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Review Article

Recent progress in engineering *Clostridium autoethanogenum* to synthesize the biochemicals and biocommodities

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ABSTRACT

Excessive mining and utilization fossil fuels has led to drastic environmental consequences, which will contribute to global warming and cause further climate change with severe consequences for the human population. The magnitude of these challenges requires several approaches to develop sustainable alternatives for chemicals and fuels production. In this context, biological processes, mainly microbial fermentation, have gained particular interest. For example, autotrophic gas-fermenting acetogenic bacteria are capable of converting CO, CO_2 and H_2 into biomass and multiple metabolites through *Wood-Ljungdahl* pathway, which can be exploited for large-scale fermentation processes to sustainably produce bulk biochemicals and biofuels (e.g. acetate and ethanol) from syngas. *Clostridium autoethanogenum* is one representative of these chemoautotrophic bacteria and considered as the model for the gas fermentation. Recently, the development of synthetic biology toolbox for this strain has enabled us to study and genetically improve their metabolic capability in gas fermentation. In this review, we will summarize the recent progress involved in the understanding of physiological mechanism and strain engineering for *C. autoethanogenum*, and provide our perspectives on the future development about the basic biology and engineering biology of this strain.

1. Introduction

The development of modern industry is still mostly depending on the non-renewable fossil fuel resources for the production of biochemicals and biofuels, which caused many environmental concerns about global warming. When addressing these challenges, microbial fermentation process has shown its great promising as they allow efficient conversion of carbonaceous substrates into target products. The first generation of biofuel such as ethanol production by bacteria and yeasts or acetone, butanol, and ethanol fermentation by Clostridia are mainly relying on the fermentation of starch and sugar materials, which are making the development of economic processes extremely challenging [1,2]. One promising route to overcome these challenges is leveraging acetogenic bacteria to sustainably produce fuels and chemicals from single carbon (C1) gases CO and CO_2 in a process called gas fermentation. Optimization of this technical route followed by industrial promotion is expected to build a new and sustainable biomanufacturing model.

Chemoautotrophic gas-fermenting bacterium, such as *Clostridium autoethanogenum* [3], *Clostridium ljungdahlii* [4], *Clostridium carboxidivorans* [5], *Clostridium ragsdalei*, *Clostridium coskatii* [6] and *Acetobacterium woodii* [7], possess the Wood-Ljungdahl pathway (WLP) of carbon fixation [2], which allows the conversion of C1-gases into the biomass precursor acetyl-CoA, acetate and other specific products, such as ethanol or butanol, while generating ATP for growth [8,9]. The WLP

