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Advances in metabolic engineering in the microbial production of fuels and chemicals from C1 gas

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The future sustainable production of chemicals and fuels from non-petrochemical sources, while at the same time reducing greenhouse gas (GHG) emissions, represent two of societys greatest challenges. Microbial chassis able to grow on waste carbon monoxide (CO) and carbon dioxide (CO_2) can provide solutions to both. Ranging from the anaerobic acetogens, through the aerobic chemoautotrophs to the photoautotrophic cyanobacteria, they are able to convert C1 gases into a range of chemicals and fuels which may be enhanced and extended through appropriate metabolic engineering. The necessary improvements will be facilitated by the increasingly sophisticated gene tools that are beginning to emerge as part of the Synthetic Biology revolution. These tools, in combination with more accurate metabolic and genome scale models, will enable C1 chassis to deliver their full potential.

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Introduction

The continued use of fossil fuels is no longer tenable. A finite resource, their extraction, processing and exploitation is wreaking havoc with the environment through pollution and global warming. The challenge facing our generation is, therefore, to identify sustainable and cleaner processes for chemical, fuel and energy production. Biological routes offer the most promising alternative where, to avoid conflict with the food chain, attention has largely focussed on using lignocellulosic biomass as the feedstock. However, its recalcitrance to deconstruction is making the development of economic processes extremely challenging. One solution is to directly capture carbon before its incorporation into lignocellulose through the use of microbial chassis able to utilize single carbon (C1) gases (CO and CO_2) as a feedstock. Such gases are an abundant, low cost waste product from a wide range of industrial processes, with total global carbon emissions from fossil fuel equivalent to approximately 9.7 gigatons in 2014 [1]. This includes sources such as steel manufacture and power generation, additionally the availability of C1 gas from the anaerobic digestion or gasification of renewable domestic or agricultural waste and residues provides a diverse range of feedstocks of C1 gas (Figure 1).

Those C1 fermenting process organisms being most actively pursued include anaerobic clostridial acetogens, the aerobic chemolithoautotrophic *Cupriavidus necator*, various photoautotrophic cyanobacteria and, in the case of CH_4, the methanotrophic *Methylococcus capsulatus*. Many naturally produce metabolites of industrial value, such as ethanol, butyrate and 2,3-butanediol. The full extent of their capability, however, resides in their potential to produce a much wider range of chemicals and fuels through their rational metabolic engineering. There are many physical process engineering and commercialisation challenges that need to be addressed in order to bring this technology to the wider market, however for the purposes of this short review we will focus on recent advancements in metabolic engineering and Synthetic Biology with respect to these organisms. Additionally we will summarise recent efforts made with C1 chassis able to grow on CO and CO_2; CH_4-utilising bacteria [2] will not be considered, other than to note current commercial activity (see Table 1).

