaldehyde, and 1 mM NADPH or 1 mM NADH. The gas phase was 100% N₂.

**Acetaldehyde dehydrogenase (CoA acetylating) (reaction 9).** The assay mixture for measurement of acetaldehyde dehydrogenase activity contained 100 mM Tris-HCl (pH 7.5), 2 mM DTT, 1.1 mM acetaldehyde, 1 mM coenzyme A, and 1 mM NADP⁺ or 1 mM NAD⁺. The gas phase was 100% N₂.

**Pyruvate:ferredoxin oxidoreductase (reaction 10).** For determination of pyruvate:ferredoxin oxidoreductase activity, the assay mixtures contained 100 mM Tris-HCl (pH 7.5), 2 mM DTT, 10 mM pyruvate, 1 mM coenzyme A, 0.1 mM thiamine pyrophosphate, ~25 μM ferredoxin, and 1 mM NADP⁺ or 1 mM NAD⁺. The gas phase was 100% N₂.

**Glyceraldehyde phosphate dehydrogenase.** For assay of glyceraldehyde phosphate dehydrogenase activity, the assay mixture contained 50 mM Tricine-NaOH (pH 8.5), 10 mM 2-mercaptoethanol, 10 mM potassium phosphate, 1 mM glyceraldehyde-3-phosphate, and 1 mM NADP⁺ or 1 mM NAD⁺. The gas phase was 100% N₂.

**RNA-seq analyses.** Transcriptome sequencing (RNA-seq) analysis was performed on 20-ml fermentation samples, either from a continuous culture sparged with 42% CO₂, 36% N₂, 20% CO₂, and 2% H₂ or from batch cultures grown on 65% H₂ and 23% CO₂ (N₂ as the balance) in a closed system (serum bottles). The samples were centrifuged at 4,000 rpm for 10 min at 4°C. The supernatants were discarded, and the pellets were stabilized by the addition of 5 ml of RNAlater (Ambion Inc.). Total RNA was isolated from the cell pellets by using a RiboPure bacteria kit (Ambion Inc.) according to the manufacturer’s standard protocol. DNA was removed using the Turbo DNA-free kit (Ambion Inc.), and RNA quality was assessed by using a 2100 Bioanalyzer (Agilent Technologies). RNA concentration was determined with a Nanodrop 2000 apparatus (Thermo Fisher Scientific, Waltham, MA). Ribodepletion was conducted using the Microbe Express kit (Ambion Inc.). cDNA libraries were prepared and sequenced by standard procedures using either SOLiD2 or Illumina HiSeq-2000 sequencing technology. Processed reads were mapped to a reference assembly, displayed, and manually inspected in the Geneious genome browser, v7.0.3 (Biomatters). Transcript abundance was estimated based on the fragments per kilobase of exon per million fragments mapped (FPKM).
Construction of strains with disrupted genes. Genes were disrupted by using the ClosTron system (47). Constructs were designed using the Web-based ClosTron design tool, and intron-targeting plasmids were synthesized by the company DNA 2.0 (Menlo Park, CA). Transformation of C. autoethanogenum was achieved by bacterial conjugation using Escherichia coli strain HB101 (R702) as donor according to previously published methods (48, 49). Briefly, a 2-ml culture of E. coli strain HB101 (R702) bearing the target plasmid was grown to an OD_{600} of 0.5, washed with phosphate-buffered saline, and mixed with 0.2 ml of a C. autoethanogenum culture grown to an OD_{600} of 0.5. The cell mixture was spotted onto 1.5% agar plates containing 5 g fructose/liter and incubated at 37°C for 24 h under 1.7 × 10^4 Pa gas (gas composition: 42% CO, 36% N_2, 20% CO_2, and 2% H_2) in gas-tight jars. Cells were subsequently transferred onto agar plates containing 5 g fructose/liter, 10 mg trimethoprim/liter for counterselection of E. coli, and antibiotics for selection of the plasmid and group II intron insertion (15 mg thiamphenicol/ml and 5 mg clarithromycin/ml, respectively). Single colonies were restreaked and then screened using colony PCR (50).

Nucleotide sequence accession numbers. The genome sequences of C. autoethanogenum and C. ljungdahlii have been deposited with GenBank under the accession numbers NC_022592.1 and NC_014328.1, respectively.

RESULTS

Clostridium autoethanogenum was grown on H_2/CO_2, at a ratio of 2.82 (65% H_2, 23% CO_2, 9% N_2) in continuous culture at pH 5.3, 37°C, in a 2-liter bioreactor (1.3-liter volume) equipped with a membrane system which allowed the retention of 90% of the cells. The apparent dilution rate that the cells experienced was 0.5 day⁻¹ (f_d = 33 h), and the apparent dilution rate of the metabolites was 4.9 day⁻¹. The steady-state concentration of cells was kept near 1.83 g/liter, that of acetate was 7.5 g/liter (0.125 M), and that of ethanol was 6.3 g/liter (0.134 M). Under these growth conditions, the formation of 2,3-butanediol and/or lactic acid, products formed when CO is the substrate, was not observed. Besides ethanol and acetic acid, the HPLC analyses revealed no other fermentation products. The culture produced (per day) 0.9 g cells (∼0.04 mol C)/liter, 36 g (0.6 mol) acetic acid/liter (0.23 μmol min⁻¹ mg of cells⁻¹), and 31 g (0.65 mol) ethanol/liter (0.25 μmol min⁻¹ mg of cells⁻¹). From these data and considering the stoichiometries given by reactions 1 and 2, a growth yield of about 0.22 g of cells per mol H_2 and a specific rate of H_2 consumption of 2.4 μmol min⁻¹ mg of cells⁻¹ were calculated.

The specific rate of H_2 consumption indicated that in cell extracts of the H_2/CO_2-grown organism, hydrogenase should have a specific activity near 4 μmol min⁻¹ mg of protein⁻¹ and that the oxidoreductases involved in CO_2 reduction to acetate and ethanol should have specific activities of about 0.8 μmol min⁻¹ mg of protein⁻¹ (assuming 50% of the cell mass to be protein). For almost all of the enzymes, this was found to be the case (Table 1), with exception of the specific activity of energy-conserving reduced ferredoxin:NAD⁺ oxidoreductase (Rnf), which was only 0.1 μmol min⁻¹ mg of protein⁻¹. The reason for the low Rnf specific activity could be that Rnf is oxygen sensitive and difficult to assay (19). This interpretation was substantiated by the finding that the rnf genes in C. autoethanogenum belong to those most highly expressed (see below), indicating a catalytic role. The specific activity of the electron-bifurcating hydrogenase was only 0.7 μmol ferredoxin reduced by H_2 per min per mg of protein, equivalent to 1.4 μmol H_2 oxidized per min per mg of protein (reaction 3), which is only one-third of that expected. The specific activity of the electron-bifurcating ferredoxin-dependent transhydrogenase (Nfn) was only 0.1 μmol min⁻¹ mg of protein⁻¹ (Table 1), but this low specific activity was consistent with the proposed function of this enzyme (see Discussion). In Table 1, the specific activities of the oxidoreductases found in cell extracts of H_2/CO_2-grown cells of C. autoethanogenum are compared with those found in fructose-grown and CO (BOF gas)-grown cells. At the time of harvest, the fructose-grown cells in the batch culture had a doubling time of near 15 h, fermented fructose at a specific rate of 0.02 μmol min⁻¹ mg of cells⁻¹ and produced acetic acid a specific rate of about 0.06 μmol min⁻¹ mg of cells⁻¹. The rate of ethanol formation was less than 10% of that of acetic acid. 2,3-Butanediol was not formed in significant amounts. At the time of harvest, the CO-grown cells in the continuous culture had a doubling time near 5 h, generating ethanol at a specific rate of 0.2 μmol min⁻¹ mg of cells⁻¹. Besides ethanol, acetic acid (0.1 μmol min⁻¹ mg of cells⁻¹) and 2,3-butanediol (0.12 μmol min⁻¹ mg of cells⁻¹) were formed. CO was consumed at a specific rate of about 2 μmol min⁻¹ mg of cells⁻¹ (46).

