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*Published in:*  
Current Opinion in Biotechnology

*Link to article, DOI:*  
[10.1016/j.copbio.2013.12.001](https://doi.org/10.1016/j.copbio.2013.12.001)

*Publication date:*  
2014

*Document Version*  
Publisher's PDF, also known as Version of record

[Link back to DTU Orbit](#)

*Citation (APA):*  
Latif, H., Zeidan, A., Nielsen, A. T., & Zengler, K. (2014). Trash to treasure: Production of biofuels and commodity chemicals via syngas fermenting microorganisms. *Current Opinion in Biotechnology*, 27, 79-87. <https://doi.org/10.1016/j.copbio.2013.12.001>

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# Trash to treasure: production of biofuels and commodity chemicals via syngas fermenting microorganisms

Haythem Latif<sup>1</sup>, Ahmad A Zeidan<sup>2</sup>, Alex T Nielsen<sup>2</sup> and Karsten Zengler<sup>1,2</sup>

Fermentation of syngas is a means through which unutilized organic waste streams can be converted biologically into biofuels and commodity chemicals. Despite recent advances, several issues remain which limit implementation of industrial-scale syngas fermentation processes. At the cellular level, the energy conservation mechanism of syngas fermenting microorganisms has not yet been entirely elucidated. Furthermore, there was a lack of genetic tools to study and ultimately enhance their metabolic capabilities. Recently, substantial progress has been made in understanding the intricate energy conservation mechanisms of these microorganisms. Given the complex relationship between energy conservation and metabolism, strain design greatly benefits from systems-level approaches. Numerous genetic manipulation tools have also been developed, paving the way for the use of metabolic engineering and systems biology approaches. Rational strain designs can now be deployed resulting in desirable phenotypic traits for large-scale production.

## Addresses

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Current Opinion in Biotechnology 2014, 27:79–87

This review comes from a themed issue on **Energy biotechnology**

Edited by **Arthur J Ragauskas** and **Korneel Rabaey**

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<http://dx.doi.org/10.1016/j.copbio.2013.12.001>

## Introduction

Heightened concerns over global warming and fossil fuel supply and prices have led to a paradigm shift in perceived routes to chemical commodity production and energy generation. The majority of the world community has set challenging targets for reductions in greenhouse gas emissions to be achieved in part through the development of sustainable routes to chemicals, fuels, and energy production. The EU has targeted a 10% share of renewable biofuels in the transportation sector by 2020 [1] while the US has mandated the production of 36

billion gallons of biofuels by 2022 [2]. The biological conversion of renewable lignocellulosic biomass such as wheat straw, spruce, switchgrass, and poplar to biofuels is expected to play a prominent role in achieving these goals. These forms of biomass address many of the concerns associated with the production of first-generation biofuels [3,4]. However, 10–35% of lignocellulosic biomass is composed of lignin [5–7], which is highly resistant to breakdown by the vast majority of microorganisms [8]. Thus, if the EU and US cellulosic biofuel targets are realized, land allocation for biofuel production will increase and megatons of organic waste will be generated.

This organic waste provides a significant resource of biomass that can be utilized for producing biofuels as well as commodity chemicals. Through gasification, virtually any form of organic matter can be converted into a mixture of carbon monoxide (CO), carbon dioxide (CO<sub>2</sub>), and hydrogen (H<sub>2</sub>), referred to as synthesis gas or syngas. Gasification involves high temperature (usually 600–900°C) partial oxidation of biomass in the presence of a gasifying agent (e.g. oxygen or steam) resulting in the production of gas with significant amounts of CO and H<sub>2</sub> [9]. Syngas can be metabolized by certain carbon-fixing microorganisms and converted to valuable multi-carbon compounds such as acetate, ethanol, butanol, butyrate, and 2,3-butanediol [10,11]. This process, known as syngas fermentation, provides an attractive means for converting low cost organic substrates and waste streams into valuable chemicals. Syngas fermentation has numerous advantages when compared to thermo-chemical processes such as Fischer-Tropsch synthesis. These include a higher tolerance for impurities such as sulfur compounds, a wider range of usable H<sub>2</sub>, CO<sub>2</sub>, and CO mixtures, a lower operating-temperature and -pressure, and higher product yield and uniformity. However, wide use of these syngas fermenting microorganisms as production hosts is currently hindered by several factors, including low volumetric product titers, product feedback inhibition, and low gas-liquid mass transfer coefficient (kLa) [12]. Though some of these challenges can be overcome, in part, through process improvements, a fundamental understanding of the biology enabling syngas fermentation is needed to guide those design strategies and to provide targets for cellular engineering. Thus, the biggest challenge facing process development for syngas fermentation may be the lack of tools and technologies that will further our understanding of the fundamental biology behind these versatile microorganisms. This review focuses on these unique microorganisms, their

metabolic and energy conservation pathways, and the genetic engineering strategies that together will guide advances in the use of syngas fermentation for the production of biofuels and commodity chemicals.