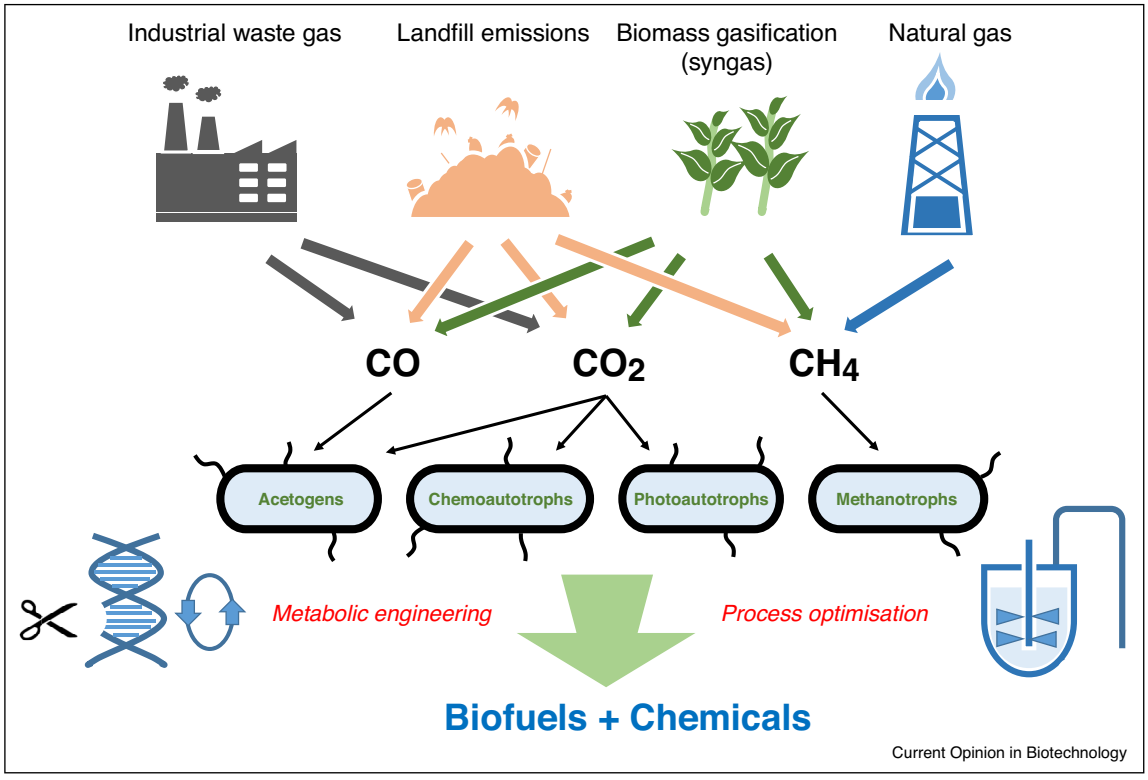
Acetogens

Obligately anaerobic acetogenic bacteria [3] employ the Wood–Ljungdahl pathway (WLP) to synthesize acetyl-CoA from either CO or $CO_2 + H_2$ (Figure 2). Acetyl-CoA can be further directed towards pyruvate, the generation of biomass, solventogenesis or acetate with, in the latter case, the generation of ATP. Acetate formation from CO and CO_2, however, does not generate net ATP, as for every mole of acetate produced one mole of ATP is consumed during the synthesis of formyl-THF from formate. Rather, net ATP generation is reliant on the Na+ or proton gradient (depending on the acetogen), formed by WLP reducing equivalents, which is coupled to a membrane bound ATPase [4]. Acetogens are particularly attractive as chemical production platforms, as the WLP is the most efficient of the known CO_2 fixation pathways.

Native products

Acetogens produce a number of native chemicals and fuels, including high value C4 compounds such as 2,3-butanediol

Figure 1



Sustainable conversion of waste gases to biofuels and chemicals through a combination of industrial process optimisation and genetic engineering approaches.

(*Clostridium ljungdahlii* and *Clostridium autoethanogenum*), butyrate and butanol (*Clostridium carboxidivorans*) and 2-oxobutyrate (*Clostridium aceticum* and *Sporomusa ovata*). Strategies explored to optimise yields include the manipulation of culture conditions, bioreactor design, adaptative evolution [28–30] and more recently electrosynthesis. The latter seeks to increase productivity through the direct or indirect acquisition of electrons. Whilst exciting, the challenges related to scalability may be unsurmountable [31,32]. Improvements to product yield through metabolic engineering have been made possible by significant improvements in available genetic tools. Exemplification of directed mutagenesis methods based on intron retargeting and allelic exchange has allowed insertional disruption, and precise in-frame deletion, of target genes [8,33]. In *C. ljungdahlii*, for example, carbon flow was successfully redirected towards acetate through in-frame deletions of *adhE1* and *adhE2* [8]. In contrast, in-frame deletion of *aor2* in *C. autoethanogenum*, resulted in an approximate 180% increase in ethanol yield over the wildtype organism [5•]. In *Acetobacterium woodii* autotrophic acetate formation was improved through overexpression of WLP genes, resulting in a strain capable of producing 51 g l^{-1} acetate in 3.8 days [13].

The efficiency of mutant generation by allelic exchange is improved through the use of CRISPR/cas9 technology as it allows the direct selection of the rare mutant alleles that arise in a population, considerably shortening mutant isolation time. The successful use of *Streptococcus pyrogenes* CRISPR/cas9 in acetogens has been reported. In *C. ljungdahlii*, in-frame deletion mutants were generated within *pta*, *adhE1*, *ctf* and *pyrE* [34•], while knockouts in *2,3-bdh* and *adh* were successfully made in *C. autoethanogenum* [35]. It may be anticipated that this type of system will figure prominently in the future engineering of acetogens.