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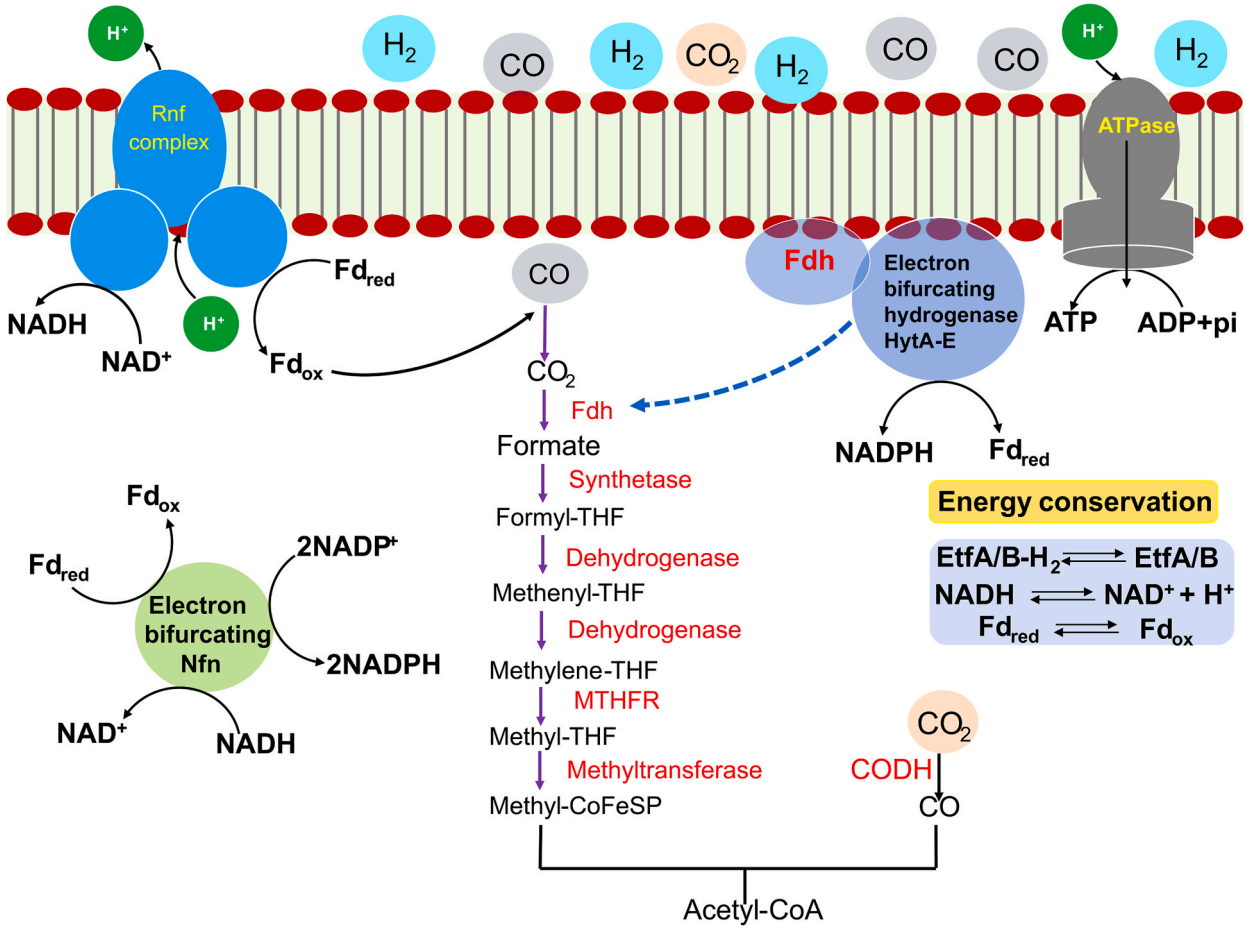

Fig. 1. Detailed mechanism of WLP and energy conservation mechanism in *C. autoethanogenum*. NAD^+: nicotinamide adenine dinucleotide; $NADP^+$: nicotinamide adenine dinucleotide phosphate. Fdh, formate dehydrogenase; THF, tetrahydrofolate; CoFeSP, corrinoid iron-sulfur protein; Acetyl-CoA, acetyl coenzyme A; CODH: carbon monoxide dehydrogenase; ADP, adenosine diphosphate; Pi, phosphate; ATP, adenosine triphosphate; Etf: electron transfer flavoprotein; The electron-bifurcating enzyme Nfn is responsible for the interconvesion of Fd, NADH and NADPH. *C. autoethanogenum* oxidizes hydrogen an enzyme complex of hydrogenase (HytA-E) and formate dehydrogenase (Fdh), achieving the reduction of ferredoxin (Fd) and NAD^+ or $NADP^+$. The membrane-bound Rnf complex and ATPase are proposed to couple the electron transfer from reduced ferredoxin (Fd^{2-}) to NAD^+ with the generation of ATP.

is known as the most efficient pathway among the six native biological carbon fixation pathways as it requires the least reaction steps and energy consumption [10]. *C. autoethanogenum* is one representative of chemoautotrophic bacteria belonging to Firmicutes, can fix gaseous carbons via WLP. Therefore, the wild-type *C. autoethanogenum* is able to utilize Cl gases derived from the gasification of domestic and agricultural wastes or different industrial off-gases and produce acetate and ethanol as main product [11]. And it is noteworthy, *C. autoethanogenum* has been applied in carbon-negative production of bulk chemicals by gas fermentation at industrial scale [12].

The genetic manipulation toolkit has already been available for the *C. autoethanogenum* [13]. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas tools have also been developed to genetically modify this organism [14,15]. These tools not only help us understand the molecular mechanism for C1 fixation and energy metabolism involved, but also allow us to improve strain performance through metabolic engineering as well as to diversify and enhance their metabolic capabilities. In this review, we will focus on the recent development of synthetic biology toolbox, biogenetics and metabolic engineering for the *C. autoethanogenum*.