### Syngas fermenting microorganisms

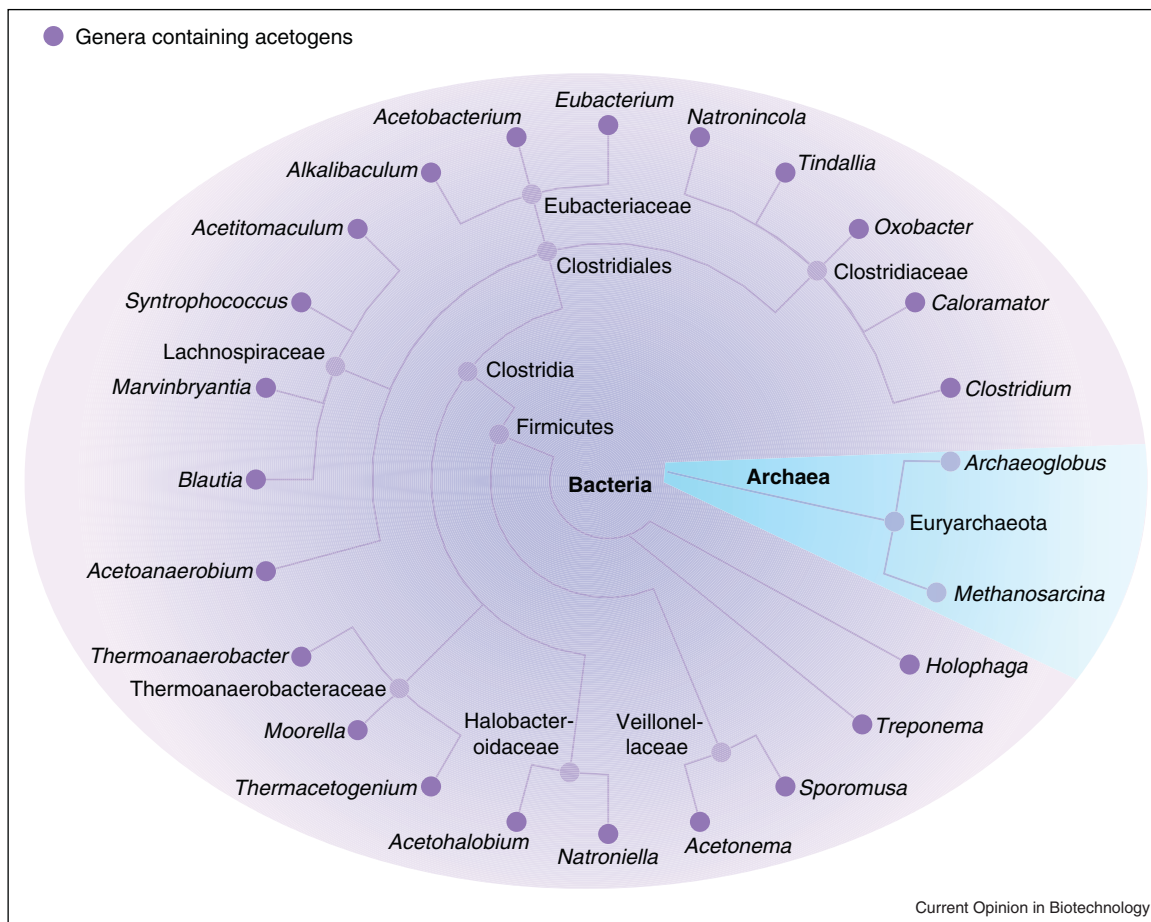
A diverse range of microorganisms that can metabolize syngas and produce multi-carbon compounds have been identified (Figure 1). These organisms are ubiquitous in numerous habitats such as soils, marine sediments, and feces, exhibit various morphologies (e.g. rods, cocci, or spirochetes), have a wide range of optimal growth temperatures (psychrophilic, mesophilic, or thermophilic), and demonstrate different tolerance toward molecular oxygen [13]. Syngas fermenting microorganisms also have diverse metabolic capabilities, resulting in the formation of a variety of native products such as acetate, ethanol, butanol, butyrate, formate, H<sub>2</sub>, H<sub>2</sub>S, and traces of methane [10,11,13–15]. Though some methanogens are

