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# Stepping on the Gas to a Circular Economy: Accelerating Development of Carbon-Negative Chemical Production from Gas Fermentation

Nick Fackler,<sup>1,\*</sup> Björn D. Heijstra,<sup>1,\*</sup> Blake J. Rasor,<sup>2</sup> Hunter Brown,<sup>2</sup> Jacob Martin,<sup>2</sup> Zhuofu Ni,<sup>2</sup> Kevin M. Shebek,<sup>2</sup> Rick R. Rosin,<sup>1</sup> Séan D. Simpson,<sup>1</sup> Keith E. Tyo,<sup>2</sup> Richard J. Giannone,<sup>3</sup> Robert L. Hettich,<sup>3</sup> Timothy J. Tschaplinski,<sup>4</sup> Ching Leang,<sup>1</sup> Steven D. Brown,<sup>1</sup> Michael C. Jewett,<sup>2,5</sup> and Michael Köpke<sup>1</sup>

<sup>1</sup>LanzaTech Inc., Skokie, Illinois 60077, USA; email: nick.fackler@lanzatech.com, bjorn.heijstra@lanzatech.com, rick.rosin@lanzatech.com, sean@lanzatech.com, ching.leang@lanzatech.com, steve.brown@lanzatech.com, michael.koepke@lanzatech.com

<sup>2</sup>Department of Chemical and Biological Engineering, Chemistry of Life Processes Institute, and Center for Synthetic Biology, Northwestern University, Evanston, Illinois 60208, USA; email: blakerasor2023@u.northwestern.edu, samuelbrown2021@u.northwestern.edu, jacobmartin2022@u.northwestern.edu, joseph.ni@u.northwestern.edu, kevinshbek2016@u.northwestern.edu, k-tyo@northwestern.edu, m-jewett@northwestern.edu

<sup>3</sup>Chemical Sciences Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831, USA; email: giannonerj@ornl.gov, hettichrl@ornl.gov

<sup>4</sup>Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831, USA; email: tschaplinstj@ornl.gov

<sup>5</sup>Robert H. Lurie Comprehensive Cancer Center and Simpson Querrey Institute, Northwestern University, Chicago, Illinois 60611, USA

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\*These authors contributed equally to this article

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gas fermentation, syngas, acetogen, *Clostridium*, cell-free, CRISPR

## Abstract

Owing to rising levels of greenhouse gases in our atmosphere and oceans, climate change poses significant environmental, economic, and social challenges globally. Technologies that enable carbon capture and conversion

of greenhouse gases into useful products will help mitigate climate change by enabling a new circular carbon economy. Gas fermentation using carbon-fixing microorganisms offers an economically viable and scalable solution with unique feedstock and product flexibility that has been commercialized recently. We review the state of the art of gas fermentation and discuss opportunities to accelerate future development and rollout. We discuss the current commercial process for conversion of waste gases to ethanol, including the underlying biology, challenges in process scale-up, and progress on genetic tool development and metabolic engineering to expand the product spectrum. We emphasize key enabling technologies to accelerate strain development for acetogens and other nonmodel organisms.

#### Greenhouse gases

**(GHGs):** gases such as CO<sub>2</sub> that absorb and emit infrared radiation in the wavelength range emitted by Earth

#### Chemo-lithoautotrophs:

organisms that obtain energy from oxidation of inorganic compounds (“chemo” for chemical reactions and “litho” for inorganic chemicals)

#### Synthesis gas

**(syngas):** a gaseous mixture composed mostly of CO, CO<sub>2</sub>, and H<sub>2</sub>, produced via partial oxidation of hydrocarbon feedstocks or steam reforming

#### Autotroph:

an organism with the ability to grow in the absence of organic carbon (“auto” for self, “troph” for food)

#### Acetogen:

an organism that uses the WLP for synthesis of acetyl-CoA, terminal electron-accepting and energy-conserving processes, and carbon fixation

## 1. INTRODUCTION

The climate crisis and rapid population growth present two of the most urgent challenges to humankind. Already, the climate crisis and environmental pollution come with a huge economic toll (1, 2) and are two of the leading causes of death (3, 4). Intensified need for energy, fuel, and chemicals demanded by our growing population over the past 100 years has led to a sharp increase in the concentration of the so-called greenhouse gas (GHG) CO<sub>2</sub> in the atmosphere. We have now reached the highest atmospheric CO<sub>2</sub> concentration our Earth has seen in approximately 3 million years, since before humans existed (2), and this level has continued to rise even despite the global restrictions on activity imposed during the coronavirus 2019 disease (COVID-19) pandemic (5). With appropriate policies, a path exists for decarbonization of the energy sector using broadly available and commercially proven renewable technologies (6, 7). However, production of chemicals and energy-dense liquid transportation fuels, particularly for the aviation and maritime sectors, will remain heavily dependent on fossil resources for the foreseeable future.

Fermentation provides a path for sustainable manufacturing of biochemicals and transportation fuels, displacing the use of fossil resources (8, 9). Traditional fermentation based on renewable sugar/starch feedstocks has been carried out at industrial scale for more than a century, including ethanol production, acetone-butanol-ethanol (ABE) fermentation, and production of amino acids or organic acids; synthetic biology has extended the product base to many additional metabolites, including 1,3-propanediol, artemisinin, farnesene, and 1,4-butanediol (8, 9). In addition to using renewable feedstocks, we must use waste resources such as agricultural, forest, and municipal wastes or off gases produced in many manufacturing processes that would end up as atmospheric GHG or environmental pollutants. Second-generation fermentation processes have been developed to tackle the complex problem of breakdown of recalcitrant lignocellulosic material (woody biomass) and use hydrolysates as feedstock, but commercialization is challenging (10). Gas fermentation provides an alternative route that offers a unique level of feedstock flexibility. During gas fermentation, a gaseous one-carbon substrate is fermented by chemolithoautotrophic microorganisms. The gaseous feedstock may be an industrial off gas or synthesis gas (syngas) produced from the gasification of biomass and municipal waste streams, available at impactful volumes throughout the globe.

## 2. BIOLOGY OF GAS FERMENTATION

A wide range of autotrophs can use either carbon oxides (CO and/or CO<sub>2</sub>) or methane (CH<sub>4</sub>). For this review, we focus on anaerobic acetogens, because they are already deployed commercially and are considered to have the most efficient of all known carbon fixation pathways (11–13).

Several excellent reviews have already been published on the development of other chemolithoautotrophic platforms for gas fermentation, including hydrogenogens (14, 15), carboxydrotrophs (15, 16), methanotrophs (17–19), and methanogens (20).

## 2.1. Metabolism

The defining feature of the acetogenic metabolism is the presence of the Wood–Ljungdahl pathway (WLP; reductive acetyl-CoA pathway) (**Figure 1**) and its key enzyme, the CO dehydrogenase/acetyl-CoA synthase (CODH/ACS) complex (21). The WLP pathway is the only known linear autotrophic carbon fixation pathway (11–13) and is considered to be the first biochemical pathway on Earth, emerging several billion years ago (long before oxygen entered the atmosphere) in deep-sea hydrothermal vents (22, 23). In fact, abiotic CO<sub>2</sub> reduction (with H<sub>2</sub>) to formate, acetate, and pyruvate has been demonstrated under mild alkaline hydrothermal conditions in the presence of just a simple hydrothermal vent alloy, awaruite (Ni<sub>3</sub>Fe) (24). In the WLP, these reactions are catalyzed by a series of metalloenzymes with tetrahydrofolate (THF) as cofactor. Via the action of formate dehydrogenase (Fdh), formyl-THF synthetase, methenyl-THF cyclohydrolase/dehydrogenase, and methylene-THF reductase (MTHFR), CO<sub>2</sub> is stepwise reduced to a methyl-group in the so-called methyl branch of the WLP. This methyl group is then transferred from the THF cofactor onto a corrinoid iron-sulfur-containing protein via a methyltransferase before being fused with a second carbon oxide molecule and CoA to form the central intermediate acetyl-CoA via the CODH/ACS complex (**Figure 1**). Pioneered by Harland G. Wood, Lars G. Ljungdahl, and others, the biochemistry and enzymology of the pathway are well understood and described in detail in several excellent reviews (25–27). On a genetic level, the ~15 genes encoding the enzymes of the WLP are typically found in one cluster of ~20 kbp (28, 29). Considering cofactors, assembly of metal centers, and electron transport, at least 200 genes are required for autotrophy (29, 30).

The energy for CO<sub>2</sub> reduction can be assimilated either from CO (which can serve as both a carbon and energy source) via biological water gas shift (catalyzed by CODH) or from hydrogen (H<sub>2</sub>) (via hydrogenases). Some acetogens can also use other C1 substrates, such as formate or methanol (or methyl groups from other sources) (31). Furthermore, functional cooperation between the WLP and the glycine synthase reductase for autotrophic growth has been demonstrated (32), and under mixotrophic conditions the WLP enables acetogens to reassimilate CO<sub>2</sub> released during sugar metabolism for increased carbon efficiency (33). A variation of the WLP using different cofactors is also present in methanogenic archaea that convert CO<sub>2</sub> to biomass and CH<sub>4</sub> (34). The WLP is unique within autotrophic carbon fixation pathways in that it allows for energy conservation (13, 35). The activation of formate in the WLP requires ATP, but this can be recouped through substrate-level phosphorylation by converting the generated acetyl-CoA to acetate. Additional energy can be generated from electron transport coupled to WLP redox reactions. The exact mechanism has long been an enigma, and a few open questions remain (35). In many acetogens, none of the WLP enzymes are membrane associated, and no cytochromes or quinones are present. In these acetogens, the Rnf complex, a ferredoxin:NAD<sup>+</sup>-oxidoreductase that was originally identified in the context of *Rhodobacter* nitrogen fixation (hence the name), plays a key role as a coupling site to generate a transmembrane Na<sup>+</sup> or H<sup>+</sup> gradient (36) (**Figure 1**). Only a decade ago, a third fundamental mechanism of energy conservation, electron bifurcation, was described (37–39). Since its elucidation, several electron-bifurcating enzymes have been characterized across several acetogens (35), including core WLP enzymes [hydrogenase (Hyd/Hyt)/Fdh complex, MTHFR] and other enzymes that play a key role in redox balancing, such as transhydrogenases (Nfn) (40–43).