1.1. Carbon fixation and native product synthesis in C. autoethanogenum

C. autoethanogenum is a strictly anaerobic, Gram-positive, spore forming, rod-like, motile bacterium. It was first isolated from rabbit faeces in 1994 under an atmosphere of carbon monoxide, nitrogen and carbon dioxide, with carbon monoxide as the sole energy source and was identified as a facultative chemolithotroph [16,17]. The whole genome of *C. autoethanogenum* DSM10061 has been published, according to this sequence, the bacterium has a chromosome length of 4,352,205 base pairs with a G + C content of 31.1 %, with 4161 predicted genes, 4042 of which are potentially protein-coding genes with 18 pseudogenes present, and 18 RNA genes [18]. Compared with its close species *C. ljungdahlii,* another model acetogenic bacteria, *C. autoethanogenum* displayed important phenotypic, genomic and metabolic differences although the two are indistinguishable at the 16S rRNA gene level [16,18–22]. For example, the two strains behave very differently at the transcriptional level [23], and *C. autoethanogenum* produced ethanol and 2,3-butanediol (2,3-BDO) production more efficiently [19,20].

Similar to other syngas-utilizing acetogenic bacteria, *C. autoethanogenum* is able to convert CO_2 and CO into low-carbon fuels and chemicals through WLP. Many excellent review articles have described the biochemistry related to this pathway in great detail [24,25]. The WLP consists of a methyl and carbonyl branch (Fig. 1). In the methyl branch, CO or CO_2 is reduced to methyl group through a sequence of tetrahydrofolate- and cobalamin-dependent reactions, which will be then combined with CO (used either directly or after enzymatic reduction of CO_2) to form acetyl coenzyme A (acetyl-CoA). In the WLP, carbon monoxide dehydrogenase (CODH) catalyzes the reversible oxidation of CO to CO_2, and acetyl-CoA synthase (ACS)