known to synthesize multi-carbon organics from syngas, the vast majority of syngas fermenting organisms are acetogens (Figure 1). Acetogens are anaerobes that assimilate CO<sub>2</sub> via the Wood–Ljungdahl (WL) pathway, also referred to as the reductive acetyl-CoA pathway. Though we focus here mainly on autotrophic conversion of syngas in acetogens, the WL pathway is also active during heterotrophic growth.

### The Wood–Ljungdahl pathway

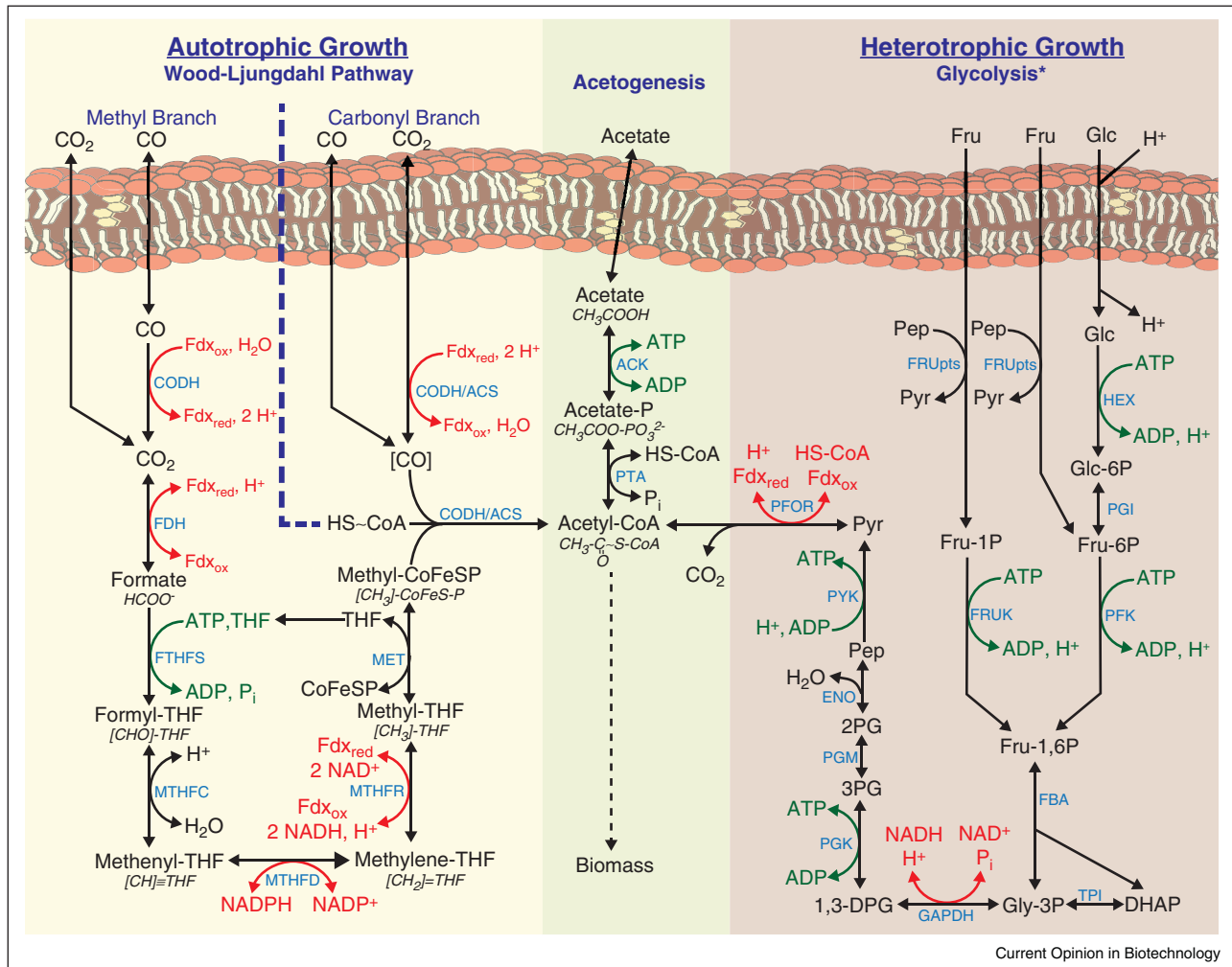
The WL pathway is hypothesized to be the most ancient CO<sub>2</sub> fixation pathway [16]. However, the apparent simplicity of this linear pathway belies the complex, interconnected energy conservation mechanisms that enable growth on syngas [13,17\*\*]. Only a short overview of the pathway will be given here since the WL pathway has been extensively reviewed elsewhere [13,18–20]. Figure 2 shows the complete WL pathway with electron