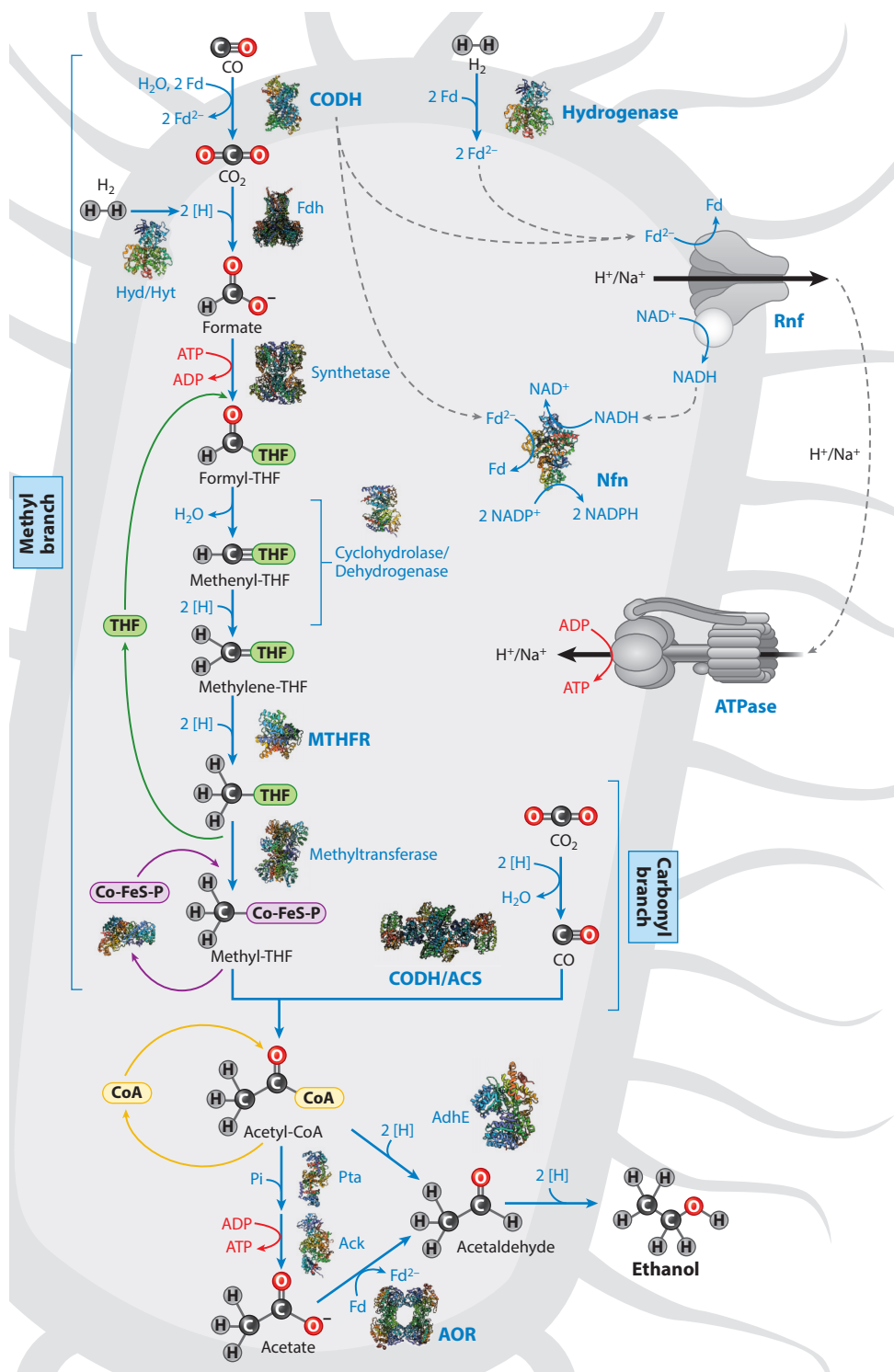
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**Electron bifurcation:**  
a third fundamental  
mechanism of energy  
conservation by  
combining endergonic  
and exergonic redox  
reactions

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**Figure 1**

Overview of the Wood–Ljungdahl pathway. Abbreviations: Ack, acetate kinase; ACS, acetyl-CoA synthase; AdhE, bifunctional aldehyde/alcohol dehydrogenase; AOR, aldehyde:ferredoxin oxidoreductase; CODH, CO dehydrogenase; Co-FeS-P, corrinoid iron sulfur protein; Hyd/Hyt, NAD/NADP-specific electron-bifurcating hydrogenase; Fdh, formate dehydrogenase; MTHFR, methylene-THF reductase; Nfn, transhydrogenase; Pta, phosphotransacetylase; Rnf, ferredoxin:NAD<sup>+</sup>-oxidoreductase; THF, tetrahydrofolate. Figure adapted from images created with BioRender.com.



In addition to acetate, some acetogens can synthesize other products natively, including ethanol, butyrate, butanol, hexanoate, hexanol, 2,3-butanediol, and lactate (21). Production follows the same pathways from acetyl-CoA as in other organisms, but some acetogens have the unique capability of reducing acids to alcohols via an aldehyde:ferredoxin oxidoreductase that can be coupled with CO and H<sub>2</sub> oxidation (44–46) (**Figure 1**).

## 2.2. Organisms

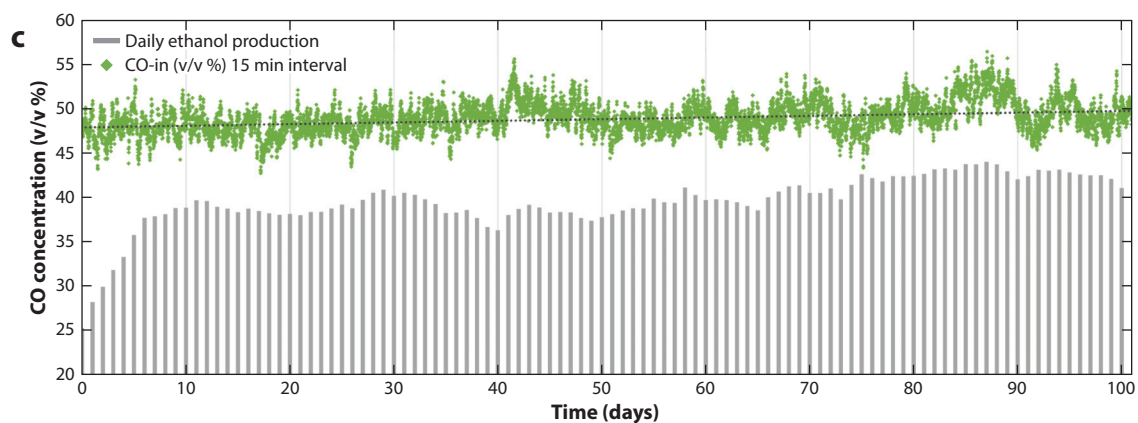
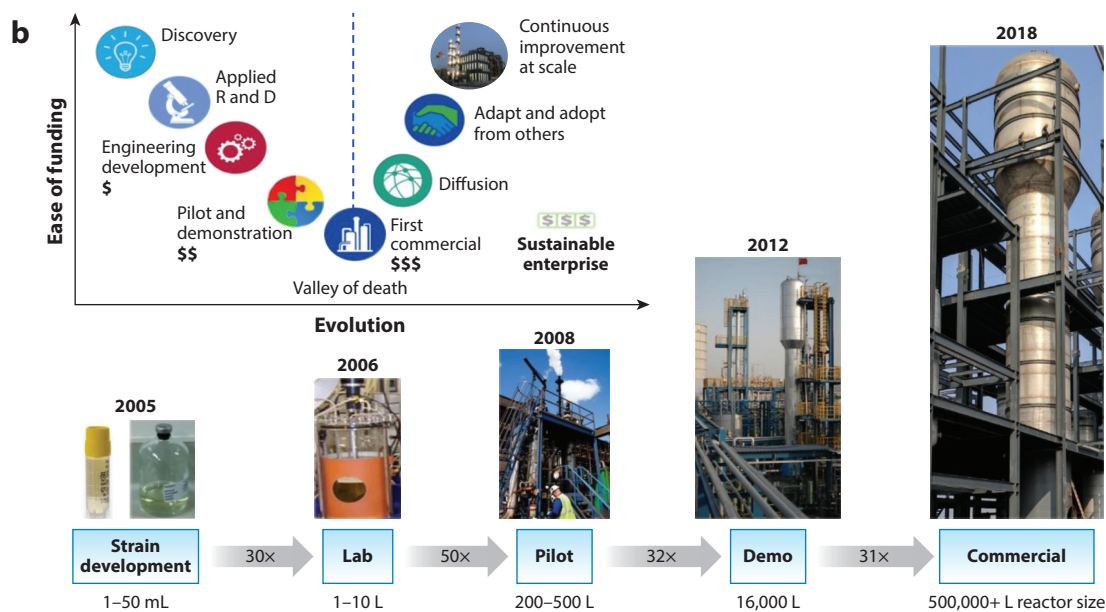
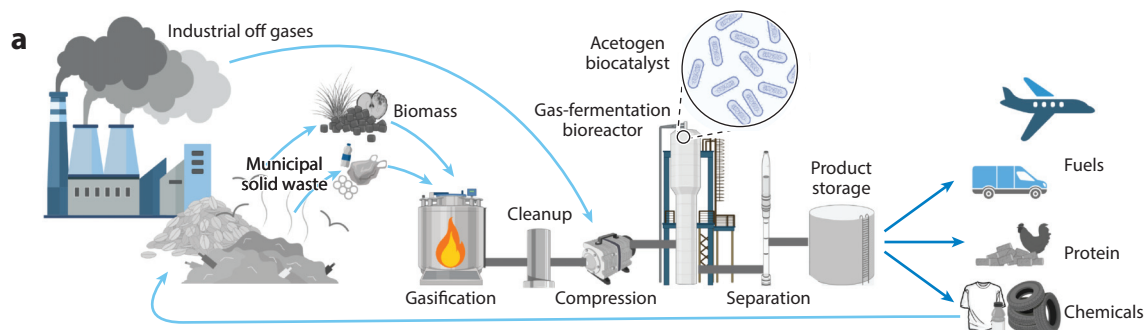
Acetogens play a central role in the global acetate cycle, being responsible for the production of at least 10<sup>32</sup> tons of acetate per year in nature (21). Acetogens are ubiquitous in anaerobic environments, such as soil, animal and human guts, sediments, the deep sea, and hot springs (21), and the WLP has also been discovered recently in a facultative aerobe candidate phylum (47). Several hundred acetogens have been isolated to date, spanning at least 25 different genera and including psychrophiles, mesophiles, thermophiles, and halophiles (21). For biotechnological applications, mostly acetogenic clostridia are considered because many species are among the fastest-growing acetogens, already make products other than acetate, and have been used industrially (e.g., ABE fermentation) for more than 100 years (48, 49). *Clostridium autoethanogenum* (50–52) and *Clostridium ljungdablii* (53–55) have emerged as acetogenic model organisms, with *Clostridium ragsdalei*, *Clostridium coskatii*, and *Clostridium carboxidivorans* also seeing significant development (56–58). Although these species are closely related on a genetic level, significant differences in performance have been observed (56, 57). Besides clostridia, a few other species, including *Acetobacterium woodii*, *Moorella thermoacetica*, and *Eubacterium limosum*, are also considered for industrial use. *A. woodii* and *M. thermoacetica* only produce acetate natively (21) but are well characterized, as much of the initial elucidation of WLP biochemistry and energetics was undertaken in these species (35).