Hydrogenase (Hyt). Cell extracts of H_2/CO_2-grown cells catalyzed the reduction of ferredoxin with H_2 only in the presence of NAD⁺ and vice versa (Table 1), indicating the presence of an electron-bifurcating, NADP- and ferredoxin-dependent hydrogenase catalyzing reaction 3. NAD⁺ was not reduced by H_2, in either the absence or presence of ferredoxin. Only in the extracts of fructose- and CO-grown cells was there some hydrogenase activity with ferredoxin alone, indicating the additional presence of a non-electron-bifurcating hydrogenase specific for ferredoxin.

\[
2 \text{H}_2 + \text{Fd}_{\text{ox}} + \text{NADP}^+ \rightleftharpoons \text{Fd}_{\text{red}}^2^- + \text{NADPH} + 3 \text{H}^+ \tag{3}
\]

The electron-bifurcating, NADP- and ferredoxin-dependent [FeFe]-hydrogenase was recently purified from CO-grown C. autoethanogenum cells and characterized as an electron-bifurcating [FeFe]-hydrogenase composed of the subunits HytABCDE1E2 (HytA-E) and catalyzing the reversible reduction of NADP⁺ and Fdₙ with 2 H₂ (reaction 3) (46). HytA is the H-cluster–harboring subunit, HytB is an iron–sulfur flavoprotein harboring the NADP binding site, and the other subunits are iron–sulfur proteins. The hydrogenase forms a tight complex with the selenium- and tungsten-dependent formate dehydrogenase FdhA (HytA-E/FdhA). The hydrogenase (HytABCDE1E2) is encoded by the genes CAETHG_2794-99 (hytCBDE1AE2), which form a transcription unit (46). RNA-seq analyses revealed that these genes belong to the most highly expressed genes in C. autoethanogenum grown on CO-rich BOF gas (42% CO, 36% N_2, 20% CO_2, and 2% H_2), with all genes among the 100 highest-expressed genes. The expression analysis revealed higher expression of the genes encoding the subunits HytE1, -A, and -E2 (405 FPKM average across all genes) over the subunits HytC, -B, and -D (312 FPKM) (Fig. 2). The genes were also highly expressed during growth of C. autoethanogenum on H_2/CO_2 (65% H_2, 9.2% N_2, 23% CO_2) and fructose, as deduced from available transcriptomics data sets for C. ljungdahlii grown on H_2/CO_2 or fructose (41).

Besides the structural genes, the other genes required for [FeFe]-hydrogenase maturation, hydE (CAETHG_1691; 47 FPKM), hydF (CAETHG_2063; 21 FPKM), and hydG (CAETHG_0339; 148 FPKM) are present and show expression under all conditions tested in C. autoethanogenum as well as in C. ljungdahlii (41).

The genome of C. autoethanogenum harbors genes for two
Table 1: Oxidoreductase activities in cell extracts of C. autoethanogenum grown on either H2/CO2, fructose, or CO

<table>
<thead>
<tr>
<th>Oxidoreductase</th>
<th>Substrates</th>
<th>Sp act U/(mg of protein) in cells grown on:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrogenase (electron bifurcating)</td>
<td>H2 + NADP^+ + Fd_rex</td>
<td>H2/CO2 1.4</td>
</tr>
<tr>
<td></td>
<td>H2 + NAD^+ + Fd_rex</td>
<td>&lt;0.1 &lt;0.1</td>
</tr>
<tr>
<td></td>
<td>H2 + Fd_rex</td>
<td>&lt;0.1 0.2</td>
</tr>
<tr>
<td></td>
<td>H2 + NADP^+</td>
<td>&lt;0.1 0.07</td>
</tr>
<tr>
<td></td>
<td>H2 + NAD^+</td>
<td>&lt;0.1 0.04</td>
</tr>
<tr>
<td>Transhydrogenase (Nfn; ferredoxin dependent)</td>
<td>NADPH + NADP^+ + Fd_rex</td>
<td>0.1 1.1</td>
</tr>
<tr>
<td></td>
<td>NADPH + Fd_rex</td>
<td>0.01 0.1</td>
</tr>
<tr>
<td></td>
<td>CO + Fd_rex + NAD^+</td>
<td>0.1 0.2</td>
</tr>
<tr>
<td></td>
<td>CO + Fd_rex + NADP^+</td>
<td>0.01 0.14</td>
</tr>
<tr>
<td></td>
<td>CO DH</td>
<td>0.01 0.02</td>
</tr>
<tr>
<td></td>
<td>CO + NADP^+</td>
<td>0.01 0.03</td>
</tr>
<tr>
<td></td>
<td>CO + NAD^+</td>
<td>0.01 0.02</td>
</tr>
<tr>
<td>Formate-H2, lyase</td>
<td>HCOO^- + H^+ (H2, formate formation)</td>
<td>0.9 3.8</td>
</tr>
<tr>
<td></td>
<td>HCOO^- + Fd_rex + H^+</td>
<td>0.9 4.0</td>
</tr>
<tr>
<td></td>
<td>HCOO^- + Fd_rex + NADP^+ + H^+</td>
<td>0.9 3.5</td>
</tr>
<tr>
<td></td>
<td>H2 + CO2 (formate formation)</td>
<td>1.0 4.0</td>
</tr>
<tr>
<td>Formate DH</td>
<td>HCOO^- + NADP^+ + Fd_rex</td>
<td>0.4 1.2</td>
</tr>
<tr>
<td></td>
<td>HCOO^- + Fd_rex + NAD^+</td>
<td>0.02 &lt;0.01</td>
</tr>
<tr>
<td></td>
<td>HCOO^- + Fd_rex + Fd_rex</td>
<td>&lt;0.01 0.7</td>
</tr>
<tr>
<td></td>
<td>HCOO^- + NADP^+</td>
<td>0.1 0.05</td>
</tr>
<tr>
<td></td>
<td>HCOO^- + NAD^+</td>
<td>&lt;0.1 0.2</td>
</tr>
<tr>
<td>Methylene-H4F reductase</td>
<td>Methylene-H4F + NADP^+</td>
<td>0.7 2.4</td>
</tr>
<tr>
<td></td>
<td>Methylene-H4F + NAD^+</td>
<td>&lt;0.01 &lt;0.01</td>
</tr>
<tr>
<td>Methylene-H4F reductase</td>
<td>Methylene-H4F + BV</td>
<td>2.8 1.0</td>
</tr>
<tr>
<td></td>
<td>Methylene-H4F + NADPH</td>
<td>&lt;0.1 &lt;0.1</td>
</tr>
<tr>
<td></td>
<td>Methylene-H4F + NADH</td>
<td>&lt;0.1 &lt;0.1</td>
</tr>
<tr>
<td></td>
<td>Methylene-H4F + NAD(P)H + Fd_rex</td>
<td>0.1 0.1 0.1</td>
</tr>
<tr>
<td>Acetaldehyde:Fd OR</td>
<td>Acetaldehyde + Fd_rex</td>
<td>8 1.5</td>
</tr>
<tr>
<td>Alcohol DH</td>
<td>Acetaldehyde + NADH</td>
<td>0.2 0.1</td>
</tr>
<tr>
<td></td>
<td>Acetaldehyde + NADPH</td>
<td>&lt;0.01 0.05</td>
</tr>
<tr>
<td>Acetaldehyde DH</td>
<td>Acetaldehyde + CoA + NADP^+</td>
<td>0.1 0.03</td>
</tr>
<tr>
<td></td>
<td>Acetaldehyde + CoA + NAD^+</td>
<td>0.04 0.08</td>
</tr>
<tr>
<td>Pyruvate:Fd OR</td>
<td>Pyruvate + CoA + Fd_rex</td>
<td>0.2 0.6</td>
</tr>
<tr>
<td></td>
<td>Pyruvate + CoA + NAD(P)^+</td>
<td>0.04 0.02</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GAP + P1 + NADP^+</td>
<td>0.1 1.6</td>
</tr>
<tr>
<td></td>
<td>GAP + P1 + NAD^+</td>
<td>0.03 0.2</td>
</tr>
</tbody>
</table>

* Underlined portions indicate substrates or products whose reduction, oxidation, or formation was monitored. Boldface indicates the relevant substrates and specific activities. The activities were determined at 37°C in cell extracts (20,000 × g supernatant) prepared with a French press. For pH and substrate concentrations, see Materials and Methods. DH, dehydrogenase; OR, oxidoreductase; Fd, ferredoxin from C. pasteurianum; BV, benzyl viologen; H4F, tetrahydrofolate; GAP, glyceroldehyde-3-phosphate.

