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Accelerate acetogenic bioproduction: Acetogens as sustainable producers of biocommodities

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Abstract

Gas fermentation using autotrophic acetogenic bacteria has been industrialized, however, its full potential remains untapped, with only native products like ethanol being produced thus far. Advancements in synthetic biology have enabled the recombinant production of diverse biocommodities to broaden their limited natural product spectrum from C1-gases. Additionally, co-culturing acetogens with other microorganisms holds the potential for expanding the product spectrum further. However, commercialization remains challenging due to complex pathway and (co)culturing optimizations. To address this, novel synthetic biology tools, including the use of high throughput biopart screenings using reporter proteins, the deployment of cell-free systems to combine best-performing enzymes, and the identification and elimination of competing pathways, can be employed. Incorporating genetically engineered strains in co-cultures improves dependencies, directs product formation, and increases resilience, enhancing bioproduction efficiency. This review emphasizes using these tools to enhance the recombinant production of biocommodities, offering promising solutions to overcome existing challenges.

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Introduction

The pressing need to mitigate the effects of greenhouse gases on the climate represents a significant challenge in our modern era. Therefore, the use of acetogenic bacteria to capture and convert greenhouse gases is outstanding. Acetogens are strictly anaerobic bacteria, which employ the Wood-Ljungdahl pathway to convert C1-carbon sources such as CO, CO_2+H_2, formate, or methanol into valuable biocommodities in the energetically most efficient way when compared to other C1-capturing pathways [1]. Despite the current commercial use of acetogens like *Clostridium autoethanogenum*, their applications remain limited due to the restricted range of naturally produced compounds, low product titers of recombinant products, and the limited availability of ATP, as these organisms operate at the thermodynamic limit of life [2,3]. To overcome this hurdle, novel synthetic approaches and molecular tools that are applicable to various acetogens are essential. Currently, several genetic manipulation methods are available for the two closely related strains *Clostridium ljungdahlii* and *C. autoethanogenum*, as well as the rapidly emergent acetogens *Acetobacterium woodii* and *Eubacterium limosum*, making them popular choices for synthetic microbial approaches [4,5].

In this review, we will discuss innovative molecular techniques for better-studied acetogens but also emphasize the potential of lesser-studied acetogens in the realm of synthetic biology and their prospects of being transformed into established biotechnological workhorses with well-established molecular tools in the future. We will commence with the implementation of reporter proteins to screen favorable bioparts, progress to the enhancement and customization of recombinant pathways, touch on the manipulation of the carbon flow with gene knockouts or knockdowns, and culminate on improving the product platform using synthetic co-cultures and less studied acetogens.

The first step to improve production: screening for bioparts using fluorescent reporter proteins

Despite the advancements in molecular tools for different acetogens, and the reported recombinant production of more than 50 products from C1-carbon sources, reaching industrial levels remains a significant challenge [6]. Initial attempts often involve enhancing transcription or translation levels by using homologous gene sequences, exchanging regulatory elements, or employing replicons to control plasmid copy numbers. However, studying novel bioparts can be cumbersome

due to the limited availability of reporter proteins. Enzyme-based reporters like CatP or GusA are often used to study gene expression and promoter strength, but their usage is time-consuming and unsuitable for real-time assays and high-throughput screenings [7–11]. Instead, fluorescent proteins (FPs) are usually preferred. Unfortunately, most FPs, such as GFP or those of the mFruit family, depend on oxygen for chromophore maturation, which makes them challenging to use in acetogens. Therefore, efforts are made to design FPs that exhibit brightly fluorescent under anaerobic conditions [12]. Flavin mononucleotide-based fluorescent proteins (FbFPs) exhibit weak green fluorescence after excitation with blue light and were successfully used in the acetogens *C. ljungdahlii* to study a temperature-sensitive origin of replication, and in *E. limosum* and the closely related strain *Butyribacterium methylotrophicum* to assess promoter strength [13–15]. Ligand-dependent self-labeling FPs such as the HaloTag and the SNAP-tag show strong, oxygen-independent fluorescence when they bind covalently to a fluorescent ligand. As the choice of ligands can be orthogonal to other FPs, they have been applied in *C. ljungdahlii* to study co-culture dynamics and cell–cell interactions, and in *E. limosum* to screen genetic bioparts [16–18]. Particularly suited for applications in anaerobic environments are novel FPs of the fluorescence-activating and absorption-shifting tag (FAST) family, which upon binding a fluorogenic ligand result in bright oxygen-independent fluorescence [19]. FAST's small size (14 kDa), immediate fluorescence after folding, and specific, rapid, and fully reversible fluorogen binding make it an ideal reporter for studying real-time molecular processes such as protein synthesis or folding [20]. Through engineering, the protein and its fluorogenic ligands have been enhanced, yielding variants with adjustable spectral, optical, and physical properties. As a result, the fluorescence of pFAST can span the visible spectrum, ranging from blue to red [21]*. Various versions of FAST were used in the acetogens *C. ljungdahlii*, *E. limosum*, *A. woodii*, and *Thermoanaerobacter kivui* to study promoter strength, synthetic co-cultures, and as a tag to track protein production of heterologous produced enzymes [22–25]. The implementation of fluorescent reporter proteins into the molecular toolbox of acetogens represents a crucial step in screening and studying regulatory bioparts, laying the basis for fine-tuning the recombinant production of biocommodities.