Synthetic pathway products

As yet, aside from those claims restricted to patent filings, examples of product expansion or enhancement through the incorporation of synthetic pathways in acetogens are relatively few. Acetone production through overexpression of the requisite genes (*ctfA/B* and *adc*) from *Clostridium acetobutylicum* has been reported in *C. aceticum* [12] and *A. woodii*. In the latter case, to concentrations of approximately 15 mM in batch and 26.4 mg l^{-1} $hour^{-1}$ in continuous culture fermentation [14•]. Conversion of acetone to isopropanol as a consequence of a native alcohol dehydrogenase (*CaADH*) has been demonstrated in *C. autoethanogenum* during both heterotrophic [36] and autotrophic [6] growth. Autotrophic production of isopropanol has similarly been achieved in *C. ljungdahlii* [11].

Table 1

Examples of targeted metabolic efforts towards selected platform chemicals and biofuels within most prominent C1 chassis of the four groupings

C1 chassis	Engineering target product	Native/recombinant product	Largest production scale	Example studies/companies
Major acetogens				
Clostridium autoethanogenum	Ethanol	Native	Industrial (15 000 l)	Lanzatech [5•]
	2,3-Butanediol	Native	Demonstration	Lanzatech
	Acetone/isopropanol	Recombinant	Proprietary	Lanzatech [6]
	Butanol	Recombinant	Proprietary	Lanzatech [7]
Clostridium ljungdahlii	Acetate	Native	Laboratory	[8]
	Butyrate	Recombinant	Laboratory	[9]
	Butanol	Recombinant	Laboratory	[10]
	Isopropanol	Recombinant	Laboratory	[11]
Clostridium aceticum	Acetone	Recombinant	Laboratory	[12]
Acetobacterium woodii	Acetate	Native	Laboratory	[13]
	Acetone	Recombinant	Laboratory	[14•]
Chemoautotroph				
Cupriavidus necator	PHB	Native	Laboratory	[15]
	Alka(e)nes	Recombinant	Laboratory	[16••]
	3HP	Recombinant	Laboratory	[17]
	Isopropanol	Recombinant	Laboratory	[18,19]
	Isobutanol/methy-1-butanol	Recombinant	Laboratory	[20]
Photoautotroph				
Synechococcus elongatus UTEX 2973	Ethanol	Recombinant	Laboratory	[21]
	1-Butanol/isobutanol	Recombinant	Laboratory	[22]
Synechococcus elongatus PCC 7942	Succinate	Recombinant	Laboratory	[23••]
Synechocystis sp. PCC 6803	Ethylene	Recombinant	Laboratory	[24]
Methanotroph				
Methylotrophus capsulatus	Propylene	Recombinant	Undisclosed	Calysta [25]
Undisclosed methanotrophic species	Isobutanol	Recombinant	Pilot plant	Intrexon [26]
	Farnesene	Recombinant	Undisclosed	Intrexon [27]

Whilst *C. carboxidivorans* naturally produces butyrate and butanol, their production in *C. autoethanogenum* [7] and *C. ljungdahlii* [10] has been demonstrated using plasmid-located genes (Figure 2) derived from *C. acetobutylicum*. In the latter case, to concentrations of approximately 2 mM at exponential phase growth in batch fermentation [10]. More recently butyrate production in *C. ljungdahlii* from CO/CO_2 was increased to 16 mM through genome integration of the butyrate pathway genes (Figure 2) concomitant with deletion of *pta* and *adhE1* [9]. Whilst further deletions were acknowledged as likely to result in higher butyrate yields, the inefficient tools available at the time precluded their generation. The advent of CRISPR/cas9 systems has the potential to overcome these limitations.

Cupriavidus necator

A number of autotrophic bacteria use oxygen as the electron acceptor during CO_2 fixation through a reductive pentose phosphate cycle. By far the most studied and developed aerobic chassis of this type is *C. necator*, formerly *Ralstonia eutropha*. A facultative chemolithoautotroph, it is able to grow heterotrophically on a range of organic carbon sources, in addition to its use of CO_2, H_2 and O_2 as sole carbon and energy sources. A broad genetic toolkit is available that allows rudimentary genome editing as well as the controlled expression of heterologous genes from a subset of vectors [37] (Figure 3).