## 3. GAS FERMENTATION PROCESS

Development of any gas fermentation process starts with selection of an organism (biocatalyst) suitable for the available gaseous substrate and desired subsequent product conversion. The catalog of available native organisms continues to grow (see Section 2.2), while in parallel the available genetic tool kit, and thus product portfolio, is expanding rapidly (see Section 4). For laboratory-scale gas fermentation research, a standard continuously stirred tank reactor is sufficient, but a reactor design that maximizes gas solubility while minimizing energetic input is essential to be economical at commercial scale (59).

### 3.1. Process Overview

Although sugar fermentations access established, conventional supply chains, they compete for this feedstock in terms of its value as food. In contrast, gas fermentation facilities require an upstream process that delivers a pressurized gas stream, with the average heating value of the gas setting its price point. This gas stream can be sourced as a by-product of existing large industrial processes, such as primary steel making and (petro)chemical refining, or can be an intentionally generated syngas (**Figure 2a**). The latter has huge potential as a hybrid thermochemical/biochemical process in which any carbon-containing waste stream could be converted into a syngas for subsequent biological conversion into products. The rise of renewable electricity further opens up an exciting integration opportunity to access an even wider range of feedstocks (see the sidebar titled Power-to-X).



(Caption appears on following page)

**Figure 2** (Figure appears on preceding page)

(a) Overview of the gas fermentation process. (b) Crossing the valley of death is the biggest challenge on the path to technology commercialization. New commercial fermentation involves developing laboratory technology; deploying pilot, demonstration, and commercial facilities; and ultimately scaling the technology across eight orders of magnitude. (c) Commercial-scale daily ethanol production levels and inflow CO gas concentration over a 100-day period. Steel mill off gas contains fluctuating amounts of CO, which is converted into ethanol by continuous gas fermentation. Data provided by the Beijing Shougang LanzaTech New Energy Science & Technology Company. Data are from a full-capacity campaign during the year 2020/2021, which lasted more than 200 days. Panel a adapted from images created with BioRender.com.

## POWER-TO-X

Renewable electricity is becoming increasingly abundant and low cost, yet storage is still a challenge (6, 7). There is increasing interest in leveraging surplus sustainable electricity for production of liquid fuels and chemicals, often referred to as Power-to-X. One approach is to generate hydrogen, syngas, CO, or formate from either water or CO<sub>2</sub> via electrolysis (11), which in turn are feedstocks for acetogens. A particular advantage gas fermentation offers over other downstream technologies is that it can handle fluctuations in the gas composition, for example, where there is inconsistency in the supply of electricity. Integrated systems of electrolyzer and gas fermentation have already been demonstrated to achieve high faradaic and carbon efficiencies (80, 196). Some acetogens can also use electricity directly in a process called microbial electrosynthesis (197, 198).

Syngas is an industrial chemical synthesis feedstock commonly used to produce ammonia, methanol, and synthetic petroleum products. By using the Fischer–Tropsch (FT) process, metal catalysts (cobalt, iron, and ruthenium) have been used commercially since 1936 to convert syngas to hydrocarbon products, preferably alkanes. Commercial FT processes mainly use fossil resources to produce hydrocarbon products at massive scale to be economical: The Shell Pearl GTL (gas-to-liquids) plant in Qatar produces up to 140,000 barrels per day (bpd) from an enormous natural gas well (60). More recently, municipal solid waste (MSW) feedstocks have been developed for FT conversion, with Fulcrum Bioenergy reporting <1,000 bpd fuel production. The growing urgency for carbon emission reduction has driven the use of sustainable resources, such as unsorted and nonrecyclable MSW and agricultural waste. These aboveground feedstocks bring economic challenges, as they are geographically highly distributed and the produced syngas has variable macro composition, with various unwanted by-products that can irreversibly inhibit catalyst reactions (61). The fluctuating syngas composition resulting from more variable feedstocks is ideally suitable for gas fermentation by the feedstock-flexible acetogenic bacteria that produce products with high selectivity (62).

The compositional variability in the macro components (CO, H<sub>2</sub>, CO<sub>2</sub>, N<sub>2</sub>, and CH<sub>4</sub>) of syngas and industrial waste gases is a consistent reality with these gas streams and reflects changes in feedstock composition (especially when using low-grade variable waste feedstocks) or process conditions. This compositional variability is also reflected in fluctuating gas contaminants such as heavy metals, aromatics (benzene, toluene, ethylbenzene, and xylenes), various sulfur species (H<sub>2</sub>S, COS, CS<sub>2</sub>), ammonia, nitric oxides, acetylene, reactive oxygen species, and hydrogen cyanide (61). Gas fermentation processes have shown elevated tolerance to typical gas contaminants compared with traditional supported-metal catalysts (63, 64). Although similar to FT catalysis, gas fermentation relies on metal-containing catalysts, in this case metalloenzymes, for conversion of gases to products; however, the catalyst poisoning risk is mitigated as these enzymes are continuously regenerated. Where sulfur irreversibly inactivates FT metal catalysts, it is an essential nutrient for gas-fermenting organisms whose enzyme comprises metal–sulfur active centers. The economic implications of key process requirements and parameters of FT catalysis versus gas fermentation

**bpd:** barrels per day

**MSW:** municipal solid waste

have been summarized elsewhere (62). In summary, continuous gas fermentation operates at low temperature and low pressure, is feedstock flexible, and has shown high tolerance to gas contaminants while maintaining high product selectivity.

Unlike traditional sugar fermentations carried out in (fed-)batch mode, gas fermentation typically operates as a continuous production system for several weeks and even months (**Figure 2c**). The continuous biocatalyst wash-out and renewal rate are an inherent feature of any biological continuous process. A continuously growing organism elevates the process tolerance to upsets and limits gas contaminant accumulation to a certain extent, thus reducing gas treatment requirements, which in turn saves operating costs. A growing organism continuously adapts to local process parameters; production titers can be elevated over time. The advantage of natural selection is knowingly used in other continuous biological systems, from long-term sourdough cultures to wastewater treatment plants, in which final clarifier sludge is recycled back to promote microbial community growth of site- and seasonally adapted organisms.

Although chemical catalytic processes are generally considered faster and more reliable than biological conversions, the latter allow near-complete conversion efficiencies, owing to the irreversible nature of biological reactions (65, 66). A well-developed biological conversion can achieve high reliability, whereas the high enzymatic conversion specificities result in higher product selectivity with the formation of fewer by-products compared with chemical processes.

### 3.2. Scale-Up and Commercialization

The journey to develop, deploy, and commercialize technology is arduous and long, and many technologies fail to cross the so-called valley of death (**Figure 2b**). Getting a new process to scale has many challenges that are not limited just to technology development. Great ideas require financing, data, and time to bridge the valley. Several recent reviews describe the biocatalysts, approaches, and companies involved in efforts to commercialize gas fermentation technology (62, 67–69). We review earlier efforts and concentrate on more recent commercially realized outcomes and practical considerations.

Briefly, syngas fermentation was a research topic in the early 1980s, and initial syngas fermentation research and development explored ways to add value to fossil resources (e.g., coal) for liquid fuel additives and provide energy security. The potential of anaerobic bioconversion technology was recognized as being ripe for scale-up research and development in the 1990s via development and application of new biotechnology approaches (70). Early applied biocatalyst development identified strain improvements for elevated productivities using model syngas and methanol-consuming anaerobes; these efforts included isolation of bacterial strains from acidic and marine environments with higher alcohol yields and tolerance. Methanol, ethanol, and isopropanol were among the top three organic solvents from syngas identified based on their octane values. In 1989, Professor James Gaddy (University of Arkansas) demonstrated the feasibility of gas fermentation technology for ethanol production. Bioengineering Resources, Inc. (BRI), piloted the University of Arkansas technology in Fayetteville, Arkansas, in 2003. Subsequently, INEOS Bio acquired the BRI rights to commercialize their technology in 2008. Then, in 2011, the INEOS New Planet BioEnergy commercial venture involved gasification waste streams from construction MSW, forestry, and agriculture, but it halted operations by 2016 with challenges related to reports of high levels of hydrogen cyanide in syngas (64). Coskata, formed in 2006, was another gas fermentation-to-ethanol company that initially used syngas from biomass gasification for cellulosic ethanol. Later, Coskata sought to use reformed methane to generate syngas with  $H_2:CO$  ratios between 2:1 and 3:1; however, it ceased operation in 2015, and its technology was later acquired by Synata Bio.