* BOF gas was the source of CO and was composed of 42% CO, 36% N2, 20% CO2, and 2% H2. Cells were from continuous cultures that were harvested in September 2012 (also October 2012 when two values are reported).

* Ferredoxin was reduced by CO via the CO dehydrogenase activity present in the cell extracts. In cell extracts prepared with a French press, most of the activity was associated with the soluble fraction. When the cell extracts were obtained by lysis of the cells with lysozyme, almost 100% of the activity was membrane associated.

Mock et al.

other multisubunit [FeFe]-hydrogenases (CAETHG_1576-78 and CAETHG_3569-71), which have subunit structures and compositions very similar to those of the electron-bifurcating and NAD- and ferredoxin-dependent [FeFe]-hydrogenase from Thermotoga maritima (51), A. woodii (21), M. thermoacetica (52), and Ruminococcus albus (53). While during growth of C. autoethanogenum on CO-rich BOF gas CAETHG_3569-71 is hardly expressed (2 FPKM), CAETHG_1576-78 is the second-highest expressed hydrogenase in C. autoethanogenum but at a significantly lower level (35 FPKM) than the characterized electron-bifurcating...
and NADP-dependent [FeFe]-hydrogenase. CAETHG_1576-78 is absent in C. ljungdahlii.

The genome of C. autoethanogenum harbors genes for two monosubunit [FeFe]-hydrogenases, CAETHG_0110 and CAETHG_3841. These generally use ferredoxin as the electron acceptor/donor, are not electron bifurcating, and show only very low expression (5 to 9 FPKM). There is also a third gene, CAETHG_0119, annotated as an [FeFe]-hydrogenase, but it lacks the sequence segments involved in H-cluster iron binding and can therefore not encode a functional [FeFe]-hydrogenase.

Genes for an [NiFe]-hydrogenase (CAETHG_0861-62) are also present and clustered with a gene for an [NiFe]-hydrogenase maturation protease (CAETHG_0860). No expression for these three genes was found under any condition investigated (<0.1 FPKM). This has also been reported for transcriptomic data sets for C. ljungdahlii grown on either H2/CO2 or fructose (41). Consistently, not all the genes required for [NiFe]-hydrogenase maturation are present. Only the genes hypECDF (CAETHG_0572-0569) were found, whereas the genes hypAB appeared to be absent. Nevertheless, the hypECDF genes were expressed at a reasonable level (35 FPKM).

None of the genes of the six putative hydrogenases contain a twin arginine (TAT) motif sequence or a transmembrane helix motif sequence, indicating that the hydrogenases are cytoplasmic enzymes.

Based on the genetic outfit (see above), C. autoethanogenum should be able to synthesize five different functional hydrogenases. This contrasts with the finding that during growth on H2/CO2, CO-rich BOF gas, or fructose, apparently only the electron-bifurcating and NADP-dependent [FeFe]-hydrogenase HytABCDE1E2 is active (Table 1). Consistently, the hyt genes could not be disrupted when we employed the Clostron technology (47), whereas, with exception of CAETHG_3841, the other [FeFe]- and [NiFe]-hydrogenases genes could be disrupted without a negative affect on growth in CO-rich BOF gas.

Ferredoxin-dependent transhydrogenase (Nfn). Extracts of H2/CO2-grown cells catalyzed the reduction of ferredoxin with NADPH only in the presence of NADP+ and vice versa (Table 1), indicating the presence of an electron-bifurcating and ferredoxin-dependent transhydrogenase (Nfn) catalyzing reversible reaction 4 (54, 55). Unlike the genes in C. kluyveri (55), the respective genes nfnA and nfnB are fused into one gene in C. autoethanogenum (CAETHG_1580) and C. ljungdahlii (CLJU_c37240). The nfn gene in CO-grown C. autoethanogenum belongs to the most highly expressed genes (926 FPKM average across all genes). Heterologous expression of this gene from C. autoethanogenum in E. coli yielded cells with Nfn activity (unpublished result).

\[
F_d^{\text{red}} + \text{NADH} + 2 \text{NADP}^+ + H^+ \rightleftharpoons F_d^{\text{ox}} + \text{NADP}^++ 2 \text{NADPH} (4)
\]

\[
F_d^{\text{red}}^{2-}:\text{NADP}^+ \text{oxidoreductase (Rnf). Cell extracts of } H_2/CO_2 \text{-grown } C. \text{autoethanogenum catalyst the reduction of } NADP^+ \text{ with reduced ferredoxin } (F_d^{\text{red}}^{2-} + NADP^+ + H^+ \rightleftharpoons F_d^{\text{ox}} + \text{NADH}). \text{Reduced ferredoxin was regenerated via the carbon monoxide dehydrogenase reaction } (CO + H_2O + F_d^{\text{ox}} + 2 H^+). \text{The Rnf activity was associated with the membrane fraction (150,000 \times g pellet) (data not shown). NADP}^+ \text{ could not substitute for NAD}^+ \text{ as the electron acceptor (Table 1). The activity was independent of the presence of sodium ions.}
\]

Rnf is generally composed of 6 different subunits (RnfA to -G), of which three are integral membrane proteins. In the genome of C. autoethanogenum, the genes are clustered in one operon, rnf-CDGEAB (CAETHG_3227-32), 193 bases downstream of the gene rseC (CAETHG_3226). The rnf genes in CO-grown cells belong to the most highly expressed genes (932 FPKM average across all genes). One of the genes encoding the Rnf complex in C. ljungdahlii has been deleted and this resulted in reduced growth on sugar and no growth on H2/CO2 or CO (56).

Carbon monoxide dehydrogenase. The specific activity of the enzyme in cell extracts of H2/CO2-grown cells was 5.6 U/mg and thus considerably higher than in those of CO-grown cells (Table 1). This can be explained by the fact that during growth of C. autoethanogenum on H2/CO2 or fructose, the function of the enzyme is to catalyze the endergonic reduction of CO2 to CO (E0' = −520 mV) with reduced ferredoxin (E0' = −400 mV), a step in the total synthesis of acetate from 2 CO2, whereas during growth

FIG 2 Expression of the hyt-fdh gene cluster and surrounding genes during growth in continuous culture on CO-rich BOF gas (42% CO, 36% N2, 20% CO2, and 2% H2). The growth conditions were the same as those for the CO-rich BOF gas-grown cells analyzed for enzyme activity in Table 1. However, RNA isolation and enzyme activity measurements were performed from different harvests. The graph indicates coverage of reads from the RNA-seq experiments, with FPKM values for the indicated fragments. HytABCDE1E2, electron-bifurcating and NADP-dependent [FeFe]-hydrogenase.
on CO the function of the enzyme is to catalyze the exergonic oxidation of CO to CO₂ with ferredoxin. The Haldane equation predicts that the catalytic efficiency (\(k_{cat}/K_m\)) of the enzyme to oxidize CO divided by the catalytic efficiency of the enzyme to reduce CO₂ is equal to the equilibrium constant (57, 58).

The *C. autoethanogenum* genome harbors three genes (one of which is split) that encode Ni-iron monoxide monodehydrogenases (CAETHG_1620-21, CAETHG_3005, and CAETHG_3899). Of these, CAETHG_1620-21 encodes the CO dehydrogenase (AscA) in complex with acetyl-CoA synthase (AscB; CAETHG_1608) within the Wood-Ljungdahl gene cluster (CAETHG_1606-21). Interestingly, the gene encoding AscA consists of two open reading frames in *C. autoethanogenum*, whereas there is only a single gene in *C. ljungdahlii* (CLJU_c37670) or *C. ragsdalei*. Of the two CO dehydrogenases outside the Wood-Ljungdahl gene cluster, CAETHG_3005 forms an operon with genes encoding a putative [4Fe-4S]-ferredoxin protein and a reductase. The same arrangement is found in the other strains.

Of the three CO dehydrogenases genes, the split gene (CAETHG_1620-21) that encodes the enzyme in complex with the acetyl-CoA synthase (1,586 FPKM) and the gene CAETHG_3005 for CO dehydrogenase show very high expression (1,129 FPKM average across all genes), while the gene CAETHG_3899 for CO dehydrogenase is hardly expressed (0.9 FPKM) during growth on CO. In *C. ljungdahlii*, the homologue of CAETHG_3005 (CLJU_c09110) was found to be highly upregulated under lithoautotrophic growth conditions (growth on H₂/CO₂) compared to that under organo-heterotrophic growth conditions (growth on fructose) (41), while the opposite was reported in a study by Tan et al. (59).