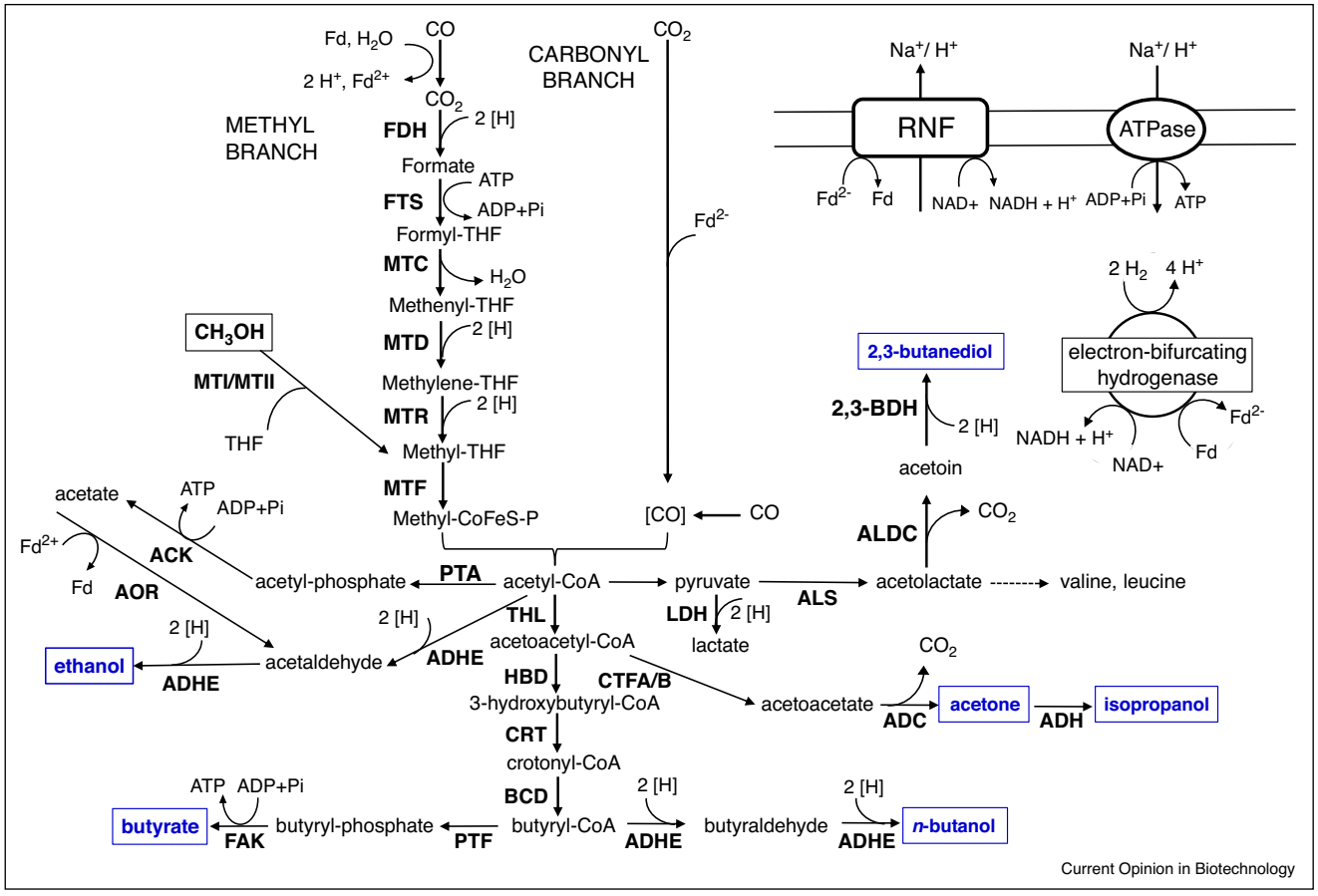
Native products

Under nutrient limitation, *C. necator* directs the majority of its reduced carbon into synthesis of the biopolymer poly[(*R*)-3-hydroxybutyrate] (PHB), an energy and carbon storage compound that can accumulate at rates of up to 1.55 g l^{-1} $hour^{-1}$, eventually representing some 70% of total cell weight [38]. PHB is formed from acetyl-CoA through the sequential activities of 3-ketothiolase (PhaA), acetoacetyl reductase (PhaB) and PHA synthase (PhaC). The practical industrial applications of PHB are as a bioplastic, however, derivatives may have more wide ranging applications in medical and pharmaceutical fields [39]. The existing high levels of production have limited the scope for substantive increases through metabolic engineering. Indeed, the plasmid-based overexpression of the native *phaCAB* operon actually reduced productivity [15].