Figure 1



Syngas fermenting organisms known to produce multi-carbon organic compounds. Shown is the taxonomic classification of the genera capable of converting syngas to multi-carbon compounds based on organisms found in [10,11,13–15]. With the exception of two archaeal genera, all of the genera that produce multi-carbon organic compounds are considered acetogens. Genera are classified based on NCBI's current taxonomic nomenclature and categorization.

Figure 2



The Wood-Ljungdahl pathway and its connection to heterotrophic metabolism. Shown is the WL pathway for *Clostridium ljungdahlii* as assessed in [21,22] with reduced ferredoxin, NADH, and NADPH serving as electron carriers during CO<sub>2</sub> fixation. The left panel shows the Methyl and Carbonyl branches of the WL pathway leading to either acetate formation or assimilation of acetyl-CoA into biomass. The right panel depicts fermentation through glycolysis for fructose and glucose. The electrons generated during glycolysis are used to produce an additional acetyl-CoA through the WL pathway. Redox reactions involving cofactor intermediates are shown in red with each cofactor reaction involving the transfer of two electrons. ATP consumption/generation reactions are shown in green. Reactants abbreviations: Acetate-P, acetate phosphate; CoA, coenzyme A; CoFeSP, corrinoid iron sulfur protein; THF, tetrahydrofolate; Fd<sub>x<sub>ox</sub></sub>, oxidized ferredoxin; Fd<sub>x<sub>red</sub></sub>, reduced ferredoxin; Pyr, pyruvate; Pep, phosphoenolpyruvate; 2PG, 2-phosphoglycerate; 3PG, 3-phosphoglycerate; 1,3-DPG, 1,3-bisphosphoglycerate; DHAP, dihydroxyacetone phosphate; Gly-3P, glycerol 3-phosphate; Fru-1,6P, fructose 1,6-bisphosphate; Fru-6P, fructose 6-phosphate; Glc-6P, glucose 6-phosphate; Fru-1P, fructose 1-phosphate; Pyr, pyruvate; Pep, phosphoenolpyruvate; Fru, fructose. Enzyme abbreviations (in blue): ACK, acetate kinase; PTA, phosphotransacetylase; MET, methyl transferase; MTHFR, methylene tetrahydrofolate reductase; MTHFD, methylene tetrahydrofolate dehydrogenase; MTHFC, methenyl tetrahydrofolate cyclohydrolase; FTHFS, formyl tetrahydrofolate synthase; FDH, formate dehydrogenase; CODH/ACS, carbon monoxide dehydrogenase/acetyl-CoA synthase; PFOR, Pyruvate ferredoxin oxidoreductase; PGK, phosphoglycerate kinase; ENO, enolase; PGM, phosphoglycerate mutase; PGK, phosphoglycerate kinase; PFK, phosphofructokinase; TPI, triose phosphate isomerase; FBA, fructose-bisphosphate aldolase; FRUK, fructokinase; PFK, phosphofructokinase; PGI, phosphoglucose isomerase; HEX, hexokinase; FRUT, fructose transporter. \*The stoichiometry shown represents conversion of a single Gly-3P molecule, thus only half of the payoff phase of glycolysis is shown.

carrier proteins and cofactors as proposed for *Clostridium ljungdahlii* [21,22].