Cell-free systems: iPROBE as a novel approach for rapid optimization of biosynthetic enzymes in acetogens

Genetic manipulation in acetogens is still complex, requiring specific transformation protocols for each strain. Consequently, characterizing multiple promoter–enzyme combinations to enhance the productivity of recombinant strains can be time-consuming, especially considering the slow growth rates of acetogens. Thus, finding an efficient and time-saving strategy to enhance productivity poses an important challenge. A promising approach to speed up pathway design is using a cell-free gene expression system for *in vitro* protein synthesis, as recently developed for *C. autoethanogenum* [26]. Another noteworthy method is the *in vitro* Prototyping and Rapid Optimization of Biosynthetic Enzymes (iPROBE) system, a cell-free system to rapidly screen for enzyme combinations to advance recombinant pathways and increase productivity [27] (Box1). Both methods circumvent the need to re-engineering strains, as only cellular lysates rather than complete genetic constructs are used.

iPROBE has been used in *C. autoethanogenum* to optimize the production of various biocommodities. In a first attempt, 54 different pathway combinations were tested *in vitro* to produce 3-hydroxybutyrate (3-HB) heterologously. Based on their TREE score, four thiolase and hydroxybutyryl-CoA dehydrogenase combinations were tested *in vivo* and resulted in the highest reported 3-HB production by an acetogen yet. Notably, the *in vivo* production correlated with results obtained in the cell-free experiments [27]. Similarly, for butanol production, a neural-network-based algorithm was trained using TREE scores of 120 pathway combinations tested *in vitro*, narrowing down the initial 314,928 possible combinations to a promising set of nine. These pathways were applied *in vivo*, leading to a remarkable over 200-fold improvement in butanol production [27]. In a further study, iPROBE was used to screen 392 enzyme combinations for the initial four reactions of the reverse β-oxidation, selectively producing C4–C6 acyl-CoA products. The *in vitro* best-performing enzymes were combined with termination enzymes to produce C4- and C6-acids, which could be further converted to the respective alcohols in *C. autoethanogenum*. Finally, 14 strains were constructed expressing optimized iPROBE pathways that could

Box 1. The four steps of the iPROBE system

In the first step, the design step, homologous enzymes are selected, which also implies the mining of homologous variants. In the second phase, the build phase, cell lysates are generated and enriched with pathway enzymes using overexpression before or cell-free protein synthesis after cell lysis. Enriched cell lysates are precisely mixed and product synthesis is activated by adding substrates and co-factors. Interestingly, the authors demonstrated an *in vitro* to *in vivo* correlation, where enzyme concentrations used in the cell-free approaches corresponded to different promoter regulatory strengths. Hence, low enzyme concentrations used *in vitro* correlate with weak promoter strengths *in vivo*, and vice versa. In the third step, the test step, the so-called TREE (Titer, Rate, and Enzyme Expression) score is calculated, which is the titer of the produced product multiplied by the liner production rate and by the protein solubility plus the total enzyme amount. The TREE score can be used to easily rank cell-free pathway performance. Finally, in the fourth step, the apply step, the best-performing cell-free pathways are assembled and used *in vivo* in the host strain [27].

produce butanol and hexanol *in vivo*. While titers were still low, hexanol was produced for the first time in *C. autoethanogenum* using iPROBE pathways [28].

The above examples show that iPROBE can rapidly speed up pathway design and optimization in *C. autoethanogenum* from month to just days. This system has the potential to be tested in less studied acetogens to improve the recombinant production of complex biocommodities.

Compete against competing pathways: gene knockouts and knockdowns for improved production

Genome engineering tools such as homologous recombination, ClosTron, transposon mutagenesis, and CRISPR-Cas-based systems were applied in various acetogens for gene mutations, deletions, and insertions and are reviewed in detail elsewhere [4,5]. While gene deletions are outstandingly important for physiological studies, they also play a key role in pathway optimization to improve native, but also recombinant production.

Due to their industrial relevance, enhancing ethanol production in *C. autoethanogenum* and *C. ljungdahlii* by mutating and deleting competing pathways has been a topic of interest in the past. Particularly, genes encoding bi-functional aldehyde/alcohol dehydrogenase or the aldehyde:ferredoxin oxidoreductase have been targeted [29—35]. These studies have yielded diverse outcomes, which range from improved to depleted ethanol production. An intriguing example of gene deletion facilitating production is the heterologous lactate production in *A. woodii*. To produce the platform chemical, the rapid lactate consumption-ability of *A. woodii* required the deletion of its lactate consumption complex, followed by heterologous expression of a non-native lactate dehydrogenase [24,36]. Regardless, generating knockout strains is still complex and time-consuming for most acetogens. Recently, the demonstration, that knocking out genes of unclear functions can massively impact growth characteristics and production in *C. autoethanogenum* showcased how complex the identification of competing enzymes can be [37]. To overcome this, CRISPR interference (CRISPRi) has emerged as an easy-to-use tool for studying gene functions in acetogens by knocking down gene expression without the need for knockout generations. Here, swapping sgRNAs enables rapid knock-down gene expression of various genes in a plasmid-based manner. CRISPRi was employed in *C. ljungdahlii* to enhance 3-HB production, resulting in a 2.3-fold increased titer by targeting the phosphotransacetylase gene, albeit only in heterotrophic conditions [38]. Additionally, downregulating the bifunctional aldehyde/alcohol dehydrogenase *adhE1* not only reduced ethanol production but also boosted recombinant butyrate production [39]. In *C. autoethanogenum*, 2,3-butanediol formation was depleted by knocking down the essential gene for its production encoding the alpha-acetolactate decarboxylase *budA*. Moreover, repression of the secondary alcohol dehydrogenase *sADH* decreased the conversion of acetone to isopropanol [40]. In *E. limosum*, a CRISPRi library targeting each gene in its genome was used to achieve a fourfold increase in autotrophic growth [41]*.