In this respect, it is of interest that the genome of *Methanosarcina acetivorans* harbors one gene for CO dehydrogenase in a cluster with a gene for acetyl-CoA synthase and an orphan gene for CO dehydrogenases, either one of which can be disrupted without affecting growth of the methanogen on CO, methanol, or acetate, indicating that each of the two CO dehydrogenases can function in vivo in both directions (60, 61).

**Formate-hydrogen lyase.** The cell extracts of H₂/CO₂ grown cells catalyzed the formation of CO₂ and H₂ from formate and the reduction of CO₂ with H₂ to formate (HCOO⁻ + H⁺ \(\rightleftharpoons\) CO₂ + H₂) at similar specific rates. The specific rates were not affected by the addition of ferredoxin or NAD(P), indicating that CO₂ was directly reduced by H₂. The enzyme complex catalyzing the formate-hydrogen lyase reaction has been purified from CO₂-grown cells and characterized (46). It is composed of 7 different subunits, namely, FdhA and HytABCDE1E2. The subunit FdhA (CAETHG_2789) is a selenium- and tungsten-containing formate dehydrogenase, and HytABCDE1E2 is the electron-bifurcating and NADP- and ferredoxin-dependent hydrogenase described above, catalyzing reaction 3. The gene *fdhA* for the selenium-dependent formate dehydrogenase clusters with genes for formate dehydrogenase biosynthesis (moaA, mob, and *fdhD*; CAETHG_2790-93). The cluster is located directly upstream of the gene cluster *hycBDE, AE* for the electron-bifurcating [FeFe]-hydrogenase HytABCDE,E2. It has been shown that the two clusters are transcribed separately (46), which was confirmed by the RNA-seq analysis (Fig. 2).

**Formate dehydrogenase.** Cell extracts of H₂/CO₂-grown C. autoethanogenum catalyzed the NADP⁺-dependent reduction of ferredoxin with formate (Table 1). NAD⁺ could not substitute for NADP⁺. The reduction of NADP⁺ and of ferredoxin with formate proceeded in a 1:1 stoichiometry and was tightly coupled (2 HCOO⁻ + NADP⁺ + FdhA \(\rightleftharpoons\) 2 CO₂ + NADPH + Fdred²⁻ + H⁺). The electron-bifurcating formate dehydrogenase has been purified from CO₂-grown C. autoethanogenum and found to be composed of the subunits FdhA and HytABCDE,E2. It is thus the same enzyme complex (HytA-E/FdhA) that catalyzes the reduction of ferredoxin and NADP⁺ with 2 H₂ (reaction 3) and the formate-hydrogen lyase reaction.

In the genome of C. autoethanogenum, there are several genes for formate dehydrogenase: the aforementioned CAETHG_2789 (*fdhA*) as well as CAETHG_0084 encode two selenium-containing enzymes, and CAETHG_2988 encodes a selenium-free enzyme. Of these, the gene for the formate dehydrogenase that forms a complex with the Hyt hydrogenase is most highly expressed (580 FPKM) (Fig. 2).

None of the formate dehydrogenases appear to have a “periplasmic” location, based on the lack of an N-terminal twin arginine motif. This is in contrast to *Moorella thermoaceticata*, in which one of three formate dehydrogenases is predicted to have a “periplasmic” location (52, 54, 62).

**Methylene-H₄F dehydrogenase (Fold).** Cell extracts of H₂/CO₂-grown C. autoethanogenum catalyzed the reduction of NADP⁺ with methylene-H₄F (reaction 5) at a specific rate of 0.7 U/mg. The activity with NAD⁺ was below the detection limit. The highest specific activity (5.7 U/mg) was found in CO₂-grown cells, and an intermediate specific activity (2.4 U/mg) was determined for fructose-grown cells, in agreement with the finding that growth on H₂/CO₂ was slowest (\(t_d = 33\) h) and on CO it was fastest (\(t_d = 5\) h).

\[
\text{NADPH} + \text{methylene-H}_4\text{F} + \text{H}^+ \rightleftharpoons \text{NADP}^+ + \text{methylene-H}_4\text{F}
\]

In the genome of C. autoethanogenum, only one gene (CAETHG_1616), for a methylene-H₄F dehydrogenase, is found. It is predicted to encode a cytoplasmic bifunctional cyclohydro-lase/dehydrogenase without a prosthetic group and is located in a cluster of genes that encode many of the other enzymes involved in the Wood-Ljungdahl pathway, including methylene-H₄F reductase (CAETHG_1614-15).

**Methylene-H₄F reductase (MetFV).** Cell extracts of H₂/CO₂-grown cells catalyze the reduction of benzyl viologen with methyl-H₄F (reaction 6). The extracts did not catalyze the oxidation of NADPH or NADH with methylene-H₄F in either the absence or the presence of oxidized ferredoxin (Table 1). It should be noted that the cell extracts catalyzed a slow methylene-H₄F-dependent reduction of ferredoxin in the absence or presence of NAD(P)H. This activity was probably due to the high formdehyde:ferredoxin oxidoreductase activity present in the cell extracts, considering that methylene-H₄F is synthesized by a spontaneous reaction of H₄F with formaldehyde and that methylene-H₄F also spontaneously dissociates into H₂ and formaldehyde. Formaldehyde:ferredoxin oxidoreductase activity is a side activity of the acetalddehyde:ferredoxin oxidoreductase (63–65), which was found to have high specific activities in the cell extracts (Table 1).

\[
2\text{e}^- + \text{methylene-H}_4\text{F} + 2\text{H}^+ \rightleftharpoons \text{methyl-H}_4\text{F}
\]

In the genome of C. autoethanogenum, only one set of genes for methylene-H₄F reductase is found, namely, *metFV* (CAETHG_1614-15), which is adjacent to the gene *fold* for meth-
ylene-H4F dehydrogenase (CAETHG_1616). Both genes are reported to be cotranscribed in C. ljungdahlii (41), and the same was confirmed in the RNA-seq analysis for C. autoethanogenum (data not shown). Heterologous expression of the metF gene from C. autoethanogenum in Escherichia coli revealed that the subunit MetF contains one FMN rather than FAD, the usual cofactor of methylene-H4F reductases. Bioinformatic analyses indicated that MetF lacks an NAD(P) binding site and favors FMN as a cofactor. MetF exhibited high methylene-H4F reductase activity with benzyl violen only when produced together with the iron-sulfur zinc protein MetV, which in part shows sequence similarity to MetF (23, 62). The nature of the electron donor(s) is still uncertain (see Discussion).

**Acetaldehyde:ferredoxin oxidoreductase.** Cell extracts of H2/CO2-grown cells exhibited very high acetaldehyde:ferredoxin oxidoreductase (AOR) activity (Table 1) that catalyzes the reversible reaction 7. The high specific activity (8 U/mg) can probably be explained by the fact that the activity was tested in the direction of ferredoxin (E0 red = −400 mV) reduction with acetaldehyde (E0′ = −580 mV) rather than in the physiological direction of acetic acid reduction with reduced ferredoxin (see the explanation given for CO dehydrogenase).

\[
\text{Fd}_{\text{red}}^{2–} + \text{CH}_3\text{CHO}^+ + 3 \text{H}^+ \rightleftharpoons \text{Fd}_{\text{ox}} + \text{CH}_3\text{CHO} + \text{H}_2\text{O} \quad (7)
\]

We tried to also determine in the cell extracts the activity in the physiological direction by measuring the acetate-dependent oxidation of NADH in the presence of ferredoxin and CO (100% in the gas phase). In this assay, the acetaldehyde:ferredoxin oxidoreductase reaction is coupled with the ferredoxin-dependent carbon monoxide dehydrogenase reaction and NAD-dependent ethanol dehydrogenase reaction. Unfortunately, the background activity was too high for an accurate determination, one likely reason being that the cell extracts contained acetate, which the cells accumulate during growth (see Discussion).