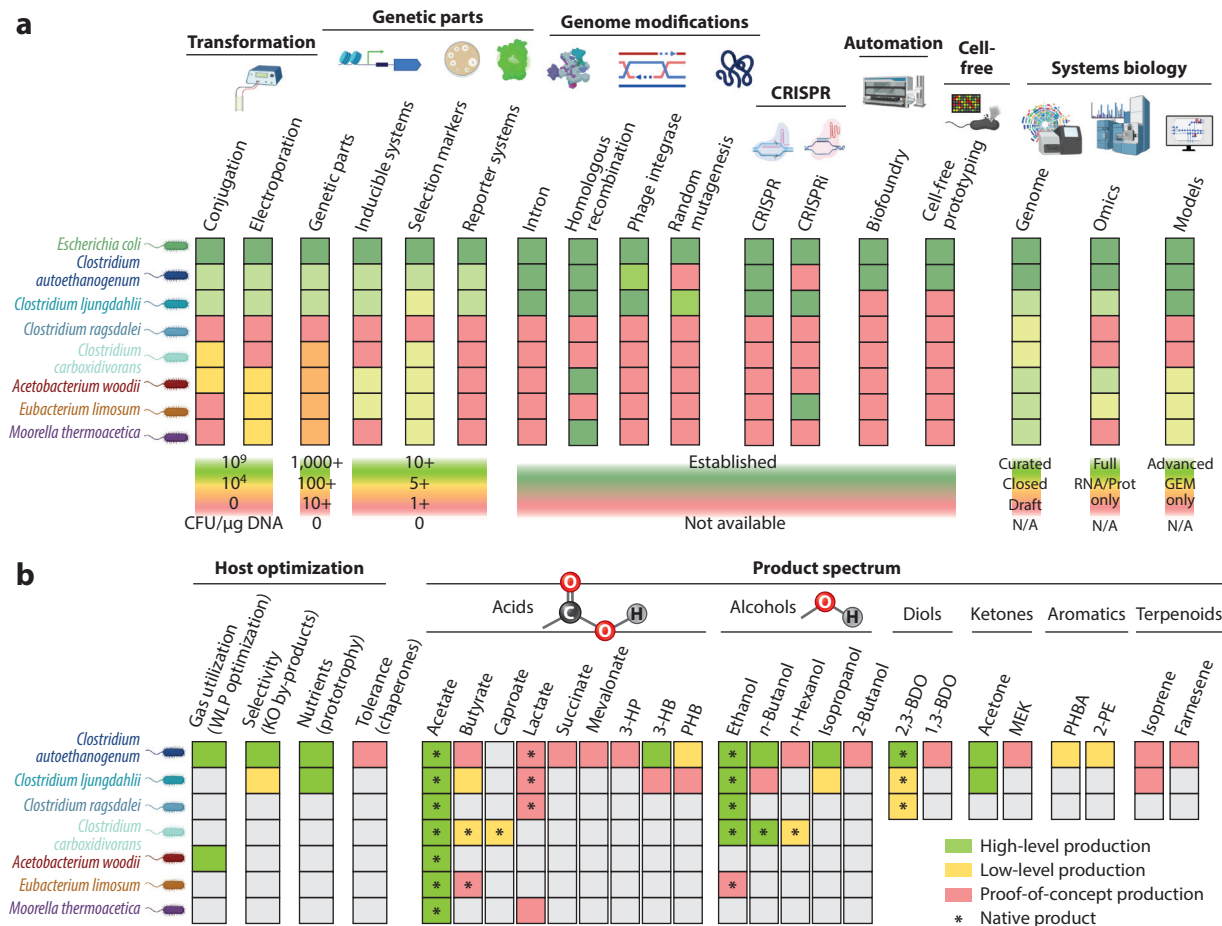
In 2005, Dr. Sean D. Simpson and Dr. Richard Foster founded LanzaTech in New Zealand, where they selected and adapted a culture *C. autoethanogenum* using classical microbiological techniques at the bottle scale and laboratory continuously stirred tank reactors with CO-rich steel-mill off gases as the carbon and energy sources. In 2008, an important milestone was reached with the installation and operation of a 500-L pilot fermenter located at a steel mill site to further develop and scale the gas fermentation process (**Figure 2b**). Past reviews (62, 64) describe the sites and scales of earlier demo units in some detail. In 2014, LanzaTech relocated its headquarters to Skokie, Illinois. In May 2018, gas fermentation successfully started continuous production (**Figure 2c**) at the Beijing Shougang LanzaTech New Energy Science & Technology Co., Ltd., a joint-venture commercial operation with a capacity of 16 million gallons per year (gpy) of ethanol. A second commercial-scale facility is due to be mechanically complete by Q2 2021 in China, with more plants due for completion soon thereafter, including at an ArcelorMittal steel mill site in Ghent, Belgium (71), and at an Indian Oil Company refinery site in India. To further highlight feedstock flexibility, two gasification projects are being built: In 2017, after three years of development, Sekisui Chemical (Japan) and LanzaTech announced high-efficiency production of ethanol from MSW. In April 2020, Sekisui (72) announced the formation of a joint venture, SEKISUI Bio Refinery CO., Ltd., and built a verification plant at one-tenth ( $\sim 20$  t/day) MSW volume processed at a standard-scale waste disposal facility. In September 2020, LanzaTech announced a partnership with Mangalore Refinery and Petrochemical Ltd., one of India's largest refiners, and Ankur Scientific to gasify agricultural residues into 5.3 million gpy of ethanol. This biomass gasification approach is distinguished by its use of simple air-blown gasifiers that permit low-cost distributed systems and biochar coproduction (73). The projects above highlight the rapid deployment of gas fermentation across various industries.

gpy: gallons per year

#### 4. CHEMICALS PRODUCTION

The initial application for ethanol produced from these first large-scale units is as a gasoline-blending component in the road transportation sector. In many of these regions, gasoline consumption is falling and ethanol production is increasing, so bio-ethanol will be used increasingly in other supply chains. This includes as a feedstock for jet and diesel fuel synthesis (69): Blends of gas fermentation-derived jet fuel have obtained ASTM certification, and a first commercial flight was undertaken in October 2018 (74). Ethanol produced from gas fermentation can also meet the specifications for chemical applications and is already used in consumer products such as cleaners (75). Moreover, ethanol is an ideal chemical building block that can be converted into a variety of downstream products via catalytic upgrading, as described by recent reviews (76–78). Many products can be made from ethanol using ethylene as an intermediate via established chemical conversions. Ethylene oxide and ethylene glycol can be used in the production of PET, the world's most-used thermoplastic. Gas fermentation-derived PET has been introduced into the market recently (79). The coupling of ethanol to produce butanol, butadiene, or paraxylene is an active research area. Any application of bio-ethanol other than for fuel blending will require purification. There is also an opportunity to biologically upgrade ethanol and acetate either in a second stage or directly as coculture, e.g., using chain-elongating microorganisms (62, 80). The biomass by-product from the gas fermentation process is a good source of protein and is currently used as such in animal feed formulations (81, 82).

Synthetic biology offers a real paradigm shift, enabling direct production of an array of molecules from gas streams using engineered biocatalysts. Just a decade ago, acetogens were thought to be genetically inaccessible, but scientists now have a comprehensive suite of tools at



**Figure 3**

Status of (a) genetic tool development and (b) metabolic engineering in industrially relevant acetogens in the literature. (a) The most advanced tools for acetogens are available for *Clostridium autoethanogenum* and *Clostridium ljungdahlia*. (b) There are several examples of successful metabolic engineering in acetogens. In each case, production has been demonstrated from gas as feedstock. Whereas several products have been demonstrated only to a proof of concept for production, in several examples in the literature, production has been optimized to a higher level of performance. Abbreviations: 2-PE, 2-phenylethanol; 3-HB, 3-hydroxybutyrate; 3-HP, 3-hydroxypropionate; BDO, butanediol; CFU, colony-forming unit; CRISPR, clustered regularly interspersed short palindromic repeats; CRISPRi, CRISPR interfering system; KO, knockout; MEK, methyl ethyl ketone; N/A, not available; PHB, polyhydroxybutyrate; PHBA, para-hydroxybenzoate; WLP, Wood-Ljungdahl pathway. Figure adapted from images created with BioRender.com.

their disposal (especially for *C. autoethanogenum* and *C. ljungdahlia*) for metabolic engineering and have greatly broadened the range of potential products from gas fermentation (Figure 3).

## 4.1. Genetic Tool Development

**4.1.1. Gene expression and genetic parts.** Introducing foreign DNA to cells is typically the first step in establishing any genetic system. Traditionally, this can be achieved by either conjugation, which normally uses *E. coli* as a donor (45, 83), or electroporation. Key hurdles to overcome include restriction-modification systems that are prevalent in acetogens. Methylation patterns can

be identified by single-molecule, real-time sequencing to devise improved transformation strategies that mimic native DNA modifications (84, 85). Today, transformation protocols exist for a range of acetogens (54, 86–88) (**Figure 3**). The best efficiency, reported at  $10^4$  CFU (colony forming units) per microgram of DNA (86), is still too low to enable transformation of linear DNA fragments or suicide plasmids, which prohibits creation of direct genetic engineering methods (e.g., plasmid assembly and genome modification) that are possible in model systems such as *E. coli* or yeast.

Since the first report of heterologous gene expression in an acetogen in 2010 (54), a suite of native and synthetic promoters (including inducible systems), terminators, and other genetic parts have been developed for acetogens (**Figure 3**). Different reporter gene systems have been developed for acetogens to validate promoter strengths. However, most of these systems require downstream processing to develop signals and cannot be applied in vivo. Recently, three anaerobic fluorescent systems were reported that allow for real-time studies and open up high-throughput possibilities (89, 90). When actively bound to a fluorogenic ligand, FAST (fluorescent-activating and absorption-shifting tag) protein is highly fluorescent under anaerobic conditions. The ligand binding is reversible in the FAST reporter system; therefore, cells can be sorted using fluorescent and washed and incubated for additional rounds of experiments. Two other proteins, HaloTag and SNAP-tag, were shown to be highly fluorescent when covalently bound to fluorogenic ligands under anaerobic conditions in *C. ljungdablii*. All three systems work orthogonally and therefore can be used to study different protein expressions in the same cell simultaneously, or to study mixed-cell populations in real time (90).