Synthetic pathway products

The ability of *C. necator* to divert so much of its carbon into PHB makes it an attractive chassis for chemical and fuel production, based on the assumption that other, more desirable products could replace PHB as the carbon sink.

Figure 2



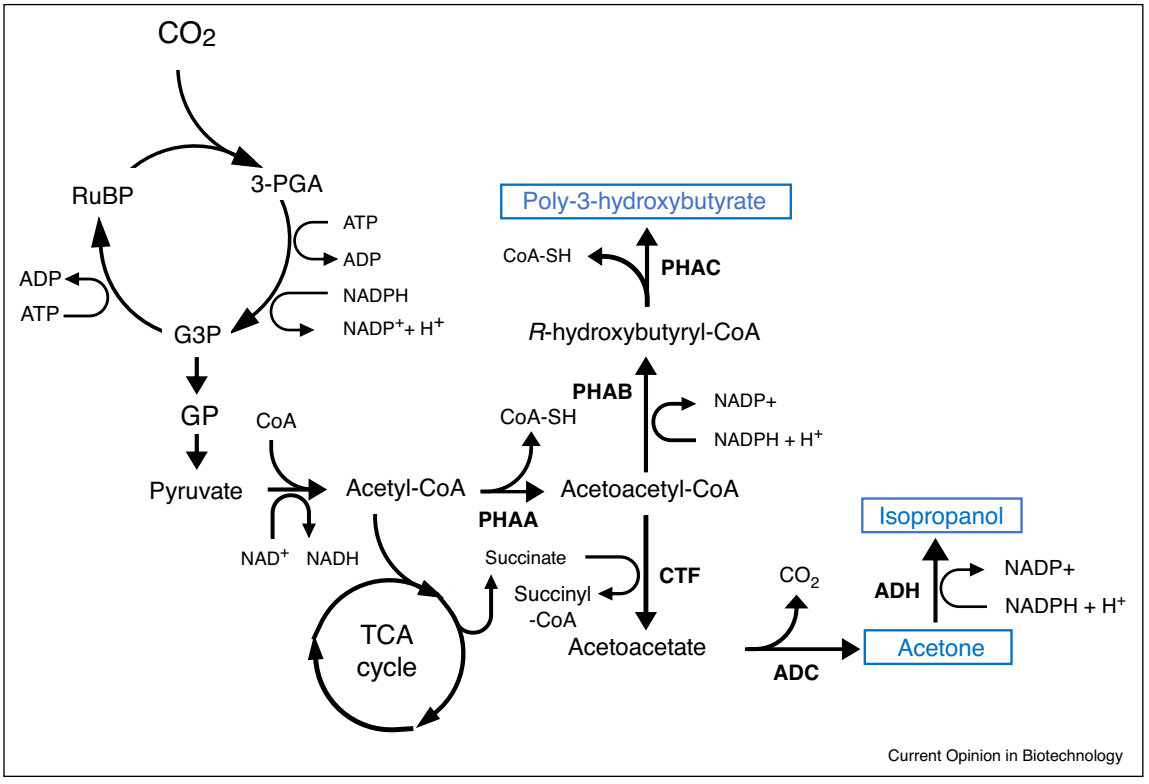
The Wood–Ljungdahl pathway of acetogens with possible products (boxed). ACK, acetate kinase; ACS, CO dehydrogenase/acetyl-CoA synthase; ADC, acetone decarboxylase; ADHE, aldehyde/alcohol dehydrogenase; ALDC, acetolactate decarboxylase; ALS, acetolactate synthase; AOR, aldehyde:ferredoxin oxidoreductase; BCD, butyryl-CoA dehydrogogenase; CoFeS-P, corrinoid iron–sulphur protein; CRT, crotonase; CTFA/B, acetoacetyl-CoA:acetate/butyrate-CoA-transferase; FAK, fatty acid kinase; Fd, oxidized ferredoxin; Fd 2−, reduced ferredoxin; FDH, formate dehydrogenase; FTS, formyl-THF synthetase; HBD, 3-hydroxybutyryl-CoA dehydrogenase; LDH, lactate dehydrogenase; MTI, methyltransferase I; MTII, methyltransferase II; MTC, methenyl-THF cyclohydrolase; MTD, methylene-THF dehydrogenase; MTF, methyltransferase; MTR, methylene-THF reductase; PFOR, pyruvate:ferredoxin oxidoreductase; PTA, phosphotransacetylase; PTF, phosphotransferase; RNF, Rnf complex THF: tetrahydrofolate; THL, thiolase, 2,3-BDH: 2,3-butanediol dehydrogenase; 2 [H], reducing equivalents (e.g. NADH or NADPH). Figure adapted from Bengelsdorf FR, Straub M, Durre P: Bacterial synthesis gas (syngas) fermentation. *Environ Technol* 2013, **34**:1639–651.