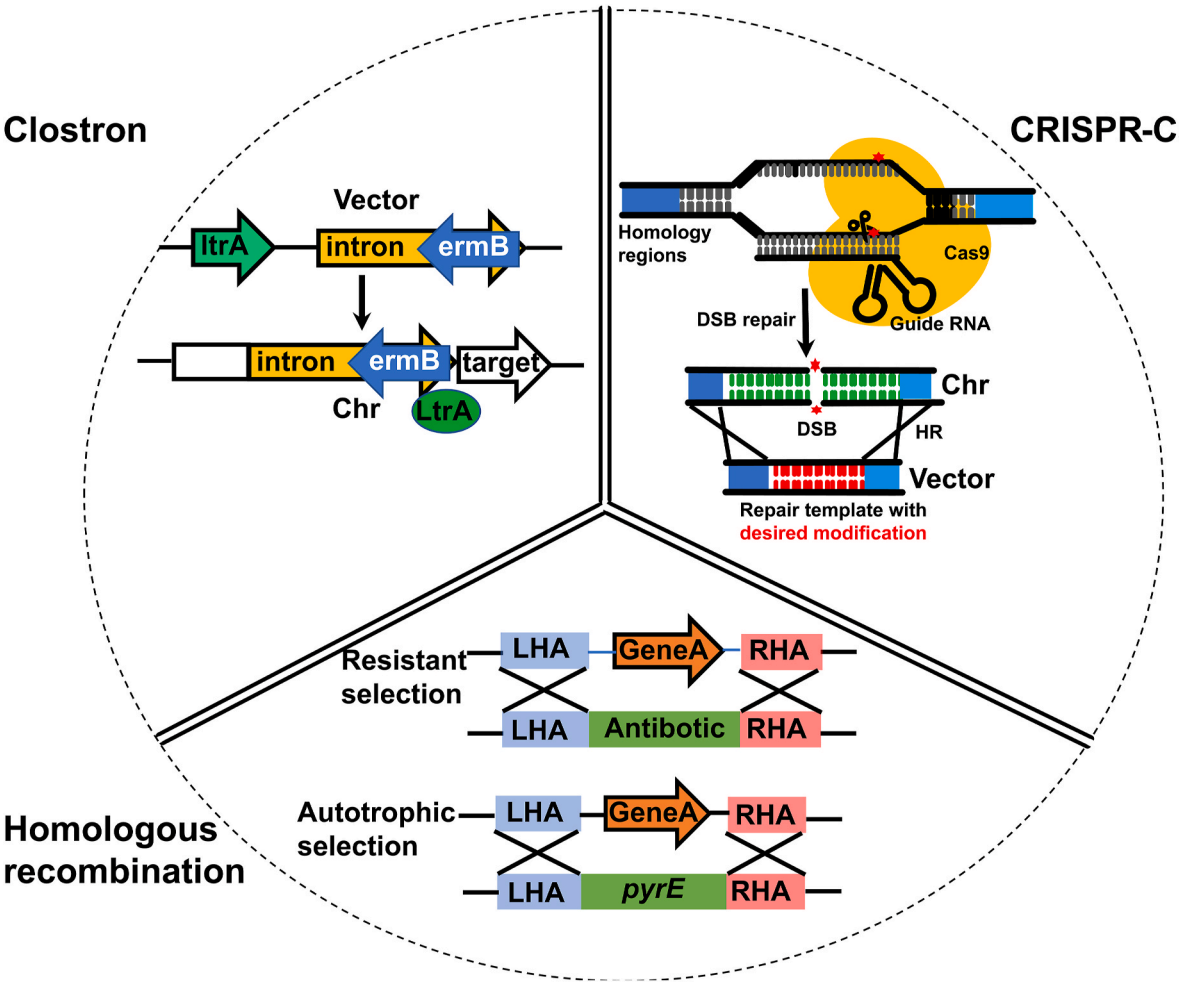

Fig. 2. The genetic tools available for the *C. autoethanogenum.* ClosTron method is most widely used tools to disrupt gene expression in *C. autoethanogenum.* The CRISPR-Cas genome editing system has been efficiently used in *C. autoethanogenum*. The homologous recombination system for gene deletion is based on double crossover. CRISPR: clusted regularly interspaced short palindromic repeats; Cas: CRISPR-associated protein; Chr: chromosome; DSB: double-stranded break; HR: homologous recombination; LHA: left homology arm; RHA: right homology arm.

combines with CODH to form a CODH/ACS complex for acetyl-CoA fixation. The overall structure of this complex adopts the classic α2β2 architecture, the α- and β-subunits catalyzed the formation of acetyl-CoA and the reduction of CO_2 to CO, respectively [26]. Both of these two enzymes are predicted to be essential to the function of WLP. Whole genome sequencing revealed the presence of three putative genes encoding CODHs: *acsA* (CAETHG_1620-162), *cooS1* (CAETHG_3005) and *cooS2* (CAETHG_3899). Mutagenesis study shows that, of the three genes, *acsA* is essential for autotrophy, by contrast, *cooS1* and *cooS2* are dispensable for autotrophy [13]. In fact, the WL pathway is energy-consuming and requires reducing equivalent supply which comes from CO or hydrogen under autotrophic growth conditions. Especially, the oxidation of hydrogen requires the participation of hydrogenases, and the Fdh/Hydrogenase complex directly utilizes the reducing force from hydrogen to reduce CO_2. Alternatively, Hydrogenase catalyzes H_2 oxidation to produce Fd^{2-} and NADPH by electron bifurcation, storing the reducing power in cofactors [27]. Besides, many genes for gluconeogenesis also plays important roles in the carbon fixation during syngas fermentation. For example, transcriptomics revealed that the largest transcriptional fold change in the glycolytic enzymes observed was for one glyceraldehyde-3-phosphate dehydrogenase (GAPDH, CAETHG_3424) during autotrophic growth. The CAETHG_3424 deficient strain could not grow autotrophically on gas as the sole carbon and energy source. It indicated that CAETHG_3424 is one critical gene for the syngas utilization [23]. Additionally, the phosphoenolpyruvate carboxykinase (PCK) is responsible for the conversion of oxaloacetate to phosphoenolpyruvate using a molecule of ATP and controls the rate-limiting step of gluconeogenesis [28]. In the genome of *C. autoethanogenum*, gene CAETHG_2721 is annotated as an ATP-dependent PCK and significantly upregulated during autotrophic growth. Disruption of CAETHG_2721 impaired growth when cultured on gas only, the growth of ΔCAETHG_2721 was restored in the presence of both gas and fructose. This result showed that CAETHG_2721 also control the rate-limiting step of gluconeogenesis in the *C. autoethanogenum* [23]. Recently, Craig Woods et al. has developed the transposon insertion sequencing techniques for high-through functional genomics in *C. autoethanogenum* and identified 758 genes (19 % of the genome) essential genes for the autotrophic growth [29].