Case example: acetone production from steel mill off-gas

Although acetogens are not capable of producing acetone naturally, recombinant production was achieved by expressing genes encoding thiolase (ThlA), CoA-transferase (CtfAB), and acetoacetate decarboxylase (Adc) [8,23,42,43]. However, low titers made them unappealing for usage at an industrial scale. Recently, a highly engineered *C. autoethanogenum* strain was designed, which produces acetone (or isopropanol, which will not be discussed in this review) with high selectivity by pathway, strain, and process optimizations [44]**.

Initially, novel acetone biosynthesis enzymes were identified by screening 272 naturally performing acetone-butanol-ethanol fermentation bacteria. This approach resulted in 30 promiscuous gene homologous, which were combined with high-, medium-, and low-strength promoters to construct a plasmid library. The resulting transcription units were assembled into expression vectors and applied for acetone production in *C. autoethanogenum*. The deletion of the native secondary alcohol dehydrogenase prevented the conversion to isopropanol. Screening the resulting 247 recombinant strains revealed varied acetone production levels based on promoter strength, emphasizing their significance as bioparts. The top-performing five pathways were genome-integrated, which resulted in the initial acetone production strain.

By identifying and deleting competing pathways, the production of this strain was improved. Deletion of the gene responsible for 2,3-butanediol formation, identified through a genome-scale model and evolutionary algorithm, resulted in the abolition of production. In addition, 13 gene candidates responsible for 3-HB production were identified using an *in silico* homology search, which could potentially act alone or in combinations to produce this byproduct. As knocking out all these genes would take several months, the authors adapted the iPROBE system to prototype knockout targets for these candidates. Cell-free acetone biosynthesis using *in vitro*-produced ThlA, CtfAB, and Adc was achieved in the presence and absence of the 13 knockout candidates. Decreased acetone production compared to the control indicated that the added knockout candidates shifted the carbon flow from

acetone to the byproduct. By using this system, three genes were identified and deleted, which abolished 3-HB production completely. Finally, iPROBE was used to evaluate enzyme importance in acetone biosynthesis and found, that high levels of CtfAB enhance production. Consequently, CtfAB was overexpressed in a plasmid-based manner.

The final engineered strain harbors four gene deletions to circumvent by production, the acetone production operon integrated into its genome, and a plasmid to additionally overexpress *ctfAB*. This strain resulted in the highest acetone pilot-scale production reported so far, with a productivity of $\sim$2.5 g L^{-1} h^{-1}.

Expanding the product platform for acetogens through synthetic co-cultures

Synthetic co-cultures containing at least one acetogenic partner can be used to convert C1 substrates into non-native products for acetogens that are more valuable. Acetogens play a crucial role as C1-utilizers and provider of intermediate substrates, such as acetate or ethanol, that other organisms within the consortia can further metabolize. With respective co-cultures the production of C4–C8 fatty acids and alcohols was successfully demonstrated, as reviewed elsewhere [45]. However, the utilization of engineered acetogens in co-cultures is scarce, possible due to the limited number of available genetic tools. The dependency on antibiotic pressure of plasmid-based recombinant strains could impede their use in co-cultures, potentially inhibiting the growth of the co-culture partner. Nevertheless, engineered acetogens have the potential to produce novel intermediate substrates for the co-culture partner, as discussed later [47] or to generate artificial dependencies. Moreover, population dynamics can be tracked in co-cultures, combining engineered FP producing strains with flow cytometry [16,46]. Engineered strains of *Clostridium acetobutylicum* and *C. ljungdahlii* were used to study protein exchange when co-cultured. Authors utilized FAST and the HaloTag and showed that upon cell fusion, the FPs can be quantified in both cells [17]. The aforementioned engineered lactate producing *A. woodii* strain was co-cultured to produce caproate [47]*. In this study, *A. woodii* was cultivated until the stationary phase to facilitate the conversion of H_2 and CO_2 to lactate. Subsequently, *Clostridium drakei* was added to the co-culture, utilizing lactate as a substrate to produce caproate. As far as we know, this represents the only reported instance of producing a platform chemical using an engineered acetogen in a synthetic co-culture. Although currently underutilized, engineered acetogens have the potential to produce favorable substrates for co-culture partners or might be engineered to be more robust. Alternatively, by combining an acetogen with a genetically accessible model organism such as *Escherichia coli*, an incredible opportunity arises to produce valuable compounds from C1 substrates in a synthetic co-culture [48,49].