**4.1.2. Genome modification and mutagenesis.** One of the earliest methods applied to acetogens for stable gene interruption was based on group II intron-directed mutagenesis (dubbed ClosTron) (91, 92). Although applicable to a wide host range, the method is limited by its cargo size (up to 1 kb in addition to a selection marker), gene modification capabilities, and potential instability introduced by multiple rounds of ClosTron insertion (93), which led to development of other tools based on homologous recombination (HR) (**Figure 3**). Low transformation efficiencies and rare integration success rates make it difficult to obtain double-crossover mutants for gene knockout or knockin in acetogens without the use of a replicative plasmid vector and mostly result in single-crossover mutants with an unstable genotype (94–97). To date, only one successful gene knockout has been reported using non-replicative vector in acetogen (86).

A suite of counterselection markers was developed to select for the rare occasion of the second crossover event in *Clostridium* species (reviewed in 64). Most counterselection methods require selection of a single-crossover event, or deletion of the gene responsible for conferring sensitivity of the marker, followed by selection of double-crossover mutants with the desired genotype and/or loss of the counterselection marker. Two methods, allele-coupled exchange and triple crossover, which are reviewed extensively elsewhere (64), combine multiple counterselection and positive selection markers to achieve long DNA integration and/or scarless gene deletions (98). Many of the counterselectable markers require either a mutant host strain or the use of a toxic agent that may inhibit growth rates. One alternative system to select and force rare double-crossover events in clostridia is to introduce double chromosome breaks using I-SceI, an intron-based endonuclease that recognizes an 18-bp sequence, originally identified in the yeast *Saccharomyces cerevisiae* (99). Owing to the long recognition site, it is very rare to find I-SceI target sites in bacterial genomes. Statistically, it occurs once in every  $6.9 \times 10^{10}$  bp. It was demonstrated successfully in different clostridia for scarless gene knockout and for introducing point mutants (100).

Clustered regularly interspersed short palindromic repeats (CRISPR)-based systems (see Section 5.1) and phage integrase-assisted integration offer promising alternatives for genome

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**Clustered regularly interspaced short palindromic repeats (CRISPR):** adaptive immunity system in prokaryotes that employs endonuclease activity against foreign nucleic acids based on guide RNA recognition

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engineering, specifically in the context of pathway prototyping (**Figure 3**). Phage integrases have previously been used in combination with HR for antibiotic cassette recycling, such as the Cre/*lox* system (101) and FLP recombinase (92). Most recently, a butyric acid synthesis pathway (8.5 kb in size) was successfully integrated into *C. ljungdablii*, facilitated by a phage integrase system (102). Chassis-independent recombinase-assisted genome engineering (CRAGE) (103, 104) was demonstrated to integrate DNA up to 48 kb in various proteobacteria and actinobacteria. The CRAGE recombinase system can be combined with a transposase delivery system to randomly insert a landing pad—consisting of mutually exclusive *lox* sites and a Cre recombinase—into a recipient strain. The genes of interest, which are also flanked by *lox* sites, are then introduced into the recipient strain with the landing pad. The pathway integration is facilitated via Cre recombinase between the *lox* sites with high efficiencies. The advantage of this system over other gene integration tools discussed above is its efficiency and ability to insert large cargo. Unlike plasmid-based systems for metabolic engineering, this system generates a stable genotype and reduces cell-to-cell variance. Another system that uses serine recombinases isolated from different phages offers similar advantages over other traditional HR tools (105). Unlike the Cre recombinase, which recognizes identical sequences for recombination, these recombinases catalyze unidirectional genome integration through two different recognition sites. A landing pad with multiple serine recombinase recognition sites can be inserted into the genome of a recipient strain, after which (pathway) genes of interest can be introduced to the chromosome via corresponding serine recombinases. This method offers a stable genotype for pathway prototyping and unlocks the potential for multiplex genome editing. Both phage recombinase systems, in combination with a transposon delivery method, can be used to probe for advantageous integration sites that may offer better production yields and titers.

Most genome modification efforts in acetogens have focused on targeted modifications. Random mutagenesis based on transposable elements (randomly inserting itself into the chromosome) offers a top-down approach to study essential genes/pathways and/or to isolate mutants for certain phenotypes but has not been deployed broadly in acetogens to date (**Figure 3**). No prior knowledge of whole genome sequences or gene structures is required for transposon insertions; however, a clear goal and screening protocol are necessary to successfully screen for mutant(s) with desired phenotypes. An acetone pathway was integrated into the *C. ljungdablii* chromosome, aided by the *Himar1* transposase (106), and previously the same transposase was shown to randomly insert the chloramphenicol acetyltransferase (*catP*) marker gene into the *C. difficile* chromosome with high efficiency (107, 108). These results suggest that the transposon system can be applied to acetogen for forward genetic studies.

## 4.2. Metabolic Engineering

Even in the context of a still-developing genetic toolbox, there are already several examples of successful metabolic engineering in acetogens to (a) reprogram metabolism to expand the product portfolio and (b) improve efficiency by eliminating competing pathway(s) and by-products or enhancing substrate use, end-product tolerance, and robustness (**Figure 3**).

Proof of concept for direct production of more than 50 different chemicals from gaseous feedstocks has been demonstrated (62, 64) (**Figure 3**). Notably, several have achieved industrially relevant performance levels with production rates in the g/L/h range and titers at the tens of g/L level. LanzaTech has developed an acetone production strain and process with commercial-ready levels of performance (109). Acetone pathway genes (*tblA*, *ctfA*, *ctfB*, *adc*) were selected from a combinatorial library with sequences identified from a commercial ABE fermentation strain collection spanning more than four decades of work (49). The pathway genes were integrated into the

*C. autoethanogenum* chromosome, and subsequently, competing pathway genes encoding enzymes for 2,3-butanediol (2,3-BDO) and 3-hydroxybutyrate (3-HB) production were deleted (109). The combinatorial library consisted of ~250 unique pathways, and the final acetone production strain included more than five genomic modifications, both the most reported in an acetogen to date. Similarly, White Dog Labs has developed a *C. ljungdablii* engineered acetone production strain that produced up to 2 g/L/h acetone using anaerobic, non-photosynthetic (ANP) mixotrophy technology (110). Isopropanol, 3-HB, and butanol (see Section 5.2) (111) are further examples produced at high titer and rates. In many cases, chromosomal integration has been critical for production and process stability and avoids the requirement of antibiotics for plasmid maintenance. It remains to be tested empirically whether one integration site is better than others regarding titer, productivity, and selectivity.

Beyond these C2-C4 molecules, feasibility for direct synthesis of more complex and longer carbon chain products has been demonstrated, including isoprene (112, 113), C6-C14 fatty alcohols, and 2-phenylethanol (114). A key challenge in optimizing fatty alcohol production was the interaction between the heterologous pathway and the cell's own amino acid synthesis metabolism. Guided by genome-scale and kinetic modeling (see Section 5.3), candidate genes/pathways generating undesirable by-products were identified and removed to further improve fatty alcohol production in the host strain (114).

Significant work has also been carried out to improve the biocatalyst, including optimizing flux through the WLP (115) or generating vitamin prototrophic strains (116). A holistic approach, including gene variants analysis (combinatorial library), multi-omics studies, and computational modeling, will greatly assist in understanding physiology and improving the process for better production yield and titer.

## 5. ENABLING TOOLS TO ACCELERATE DEVELOPMENT

A range of new enabling tools are now available to accelerate development of next-generation strains and enable more parallelized development (**Figure 4a**). CRISPR-based engineering tools have largely been heralded as a breakthrough technology and open up new engineering opportunities for previously difficult-to-engineer organisms, including multiplexed genome engineering. To develop efficient production strains, exploration of a large design space is critical. This can be achieved by implementation of high-throughput, automated workflows realized in biofoundries, which is not trivial given the requirement for anaerobic conditions and gaseous substrates. Advances in cell-free technologies now enable pathway prototyping in vitro to inform in vivo design. Predictive models, enabled by the growing pool of omics data, help to further reduce the design space. Retrobiosynthesis further allows generation of optimized pathways. Together, these tools form a powerful approach to enhance the speed of engineering acetogens and are broadly applicable to other nonmodel organisms.

### 5.1. CRISPR Genome Editing

Most characterized CRISPR systems have protospacer-adjacent motif (PAM) requirements that restrict nuclease activity unless a short, specific sequence is present in regions immediately flanking those recognized by the guide RNA (117). The CRISPR system from *Streptococcus pyogenes*, well-known as Cas9, has been engineered with a simplified (single) guide RNA (sgRNA) system and repurposed toward creating a double-stranded DNA break (DSB) proximal to DNA sequences that meet its sgRNA and 5'-NGG-3' PAM requirement (118). Owing to Cas9's ease of use and relative orthogonality, this system gained traction in the genome engineering community, and

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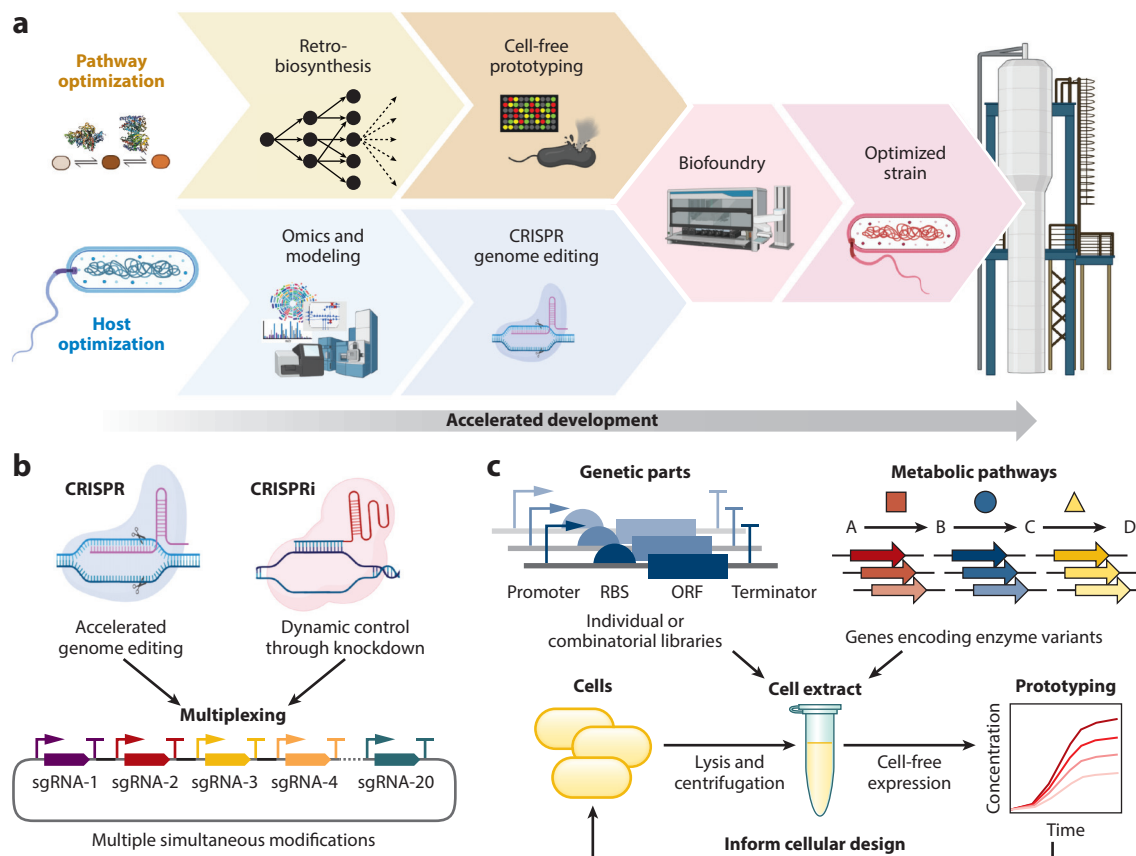
**Omics:** studies of the genome (genomics), transcriptome (transcriptomics), proteome (proteomics), metabolome (metabolomics), or flux (fluxomics)