Like *C. ljungdahlii,* a variety of compounds can be produced from acetyl-CoA, acetate and ethanol are main products in the *C. autoethanogenum* (Fig. 3). The formation of acetate is directly derived from acetyl-CoA through the pathway with phosphotransacetylase and acetate kinase. For the synthesis of ethanol, there are two pathways proposed: (1) The classic pathway via a bi-functional aldehyde/alcohol dehydrogenase (AdhE). (2) Acetate reduction to acetaldehyde and further to ethanol via an aldehyde: ferredoxin oxidoreductase (AOR) and alcohol dehydrogenase [30]. Fungmin Liew et al. demonstrated that AOR-based pathway is critical to ethanol formation in *C. autoethanogenum* [31]. During gas fermentation *C. autoethanogenum* is also known to synthesize other two valuable chemical compound,

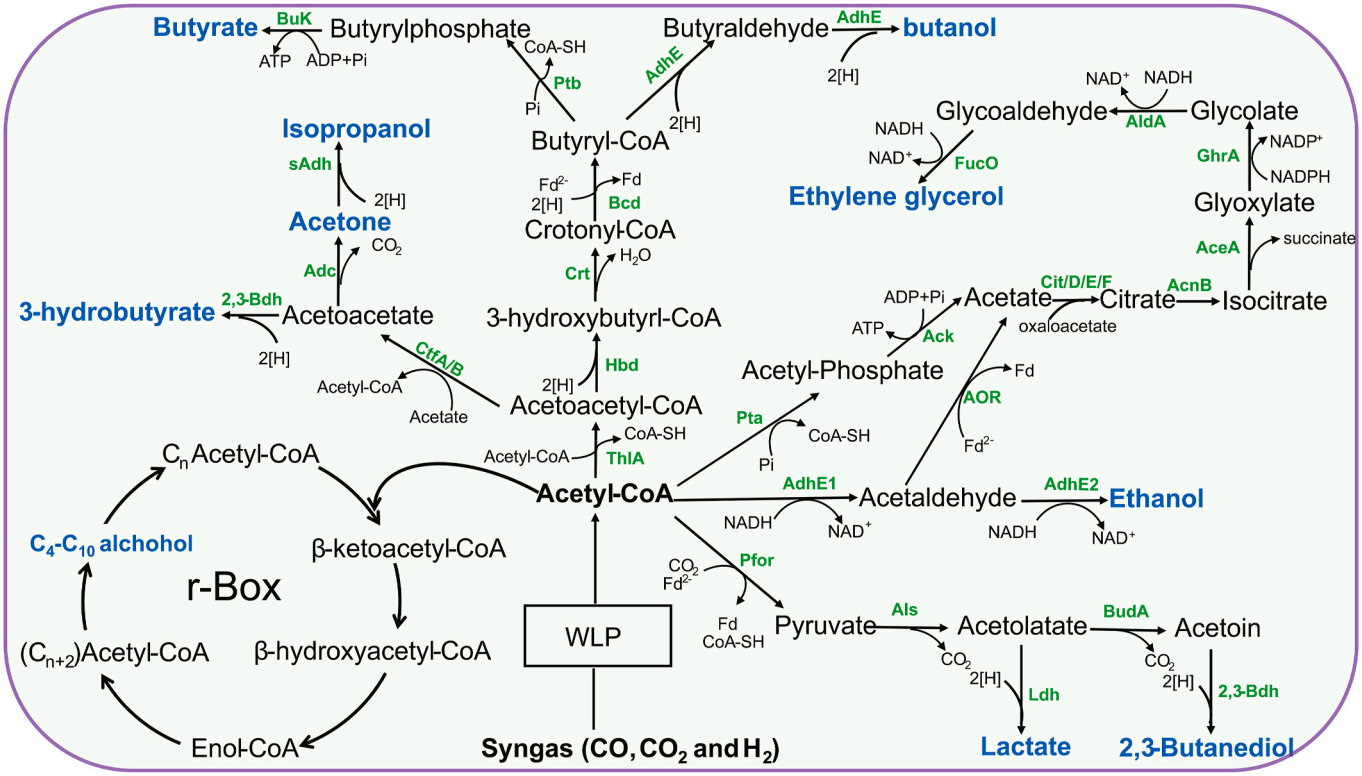

Fig. 3. The main products of wild-type or engineered *C. autoethanogenum.* NAD^+: nicotinamide adenine dinucleotide; $NADP^+$: nicotinamide adenine dinucleotide phosphate. ThlA, thiolase; CoA-SH: Coenzyme A; Acetyl-CoA: Acetyl Coenzyme A; Hbd: 3-hydroxybutyryl-CoA dehydrogenase; Crt: crotonase; Bcd: butyryl-CoA dehydrogenase; Fd^{2-}: reduced ferredoxin; Fd: ferredoxin; Ptb: phosphotransbutyrylase; Pi:phosphate; BuK: butyrate kinase; ATP: adenosine triphosphate; ADP: adenosine diphosphate; AdhE: aldehyde/alcohol dehydrogenase; CtfA/B: electron-transferring flavoprotein A and electron-transferring flavoprotein B; 2,3-Bdh: 2,3-butanediol dehydrogenase; Adc:acetoacetate decarboxylase; sAdh: secondary alcohol dehydrogenase; Pfor: pyruvate:ferredoxin oxidoreductase; Als: acetolactate synthase; BudA: acetoin decarboxylase; Ldh: lactate dehydrogenase; Pta: phosphotransacetylase; AOR: aldehyde:ferredoxin oxidoreductase; Ack: acetate kinase; Cit/D/E/F: citrate lyase; AcnB: aconitase; AceA: isocitrate lyase; GhrA: glyoxylate reductase; AldA: Glycolaldehyde dehydrogenase; fucO: lactaldehyde reductase. r-Box: reverse β-oxidation.

lactate and 2,3-BDO (Fig. 3). The lactate is produced through direct reduction of pyruvate catalyzed by the lactate dehydrogenase (CAETHG_1147). The synthetic pathway for 2,3-BDO includes three key enzyme, acetolactate synthase (CAETHG_1740), acetolactate decarboxylase (CAETHG_2932) and 2,3-BDO dehydrogenase (CAETHG_0385), all of the genes for those enzymes have been identified in the genome (Fig. 3). When reconstructing the complete pathway through expressing those enzymes in the *E.coli*, under anaerobic conditions, the resulting *E. coli* strain is able to produce 2,3-BDO comparable to the level produced by *C. autoethanogenum* during growth on CO containing waste gases [11].