The genome of C. autoethanogenum encodes two putative AORs (CAETHG_0092 and CAETHG_0102) that share 79% identity on the amino acid level and are both highly expressed (1,558 and 353 FPKM) when the bacterium is grown on CO. The acetaldehyde:ferredoxin oxidoreductases are molybdenum- or tungsten iron-sulfur proteins that have been characterized for other acetogenic bacteria and shown to catalyze the reversible reduction of acetic acid to acetaldehyde (63–65). A role in ethanol formation from acetic acid during growth of C. autoethanogenum on CO has therefore been proposed (46). The high specific activity of acetaldehyde:ferredoxin oxidoreductase in extracts of H2/CO2-grown cells (Table 1) indicates that the enzyme also plays a critical role in the formation of ethanol from H2 and CO2. Gene expression profiles obtained with C. ljungdahlii point in the same direction (66).

In addition to the two genes for the two acetaldehyde:ferredoxin oxidoreductases, the genome of C. autoethanogenum contains two genes annotated as aldehyde oxidoreductases. Careful inspection revealed, however, that the two genes most probably encode xanthine dehydrogenases rather than acetaldehyde:ferredoxin oxidoreductases.

**Alcohol dehydrogenase and acetaldehyde dehydrogenase.** Cell extracts of H2/CO2-grown cells catalyzed the reduction of acetaldehyde with NADH rather than with NADPH (reaction 8) and the reduction of NADP+ with acetaldehyde in a CoA-depen-
dent reaction (reaction 9). Some acetaldehyde dehydrogenase activity (CoA acetylation) was found, also with NAD+ (Table 1).

\[
\text{CH}_3\text{CHO} + \text{NADH} + \text{H}^+ \rightleftharpoons \text{CH}_3\text{CH}_2\text{OH} + \text{H}_2\text{O} + \text{NAD}^+ \quad (8)
\]

\[
\text{CH}_3\text{CO}-\text{CoA} + \text{NAD}(P)\text{H} + \text{H}^+ \rightleftharpoons \text{CH}_3\text{CHO} + \text{NAD}(P)^+ + \text{CoA} \quad (9)
\]

In the genome of C. autoethanogenum, there are two adjacent open reading frames (CAETHG_3747 and CAETHG_3748) for bifunctional alcohol dehydrogenase/acetaldehyde dehydrogenase (CoA acetylation; AdhE). It is not yet known whether these enzymes are NAD or NADP dependent. In C. ljungdahlii, the genes for the putative bifunctional alcohol/acetdehyde dehydrogenases, adhE1 (CLJU_c16510) and adhE2 (CLJU_c16520), are arranged the same way and have been deleted individually or together. In C. ljungdahlii, deletion of adhE1 but not adhE2 diminished ethanol production from fructose with proportional carbon recovery in acetate. The double deletion mutant had a phenotype similar to that of the adhE1-deficient strain (67). RNA-seq data indicated that only the adhE1 (61 FPKM) and not the adhE2 gene (0.4 FPKM) was transcribed in C. autoethanogenum, and data from C. ljungdahlii (41) suggest the same, explaining the observed effect.

Additionally, the genome of C. autoethanogenum contains open reading frames for three putative aldehyde dehydrogenases (CoA acetylation) that catalyze reaction 9 (CAETHG_1819, CAETHG_1830, and CAETHG_3287). All three are clustered with genes for microcompartment proteins and genes for ethanolamine or propanediol utilization. Genes CAETHG_1819 and CAETHG_3287 share 98% sequence identity on the nucleotide level. In each of the two microcompartment protein gene clusters, there is also a gene for an alcohol dehydrogenase (CAETHG_1813 and CAETHG_3279). The coenzyme specificities have not been determined for any of these dehydrogenases.

Besides the two adhE genes and the two genes for alcohol dehydrogenase in the microcompartment protein gene clusters, there are additional genes annotated as alcohol dehydrogenases. Of these orphan alcohol dehydrogenases, three have been purified and characterized in detail. These are the primary:secondary alcohol dehydrogenase of C. autoethanogenum encoded by CAETHG_0553 (10), as well as two butanol dehydrogenases of C. ljungdahlii encoded by CLJU_c24880 (homologue of CAETHG_0555) and CLJU_c39950 (homologue of CAETHG_1841) (68). All three alcohol dehydrogenases have been shown to have activity with acetaldehyde/ethanol and to prefer NAD(P)H rather than NADH as a cofactor. It is thus unlikely that they play a major role in ethanol formation during growth on H2/CO2, where mainly NADH-dependent alcohol dehydrogenase activity was measured in cell extracts (Table 1).

**Pyruvate:ferredoxin oxidoreductase.** Cell extracts of H2/CO2 or CO-grown C. autoethanogenum catalyzed the CoA-dependent reduction of ferredoxin with pyruvate at a specific rate of 0.2 U/mg and of cell extracts of fructose-grown cells at a specific rate of 0.6 U/mg (Table 1). During growth on H2/CO2 or on CO, the function of the enzyme was anabolic and it catalyzed the reduction of acetyl-CoA plus CO2 to pyruvate (E0′ = −500 mV) with reduced ferredoxin (E0 red = −400 mV) (reaction 10), which under standard conditions is an endergonic reaction.

\[
\text{CH}_3\text{CO}^-\text{CoA} + \text{CO}_2 + \text{Fd}_{\text{red}}^{2–} + \text{H}^+ \rightleftharpoons \text{CH}_3\text{COCOO}^- + \text{Fd}_{\text{ox}} + \text{CoA} \quad (10)
\]
During growth on fructose, the function of the enzyme is catabolic and it catalyzes the reverse reaction. In the genome of *C. autoethanogenum*, there are two copies of the gene for pyruvate:ferredoxin oxidoreductase (CAETHG_0928 and CAETHG_3029).

Glyceraldehyde-phosphate dehydrogenase. Cell extracts catalyzed the readily reversible phosphate-dependent reduction of \( \text{NAD}^+ \) rather than that of \( \text{NADP}^+ \) with GAP (reaction 11). The specific activities (Table 1) are sufficient to account for the purely anabolic function of the enzyme when *C. autoethanogenum* is grown on \( \text{H}_2/\text{CO}_2 \) (0.1 U/mg) or \( \text{CO} \) (0.4 U/mg) and for the purely catabolic function when the bacterium is grown on fructose (1.6 U/mg) (Table 1).

\[
\text{GAP} + \text{P} + \text{NAD}^+ \rightleftharpoons \text{1,3-bis-phosphoglycerate}^- + \text{NADH} + \text{H}^+ \quad (11)
\]

The genome of *C. autoethanogenum* contains two open reading frames for GAP dehydrogenase (GAPDH; CAETHG_1760 and CAETHG_3424), one of which probably is NAD specific and the other NADP specific. This is indicated by the finding that in extracts of \( \text{H}_2/\text{CO}_2 \)- and fructose-grown cells, there was some GAPDH activity also with NADP (Table 1).

**DISCUSSION**

**\( \text{H}_2 \) activation.** The results indicated that during growth of *C. autoethanogenum* on \( \text{H}_2 \) and \( \text{CO}_2 \), probably only one of the six hydrogenases encoded by the genome is active, namely, the electron-bifurcating, NADP- and ferredoxin-dependent \([\text{FeFe}]\)-hydrogenase HytABCDE\(_1\)\(\_E_2\) catalyzing reaction 3. This hydrogenase appears to provide the cells not only with electrons for the reduction of NADP and ferredoxin but also, together with formate dehydrogenase, with electrons for the reduction of \( \text{CO}_2 \), as shown in Fig. 3 and 4. The hydrogenase differs in several respects from the electron-bifurcating \([\text{FeFe}]\)-hydrogenase HydABCD present in \( \text{H}_2/\text{CO}_2 \)-grown *A. woodii*: HydABCD is NAD rather than NADP specific, is composed of four rather than of six different subunits, and is not involved in \( \text{CO}_2 \) reduction to formate (12).

An electron-bifurcating, NAD- and ferredoxin-dependent hy-
dogenous activity was observed in neither H₂/CO₂-grown cells nor in fructose- nor CO-grown cells (Table 1). However, such a hydrogenase (most likely CAETHG_3569-71) could have a function when the cells are grown at lower H₂ concentrations than the H₂ concentration used to grow the cells analyzed for enzyme activity. At an H₂ concentration below 1%, the coupled reduction of NADP and ferredoxin with H₂ is thermodynamically no longer possible (see the introduction), because then the redox potentials of the 2H⁻/H₂ couple and that of the NADP⁻/NADPH couple are almost identical, whereas the redox potential of the NAD⁺/NADH couple is still more positive by 60 mV.