**Biofoundry:** biological foundry that makes use of automation and high-throughput equipment to accelerate engineering of biology

**Protospacer-adjacent motif (PAM):** a short DNA sequence that is essential for Cas activity and helps protect endogenous systems from targeting native DNA

**Guide RNA:** RNA that programs Cas9 nucleases to cut at a specific genomic location

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**Figure 4**

(a) New enabling tools are now available to accelerate strain development. (b) CRISPR technologies speed up host engineering, in particular where multiplexing can be realized. (c) Cell-free prototyping accelerates part and pathway prototyping. Cell extracts enable rapid prototyping of genetic parts and metabolic pathways using small-scale, high-throughput experiments to provide a more efficient testing platform than slow-growing cells, which can result in highly optimized strains in significantly less time. Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeats; CRISPRi, CRISPR interfering system; ORF, open reading frame; RBS, ribosomal binding site; sgRNA, single guide RNA. Panels a and b adapted from images created with BioRender.com.

efforts to reduce or remove Cas9 enzymatic activity, such as inactivating the nuclease domain (Cas9-Nickase), have led to additional tools, such as generation of single-stranded DNA nicks, gene silencing via CRISPR interference (CRISPRi), and gene activation via CRISPR activation (CRISPRa) (119–121).

Toward targeted genome editing, Cas9 can be employed to generate a DSB at a specific location that opens that site for modification. Generally, cells have two mechanisms to repair DSBs in DNA: HR, which requires the presence of template DNA, and nonhomologous end-joining (NHEJ), so-named because it does not require a homologous template to join ends of DNA (122). NHEJ is the preferred DSB repair mechanism for eukaryotes, but few prokaryotic NHEJ systems have been identified to date (122). Lack of NHEJ creates a burden on prokaryotic genome editing by Cas9, in that it requires (depending on the strain) > 50 bp of template DNA per target for HR to be transformed alongside Cas9; otherwise, DSBs in prokaryotes are lethal. Cas9-Nickase uses a mutated Cas9 to generate a single-stranded nick in the DNA sequence without eliciting the severe

lethality brought by a DSB (123). Both Cas9 and Nickase have been employed, alongside homologous template DNA, to force HR enabling site-specific chromosomal mutagenesis, knockin and knockout. This general method has been used at 50–100% efficiency and in a rapid workflow in several acetogens, including *C. autoethanogenum* (124), *C. ljungdablii* (125), and *E. limosum* (126), as well as many other clostridia species (127) (**Figure 4b**). Gene transfer has been managed mostly through HR of replication-defective plasmids, which has been notoriously inefficient, unstable, and difficult to reproduce (98). The employment of Cas9 as a tool for increased HR efficiencies is a major advancement for editing intractable bacterial genomes.

Because prokaryotic immune systems are the source of the CRISPR revolution, some research groups have recognized that native Cas systems can be hijacked for genome engineering. It has been estimated that ~50% of bacteria and 74% of clostridia harbor endogenous CRISPR-Cas systems (128, 129). Repurposing these systems involves identification of Cas enzymes and PAM requirements and engineering of a CRISPR array or the more complicated process of designing a synthetic sgRNA (129). When the endogenous system is provided a targeted CRISPR array, or a correctly designed sgRNA, and template DNA for HR, it will recode at the directed site; this has been demonstrated in several clostridia and several model prokaryotic organisms (129–131). When it comes to bacterial genome engineering, endogenous Cas systems are somewhat preferred, as the constitutive presence of the native enzymes decreases the burden on the length of DNA necessary for transformation, reduces toxicity caused by off-target Cas9 activity, and removes the metabolic cost of heterologous protein expression (132–134).

The Cas9 enzyme has been engineered by two point mutations (D10A and H840A) to lose all endonuclease activity while maintaining DNA-binding specificity (120). This catalytically dead mutant (dCas9), co-expressed with sgRNA, is well-known as the CRISPRi (120). When sgRNA directs dCas9 just upstream of protein-coding regions (e.g., the promoter region), dCas9 can sterically block RNA polymerase and *trans*-acting transcription factors from initiating transcription, thus preventing gene expression (120). When targeted to the non-template (coding) strand of an intragenic region, CRISPRi prevents transcriptional elongation; however, this process tends to be less effective at reducing gene expression than employing dCas9 to sterically prevent initiation by RNA polymerase (120, 135). Lacking endonuclease activity, deployment of CRISPRi neither results in heritable genomic alterations nor necessitates inclusion of HR template DNA. Plasmid-based dCAS-sgRNA expression systems can be directed to a genomic position through a simple ~20-bp change of the sgRNA, which can be generated via polymerase chain reaction (136). The degree of gene-expression knockdown by CRISPRi can be engineered by altering sgRNA expression, length of sgRNA complementation, dCas9 expression, and sgRNA targeting location relative to the gene of interest and by employing multiple sgRNAs to stack an effect (120, 135). The tunability and inducibility of CRISPRi knockdown serve to prevent total loss of gene function, which enables perturbation of essential genes without causing cell death and allows for elucidation of segments of the genome that were once inaccessible (137) (**Figure 4b**). Polar effects on genes organized in operons represent one drawback of the CRISPRi system (138). Thus far, CRISPRi has been demonstrated in acetogens *C. autoethanogenum* (139) and *C. ljungdablii* (140) [among other clostridia (127)] and *E. limosum* (126). An endogenous Cas system has been rendered inactive and repurposed for gene silencing in *Escherichia coli* through deletion of catalytic parts of the Cas complex (141). Its ease of use, undemanding cloning requirements, and tunable knockdown capabilities have contributed to make CRISPRi an ideal system for investigating gene function in bacteria.

CRISPRi's effectiveness, and orthogonality have facilitated deployment of the system as a genome-wide library screening tool for studying functional genomics in bacteria (142–144). CRISPRi libraries allow simultaneous, unbiased targeting of every gene through a single screening

process, which has been used to create essential gene network maps and characterize gene function under a wide variety of conditions (142–144). CRISPRi libraries outperform similar gene-phenotype mapping methods, namely transposon insertion sequencing (Tn-seq), as they do not carry a bias toward long-coding genes and offer flexibility in design, enabling study of a subset of genes rather than always being applied at a genome-wide scale (143). The off-target effects of Cas9 systems are well-reported; less reported and seemingly more difficult to observe are off-target effects of CRISPRi systems (144). Using a CRISPRi library in *E. coli*, Cui et al. (144) reported that sgRNAs with as little as 9 bp of complement can elicit off-target effects. Increased confidence in gene-phenotype specificity can be reached through strict sgRNA design principles that restrict off-target responses in the upstream regions of other genes and through redundant gene targeting by multiple sgRNAs in a CRISPRi library.

The CRISPRi system has been modified for purposes outside of single-gene repression in prokaryotes. Recently, the creation of nonrepetitive sgRNA handles enabled targeting of more than a dozen operons within a single cell, which was demonstrated by redirecting carbon use in *E. coli* (145). The ability to interrogate multiple knockdown genotypes through a single transformation enables discoveries that previously would have required generations of passaging, creation and curation of multiple plasmids, and weeks of time to achieve in bacteria (**Figure 4b**).

One major drawback of Cas9 for engineering AT-rich organisms such as clostridia is the low frequency of the Cas9 PAM (5'-NGG'3'), resulting in PAM deserts in the genome (146, 147). Hundreds of other Cas proteins have been verified through reviews of metagenomic pipelines, and hundreds of thousands of putative Cas proteins have been annotated (148). Many of these Cas proteins could be repurposed as tools, similarly to the way Cas9 has been described in this review and highlighted with a few descriptions of endogenous Cas redeployment. A frequently adapted system is Cas12a (Cpf1) from *Acidaminococcus* sp., for which the PAM recognition site (5'-TTTN-3') is well-suited for clostridia (146, 147). The CRISPR/Cpf1 system has been used for genome editing in several clostridia (127), including the acetogen *C. ljungdablii* (149). Unlike Cas9, which exhibits no activity past binding to its sgRNA, Cpf1 processes its CRISPR RNAs into sgRNA, which enables a singly expressed handle alongside multiple 20-bp spacer sequences to become many sgRNAs, making this system amenable to multiplexing (150).

The field of Cas enzymes is expected to continue to grow, with companies and academics seeking to mine data for new and interesting enzymes. Extra-small Cas enzymes, Cas enzymes without PAM requirements, and Cas enzymes able to execute new functions (such as DNA shearing versus cutting) have all been discovered but have yet to be adopted by the genomic engineering community. Novel and interesting enzymes likely will continue to emerge in the coming years.