Applying genetic tools for less studied acetogens with unique features

Unique metabolic features make several acetogens intriguing for synthetic biology approaches, despite their limited exploration in terms of genetic tools. Thermophilic acetogens like *Moorella thermoacetica* and *T. kivui* are outstanding from an industrial perspective due to their optimal growth temperature at 55° and 66°, respectively, which limits the risk for contaminations or cooling costs during fermentation and facilitates the separation of volatile products. In addition, *T. kivui* is exceptional as it can take up foreign DNA naturally, circumventing the need for time-intensive and often arduous electroporation or conjugation protocols [50]. Although suicide vectors for gene deletions and insertions based on homologous recombination were applied for both thermophiles, making use of replicating shuttle vectors is challenging due to the sparse amount of functional Gram-positive replicons and thermostable bioparts [50–52]. Kanamycin serves thermostable antibiotic and respective resistance gene was already used in both thermophiles [50,52]. Functional Gram-positive replicons were recently reported for *M. thermoacetica* and *T. kivui* [25,53]. Ethanol production was achieved in *M. thermoacetica* by constructing a replicating shuttle vector to overexpress the native alcohol dehydrogenase *aldh* or the non-native bifunctional aldehyde/alcohol dehydrogenase *adhE1* of *C. autoethanogenum*. In *T. kivui*, an replicating shuttle vector was used to establish a promoter library, making use of the reporter protein pFAST [25]. Moreover, acetone production was achieved by genomic integration of respective pathway into the genome of *M. thermoacetica* [54,55]. As the strain can grow above the boiling point of acetone, recombinant production seems promising in an industrial perspective.

With its toolbox expanding rapidly, the aforementioned *E. limosum* is gradually stepping into the spotlight (Table 1). Despite most other acetogens, *E. limosum* can utilize a broad range of substrates, including the C1 sources, CO_2, CO, methanol, and formate, to produce acetate, butyrate, and traces of hexanoate and butanol [56]. Several constitutive and inducible promoters and genetic bioparts were investigated using enzymatic but also fluorescent reporter proteins [14,18,23,57,58]. CRISPR-Cas9 was applied to knock out genes of the Wood-Ljungdahl pathway and genes encoding hypothetical proteins [14,58], while its native homologous recombination machinery was used to delete biofilm synthesis genes by transforming linear DNA with homologous overhangs [59]*. The deletion of respective genes not only showcases the functionality of this tool, but also improves handling of the strain in further

Table 1

Genetic toolbox of *Eubacterium limosum* DSM 20543^T and closely related strains (KIST612†, B2‡, and DSM 3468*).

Reporter proteins			Genetic manipulation			Recombinant production			
Reporter	Purpose	Literature	Tool	Target	Literature	Product	Substrate	Amount	Literature
FbFP	Inducible promoter screening	[14] [15] *	CRISPR-Cas9	*folD*, *acsC*, hypothetical protein	[14]	Acetone	Methanol	1.6 mM	[23] ‡
β-glucuronidase (GUS)	Promoter screening	[58] †		*pyrF*	[58] †				
FAST	Promoter screening, fluorescent tag, population dynamics	[23,46] ‡	CRISPRi	*fhs1*, *folD*, *acsC*, *acsD*, *ptsF*	[14]	Butanol	Methanol	0.6 mM	[23] ‡
eGFP HaloTag SNAP-tag	Biopart screening (promoters, 3′ UTRs, 5′ UTRs)	[18]		All genes using a CRISPRi library	[41]	Butanol	Formate /methanol	1.5 mM	[61] *
Chloramphenicol acetyltransferase (CatP)	Promoter screening	[57]	Native homologues recombination	*sinI*, *tasA*, *epsFML*, *epsABC*/*ptkA*/*tmkA*	[59]	Acetoin	H_2/CO_2	1.3 mM	[18,41]
			Genome editing based on RelBE-family toxin-antitoxin	*hisI*, *mtaA*, *mtaC*, *mtcB*	[60] ‡				

engineering approaches. In addition, an inducible toxin-antitoxin system was established and used to generate various gene knockouts, focusing on methanol metabolism [60]. As mentioned above, a genome scale CRISPRi screening identified essential genes for autotrophic acetogenesis and genes that enhanced growth when repressed [40]*. The CRISPRi library successfully pinpointed three genes for enhancing autotrophic growth and, upon repression, led to a significant fourfold increase in autotrophic growth. Although the recombinant production of biocommodities at an industrial scale is currently lacking, the rapid establishment of these powerful tools holds the potential to transform *E. limosum* into a biotechnological workhorse.

Conclusion

The number of molecular tools increases for acetogens, and first steps are made to improve production. However, demonstrating the ability of metabolically engineered acetogens to produce significant amounts of certain products, while reducing greenhouse gases at an industrial scale remains to be achieved. So far, promising and closest to use industrially seems *C. autoethanogenum* to produce acetone or isopropanol. Moreover, the rapidly increasing molecular toolbox of *E. limosum* commence high expectations, although producing chemicals recombinantly seems far from being commercialized.

Authors' contributions

Maximilian Flaiz: Conceptualization, Writing - original draft. **Diana Z. Sousa**: Conceptualization, Writing - review & editing, Funding Acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data were used for the research described in the article.

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References

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest
** of outstanding interest

1. Claassens NJ, Cotton CAR, Kopljar D, Bar-Even A: **Making quantitative sense of electromicrobial production**. *Nat Catal* 2019, **2**:437–447, https://doi.org/10.1038/s41929-019-0272-0.

2. Katsyv A, Müller V: **Overcoming energetic barriers in acetogenic C1 conversion**. *Front Bioeng Biotechnol* 2020, **8**, 621166, https://doi.org/10.3389/fbioe.2020.621166.