The WL pathway consists of two branches: the methyl branch and the carbonyl branch. These two branches provide reduced, single-carbon molecules that contribute

to the formation of acetyl-CoA, which can then be assimilated into cellular biomass or converted to acetate. For simplicity, the case of autotrophic growth on CO<sub>2</sub> and H<sub>2</sub> will be examined first. On the methyl branch, a six-electron reduction of CO<sub>2</sub> yields a methyl moiety while the carbonyl branch reduces CO<sub>2</sub> to CO which is bound to

the carbon monoxide dehydrogenase/acetyl-CoA synthase complex (CODH/ACS). CODH/ACS unites the two branches by reacting these products with coenzyme A to yield acetyl-CoA. Thus, a total of one ATP (needed for formate fixation) and eight electrons are needed to fix two molecules of CO<sub>2</sub>. With H<sub>2</sub> as the sole electron donor, hydrogenases reduce cofactor intermediates (e.g. reduced ferredoxin, NAD(P)H) that are subsequently used to reduce CO<sub>2</sub>. CO, typically the most abundant carbon species in syngas [23], is often able to sustain autotrophic growth through the WL pathway as the sole electron donor. In this case, CODH carries out a water-gas shift reaction where water and CO yield CO<sub>2</sub>, two protons, and two electrons providing the necessary inputs for the formate dehydrogenase reaction on the methyl branch. CO is bound directly by CODH/ACS on the carbonyl branch requiring no reduction. Thus, the conversion of two CO to acetyl-CoA requires one ATP and four electrons. The formation of acetate from acetyl-CoA yields one molecule of ATP through substrate level phosphorylation (SLP). As a whole, the WL pathway generates no net ATP through SLP and the transfer of four or eight electrons from reduced ferredoxin, NADH, or NADPH to fix syngas.

During syngas fermentation electrons are donated by H<sub>2</sub> or CO. However, the WL pathway is capable of sourcing electrons from many compounds under heterotrophic conditions including alcohols, organic acids, and simple sugars [13]. This enables acetogens to have near complete stoichiometric conversion of hexoses to acetate. From glycolysis, acetogens typically synthesize two acetates, four ATPs (via SLP), and two CO<sub>2</sub>'s from one mole of hexose. The CO<sub>2</sub> generated can be fixed by the WL pathway using the eight electrons generated during glycolysis. Thus, a third mole of acetate is produced but no net ATP is synthesized via SLP. The overall conversion of hexose yields three moles of acetate and four moles of ATP via SLP.

### Energy conservation in acetogens

Since the WL pathway yields no net ATP through SLP, a chemiosmotic gradient is necessary to drive ATP synthesis under autotrophic conditions, such as syngas fermentation. Some acetogens utilize anaerobic respiration (also known as electron transport phosphorylation, ETP), for energy conservation such as *Moorella thermoacetica* [24–26]. However, many acetogens do not possess electron transport chain proteins. Recently, flavin-based electron bifurcation (FBEB) has been described as an alternative means for energy conservation in certain bacteria [17\*\*]. FBEB couples exergonic redox reactions with endergonic redox reactions. Certain FBEB complexes couple this redox reaction with the translocation of cations thereby forming a chemiosmotic gradient. An example of this is the Rnf complex, which is found in many microorganisms. The Rnf complex is a membrane associated FBEB