## 5.2. Automation

Automation and biofoundries have transformed the speed at which model organisms can be engineered (151) but require adaptation for other systems. A particular challenge in working with acetogens is the requirement for anaerobic conditions and gaseous substrates. Only very few high-throughput workflows exist for acetogens or clostridia, and the largest reported combinatorial pathway or promoter libraries are all within 250 designs (109, 152, 153). Automation equipment typically requires a significant footprint, yet maintaining a large oxygen-free space is challenging. Although genetic modifications can be carried out using heterotrophic substrates, screening is ideally performed under relevant conditions using gaseous substrates, which adds additional complexity, in particular in working with flammable and toxic gases such as H<sub>2</sub> and CO. Yet, the first fully integrated anaerobic biofoundry for acetogens, capable of generating and screening thousands of strains per cycle, is now in operation (62).

### 5.3. Cell-Free Prototyping

Strain engineering efforts can be expedited through use of cell-free systems, in which biological machinery is harvested after cell lysis to separate biochemical functions from the cells' semipermeable membranes and viability constraints. The open reaction environment and lack of growth requirements in cell-free systems enable high-throughput testing of genetic parts, individual enzymes, and metabolic pathways without inefficient DNA transformation or conjugation techniques (**Figure 4**). This flexibility has enabled myriad cell-free applications in *E. coli* extract for gene expression (154). Particularly useful examples of cell-free prototyping to accelerate strain development include (a) high-throughput characterization of genetic regulatory elements and circuits to identify reliable expression architectures (155, 156); (b) testing of CRISPR system components, including nucleases, guide RNAs, anti-CRISPR proteins, and PAMs (157); and (c) prototyping of metabolic pathways with the ability to test hundreds to thousands of enzyme variants and combinations in weeks rather than months (111, 158).

Metabolic prototyping efforts enable small-scale optimization prior to strain development. This is exemplified by the cell-free conversion of whey permeate waste to polyhydroxybutyrate (PHB), in which several PHB operons were tested in vitro prior to expression in vivo with *E. coli* grown on the same industrial waste (158). The cell-free reactions successfully identified the highest-yielding PHB operon in vivo and indicated a potentially beneficial accessory enzyme. Cell-free prototyping has also proven useful in the development of nonmodel industrial strains, as demonstrated by the successful use of *E. coli* extract to inform engineering of *C. autoethanogenum* for the production of 3-HB and butanol (111). Enzyme homologs from several species were produced through cell-free gene expression and combined to reconstitute the biosynthetic pathways in vitro. In total, more than 50 pathway combinations were evaluated for 3-HB, and more than 200 pathways were evaluated for butanol. The most productive 3-HB strain was then grown on syngas at pilot scale and exhibited a 20-fold improvement in product titer without any genomic modifications to increase pathway flux. These endeavors provide powerful examples of industrially relevant improvements made in production strains through rapid cell-free prototyping. To facilitate, the workflow a new modular vector system has been developed that enable seamless shuttling of DNA between in vitro and in vivo experiments (159).

In addition to *E. coli* extract for in vitro gene expression and biochemical transformations, cell-free systems have been developed using nonmodel organisms from diverse bacterial genera, including *Bacillus* (158), *Clostridium* (160), *Pseudomonas* (161, 162), *Streptomyces* (163, 164), and *Vibrio* (165). A cell-free platform for *Bacillus megaterium* provided the first large-scale analysis of ribosome binding sites and promoters for this underused species, with a lengthy transformation procedure but significant advantages for biotechnology, such as a native secretion system (166). Cell-free prototyping can also capitalize on the microbial world's rich metabolic diversity by employing mixtures of cell extracts to create hybrid systems with unique biotransformation capabilities (167). Most recently, a *C. autoethanogenum* cell-free platform was established that can produce up to 320 µg of protein per ml in semicontinuous transcription/translation reactions, which enabled rapid screening of genetic parts (160). Together, cell-free systems from *E. coli* and nonmodel organisms will expedite engineering efforts in gas-fermenting microbes by increasing the throughput of genetic part characterization and accelerating development cycles for metabolic pathway optimization to more efficiently produce industrial strains that convert waste gas to chemical products.

### 5.4. Omics and Modeling

The expansion of systems biology approaches over the past decade laid the groundwork for greatly increasing the understanding of acetogens on both molecular and systems levels. The foundational

element was completion of whole genome sequencing/assembly for a variety of acetogens, which permitted data mining and comparisons of the molecular machinery these organisms deploy (29). The extension into functional genomics (transcriptomics, proteomics, and metabolomics) complements genomics by providing methods to examine the discrete and operational metabolic activities under a variety of growth conditions and states. For example, the first integrated omics study including transcriptome, metabolome, and proteome analyses of *C. autoethanogenum* was published in 2016 (50). This study demonstrated that the ATP pool remains constant during autotrophic growth on gas and heterotrophic growth on fructose and dissected the underlying mechanisms.

Recent advancements in high-performance mass spectrometry have greatly enhanced metabolomic and proteomic interrogation of the metabolic functions underpinning microbial systems (168), including acetogens. Improvements in sample preparation techniques, mass spectrometry measurements that exploit high mass accuracies and enhanced measurement throughput (with greater precision), and an expanded bioinformatic tool set provide an unprecedented view of the wide dynamic range of biomolecules that control metabolic processes. These tools have uncovered a variety of functional details describing acetogen metabolism, including H<sub>2</sub>-driven metabolic rearrangements in gas-fermenting *C. autoethanogenum* (169), thermodynamic-level control of oscillations of acetogenic metabolism in *C. autoethanogenum* (44), thermodynamic control of ethanol production in *C. ljungdablii* (170), and arginine deiminase pathway boost for *C. autoethanogenum* growth (171).

The growth and continued widespread use of multi-omics analyses represent a powerful tool to facilitate the construction of metabolic models (172). Traditionally, model construction has relied heavily on either exhaustive fluxomics data sets (173) or literature values for kinetic parameters (174). However, in recent years, several models have been constructed to capture steady-state fermentation, which instead are sufficiently characterized by multi-omics data sets. These data sets are generally more economical to obtain and yet, by measuring metabolite, enzyme, and transcript levels, perform a similar task to fluxomics in characterizing reaction kinetics (172). These have included models of acetogen fermentation (175), including one of the first macromolecular expression (ME) models (176), as well as large-scale models of other, less-characterized organisms (177), which have been used to provide engineering predictions and recommendations for strain optimization (172, 174–177). Modeling of the metabolic behavior of cell-free systems presents another challenge for which multi-omics are particularly well-suited (178). Because these systems do not readily support a steady state, they necessitate dynamic models with more degrees of freedom, which therefore require more data to construct (178). Moreover, because multi-omics can capture broad system behavior in addition to the behavior of an engineered pathway, models incorporating these system-level effects will uncover interactions between the engineered pathway and native metabolism, which will impact its translation to in vivo cellular metabolism but would be overlooked with in vitro assays or narrower measurements.

Recent work has seen success in modeling dynamic cell-free metabolism that encompasses more than 200 reactions (30). However, this work focused on capturing the dynamics of protein synthesis rather than flux in heterologous pathways and was carried out in *E. coli* cell-free extracts. Both of these factors allowed this work to forego proteomics measurements and instead use literature values for kinetic parameters, most of which have been painstakingly gathered from purified in vitro kinetic assays, but which have further been shown to be unreliable when translated naïvely into a model (179). In contrast, research modeling cell-free extracts from nonmodel systems lacks access to these literature values and must instead make use of detailed multi-omics data sets, particularly proteomics and metabolomics. By obtaining system-wide time-course measurements of enzyme and metabolite levels, these multi-omics analyses will effectively act as individualized assays

for each organism and condition, allowing accurate identification of experiment-specific kinetic parameters.

### 5.5. Retrobiosynthesis and Pathway Discovery

Retrobiosynthesis enables the identification of novel pathways. On one hand, it can be applied to identify pathways to a target molecule of interest. One example is the discovery of pathways that could lead to the production of mono-ethylene glycol from syngas in acetogens (180). On the other hand, retrobiosynthetic approaches have been developed to generate novel pathways for carbon fixation, which improve upon natural carbon fixation cycles through more favorable thermodynamics, higher catalytic rates, and lower energy requirements. Efforts to design these pathways by hand have been successful; however, this process can be enhanced through computational approaches (177). Using a set of approximately 5,000 naturally occurring enzymes, Bar-Even et al. (13, 181, 182) computationally generated a large number of synthetic, C1 fixation pathways and analyzed their performance based on ATP efficiency, kinetics, and thermodynamic feasibility. Of these, a malonyl-CoA-oxaloacetate-glyoxylate pathway, which uses phosphoenolpyruvate carboxylase as the only carboxylation enzyme, has the most promise (182). This same principle was later used to generate the crotonyl-CoA/ethylmalonyl-CoA/hydroxybutyryl-CoA cycle (183). This cycle was successfully demonstrated *in vitro* using enzymes from 9 different organisms from each domain of life, as well as an engineered enzyme. These successes highlight the potential of computational tools for generating and improving C1 fixation pathways. In fact, the computationally predicted reductive glycine pathway (181) was recently discovered in a naturally occurring microorganism, becoming the seventh known CO<sub>2</sub> fixation pathway (184).

However, current efforts in designing C1 fixation pathways have relied on knowledge of known enzymatic reactions. Because up to one-third of all enzymes may exhibit enzyme-substrate promiscuity (185), enzymes could catalyze non-native reactions that are currently unknown in nature. By considering this larger space of novel reactions, new pathways with even further improved carbon fixation pathways might be found. Reaction rule-based retrobiosynthetic platforms have been described in the literature to achieve this purpose (186, 187). First, reaction rules are abstracted from known reactions to capture all generalized patterns of enzymatic transformations, which can be applied on a set of reactants to enumerate novel reactions with the same transformation. Reaction rules can be strategically selected to reflect the conditions of the intended organisms, for example, eliminating reactions involving oxygen in obligate anaerobes. Then, rules can be applied iteratively to a set of starting compounds to generate a large reaction network, which may contain many possible pathways for carbon fixation. The resulting predictions require further analysis to identify the most promising candidates for experimental validation.