3. Schuchmann K, Müller V: **Autotrophy at the thermodynamic limit of life: a model for energy conservation in acetogenic bacteria**. *Nat Rev Microbiol* 2014, **12**:809–821, https://doi.org/10.1038/nrmicro3365.

4. Jin S, Bae J, Song Y, Pearcy N, Shin J, Kang S, Minton NP, Soucaille P, Cho B-K: **Synthetic biology on acetogenic bacteria for highly efficient conversion of C1 gases to biochemicals**. *Int J Mol Sci* 2020, **21**:7639, https://doi.org/10.3390/ijms21207639.

5. Bourgade B, Minton NP, Islam MA: **Genetic and metabolic engineering challenges of C1-gas fermenting acetogenic chassis organisms**. *FEMS Microbiol Rev* 2021, **45**, https://doi.org/10.1093/femsre/fuab008. fuab008.

6. Köpke M, Simpson SD: **Pollution to products: recycling of 'above ground' carbon by gas fermentation**. *Curr Opin Biotechnol* 2020, **65**:180–189, https://doi.org/10.1016/j.copbio.2020.02.017.

7. Beck MH, Flaiz M, Bengelsdorf FR, Dürre P: **Induced heterologous expression of the arginine deiminase pathway promotes growth advantages in the strict anaerobe *Acetobacterium woodii***. *Appl Microbiol Biotechnol* 2020, **104**:687–699, https://doi.org/10.1007/s00253-019-10248-9.

8. Banerjee A, Leang C, Ueki T, Nevin KP, Lovley DR: **Lactose-inducible system for metabolic engineering of *Clostridium ljungdahlii***. *Appl Environ Microbiol* 2014, **80**:2410–2416, https://doi.org/10.1128/AEM.03666-13.

9. Nagaraju S, Davies NK, Walker DJF, Köpke M, Simpson SD: **Genome editing of *Clostridium autoethanogenum* using CRISPR/Cas9**. *Biotechnol Biofuels* 2016, **9**:219, https://doi.org/10.1186/s13068-016-0638-3.

10. Chowdhury NP, Alberti L, Linder M, Müller V: **Exploring bacterial microcompartments in the acetogenic bacterium *Acetobacterium woodii***. *Front Microbiol* 2020, **11**, 593467, https://doi.org/10.3389/fmicb.2020.593467.

11. Song Y, Shin J, Jin S, Lee J-K, Kim DR, Kim SC, Cho S, Cho B-K: **Genome-scale analysis of syngas fermenting acetogenic bacteria reveals the translational regulation for its autotrophic growth**. *BMC Genom* 2018, **19**:837, https://doi.org/10.1186/s12864-018-5238-0.

12. Streett H, Charubin K, Papoutsakis ET: **Anaerobic fluorescent reporters for cell identification, microbial cell biology and high-throughput screening of microbiota and genomic libraries**. *Curr Opin Biotechnol* 2021, **71**:151–163, https://doi.org/10.1016/j.copbio.2021.07.005.

13. Molitor B, Kirchner K, Henrich AW, Schmitz S, Rosenbaum MA: **Expanding the molecular toolkit for the homoacetogen *Clostridium ljungdahlii***. *Sci Rep* 2016, **6**, 31518, https://doi.org/10.1038/srep31518.

14. Shin J, Kang S, Song Y, Jin S, Lee JS, Lee J-K, Kim DR, Kim SC, Cho S, Cho B-K: **Genome engineering of *Eubacterium limosum* using expanded genetic tools and the CRISPR-Cas9 system**. *ACS Synth Biol* 2019, **8**:2059–2068, https://doi.org/10.1021/acssynbio.9b00150.

15. Wang X, Qin J, Ma C, Wang J, Wang X, Xu S, Feng J, Chen K, Ouyang P: **Methanol assimilation with CO_2 Reduction in *Butyribacterium methylotrophicum* and development of genetic toolkits for its engineering**. *ACS Sustainable Chem Eng* 2021, **9**:12079, https://doi.org/10.1021/acssuschemeng.1c02365.90.

16. Charubin K, Streett H, Papoutsakis ET: **Development of strong anaerobic fluorescent reporters for *Clostridium acetobutylicum* and *Clostridium ljungdahlii* using HaloTag and SNAP-tag proteins**. *Appl Environ Microbiol* 2020, **86**, https://doi.org/10.1128/AEM.01271-20.

17. Charubin K, Modla S, Caplan JL, Papoutsakis ET: **Interspecies microbial fusion and large-scale exchange of cytoplasmic proteins and RNA in a syntrophic *Clostridium* coculture**. *mBio* 2020, **11**, https://doi.org/10.1128/mBio.02030-20.

18. Song Y, Bae J, Jin S, Lee H, Kang S, Lee J, Shin J, Cho S, Cho B-K: **Development of highly characterized genetic bioparts for efficient gene expression in CO_2-fixing *Eubacterium limosum***. *Metab Eng* 2022, **72**:215–226, https://doi.org/10.1016/j.ymben.2022.03.016.

19. Gautier A: **Fluorescence-activating and absorption-shifting tags for advanced imaging and biosensing**. *Acc Chem Res* 2022, **55**:3125–3135, https://doi.org/10.1021/acs.accounts.2c00098.