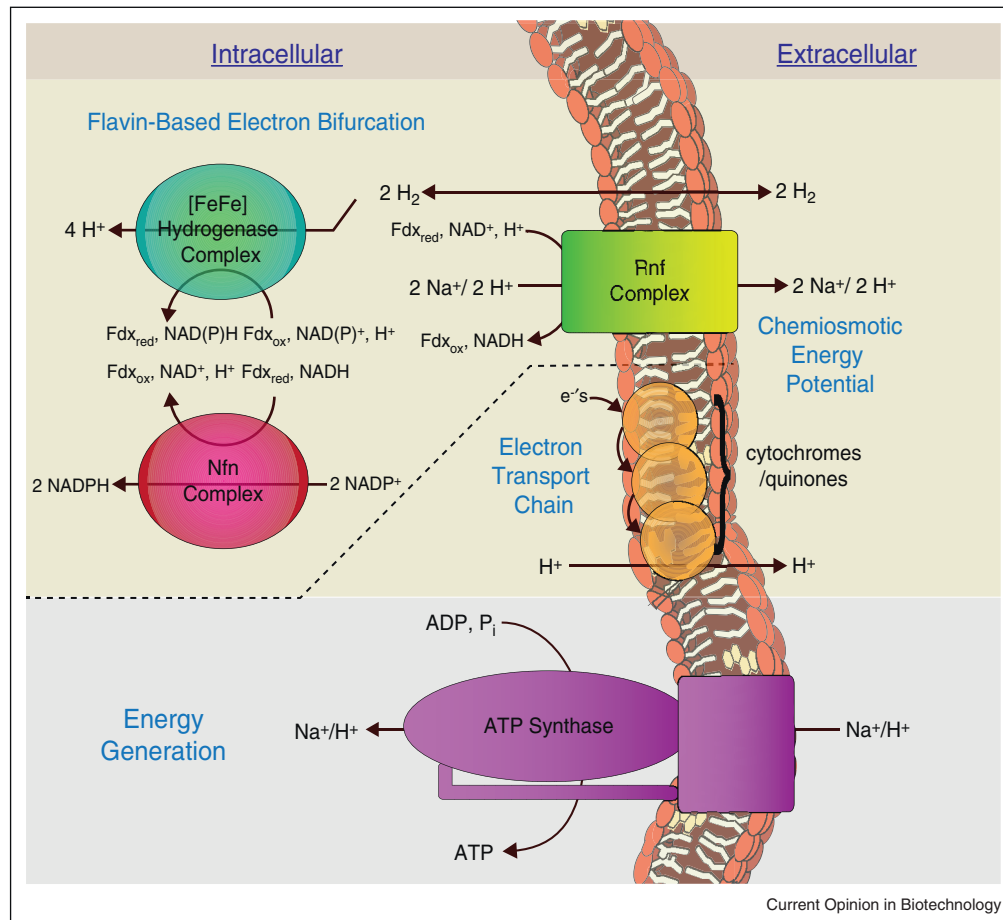
system that pumps Na<sup>+</sup> or H<sup>+</sup> ions out of the cell. This is driven by coupling the oxidation of reduced ferredoxin with the reduction of NAD<sup>+</sup> [27] (Figure 3). In the acetogen *Acetobacterium woodii*, a Na<sup>+</sup> gradient generated by the Rnf complex is converted to ATP by a Na<sup>+</sup> F<sub>1</sub>F<sub>0</sub> ATP synthase [28–31]. A proton translocating Rnf complex in *C. ljungdahlii* has recently been shown to be essential for autotrophic growth [32\*]. Studies have also revealed soluble FBEB complexes in acetogens. A [FeFe]-hydrogenase (Figure 3) that reduces ferredoxin and NAD<sup>+</sup> through the oxidation of H<sub>2</sub> has been identified in *A. woodii* [33] and *M. thermoacetica* [34\*,35]. Recently, an NADP-specific [FeFe]-hydrogenase has been discovered in *Clostridium autoethanogenum* [36]. An Nfn complex similar to that of *Clostridium kluyveri* [37] (Figure 3) was identified in *M. thermoacetica* as well [34\*]. This complex reduces NADP<sup>+</sup> while oxidizing NADH and ferredoxin. Furthermore, it has been suggested that the WL reaction catalyzed by methylene tetrahydrofolate reductase (MTHFR) is also an electron bifurcation reaction in *A. woodii* [31] and *C. ljungdahlii* [21,22] (Figure 2).

Together, membrane bound and cytoplasmic FBEB reactions create an intricate network of redox reactions that collectively contribute to energy conservation and redox homeostasis. For example, it is thought that energy conservation during hydrogen-dependent caffeine respiration in *A. woodii* is governed by three FBEB complexes working in concert; the membrane associated Rnf, the soluble [FeFe]-hydrogenase, and the soluble caffeine-CoA reductase-Etf complex responsible for oxidation of NADH and reduction of ferredoxin [38]. FBEB reactions metabolically ‘hard-wire’ the redox state of the different cofactor pools and electron carrier proteins. Thus, these reactions can be envisioned as moving together in response to changes in metabolism and energy conservation.

### Advances in genetic manipulation tools

A lack of versatile genetic tools for manipulating syngas fermenters has hindered the ability to engineer this group of microorganisms. However, there has been remarkable progress in the development of genetic systems for numerous industrially important anaerobes, especially clostridia, in recent years. Protocols for gene deletion via double crossover homologous recombination were recently developed for the syngas fermenting *C. ljungdahlii* [39\*] and *M. thermoacetica* [40–42]. Uracil-auxotrophic mutants of *M. thermoacetica* [41\*] and *C. thermocellum* [43] were constructed allowing for both positive- and counter-selection of desired recombinants. Moreover, methods employing bacterial group II intron for gene deletion [44,45,46\*\*] and a *Bacillus subtilis* resolvase [47–49] were developed as universal genetic tools for *Clostridium* species. Genetic systems were also established based on replicative plasmids capable of double crossover chromosomal integration [50], inducible