1.2. Energy metabolism in C. autoethanogenum

The WLP is a net energy-consuming process and takes in one ATP molecule and eight reducing equivalents. In acetogenic bacteria, energy conservation is mainly relying on the membrane-associated reduced ferredoxin: NAD^+ oxidoreductase (Rnf), which can convert reduced ferredoxin (Fd^{2-}) to Fd by coupling the translocation of protons across the cell membrane. The proton gradient generated will drive the ATPase to synthesize ATP for the carbon fixation. Besides, electron bifurcation is another mechanism of energetic coupling that saves cellular ATP for the bacteria, in which electron bifurcating enzymes deliver the electrons from the electron donor to two different electron acceptors coupling endergonic redox reactions to exergonic redox reactions [32] to overcome thermodynamic barriers and minimize free energy waste [33]. When oxidizing hydrogen, the electron-bifurcating and ferredoxin-dependent transhydrogenase (Nfn) or hydrogenase will produce Fd^{2-} and NADH/NADPH. Although there are six various types of hydrogenase systems encoded in the genome, the cells appear to contain only one active hydrogenase HytA-E1/E2 (CAETHG_2794-99) [27]. When CO is used as the sole carbon source, CO dehydrogenase will oxidize partial CO to CO_2 by coupling the formation of Fd^{2-} and NADH/NADPH [34] (Fig. 1).

Most acetogens can reduce CO_2 with H_2 to acetic acid via the WLP. By contrast, a few species such as *C. autoethanogenum*, *C. ljundahlii* and *C. ragsdalei* grow optimally between pH 5 and pH 5.5, and is able to form ethanol and acetate when fermenting H_2 and CO_2 [35]. With *C. autoethanogenum* as model, Johanna Mock et al. investigated the underlying special energy conservation mechanisms for its growth during ethanol formation from H_2 and CO_2. It is shown that the presence of Rnf, Nfn and AOR with very high specific activities in H_2/CO_2-grown cells is key point to understand the energy metabolism of *C. autoethanogenum* [27].

1.3. Advances in genetic tools for C. autoethanogenum

An efficient DNA transfer method to introduce and express foreign DNA molecules is prerequisite to genetically engineer acetogens. Conjugation is widely used for DNA transfer in *C. autoethanogenum,* which relies on cell-to-cell contact between the donor strain, usually *Escherichia coli* and the receiving host [36,37]. ClosTron, a group II-intron-based retrohoming gene inactivation method, has been widely used for gene inactivation in *C. autoethanogenum* [13,27]. ClosTron can be well used for the single gene mutation, but generating double genes mutation is proved to be impossible for this technique [31] (Fig. 2).