**Methylene-H₄F reduction to methyl-H₄F.** There is a second important difference between *C. autoethanogenum* and *A. woodii*. In *A. woodii*, the reduction of methylene-H₄F to methyl-H₄F is catalyzed by an NAD-specific methylene-H₄F reductase composed of the subunits MetF, MetV, and RnfC2 (23). MetF contains FMN and is the site of methylene-H₄ reduction, RnfC2 contains FMN and iron-sulfur clusters and is the site of NADH oxidation, and MetV is an iron-sulfur protein that mediates electron transfer from RnfC2 to MetF. In *C. autoethanogenum*, only the genes for MetFV and not those for RnfC2 are present, which is in agreement with the observation that in cell extracts, methylene-H₄F reductase activity is only found with viologen dyes (Table 1).

It has been proposed (9) that in *C. ljungdahlii* the subunits MetFV form a complex with the electron transfer flavoproteins (EtfAB) and that the complex of MetFV and EtfAB mediates the reduction of methylene-H₄ to methyl-H₄F (Eₐ = −200 mV) (69) with NADH (Eₐ = −320 mV) and couples this exergonic reaction with the endergonic reduction of ferredoxin (Eₐ = −400 mV) with NADH (reaction 12). The proposal is substantiated by the finding that in anaerobic bacteria the complexes of EtfAB with butyryl-CoA dehydrogenase (43, 70), with caffeyl-CoA reductase (71) and with lactate dehydrogenase (72) catalyze electron-bifurcating reactions, in which the exergonic reduction of cytochrome-CoA, caffeyl-CoA and pyruvate, respectively, with NADH are coupled with the endergonic reduction of ferredoxin with NADH. In these complexes EtfAB is assumed to be the site of electron bifurcation (38, 70).

\[
\text{Methylene-H}_4\text{F} + 2 \text{NADH} + \text{Fd}_{\text{ox}} \rightarrow \text{methyl-H}_4\text{F} + 2 \text{NAD}^+ + \text{Fd}_{\text{red}}^2^- \quad (12)
\]

MetFV alone catalyzes only the reduction of methylene-H₄F with reduced viologen dyes (62), and EtfAB alone only catalyzes the reduction of viologen dyes with NADH (70). Both activities are found in cell extracts of *C. autoethanogenum* but a ferredoxin-dependent reduction of methylene-H₄F with NADH (reaction 12) or NADPH has not yet been demonstrated, maybe because MetFV and EtfAB only form a loose complex which dissociates in the cell extracts. In the genome of *C. autoethanogenum*, the genes metFV (CAETHG_3551-52), of which there exist no paralogues, are not colocated with the genes etfA, of which there exist five paralogues: CAETHG_0115-0116 (clustered with an FMN/FAD-containing dehydrogenase and a reductase); CAETHG_0245-0246 (clustered with an FMN/FAD binding protein); CAETHG_1785-86 (clustered with an acyl-CoA dehydrogenase, transferase, and ligase); CAETHG_1868-69 (clustered with a FixC protein); and CAETHG_3471-72 (clustered with an FMN/FAD binding protein). It could also be that in vivo methylene-H₄F reductase forms a complex with both EtfAB and carbon monoxide dehydrogenase and that only this supercomplex couples the exergonic reduction of methylene-H₄F to methyl-H₄F with the endergonic reduction of CO₂ to CO (Fig. 3 and 4). A further possibility is that an iron-sulfur protein other than ferredoxin or a specific ferredoxin is the low-potential electron acceptor in reaction 12 and that therefore activity with ferredoxin from *C. pasteurianum* was not found.

As explained below, it is also possible that methylene-H₄F reductase in *C. autoethanogenum* is not electron bifurcating and is either NAD or NADP specific, as EtfAB can be involved in electron transfer from NADH to a more positive electron acceptor without electron bifurcation, as has been reported for the propionyl-CoA dehydrogenase/EtfAB complex from *Clostridium propionibacterium* (38). Finally, it has to be considered that in *C. autoethanogenum*, depending on the growth conditions (low or high H₂ concentrations) (Table 2), MetFV could form complexes with different diaphorases (flavoproteins like EtfA and RnfC2) that catalyze the reduction of dyes with NAD(P)H. Dependent on the diaphorase, the complex could be electron bifurcating or not electron bifurcating.

**Acetic acid reduction to acetaldehyde.** There are three other important differences between *C. autoethanogenum* and *A. woodii* with respect to their oxidoreductase composition. In *C. autoethanogenum*, the methylene-H₄F dehydrogenase is NADP specific, whereas this enzyme in *A. woodii* is NAD specific (12). In *C. autoethanogenum*, there is an electron-bifurcating and ferredoxin-dependent transhydrogenase (Nfn) (46) which is not present in *A. woodii* (27). Most importantly, in *C. autoethanogenum* there is an active acetaldehyde:ferredoxin oxidoreductase (AOR) which is not found in *A. woodii* (27). A putative formaldehyde:ferredoxin oxidoreductase gene in *A. woodii* lacks the part for the important C-terminal domain (Awo_c12420).

In *C. autoethanogenum*, acetaldehyde:ferredoxin oxidoreductase can catalyze the direct reduction of acetic acid (pK ~4.7) to acetaldehyde (65–65) without prior activation of the acetic acid to acetyl-CoA, despite the fact that under standard conditions (1 M concentrations of substrates and products except for the proton concentration of 10⁻⁷ M) the reduction of acetate to acetaldehyde (Eₐ = −580 mV) with reduced ferredoxin (Eₐ = −400 mV) is endergonic by +35 kJ/mol (31). The bioreduction of carboxylic acids has recently been reviewed (73).

The internal pH of *C. autoethanogenum* was determined to be near 6 when grown on BOF gas at pH 5.3, based on measurements of the intracellular and extracellular acetate concentrations (unpublished results). An internal pH of near 6 has also been observed in other anaerobes when they are grown at an extracellular pH near 5 (29, 74–78). At pH 6, the thermodynamics of direct acetate reduction are more favorable by ~17.1 kJ/mol, since in reaction 7 three protons are consumed. Thus, at pH 6, reaction 7 already is exergonic when the intracellular acetate concentration is 1,000-fold higher than the extracellular acetaldehyde concentration. Such a high ratio is easily reached in the cell when the intracellular pH is 6 and the extracellular is pH 5. Then the intracellular acetate concentration will be 10 times higher than the extracellular acetate concentration, since undissociated acetic acid can freely diffuse through the cytoplasmic membrane but not the anion. For example, at an extracellular acetate concentration of 0.01 M (0.6 g/liter), the intracellular acetate concentration will be 0.1 M and reaction 7 will be already exergonic at an acetaldehyde concentration of 0.1 mM. However, the intracellular acetaldehyde concentration is most probably much lower than 0.1 mM due to the
TABLE 2 ATP gains predicted from metabolic schemes for C. autoethanogenum

<table>
<thead>
<tr>
<th>Scenario and fermentation product</th>
<th>Methylene-H4F reductase</th>
<th>Acetaldehyde dehydrogenase (CoA acetylating)</th>
<th>Acetaldehyde:ferredoxin oxidoreductase</th>
<th>ATP gain</th>
<th>Theoretical H2 threshold (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Scenario 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>2 NADH + Fd_{ox}</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Ethanol</td>
<td>2 NADH + Fd_{ox}</td>
<td>+</td>
<td>-</td>
<td>0.5</td>
<td>~1</td>
</tr>
<tr>
<td>Ethanol</td>
<td>2 NADH + Fd_{ox}</td>
<td>-</td>
<td>+</td>
<td>1.2</td>
<td>&gt;10</td>
</tr>
<tr>
<td><strong>Scenario 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>2 NADPH + Fd_{ox}</td>
<td>-</td>
<td>-</td>
<td>0.4</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Ethanol</td>
<td>2 NADPH + Fd_{ox}</td>
<td>+</td>
<td>-</td>
<td>-0.04</td>
<td>0</td>
</tr>
<tr>
<td>Ethanol</td>
<td>2 NADPH + Fd_{ox}</td>
<td>-</td>
<td>+</td>
<td>0.7</td>
<td>&gt;1</td>
</tr>
<tr>
<td><strong>Scenario 3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>NADH</td>
<td>-</td>
<td>-</td>
<td>0.4</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Ethanol</td>
<td>NADH</td>
<td>+</td>
<td>-</td>
<td>-0.03</td>
<td>0</td>
</tr>
<tr>
<td>Ethanol</td>
<td>NADH</td>
<td>-</td>
<td>+</td>
<td>0.7</td>
<td>&gt;1</td>
</tr>
<tr>
<td><strong>Scenario 4</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>NADPH</td>
<td>-</td>
<td>-</td>
<td>0.14</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Ethanol</td>
<td>NADPH</td>
<td>+</td>
<td>-</td>
<td>-0.3</td>
<td>0</td>
</tr>
<tr>
<td>Ethanol</td>
<td>NADPH</td>
<td>-</td>
<td>+</td>
<td>0.4</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