Figure 3



Energy conservation during syngas fermentation. Shown are examples of protein complexes found in acetogens that conserve energy during syngas fermentation. Syngas fermentation produces no net ATP through substrate level phosphorylation. Thus, a chemiosmotic gradient is utilized to drive ATP synthesis. This gradient is achieved predominantly through ion translocation via electron transport chain proteins or through membrane-bound flavin-based electron bifurcation (FBEB) complexes such as the Rnf. Soluble FBEB complexes like the [FeFe]-hydrogenase and Nfn complex have also been identified in acetogens. These, as well as other complexes (not shown) are important for maintaining cellular redox balance. Energy generation occurs through the ATP synthase. Acetogens such as *Clostridium ljungdahlii* and *Moorella thermoacetica* utilize a  $H^+$  gradient while others like *Acetobacterium woodii* use  $Na^+$ .

counter-selection for markerless gene deletions/insertions at any desired genomic loci [51], coupled expression of heterologous selectable markers to a chromosomal promoter to select for double crossover events [52], and antisense RNA for protein down-regulation [53]. Reverse genetic tools in the form of transposon mutant libraries were generated using a *mariner*-based system in the autotrophic pathogen *C. difficile* and in *C. perfringens* [54,55] and a *Tn1545*-based system in *C. cellulolyticum* [56].

### Strain engineering to obtain desired production phenotypes

With the availability of such genetic tools and the recent advances in whole-genome sequencing, combined metabolic engineering and synthetic biology approaches can

be applied to accelerate the development of syngas fermentation processes. Boosting the yield and productivity of syngas fermenters and broadening the spectrum of chemicals and fuels that can be produced are immediate goals. As a proof of concept, the deletion of the bifunctional aldehyde/alcohol dehydrogenase in *C. ljungdahlii* resulted in increased acetate yield on the expense of ethanol [39]. The functionality of a construct harboring the known acetone pathway of *C. acetobutylicum* was demonstrated in *C. acetivum*, enabling the latter to produce 8 mg/L of acetone from a mixture of  $H_2$  and  $CO_2$  [11,57]. Similarly, plasmids bearing heterologous genes for the butanol synthesis pathway of *C. acetobutylicum* were introduced into *C. ljungdahlii* allowing for low levels of butanol production [21]. Butanol was subsequently

converted to butyrate at the end of the fermentation, indicating the need for further genetic modifications to prevent the loss of butanol. Yet, the reversibility of this and similar alcohol forming enzymatic reactions was exploited by providing external short-chain carboxylic acids and syngas to *C. ljungdahlii* for the production of alcohols [58].

In addition to improved yield and product-range of acetogens, engineering strategies targeting other limitations identified at industrial-scale may be considered. Among these limitations are low syngas kLa, low cell-biomass, sporulation, as well as substrate and product inhibition. Engineering syngas fermenting microorganisms for enhanced biofilm development can help overcome their inherently low biomass yield while enabling the use of reactors with enhanced gas mass transfer rates such as airlift reactors [59] or membrane biofilm reactors [60] as well as other types of biofilm-based reactors suitable for syngas fermentation [15,57]. More effective biofilms can be formed by increased exopolysaccharide production [61] or by manipulating other factors that are known to modulate biofilm formation in *Clostridium* species [62]. Longer fermentation increases the possibility of spore formation. Inactivating Spo0A, the master regulator of sporulation in *Clostridium* species [63\*], was thought to be the obvious strategy for abolishing sporulation [64]. However, Spo0A's involvement in solvent production [65,66] and biofilm formation [62,63\*,67] will necessitate tuned expression rather than complete inactivation. Alternatively, sporulation regulators downstream to Spo0A can be targeted for creating asporogenous strains, as was demonstrated for *C. acetobutylicum* [64] and *C. phytofermentans* [68].

Phenotypes with improved fitness for syngas fermentation can also be obtained by adaptive laboratory evolution (ALE) [69–71]. Various ALE approaches can be employed to obtain highly desirable industrial traits such as optimal growth during syngas fermentation and tolerance to high concentrations of substrates (e.g. CO) or products (e.g. ethanol). For instance, ALE approaches were used to generate strains of *Butyribacterium methylo-trophicum* that were adapted to growth in a pure CO-headspace [72]. Ethanol tolerant strains of *Escherichia coli* and subsequent genome-scale analysis provided insights into the metabolic and regulatory mechanisms that caused that phenotype to emerge [73,74]. A similar approach can be applied to syngas fermenting production strains.