Table 1
Strain engineering of *C. autoethanogenum*.

Year	Genetic manipulations	Results	Scale	References
2011	Expressing *C. acetobutylicum* butanol synthesis pathway genes	1.54 g/L Butanol 0.31 g/L Butyrate	Lab	[21,60]
2013	Expressing diol dehydratase genes from *Klebsiella opylova*	1.4 g/L 1-propanol 0.012 g/L 2-butanol	Lab	[61]
2013	Expressing exogenous mevalonate pathway enzymes and/or DXS pathway enzymes	not quantified Isoprene	Lab	[62]
2014	Expressing native PFOR, alsS and alsD	9 g/L 2,3-BDO, 33 % selectivity	Pilot	[63]
2017	Inactivating aldehyde/alcohol dehydrogenase (AdhE)	2.46 g/L ethanol	Lab	[31]
2019	Expressing the PHB synthetic genes from *Cupriavidus necator*	10 % (w/w) of CDW	Lab	[64]
2019	Expressing operon *aceA-ghrA- aldA* and *fucO* from *E. coli*	0.029 g ethylene glycol/g fructose	Lab	[46]
2021	Multiple strategies in metabolic engineering	10 g/L/h ethanol, 95 % selectivity	Commercial	[14]
2020	Expressing the 3-hydroxybutyrate and butanol synthetic pathway optimized by the CFS	14.63 g/L 3-hydroxybutyrate 1. 63 g/L butanol 0.5 g/L 1,3-butanediol	Lab	[57]
2021	Expressing heterologous phenypyruvate decarboxylase, phenyacetaldehyde reductase and native key enzyme for the phenypyruvate synthesis pathway	0.28 g/L 2-phenylethanol	Lab	[58]
2022	Expressing the 1-hexanol synthetic pathway optimized by the CFS	0.26 g/L 1-hexanol	Lab	[59]
2022	Expressing the acetone or isopropanol synthetic pathway optimized by the CFS	3 g/L/h acetone, 90 % selectivity 3 g/L/h isopropanol, 90 % selectivity	Pilot	[12]
2022	Expressing the β-alanine pyruvate aminotransferase/ γ-aminobutyrate transaminases, malonic semialdehyde reductase, aspartate decarboxylase/2,3-alanine aminomutase from multiple microorganisms	0.358 g/L/d 3-hydroxypropionate 0.0822 mg/L/d 1,3-propanediol	Lab	[65]

Fungmin Liew et al. developed an allelic exchange method for *C. autoethanogenum,* which utilizes a pseudo-suicide vector reliant on the pCD6 replicon [36] and counter selection marker composed of an orotate phosphoribosyltransferase gene from *C. acetobutylicum* [38] (Fig. 2). This system was successfully used for making double mutation such as *adhE1*+*adhE2* and *aor1*+*aor2* [31].

CRISPRs are adaptive immune systems evolved by bacteria to protect against exogenous genetic elements, such as phages and plasmids [39], and CRISPR-Cas-based genetic tools have been adapted for many organisms including some *Clostridium* [40–43]. Due to toxicity caused by uncontrolled Cas9 protein expression, CRISPR-Cas9-mediated desired gene deletion is unsuccessful in *C. autoethanogenum*. To address this, Shilpa Nagaraju et al. constructed and screened a small library of tetracycline-inducible promoters that can be used to finely tune gene expression [15]. With a new inducible promoter, the efficiency of target gene deletion in *C. autoethanogenum* was improved to over 50 %, making it a viable tool for engineering *C. autoethanogenum* [15] (Fig. 2). To increase the pace of engineering progress, Nicholas Fackler et al. developed an inducible CRISPR interference (CRISPRi) system for *C. autoethanogenum*, which is used to repress the expression of crucial genes for the native production of 2,3-BDO [14]. More recently, a novel riboswitch-based editing tool, RiboCas, has been engineered to overcome excessive Cas9 toxicity by tightly repressing cas9 expression using a theophylline-inducible riboswitch [44]. Originally demonstrated in four non-acetogenic clostridial species, it has now been shown to function effectively for the generation of mutants in *C. autoethanogenum* [45,46]. Compared to Cas9, the utilization of Cas12a proteins is still not implemented in *C. autoethanogenum*, although they were more adapted to Clostridium genomes for the T-rich recognition and lower toxicity [47]. Based on their successful application in redirecting carbon flux of the closely related acetogenic bacterium (*C. ljungdahlii*) [48], Cas12a-based genome editing of *C. autoethanogenum* may be more applicable and promising.