α ATP gains (in moles of ATP per mole of product) were predicted from the metabolic schemes for C. autoethanogenum (see Fig. 3 and 4 for illustration of results with Clostridium autoethanogenum growing on H2 and CO2). In all scenarios, H2 activation is assumed to be catalyzed by electron-bifurcating and NADP dependent (Fe/S)-hydrogenase (HysA-E), and ethanol formation from acetaldehyde is assumed to be catalyzed by a NAD-dependent alcohol dehydrogenase. Either a NADP-dependent CoA-acetylating acetaldehyde dehydrogenase or an acetaldehyde:ferredoxin oxidoreductase is involved in acetaldehyde formation. In scenario 1 (see Fig. 3 and 4), the methylene-H4F reductase is assumed to be electron bifurcating and NAD dependent. In scenario 2, methylene-H4F reductase is assumed to be electron bifurcating and NADP dependent. In scenario 3, methylene-H4F reductase is assumed to be non-electron bifurcating and NAD dependent. In scenario 4, methylene-H4F reductase is assumed to be non-electron bifurcating and NADP dependent. From the ATP gains, the H2 thresholds were calculated. The H2 threshold is the minimal H2 concentration in the gas phase in equilibrium with the liquid phase at which the fermentation is sufficiently exergonic to allow for the predicted ATP gain (see Discussion). +, involved; -, not involved; <, somewhat below threshold value; >, somewhat above threshold value.

The exergonic reduction of acetaldehyde to ethanol (E0' = −200 mV) with NADH (E0' = −320 mV).

Many bacteria contain a monocarboxylate permease for acetate transport into the cells when the extracellular pH is high, the acetate concentration is low, and thus the acetic acid concentration is very low (79–81). This indicates that diffusion of acetic acid into the cells can become rate limiting. In the genome of C. autoethanogenum, a gene for such a transporter is not apparent.

The acetaldehyde:ferredoxin oxidoreductase has been shown to only catalyze the reduction of the undissociated acetic acid (pKa = 4.7) rather than the dissociated acetate (64, 73). At an intracellular pH of 6 and an intracellular acetic acid concentration of 0.1 M, as assumed in the example above, the intracellular concentration of acetic acid is 5 mM and thus in the order of the reported K_m of 6 mM acetic acid for the acetaldehyde:ferredoxin oxidoreductase (64).

With a recombinant strain of Pyrococcus furiosus it was recently demonstrated that the acetaldehyde:ferredoxin oxidoreductase is involved in ethanol formation from acetic acid (82).

Aldehyde:ferredoxin oxidoreductase are tungsten- or molybdenum iron-sulfur proteins (63–65). It has therefore been considered that ethanol formation from H2 and CO2 in C. autoethanogenum could be steered via the tungstate and/or molybdate concentration. However, since also the formate dehydrogenase catalyzing the first step in the Wood-Ljungdahl pathway is a tungsten- and/or molybdenum iron-sulfur protein (46), this proved not to be possible.

**Calculation of ATP gains and H2 thresholds.** For the calculation of ATP gains (mole ATP per mole of acetate or ethanol formed from H2 and CO2), four reactions have to be considered: (i) the formyl-H4F synthetase reaction in which 1 ATP is consumed; (ii) the acetate kinase reaction, in which 1 ATP is formed by substrate level phosphorylation; (iii) the reduced ferredoxin: NAD oxidoreductase reaction, in which two protons are electrogenicly translocated via the Rnf complex; (iv) the ATP synthetase (F1F0) reaction, in which depending on the number of subunits in the c-ring between 3 and 4 vectorial protons are required to drive the synthesis of 1 ATP (83). The c-ring of the ATP synthase of Clostridium paradoxum has been shown to be composed of 11 identical subunits, indicating that in clostridia 3.66 (11/3) protons are required to drive the synthesis of 1 ATP (84, 85). This stoichiometry was used in all our calculations (Fig. 3 and 4; Table 2). It is a little bit higher than that used in the metabolic scheme for the energy metabolism of A. woodii (Fig. 1), whose ATP synthase most probably requires 3.33 (10/3) sodium ions for 1 ATP (20), and it is a little bit lower than the stoichiometry of 4 (12/3) generally assumed for the ATP synthase of bacteria (12). All three stoichiometries are compatible with the conclusions drawn in the following for the energy metabolism of C. autoethanogenum.

From the ATP gains, H2 thresholds can be calculated (86), considering that to drive the synthesis of 1 ATP under the irreversible conditions of the living cell, between −60 kJ/mol and −80 kJ/mol are required (29, 31).

**Different scenarios.** In the following discussion, we first assume that in C. autoethanogenum, methylene-H4F reductase catalyzes the electron-bifurcating reaction 12 with NADH as electron donor. The metabolic scheme in Fig. 3 additionally assumes that only acetic acid is formed as the product from H2 and CO2. All the
enzymes required are present in \( \text{H}_2/\text{CO}_2 \)-grown cells (Table 1). The scheme predicts that per mole of acetate about 1 ATP is generated, for which, under the conditions in the growing cells, at least \(-60 \text{ kJ/mol acetate are required (see above)}\). At pH 5.3, an acetate concentration in the growth medium near 0.1 M and a \( \text{CO}_2 \) concentration in the gas phase of 23% (growth conditions of the cells described in the Results section), acetate formation from \( \text{H}_2/\text{CO}_2 \) (reaction 1) is associated with a free energy change of about \(-60 \text{ kJ/mol} \), when the \( \text{H}_2 \) concentration in the gas phase in equilibrium with the liquid phase is somewhat below 10% (the theoretical \( \text{H}_2 \) threshold) (Table 2). The much smaller ATP gain per mol acetate in \( \text{A. woodii} \) (0.3 mol ATP/mol acetate) (Fig. 1) allows this acetogen to grow at much lower \( \text{H}_2 \) concentrations (at about 0.1%), which can explain why \( \text{A. woodii} \) is much easier to cultivate on \( \text{H}_2/\text{CO}_2 \) than \( \text{C. autoethanogenum} \), with an ATP gain near 1 mol ATP/mol acetate and an \( \text{H}_2 \) threshold of \(<10% \) (Table 2).

Schemes of the energy metabolism of \( \text{C. autoethanogenum} \) are given in Fig. 4A and B, under the assumption of an electron-bifurcating, NAD- and ferredoxin-dependent methylene-\( \text{H}_2 \)-F reductase and that only ethanol is formed as the product from \( \text{H}_2 \) and \( \text{CO}_2 \). The scheme in Fig. 4A involves ethanol formation via an NADP-specific acetaldehyde dehydrogenase (CoA acetylating) and NAD-specific ethanol dehydrogenase, and the scheme in Fig. 4B involves acetaldehyde:ferredoxin oxidoreductase and NAD-specific ethanol dehydrogenase (Table 1). Ethanol formation via acetyl-CoA reduction to acetaldehyde is associated with the formation of 0.5 mol ATP, requiring reaction 2 to be exergonic by at least \(-30 \text{ kJ/mol} \). Under the growth conditions described in the Results section, this is the case when the \( \text{H}_2 \) concentration in the gas phase in equilibrium with the liquid phase is somewhere near 1% (Table 2). Ethanol formation via acetic acid reduction to acetaldehyde involving acetaldehyde:ferredoxin oxidoreductase is associated with the net formation of 1.2 mol ATP, requiring reaction 2 to be exergonic by at least \(-70 \text{ kJ/mol} \). Under the growth conditions described in the Results section, this is the case when the \( \text{H}_2 \) concentration in the gas phase in equilibrium with the liquid phase is higher than 10% (Table 2).