Accordingly, isopropanol production at up to final concentrations of 3.44 g l^{-1} have been achieved in heterotrophic batch conditions by overexpressing codon optimised clostridial genes (Figure 2) in a *phaB*|*phaC* double mutant [18]. The observed slow growth rates were subsequently alleviated by overexpression of native *GroESL* genes leading to final isopropanol concentrations of 9.8 g l^{-1} in fed batch cultures using fructose as carbon source. During auxotrophic growth, isopropanol reached final concentrations of 250 mg l^{-1} in only 12 hours [19].

Recombinant fructose grown strains producing appreciable titres of isobutanol (270 mg l^{-1}) and of 3-methyl-1-butanol (40 mg l^{-1}) have also been engineered through heterologous overexpression of ketoisovalerate decarboxylase and alcohol dehydrogenase encoding genes from *Lactococcus lactis* and *Escherichia coli*, respectively, in combination with the disruption of competing pathways. These included deletion of the *phaCAB* operon, and the circumvention of three potential carbon sinks through deletion of *ilvE*, *bkdAB* and *aceE*. Additionally, the native branched-chain amino acid biosynthesis pathway genes, which generate the precursors to the introduced synthetic pathway, were overexpressed.

Alkanes and alkenes are the predominant components of diesel, petrol and jet fuel, and are therefore attractive biofuel targets. Heterologous expression of an alkane synthesis pathway from *Synechococcus elongatus*, comprising

Figure 3



Simplified overview of autotrophic native poly-3-hydroxybutyrate and recombinant acetone/isopropanol production pathways in *Cupriavidus necator*. Abbreviations: 3-PGA (3-phosphoglyceric acid), G3P (glyceraldehyde-3-phosphate), RuBP (ribulose bisphosphate), GP (glycerate-3-phosphate), CoA (coenzyme a), TCA (tricarboxylic acid), ATP (adenosine triphosphate), ADP (adenosine diphosphate), NAD (nicotinamide adenine dinucleotide). Native enzymes; PHAA (acetyl-CoA acetyltransferase), PHAB (acetoacetyl-CoA reductase), PHAC (polyhydroxyalkanoic acid synthase), CTF (acetoacetyl-CoA transferase). Recombinant enzymes; ADC (Acetoacetate decarboxylase), ADH (alcohol dehydrogenase).

genes encoding an acyl-ACP reductase and an aldehyde deformylating oxygenase, autotrophic alkane production was demonstrated in *C. necator*. Through knockout mediated redirection of carbon flow from PHA synthesis, 435 mg l^{-1} of alka(e)nes and 670 mg l^{-1} total hydrocarbons were achieved in batch growth on heterotrophic carbon sources, while on gas ($H_2/O_2/CO_2$ composition of 60:2:10) a final concentration of 4.4 mg l^{-1} was achieved. The latter represents the first demonstration of non-native alka(e)ne production from C1 gas [16••].