1.4. Strain engineering

A variety of strategies for manipulating the culture conditions were explored to optimize the yields of the native chemicals and fuels in *C. autoethanogenum*, which includes tuning pH value, adding the amino acids, optimizing the basic composition and modulating the composition of gas [49–52]. To improve our understanding of this organism's metabolism, Marcellin et al. has constructed genome metabolic model of *C. autoethanogenum* by combining multiomics and experimental data [23]. Moreover, Kaspar Valgepea et al. performed a systems-level steady-state gas fermentation study to build quantitative links between carbon, energy, and redox metabolism of this microorganism [53]. These studies have significantly accelerated the metabolic engineering of *C. autoethanogenum* as cell factories, which has been engineered to produce a wide range of biofuels and industrially relevant chemicals including primary and secondary alcohols, acids and terpenes (Table 1). Notably, Lanzatech has genetically manipulated *C. autoethanogenum* through multiple metabolic engineering strategies and constructed an engineered strain capable of producing 10 g/L/h ethanol and 95 % selectivity under the optimal syngas fermentation condition, which resulted in one commercialized process for the production of bioethanol.

Recently, the cell-free systems (CFS) are emerging as powerful platforms for synthetic biology applications, which allows for testing of hundreds to thousands of different designs within days. Particularly, this innovative way is used to inform strain design in absence of high-throughput capabilities [54]. Antje Krüger et al. developed and optimized one batch CFS platform for *C. autoethanogenum*, with which protein synthesis can reach up to 260 μg/mL. A key feature of the platform is that both circular and linear DNA templates can be applied directly to the CFS reaction to program protein synthesis [55,56]. The CFS has been successfully used to engineer this microorganism for the production of multiple chemicals (Table 1). For example, Ashty S. Karim et al. screened 54 different cell-free pathways for 3-hydroxybutyrate production with CFS, which improved in vivo 3-HB production in *Clostridium* by 20-fold up to 15 g/L in a 1.5 L continuous system. The same group also optimized a six-step butanol pathway across 205 permutations and increased the production of butanol up to 22 mM in the batch ferment, which is the 10 folds than the highest yield previously reported for the engineered acetogenic bacteria [57]. Bastian Vögeli

et al. have optimized and implemented reverse β-oxidation pathway in the *C. autoethanogenum* by the CFS platform, which generated *C. autoethanogenum* strains able to produce 1-hexanol from syngas, achieving a titer of 0.26 g/L in a 1.5 L continuous fermentation [58]. Most recently, combining genome mining and kinetic modeling prototyping cell-free system to optimize metabolic flux, Fungmin Eric Liews et al. generated one engineered strain capable of producing either acetone or isopropanol using syngas as a feedstock with productivity of over 3 g/L/h and 90 % selectivity in continuous pilot scale production, and life cycle analysis confirmed a negative carbon footprint for the products [12].

2. Conclusions and outlook

Microbial gas fermentation is a promising technology to address drastic environmental consequences caused by modern industry. *C. autoethanogenum* represents one promising platform for sustainable production of bulk chemicals from syngas. To further understand the basic biology for this strain, the high-throughput functional genomics tools remains to be developed for investigating the gene interaction network to provide a new understanding of the relationship between genotype and phenotype. The uncovering of genome-wide gene regulatory networks is also a key tool allowing us to understand the complex mechanisms of transcriptional gene regulation and to better control the expressional behavior of native and foreign genes. For the engineering biology, metabolic engineering efforts in *C. autoethanogenum* are constrained by their energy requirements, and computational approaches will be of great advantage in the metabolic pathway design and enzyme engineering. Moreover, the recent development of synthetic biology tools, such as combinatorial libraries of DNA parts and regulatory circuits, also allows for optimizing the expression of synthetic pathway and balancing metabolic fluxes. In general, metabolic engineering in the *C. autoethanogenum* is still in its infancy. Alternatively, constructing the synthetic microbial communities in which sygnas-untilizing *C. autoethanogenum* produce the organic carbon resource for the other heterotrophic member, can take advantage of syngas fermentation and expand the spectrum of products.

Author contributions

Sai Wan and Mingchi Lai contribute equally to the article.

Declaration of competing interest

The authors declare no conflict of interest.

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