20. Plamont M-A, Billon-Denis E, Maurin S, Gauron C, Pimenta FM, Specht CG, Shi J, Quérard J, Pan B, Rossignol J, *et al.*: **Small fluorescence-activating and absorption-shifting tag for tunable protein imaging *in vivo***. *Proc Natl Acad Sci USA* 2016, **113**:497–502, https://doi.org/10.1073/pnas.1513094113.

21. * Benaissa H, Ounoughi K, Aujard I, Fischer E, Goïame R, Nguyen J, Tebo AG, Li C, Le Saux T, Bertolin G, *et al.*: **Engineering of a fluorescent chemogenetic reporter with tunable color for advanced live-cell imaging**. *Nat Commun* 2021, **12**:6989, https://doi.org/10.1038/s41467-021-27334-0.
In this study authors generated the oxygen-independent fluorescent protein pFAST by mutagenesis of FAST, resulting in optimized fluorescence brightness. They also designed fluorogens covering the entire spectrum from blue to red. These advancements are crucial for multicolor approaches in anaerobic microorganisms, which still lack a great number of brightly fluorescent reporter proteins.

22. Streett HE, Kalis KM, Papoutsakis ET: **A strongly fluorescing anaerobic reporter and protein-tagging system for *Clostridium* organisms based on the fluorescence-activating and absorption-shifting tag protein (FAST)**. *Appl Environ Microbiol* 2019, **85**, https://doi.org/10.1128/AEM.00622-19.

23. Flaiz M, Ludwig G, Bengelsdorf FR, Dürre P: **Production of the biocommodities butanol and acetone from methanol with fluorescent FAST-tagged proteins using metabolically engineered strains of *Eubacterium limosum***. *Biotechnol Biofuels* 2021, **14**:117, https://doi.org/10.1186/s13068-021-01966-2.

24. Mook A, Beck MH, Baker JP, Minton NP, Dürre P, Bengelsdorf FR: **Autotrophic lactate production from H_2 + CO_2 using recombinant and fluorescent FAST-tagged *Acetobacterium woodii* strains**. *Appl Microbiol Biotechnol* 2022, **106**:1447–1458, https://doi.org/10.1007/s00253-022-11770-z.

25. Hocq R, Bottone S, Gautier A, Pflügl S: **A fluorescent reporter system for anaerobic thermophiles**. *Front Bioeng Biotechnol* 2023, **11**, 1226889, https://doi.org/10.3389/fbioe.2023.1226889.

26. Krüger A, Mueller AP, Rybnicky GA, Engle NL, Yang ZK, Tschaplinski TJ, Simpson SD, Köpke M, Jewett MC: **Development of a clostridia-based cell-free system for prototyping genetic parts and metabolic pathways**. *Metab Eng* 2020, **62**:95–105, https://doi.org/10.1016/j.ymben.2020.06.004.

27. Karim AS, Dudley QM, Juminaga A, Yuan Y, Crowe SA, Heggestad JT, Garg S, Abdalla T, Grubbe WS, Rasor BJ, *et al.*: ***In vitro* prototyping and rapid optimization of biosynthetic enzymes for cell design**. *Nat Chem Biol* 2020, **16**:912–919, https://doi.org/10.1038/s41589-020-0559-0.

28. Vögeli B, Schulz L, Garg S, Tarasava K, Clomburg JM, Lee SH, Gonnot A, Moully EH, Kimmel BR, Tran L, *et al.*: **Cell-free prototyping enables implementation of optimized reverse β-oxidation pathways in heterotrophic and autotrophic bacteria**. *Nat Commun* 2022, **13**:3058, https://doi.org/10.1038/s41467-022-30571-6.

29. Liew F, Henstra AM, Köpke M, Winzer K, Simpson SD, Minton NP: **Metabolic engineering of *Clostridium autoethanogenum* for selective alcohol production**. *Metab Eng* 2017, **40**:104–114, https://doi.org/10.1016/j.ymben.2017.01.007.

30. Leang C, Ueki T, Nevin KP, Lovley DR: **A genetic system for *Clostridium ljungdahlii*: a chassis for autotrophic production of biocommodities and a model homoacetogen**. *Appl Environ Microbiol* 2013, **79**:1102–1109, https://doi.org/10.1128/AEM.02891-12.

31. Huang H, Chai C, Li N, Rowe P, Minton NP, Yang S, Jiang W, Gu Y: **CRISPR/Cas9-based efficient genome editing in *Clostridium ljungdahlii*, an autotrophic gas-fermenting bacterium**. *ACS Synth Biol* 2016, **5**:1355–1361, https://doi.org/10.1021/acssynbio.6b00044.

32. Xia P-F, Casini I, Schulz S, Klask C-M, Angenent LT, Molitor B: **Reprogramming acetogenic bacteria with CRISPR-targeted base editing via deamination**. *ACS Synth Biol* 2020, **9**: 2162–2171, https://doi.org/10.1021/acssynbio.0c00226.

33. Liu Z-Y, Jia D-C, Zhang K-D, Zhu H-F, Zhang Q, Jiang W-H, Gu Y, Li F-L: **Ethanol metabolism dynamics in *Clostridium ljungdahlii* grown on carbon monoxide**. *Appl Environ Microbiol* 2020, **86**:7300, https://doi.org/10.1128/AEM.00730-20. 20.

34. Lo J, Humphreys JR, Jack J, Urban C, Magnusson L, Xiong W, Gu Y, Ren ZJ, Maness P-C: **The metabolism of *Clostridium ljungdahlii* in phosphotransacetylase negative strains and development of an ethanologenic strain**. *Front Bioeng Biotechnol* 2020, **8**, 560726, https://doi.org/10.3389/fbioe.2020.560726.