Key criteria to evaluate the potential performance of a pathway include thermodynamics, maximum theoretical yield, and enzyme availability (188–190). Advances in thermodynamic analysis have significantly improved pathway assessment in the process of designing new pathways. eQuilibrator, a Gibbs free energy calculator, allows for the calculation of thermodynamic properties of metabolites, reactions, and pathways to determine potential bottlenecks and infeasible reactions (191, 192). Flux balance analysis provides a method for predicting the maximum theoretical yield of a given pathway inserted into the metabolism of an organism (193, 194). Furthermore, tools have been developed to determine whether there are known enzymes available for predicted reactions based on enzyme promiscuity. SimZyme proposes enzymes for novel reactions based on possible promiscuous enzymes able to carry out the intended transformation (195). The given criteria of an individual pathway can then be scored and used to give a ranking system to determine the highest-performing pathways (188). Combining network generation and analysis tools

provides a powerful method for the design of novel synthetic pathways and offers the opportunity to create high-performing carbon fixation pathways.

## 6. SUMMARY AND OUTLOOK

A clear consequence of the deepening climate crisis is that the predictability of agricultural output throughout the world will deteriorate. This, coupled with the overwhelming need to reduce reliance on fossil resources, makes imperative the development and deployment of gas-fermentation processes that do not rely on the use of specific farmed carbohydrates but allow the recycling of carbon in waste streams from industrial processes, society, or agriculture. Waste resources from industry, society, and agriculture are available on a vast scale globally and can displace fossil resources as the basis for the materials, chemicals, and fuels upon which society relies. In the use of these compositionally varied and variable resources, biological systems are inherently advantaged over the traditional thermochemical processes used by the petrochemical industry to produce fuel and chemical molecules from fossil resources. This advantage stems from both their inherent capacity to convert chaotic inputs into simple outputs and their elevated tolerance to the typical contaminating compounds found in these waste streams. These traits allow commercial production of sustainable molecules in biorefineries at an order-of-magnitude-smaller scale than is possible today with traditional petrochemical refineries. This is commercially important because, although waste streams are plentiful globally, they are dispersed, available only at delivered volumes of hundreds or low thousands of tons per day in any given location. This is in contrast to fossil feedstocks, which are delivered to refineries at rates of hundreds of thousands of tons per day. With a reduced need to either harmonize the composition of these waste streams or remove contaminating molecules to achieve commercial production rates and yields, the capital costs of industrial bioconversion processes are viable at the reduced scales that match with the availability of these waste resources at a given location.

Additionally, the microbe chassis used in gas fermentation processes are now programmable, thanks to the comprehensive suite of synthetic biology tools that have now been reduced to practice in these chassis. This in turn enables commercial manufacturing of an increasingly diverse array of sustainable chemical products from waste streams. This ensures not only that the maximum value can be added to these otherwise low-value resources but also that sustainable solutions for a full spectrum of chemically different polymers, materials, fibers, solvents, and fuels can be delivered at an impactful scale.

The challenge we face is that although processes that use fossil resources have been fully matured over the past 150 years, we are at the start of an urgent journey to implement processes that deliver the products society demands while also achieving carbon circularity. The first commercial gas fermentation process for ethanol production, built by Beijing Shougang LanzaTech New Energy Science & Technology Co., Ltd., has been operating successfully for more than two years. These new manufacturing processes recognize that going forward, products must check three boxes: They must be functional, cost effective, and sustainable. We must reimagine the by-products of industry, society, and agriculture as resources and scale the technologies that can accept these as inputs to deliver sustainable products that avoid the use of fossil resources and further GHG emissions. The prize for doing so is industrial transformation to manufacturing ecosystems free of waste streams, and ultimately the ability to directly harness the sustainable electrons as an energy source for the biosynthesis of products at an unprecedented scale using the ultimate carbon waste: CO<sub>2</sub>.

## SUMMARY POINTS

1. Carbon recycling and a circular economy are critical to reducing atmospheric greenhouse gas emissions. Gas fermentation offers unique feedstock and product flexibility at impactful quantities.
2. The gas fermentation field has developed rapidly over the past decade, and the first commercial plant has been operating successfully since 2018.
3. A decade ago, acetogens were considered genetically inaccessible. Today, an array of genetic tools are available to researchers.
4. Gas fermentation has led to the direct production of more than 50 products, with the first wave of products currently being scaled up and rolled out.
5. CRISPR-Cas9 genome engineering has successfully been applied to gas fermenting microbes, opening new opportunities, including the use of mutant Cas enzymes for controlled gene expression.
6. Cell-free pathway prototyping can accelerate strain engineering (in particular for non-model organisms, such as acetogens) for increased product titers and new chemical products.
7. Integration of multi-omics data sets in the construction of metabolic models provides engineering predictions and recommendations for acetogen strain optimization.
8. Computational tools for pathway discovery allow for the design of C1 fixation pathways that improve upon natural carbon fixation cycles.

## FUTURE ISSUES

1. To meet any climate and decarbonization targets, the scope of all policy frameworks should be broad and technology neutral, with a focus on outcomes rather than prescribed inputs. Policy must focus on sustainable solutions that can deliver carbon savings, and all possible solutions must be pursued to create a resilient and climate-secure future. Individual governments urgently need to set universal carbon tax pricing without exemptions for large-emission industries. Currently, exemptions are in place in all jurisdictions (except Singapore) for industries that present noncompetitive trade status. Binding global agreements are required to provide an equal economic playing field.
2. Speed to deployment of new technologies is crucial as we face an ever-dwindling carbon budget and increased impacts of climate change globally. We must deploy sustainable solutions that can combat climate change in the same way we are approaching COVID vaccines: by pursuing all possible technology solutions with an eye to the future. This model has been followed for electric vehicles, enabling this market to grow and driving down production costs, in parallel with grid transition to 100% renewable electricity. If electric vehicle development and deployment had been forced to wait until the grid was sufficiently green to fully realize their anticipated benefits, we would now have decades to wait for the sector to emerge. Similarly, we do not have the luxury of time before starting to put steel in the ground for production of new sustainable chemicals and must support all policy and financial frameworks that allow this sector to flourish.

3. Gas-fermentation products present significant carbon savings relative to fresh fossil feedstocks. Locking carbon into chemicals that produce durable goods supports a new circular carbon economy, resulting in carbon-negative products. When durable goods reach the end of their useful life, gas fermentation allows this carbon to go back into the material cycle; thus, waste carbon becomes a thing of the past and is permanently sequestered into the product cycle.
4. Significant advances have been made in understanding of the energetics and molecular basis of acetogens over the past decade (see Sections 2.1 and 5.3), yet open questions remain. For example, the reduction step from methylene-tetrahydrofolate to methyl-tetrahydrofolate is the most exergonic step in the Wood–Ljungdahl pathway and therefore may be associated with energy conservation. In some acetogens, methylene-tetrahydrofolate reductase activity has so far been demonstrated only with artificial electron donors. A complete understanding of energy metabolism is important to develop effective engineering strategies.
5. Traditionally, most fermentation processes are operated in batch or fed-batch mode, but there is increased interest in continuous manufacturing, which offers several advantages. Gas fermentation is typically operated continuously, as the gas must be provided constantly, and long, continuous campaigns have been demonstrated across scales (Section 3). Yet in general there is little research around the unique challenges of continuous over batch fermentations.
6. Low transformation efficiencies in acetogens limit the adaptation of some applications developed in model systems to acetogen and necessitate extra steps. Significant optimization has already gone into developing and optimizing more efficient transformation protocols (see Section 4.1), but new tools, such as methylome analysis, offer opportunities for improvements and accelerate research. The recent development of anaerobic fluorescence markers opens up new high-throughput screening opportunities, but workflows require development.
7. Although the cell-free system demonstrates a strong correlation with *in vivo* pathway performance, future efforts to mimic physiochemical conditions of the organism of interest (for example, cofactors) and various conditions that mimic the phase of fermentation used during biochemical production (for example, batch versus semicontinuous) could be explored. A key opportunity is the development of anaerobic cell-free systems.
8. Key challenges in retrosynthetic design of pathways are related to the need for better cheminformatics algorithms and enzyme activity data. Current cheminformatics algorithms suffer from both not representing the full repertoire of biological reactions and, at the same time, including unhelpful redundancies and mispredictions. We need to more efficiently mine known metabolism to abstract generalized reaction rules. Further, enzyme substrate promiscuity models are only as good as the data on which they are trained. Data are available through repositories like the Braunschweig Enzyme Database (BRENDA) but are limited by multiple factors: (a) The data often test many highly similar compounds, but not more diverse compounds that would inform the breadth of promiscuity, and (b) negative data (as in enzyme-substrate pairs that have no activity) are rarely extracted from the primary literature sources. Negative data are essential to developing predictive models of promiscuity.

## DISCLOSURE STATEMENT

N.F., B.D.H., R.R.R., S.D.S., C.L., S.D.B., and M.K. are employees of LanzaTech, which has commercial interest in gas fermentation. M.C.J. consults for and has joint funding with LanzaTech. K.E.T. sits on the Scientific Advisory Board and consults for Manus Biosynthesis and holds patents related to synthesis of biochemicals. S.D.B. is a former ORNL senior staff scientist; formerly held University of Tennessee joint and adjunct faculty appointments; and has current and prior research support from the US Department of Energy (DOE), Biological and Environmental Research, Office of Energy Efficiency and Renewable Energy Bioenergy Technologies program offices. S.D.S. is a LanzaTech board member and has a significant patent portfolio in gas fermentation. M.K. is the inventor of more than 100 patents related to gas fermentation assigned to LanzaTech and an adjunct faculty member at Northwestern University, has received current and prior research support from the DOE and the Australian Research Council, and is a council member of the Engineering Biology Research Consortium and scientific advisory board member of the European Research Agency GasFermTEC project and Northwestern University Master Biotech program.

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## RELATED RESOURCES

1. Department of Energy (DOE) Office of Sciences, Genomic Science Program, Biosystems Design Research Page that among other projects provides background of the clostridia Foundry for Biosystems Design (cBioFAB), that integrates computational modeling, systems-level analyses, and cell-free engineering technologies: <https://genomicscience.energy.gov/biosystemsdesign/index.shtml>; <https://www.energy.gov/science/ber/articles/cell-free-technology-accelerates-industrial-biotechnology>
2. Joint Genome Institute (JGI) Community Science Program (CSP) page that provide the scientific community at large with access to high-throughput sequencing and other resources: <https://jgi.doe.gov/user-programs/program-info/csp-overview/>
3. In memory of Arren Bar-Even, 1980–2020: <https://arren-bar-even.muchloved.com/>.