### Rational strain design and process optimization through a systems-level approach

Currently, little is known about the possibility of completely redirecting the metabolic fluxes from acetate to other products during syngas fermentation or its effect

on cellular energetics. Recently, Tyrurin and co-workers reported in a series of publications on completely abolishing acetate production in an undisclosed *Clostridium* strain, in favor of acetone [75], ethanol [76,77], butanol [78], mevalonate [78], methanol, or formate [79] production during autotrophic growth on syngas. However, eliminating acetate synthesis by deletion of the phosphotransacetylase and/or acetate kinase (*ack*) genes presumably prevents the concurrent synthesis of ATP (via SLP) by Ack. The impact this change has on the growth energetics in these strains is not yet fully understood.

Perhaps the most effective way for rational strain design is through the use of genome-scale metabolic models. Genome-scale models have successfully been implemented in rational strain design [80,81]. Recently, the first comprehensive reconstruction of metabolism in an acetogen has been generated [22]. Using this *C. ljungdahlii* model, simulation of a  $\Delta ack$  mutant predicted conditional essentiality. *In silico* growth was observed under heterotrophic conditions and under autotrophic growth with CO as the electron donor. However, growth on H<sub>2</sub> as the sole electron donor was essential depending on the cofactor specificity of the [FeFe]-hydrogenase [22]. This conditional essentiality is attributed to differences in the redox state of the different electron carrier pools and their ability to contribute to FBEB-based energy conservation to compensate for the lost ATP generated during acetate synthesis. Thus, it is extremely important to detail the exact mechanisms of energy conservation, including the stoichiometry of the ATPase reaction, in the metabolic network reconstruction for more realistic phenotype predictions. Another model of the WL pathway in *C. ljungdahlii* was used to determine the ATP yield per mol CO consumed and the proton translocation per electron transfer of the Rnf complex [58]. Lastly, metabolic modeling has been used to optimize media formulations, for instance, based on energy demands [82]. These models can help optimize and reduce the cost of media, which is a challenge the needs to be addressed before commercial deployment of syngas fermentation [83,84].

### Summary and opportunities

Fermentation of syngas into biochemicals using acetogenic microorganisms offers an important economic potential for biofuel and commodity chemical production. Gasification allows for the processing of virtually all types of organic waste (e.g. industrial or municipal) into syngas. The potential of syngas fermentation is evident by the advent of large-scale projects. LanzaTech is working with steel manufactures [85] and coal producers [86] in China to make liquid fuels. BioMCN is converting glycerine to syngas which is fermented into bio-methanol and has constructed a 200 000 ton/year pilot scale production unit [87]. Coskata [88] is commercializing the production of

fuels and chemicals using a wide variety of biomass sources through syngas fermentation. They have built a demonstration-scale production facility as part of a feasibility study. Lastly, SYNPOL [89], a large research project funded by the EU, is focused on the production of biopolymers via syngas fermentation. In order to fully utilize cheap carbon sources, however, construction of novel and optimized syngas fermenting strains for the production of biochemicals and biofuels is needed. Recent advances in our knowledge of energy conservation in acetogens and the development of new molecular biology tools provide a foundation for strain design strategies.

Recently developed genetic tools can be leveraged to achieve desirable phenotypic traits. Production of compounds of interest must be maximized under the conditions optimal for syngas fermentation. Further improvements will likely involve media optimization and evolution of strains for growth in minimal media. The formation of enhanced biofilm formers for some types of fermentations is highly desirable. Tolerance toward inhibitory substrate and product concentrations as well as other complex phenotypes may be achieved through ALE approaches. Lastly, genome-scale models for acetogens provide a valuable tool to account for the intricacies of acetogen metabolism when designing optimal production strains. With many of these enabling technologies now available, a significant growth in the research and metabolic engineering of acetogens can be expected to better utilize syngas for the production of both biofuels and commodity chemicals.

## Acknowledgements

AAZ, ATN, and KZ acknowledge support from The Novo Nordisk Foundation. HL is supported through the National Science Foundation Graduate Research Fellowship under grant DGE1144086.

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- of special interest
- of outstanding interest

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