Cyanobacteria

Cyanobacteria are a diverse group of photosynthetic organisms capable of growth using sunlight and CO_2 as their source of energy and carbon, respectively. Cyanobacteria more suited to biotechnical applications then other photosynthetic organisms due to their comparatively rapid growth rate and well-developed genetic tools. The major challenges for the implementation of a photoautotrophic microbial platform for fuel and chemical production is how to supply dense cultures with sufficient sunlight to support fermentation at scale. Nevertheless, they remain an exciting microbial chassis, capable of producing industrially relevant chemicals and fuels at high titres.

Native products

Cyanobacteria have a broad spectrum of natively produced secondary metabolites, including amino acids, fatty acids, macrolides, lipopeptides and amides [40], some of which have potential pharmaceutical applications. Strategies to improve production of native compounds have largely focussed on either increasing the efficiency of photosynthesis, through overexpression of the carbon fixing enzyme RuBisCO, or reducing the light harvesting capabilities of the organism to avoid the issue of excess light absorption above the optimal values. The latter is to allow deeper light penetration into high optical density cultures [41]. Genome editing through homologous recombination and suicide plasmids has been established in the most developed strains, predominantly *Synechocystis* and *Synechococcus*. Additionally the *Streptococcus pyogenes* CRISPR/Cas9 system has recently been exemplified in *S. elongatus* UTEX 2973 through in-frame deletion of the

nblA gene [42]. NblA is involved in the degradation of phycobilisomes; primary antenna protein complexes associated with photosystem II.

Synthetic pathway products

The production of a wide range of biofuels and industrially relevant chemicals has been demonstrated through expression of heterologous genes within cyanobacteria, including ethanol [21], 1-butanol, isobutanol [21] and ethylene [24] to name a few. Recently a CRISPR-Cas9-assisted simultaneous *glgc* knockout and *gltA/ppc* knockin for succinate production was demonstrated in *S. elongatus* PCC 7942 [23••], paving the way for the rapid generation of further stable strains where the overexpressed genes are localised to the chromosome.

Metabolic and genome scale modelling

As our capability to genetically modify the range of C1 chassis available becomes more effective, so must our ability to identify the most rational gene targets for modification. The generation of sophisticated metabolic and genome scale models, in combination with the collection of proteomic and metabolomic datasets, is increasingly allowing *in silico* engineering studies to elucidate exciting, and often un-intuitive, potential targets for manipulation within industrially relevant organisms. Chen and co-workers recently published a list of proposed targets for deletion and overexpression in *C. ljungdahlii* to increase the productivity of native and non-native products, using a genome-scale metabolic reconstruction of the organisms' metabolism [43]. In parallel, a study by Richter *et al.* proposed a different model for product regulation, based predominantly on thermodynamics, nutrient limitation and pH manipulation, rather than genetic regulation [44••]. Numerous metabolic models have been proposed for *C. necator* [45–47], with Park and co-workers proposing the first genome scale model capable of *in silico* engineering and culture condition strategies towards enhanced 2-methylcitric acid production, as well as enhanced PHA and PHB production [45].

Conclusions

Progress towards the development of C1 fermenting microorganisms as chassis for the production of chemicals and fuels is gathering pace as available Synthetic Biology tools and genome scale models become ever more sophisticated. As novel technologies such as CRISPR/cas9 mutagenesis are implemented across the broad range of chassis, we can expect to see a rapid increase in the rate of generation of central metabolic pathway mutants, and elucidation of the intricacies of the pathways. Heterologous expression systems will continue to be developed and modularised, with stable expression of pathways from the chromosome rather than plasmid being a logical step to expect from the developing chassis organisms, increasing industrial applications. Significantly progress is being made on all fronts with the leading chassis, but presently the most commercially advanced system is the ethanol production process being pursued by LanzaTech based on the anaerobic acetogen *C. autoethanogenum* and the use of steel mill off-gas. Full scale production plants are planned or under construction at steel mills in China, Taiwan and Belgium. The production of other products at a commercial scale appears to be planned for an undetermined time in the future and are likely to be confined to reduced products whose synthesis requires minimal input of ATP in keeping with the anaerobic nature of this chassis. Those products that need significant ATP will require the use of an aerobic chassis, such as *Cupriavidus* or Cyanobacteria. In both cases, scale remains an issue. The former because of the more challenging issues of using an explosive gas mixture that combines O_2 with H_2 and CO_2, while the latter will always be compromised by the difficulties of providing sufficient light input at scale.

Acknowledgements

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