The metabolic schemes in Figs. 3 and 4 predict that per 4 \( \text{H}_2 \) only 0.25 ferredoxin (Fig. 3) and per 6 \( \text{H}_2 \) only 0.25 ferredoxin (Fig. 4A) or 0.75 ferredoxin (Fig. 4B) are reduced in the transhydrogenase reaction catalyzed by Nfn. This stoichiometry can explain why only relatively low specific activities of this enzyme were found in \( \text{H}_2/\text{CO}_2 \)-grown cells of \( \text{C. autoethanogenum} \) (Table 1).

In Table 2, scenarios are also considered under the assumption that methylene-\( \text{H}_2 \)-F reduction is electron bifurcating but NADP rather than NAD specific (Table 2, scenario 2) and that methylene-\( \text{H}_2 \)-F reduction is not electron bifurcating and is either NAD specific (Table 2, scenario 3) or NADP specific (Table 2, scenario 4). Notably, the formation of acetic acid from \( \text{H}_2 \) and \( \text{CO}_2 \) is always associated with net ATP synthesis regardless of the methylene-\( \text{H}_2 \)-F reductase involved. This is also the case for ethanol formation from \( \text{H}_2 \) and \( \text{CO}_2 \) when ethanol is formed via acetic acid reduction to acetaldehyde involving acetaldehyde:ferredoxin oxidoreductase. However, the ATP gains are much lower than the scenario where the methylene-\( \text{H}_2 \)-F reductase is assumed to be electron bifurcating and NAD specific (Table 2). Lower ATP gains would allow \( \text{C. autoethanogenum} \) to grow at lower \( \text{H}_2 \) concentrations (\( \text{H}_2 \) thresholds), which could be of advantage, but which would only be possible if the electron-bifurcating [FeFe]-hydrogenase would be NAD\(^+\) rather than NADP\(^+\) specific, based on the thermodynamic reasons discussed above. The fact that it is extremely difficult to grow \( \text{C. autoethanogenum} \) on \( \text{H}_2 \) and \( \text{CO}_2 \) argues against a low \( \text{H}_2 \) threshold, although the difficulties may have other reasons.

More revealing is, however, the finding that ethanol formation from \( \text{H}_2 \) and \( \text{CO}_2 \) via acetyl-CoA reduction to acetaldehyde involving CoA-linked acetaldehyde dehydrogenase is only associated with energy conservation when the methylene-\( \text{H}_2 \)-F reductase is assumed to be electron bifurcating and NAD specific (Fig. 4A), while in any other case the ATP gain is negative (ATP is net consumed rather than formed) (Table 2). Therefore, we conclude that at least in the case of ethanol formation from \( \text{H}_2 \) and \( \text{CO}_2 \) via acetyl-CoA reduction to acetaldehyde, the methylene-\( \text{H}_2 \)-F reductase must be electron bifurcating and NAD specific, because only in this case would the ATP gain be positive and able to support growth.

There is a caveat, however, for this scenario: it is possible that ethanol formation from \( \text{H}_2 \) and \( \text{CO}_2 \) in \( \text{C. autoethanogenum} \) does not at all involve acetyl-CoA reduction to acetaldehyde and that the CoA-linked acetaldehyde dehydrogenase (AdhE) activity found in the \( \text{H}_2/\text{CO}_2 \)-grown cells (Table 1) is only there to allow the cells to reuse the ethanol formed by coupling ethanol oxidation to acetate to the reduction of 2 \( \text{CO}_2 \) to acetate when the ethanol concentration is high and the \( \text{H}_2 \) concentration is low. Indeed, under the latter conditions, the ethanol concentration in cultures of \( \text{C. autoethanogenum} \) growing on BOF gas can decrease again (unpublished results) and, similarly, butanol oxidation to butyrate has been reported in \( \text{C. ljungdahlii} \) (9, 88). Growth on ethanol and \( \text{CO}_2 \) has also been observed for \( \text{A. woodii} \) (35). A counterargument is that ethanol formation from CO-rich BOF gas appears to be favored when the pH in the growth medium is pH 6 rather than pH 5.5 (87, 88), i.e., conditions under which ethanol formation via acetic acid reduction to acetaldehyde is less favored (see above). However, these findings were not backed by a more recent study that showed that during growth of \( \text{C. autoethanogenum} \) on \( \text{CO}_2 \) at pH 4.7, mainly ethanol was formed, whereas at pH 6 ethanol and acetate were generated in almost equal amounts (89).

When \( \text{C. autoethanogenum} \) is grown on \( \text{H}_2 \) and \( \text{CO}_2 \), both \( \text{H}_2 \) and \( \text{CO}_2 \) are consumed (90), sometimes resulting in a decrease in the \( \text{H}_2 \) concentration in the gas phase to below the concentration of 1%. This observation does not necessarily argue for a low \( \text{H}_2 \) threshold during growth on \( \text{H}_2 \) and \( \text{CO}_2 \), since under mixotrophic growth conditions the \( \text{H}_2 \) threshold can be lower, which has been clearly shown for \( \text{A. woodii} \) growing on \( \text{H}_2 \), \( \text{CO}_2 \), and lactate (26).

It was mentioned above that at \( \text{H}_2 \) concentrations below 1% the coupled reduction of NADP and ferredoxin with \( \text{H}_2 \) is thermodynamically no longer possible and that therefore at such low \( \text{H}_2 \) concentrations an electron-bifurcating, NAD- and ferredoxin-dependent [FeFe]-hydrogenase (HydABC) or one of the nonbifurcating ferredoxin-only-dependent [FeFe]-hydrogenases should come into action. Such scenarios were not discussed because they involve too many unknowns.

**Conclusion.** In a recent review on the bioenergetics of acetogens (12), it was stated that “open questions in the bioenergetics of \( \text{C. ljungdahlii} \) (growing on \( \text{H}_2 \) and \( \text{CO}_2 \)) concern the electron acceptor of the hydrogenase (either ferredoxin and NAD or ferredoxin and NADP), the electron donor for the formate dehydrogenase (ferredoxin, hydrogen, or ferredoxin and NADPH), and...
whether or not the methylene-H$_2$F reductase is electron bifurcating and reduces ferredoxin.” Our results indicate that in C. auto-ethanogenum, a close relative of C. ljungdahlii, ferredoxin and NADP are the electron acceptors for H$_2$ oxidation (reaction 3) and that CO$_2$ is reduced with H$_2$ in a reaction that does not involve ferredoxin and/or NAD(P)H. But the third open question, whether or not the methylene-H$_2$F reductase is electron bifurcating and “reduces ferredoxin,” remains to be answered. In Moorella thermoacetica, there is indirect evidence for an electron-bifurcating and NAD-specific methylene-H$_2$F reductase (62).

ACKNOWLEDGMENTS

This work was supported by the Max Planck Society and the Fonds der Chemischen Industrie. We thank the following investors in LanzaTech’s technology: Stephen Tindall, Khola Ventures, Qiming Venture Partners, Softbank China, the Malaysian Life Sciences Capital Fund, Mitsui, Primetals, CIICC Growth Capital Fund I, L.P., and the New Zealand Superannuation Fund.

REFERENCES


60. Bertsch J, Parthasarathy A, Buckel W, Müller V. 2013. An electron-


74. Rieling V, Thauer RK, Jungermann K. 1975. Internal-alkaline pH gradient, sensitive to uncoupler and ATPase inhibitor, in growing Clostri


77. Menzel U, Gottschalk G. 1985. The internal pH of Acetobacterium wiera


81. Allegre

82. Ferguson SA, Keis S, Cook GM. 2006. Biochemical and molecular characteriza
tion of a Na⁺–translocating F₁Fo-ATPase from the thermoalkali
philic bacterium Clostridium parado


ters on growth of Clostridium ljungdahlii and Clostri
dium autoethano

85. Yon
ess H, Naja
pour G, Mohamed AR. 2005. Ethanol and acetate production from synthesis gas via fermentation processes using anaerobic bic


ters on growth of Clostridium ljungdahlii and Clostri
dium autoethano

88. Yon
ess H, Naja
pour G, Mohamed AR. 2005. Ethanol and acetate production from synthesis gas via fermentation processes using anaerobic bic