35. Dykstra JC, van Oort J, Yazdi AT, Vossen E, Patinios C, van der Oost J, Sousa DZ, Kengen SWM: **Metabolic engineering of *Clostridium autoethanogenum* for ethyl acetate production from CO**. *Microb Cell Factories* 2022, **21**:243, https://doi.org/10.1186/s12934-022-01964-5.

36. Schoelmerich MC, Katsyv A, Sung W, Mijic V, Wiechmann A, Kottenhahn P, Baker J, Minton NP, Müller V: **Regulation of lactate metabolism in the acetogenic bacterium *Acetobacterium woodii***. *Environ Microbiol* 2018, **20**:4587–4595, https://doi.org/10.1111/1462-2920.14412.

37. Nwaokorie UJ, Reinmets K, De Lima LA, Pawar PR, Shaikh KM, Harris A, Köpke M, Valgepea K: **Deletion of genes linked to the C1-fixing gene cluster affects growth, by-products, and proteome of *Clostridium autoethanogenum***. *Front Bioeng Biotechnol* 2023, **11**, 1167892, https://doi.org/10.3389/fbioe.2023.1167892.

38. Woolston BM, Emerson DF, Currie DH, Stephanopoulos G: **Rediverting carbon flux in *Clostridium ljungdahlii* using CRISPR interference (CRISPRi)**. *Metab Eng* 2018, **48**:243–253, https://doi.org/10.1016/j.ymben.2018.06.006.

39. Zhao R, Liu Y, Zhang H, Chai C, Wang J, Jiang W, Gu Y: **CRISPR-Cas12a-mediated gene deletion and regulation in *Clostridium ljungdahlii* and its application in carbon flux redirection in synthesis gas fermentation**. *ACS Synth Biol* 2019, **8**:2270–2279, https://doi.org/10.1021/acssynbio.9b00033.

40. Fackler N, Heffernan J, Juminaga A, Doser D, Nagaraju S, Gonzalez-Garcia RA, Simpson SD, Marcellin E, Köpke M: **Transcriptional control of *Clostridium autoethanogenum* using CRISPRi**. *Synth Biol* 2021, **6**:ysab008, https://doi.org/10.1093/synbio/ysab008.

41. * Shin J, Bae J, Lee H, Kang S, Jin S, Song Y, Cho S, Cho B-K: **Genome-wide CRISPRi screen identifies enhanced autolithotrophic phenotypes in acetogenic bacterium *Eubacterium limosum***. *Proc Natl Acad Sci USA* 2023, **120**, e2216244120, https://doi.org/10.1073/pnas.2216244120.
Authors constructed a CRISPRi-based sgRNA library to repress each gene in the genome of *Eubacterium limosum*. This library serves as an outstanding tool to explore physiological properties of the organism, but also to identify the purpose of genes with unknown function. The results from this study provide a foundation for addressing energy limitations in acetogens by developing strains with improved autotrophic growth.

42. Hoffmeister S, Gerdom M, Bengelsdorf FR, Linder S, Flüchter S, Öztürk H, Blümke W, May A, Fischer R-J, Bahl H, *et al.*: **Acetone production with metabolically engineered strains of *Acetobacterium woodii***. *Metab Eng* 2016, **36**:37–47, https://doi.org/10.1016/j.ymben.2016.03.001.

43. Moreira JPC, Heap JT, Alves JI, Domingues L: **Developing a genetic engineering method for *Acetobacterium wieringae* to expand one-carbon valorization pathways**. *Biotechnol Biofuels Bioprod* 2023, **16**:24, https://doi.org/10.1186/s13068-023-02259-6.

44. ** Liew FE, Nogle R, Abdalla T, Rasor BJ, Canter C, Jensen RO, Wang L, Strutz J, Chirania P, De Tissera S, *et al.*: **Carbon-negative production of acetone and isopropanol by gas fermentation at industrial pilot scale**. *Nat Biotechnol* 2022, **40**: 335–344, https://doi.org/10.1038/s41587-021-01195-w.
Authors show selective and carbon negative pilot-scale production of acetone and isopropanol using a highly engineered strain of *Clostridium autoethanogenum*. Authors used genome data mining, various genome engineering tools, omics, and iPROBE to improve production massively. This publication combines various tools available for *C. autoethanogenum* to construct an engineered strain capable of being used at industrial scale.

45. Diender M, Parera Olm I, Sousa DZ: **Synthetic co-cultures: novel avenues for bio-based processes**. *Curr Opin Biotechnol* 2021, **67**:72–79, https://doi.org/10.1016/j.copbio.2021.01.006.

46. Flaiz M, Baur T, Gaibler J, Kröly C, Dürre P: **Establishment of green- and red-fluorescent reporter proteins based on the fluorescence-activating and absorption-shifting tag for use in acetogenic and solventogenic anaerobes**. *ACS Synth Biol* 2022, **11**:953–967, https://doi.org/10.1021/acssynbio.1c00554.

47. * Herzog J, Mook A, Guhl L, Bäumler M, Beck MH, Weuster-Botz D, Bengelsdorf FR, Zeng A: **Novel synthetic co-culture of *Acetobacterium woodii* and *Clostridium drakei* using CO_2 and *in situ* generated H_2 for the production of caproic acid via lactic acid**. *Eng Life Sci* 2023, **23**, https://doi.org/10.1002/elsc.202100169.
Authors co-cultivated the acetogen *Acetobacterium woodii*, which was genetically modified to recombinantly produce lactate, and *Clostridium drakei* to produce caproate. This is the first report in which a genetically modified acetogen was used in a synthetic co-culture to produce a platform chemical from H_2/CO_2.

48. Cha S, Lim HG, Kwon S, Kim D, Kang CW, Jung GY: **Design of mutualistic microbial consortia for stable conversion of carbon monoxide to value-added chemicals**. *Metab Eng* 2021, **64**:146–153, https://doi.org/10.1016/j.ymben.2021.02.001.

49. Stark C, Münßinger S, Rosenau F, Eikmanns BJ, Schwentner A: **The potential of sequential fermentations in converting C1 Substrates to higher-value products**. *Front Microbiol* 2022, **13**, 907577, https://doi.org/10.3389/fmicb.2022.907577.

50. Basen M, Geiger I, Henke L, Müller V: **A genetic system for the thermophilic acetogenic bacterium *Thermoanaerobacter kivui***. *Appl Environ Microbiol* 2018, **84**, https://doi.org/10.1128/AEM.02210-17.

51. Kita A, Iwasaki Y, Sakai S, Okuto S, Takaoka K, Suzuki T, Yano S, Sawayama S, Tajima T, Kato J, *et al.*: **Development of genetic transformation and heterologous expression system in carboxydotrophic thermophilic acetogen *Moorella thermoacetica***. *J Biosci Bioeng* 2013, **115**:347–352, https://doi.org/10.1016/j.jbiosc.2012.10.013.

52. Iwasaki Y, Kita A, Yoshida K, Tajima T, Yano S, Shou T, Saito M, Kato J, Murakami K, Nakashimada Y: **Homolactic acid fermentation by the genetically engineered thermophilic homoacetogen *Moorella thermoacetica* ATCC 39073**. *Appl Environ Microbiol* 2017, **83**:e00247, https://doi.org/10.1128/AEM.00247-17. 17.

53. Bourgade B, Millard J, Humphreys CM, Minton NP, Islam MA: **Enabling ethanologenesis in *Moorella thermoacetica* through construction of a replicating shuttle vector**. *Fermentation* 2022, **8**:585, https://doi.org/10.3390/fermentation8110585.

54. Kato J, Takemura K, Kato S, Fujii T, Wada K, Iwasaki Y, Aoi Y, Matsushika A, Murakami K, Nakashimada Y: **Metabolic engineering of *Moorella thermoacetica* for thermophilic bioconversion of gaseous substrates to a volatile chemical**. *Amb Express* 2021, **11**:59, https://doi.org/10.1186/s13568-021-01220-w.

55. Takemura K, Kato J, Kato S, Fujii T, Wada K, Iwasaki Y, Aoi Y, Matsushika A, Morita T, Murakami K, *et al.*: **Enhancing acetone production from H_2 and CO_2 using supplemental electron acceptors in an engineered *Moorella thermoacetica***. *J Biosci Bioeng* 2023, https://doi.org/10.1016/j.jbiosc.2023.04.001. S1389172323001123.

56. Wood JC, Marcellin E, Plan MR, Virdis B: **High methanol-to-formate ratios induce butanol production in *Eubacterium limosum***. *Microb Biotechnol* 2021:1751–7915, https://doi.org/10.1111/1751-7915.13963. 13963.

57. Sanford PA, Woolston BM: **Expanding the genetic engineering toolbox for the metabolically flexible acetogen *Eubacterium limosum***. *J Ind Microbiol Biotechnol* 2022, **49**, https://doi.org/10.1093/jimb/kuac019. kuac019.

58. Jeong J, Kim J-Y, Park B, Choi I-G, Chang IS: **Genetic engineering system for syngas-utilizing acetogen, *Eubacterium limosum* KIST612**. *Bioresour Technol Rep* 2020, **11**, 100452, https://doi.org/10.1016/j.biteb.2020.100452.

59. * Sanford PA, Miller KG, Hoyt KO, Woolston BM: **Deletion of biofilm synthesis in *Eubacterium limosum* ATCC 8486 improves handling and transformation efficiency**. *FEMS Microbiol Lett* 2023, https://doi.org/10.1093/femsle/fnad030. fnad030.
This study describes a genome engineering tool based on homologous recombination to rapidly generate knockouts in *Eubacterium limosum*. In addition, authors identified and knocked out genes required for extracellular polymeric substance formation. An easy-to-use knockout system forms a crucial step to transform *E. limosum* into an acetogenic model organism.

60. Millard J, Agius A, Zhang Y, Soucaille P, Minton NP: **Exploitation of a type 1 toxin–antitoxin system as an inducible counter-selective marker for genome editing in the acetogen *Eubacterium limosum***. *Microorganisms* 2023, **11**:1256, https://doi.org/10.3390/microorganisms11051256.

61. Humphreys JR, Hebdon SD, Rohrer H, Magnusson L, Urban C, Chen Y-P, Lo J: **Establishing *Butyribacterium methylotrophicum* as a platform organism for the production of biocommodities from liquid C1 metabolites**. *Appl Environ Microbiol* 2022, **88**:23933, https://doi.org/10.1128/aem.02393-21. 21.