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Analytical tools for unravelling the metabolism of gas-fermenting Clostridia

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Acetogens harness the Wood-Ljungdahl Pathway, a unique metabolic pathway for C1 capture close to the thermodynamic limit. Gas fermentation using acetogens is already used for CO-to-ethanol conversion at industrial-scale and has the potential to valorise a range of C1 and waste substrates to short-chain and medium-chain carboxylic acids and alcohols. Advances in analytical quantification and metabolic modelling have helped guide industrial gas fermentation designs. Further advances in the measurements of difficult to measure metabolites are required to improve kinetic modelling and understand the regulation of acetogen metabolism. This will help guide future synthetic biology designs needed to realise the full potential of gas fermentation in stimulating a circular bioeconomy.

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Introduction

New biotechnologies focusing on reducing climate change and reversing historical environmental damage are required. Gas fermentation using acetogens is a platform with unique feedstock and product flexibility when compared to other gas-to-liquid technologies. Gas fermentation capitalizes on the Wood-Ljungdahl pathway (WLP), which is the most efficient CO_2 fixation pathway known for production of fuels and chemicals [1]. Acetogens grow efficiently on a wide range of syngas mixtures, pure CO, and mixtures of H_2 and CO_2; and commercial-scale CO-to-ethanol gas fermentation has been successfully deployed by LanzaTech [2].

Large uptake of gas fermentation technology can stimulate the circular bioeconomy in multiple areas due to its flexibility of process integration and the potential of expanding the product spectrum through synthetic biology [1–3]. Gas fermentation readily integrates with thermochemical gasification of solid biomass or waste and catalytic electrolysis (for both H_2 and CO) to enhance the potential impact on the circular economy [2]. The potential of these strategies has been demonstrated through techno-economic and life cycle analysis [4–7] as well as experimentally [8,9]. Downstream process integration is also being developed with both biological [1,2] and chemical [10] upgrading to a wide range of valuable fuels and chemicals. To realise the full potential of acetogens as biocatalysts and establish viable industrial bioprocesses, a deep understanding of both metabolism and process is needed to ensure efficiency, consistency, and robustness.

Fackler *et al.* [10] recently covered many industrially important factors for gas fermentation and what advances could help address current and future issues. It was observed that omics and modelling studies had helped elucidate energetics, thus aiding the development of engineering strategies. Because of the limited energy availability in gaseous substrates under anaerobic conditions, acetogens have evolved various mechanisms to extract and efficiently use energy [11]. Many metabolic aspects (e.g. redox homeostasis, metabolic regulation, and the effect of adding synthetic pathways) remain poorly understood and detailed metabolic characterization continues to be an active research area.

Genomics and genome scale metabolic reconstructions

The genomes of many acetogens have been sequenced, closed, and annotated. The annotated genomes have been used to generate genome scale metabolic reconstructions (GSMRs) for at least 6 acetogens (Table 1). GSMRs map genes to metabolic reactions and are extensively curated to ensure that the network supports all known metabolic functions (e.g. generation of biomass components from syngas) [12]. GSMRs are essential tools in the physiological interpretation of omics data and

Table 1

Gas fermenting Clostridia with in-depth experimental and computational study, and their application

Organism	GSMR	Omics	Applications	References
Acetobacterium woodii	M[a,b]	Phenome, transcriptome, translatome proteome.	FBA	[25,62,63]
Clostridium autoethanogenum	M	Phenome, metabolome, transcriptome, proteome.	FBA, KEM, TFA, PDM	[15,29,30,31••,32••,36,37•,41]
Clostridium carboxidivorans		Phenome, metabolome		[40•]
Clostridium drakei	M	Phenome, metabolome, transcriptome.	FBA	[16••]
Clostridium ljungdahlii	M, ME	Phenome, metabolome, transcriptome, translatome, proteome.	FBA, PDM, PM	[17,18•,26,33,38,56]
Eubacterium limosum	M[a,c]	Phenome, transcriptome, translatome, proteome.		[27,28,57,64]
Moorella thermoacetica	M	Phenome, metabolome	FBA	[19,39]

GSMR indicates the type of genome scale metabolic reconstructions available for mapping genes to function. Phenome indicates product concentrations and gas uptakes, while metabolome indicates intracellular metabolomics. M — GEM of metabolism (M-models); ME — GEM of metabolism and macromolecular expression (ME-models); FBA — flux balance analysis; KEM — kinetic ensemble model using M-models; TFA — thermodynamic flux analysis; PDM — physically dynamic model using stoichiometries from M-models; PM — predictive model using a ME-model.

[a] Not publicly available/peer reviewed.

[b] Neuendorf *et al.* [62] uses a core M-model, while Mesfin *et al.* [63] conference proceedings indicates a M-model is under development.

[c] Bae *et al.* [64] presentation (unpublished) indicates a M-model is under development. Partially-curated (automated) M-models are also available for *E. limosum* and some other acetogens not listed here in the AGORA collection [58].

underpin many constraint-based modelling techniques used to mine omics data.

Genome-scale models of metabolism (M-models) are available for four acetogens (Table 1); *Clostridium autoethanogenum* (iCLAU786 [13] and updated version [14], and MetaCLAU [15]), *Clostridium drakei* (iSL771 [16••]), *Clostridium ljungdahlii* (iHN637 [17], and iJL680 [18•]), and *Moorella thermoacetica* (iAI558 [19]) (see below for ME-models). Norman *et al.* [32••] discussed the differences between three models for *C. autoethanogenum* and *C. ljungdahlii* (iCLAU786, MetaCLAU and iHN637) with a focus on reducing inconsistent aspects of the *C. autoethanogenum* model (e.g. inconsistent enzyme subsets). The M-models for acetogens typically incorporate around 600–800 genes and have around 750–1000 metabolites and reactions [20]. Fully curated M-models for *Acetobacterium woodii* and *Eubacterium limosum* may soon be available (Table 1) and these would complement comparisons between organisms. Fackler *et al.* [10], Jin *et al.* [3] and Vees *et al.* [20] have recently discussed the role metabolic models (and omics analysis) of gas fermenting Clostridia have for industrial application, synthetic biology and industrial bioprocesses respectively.

Phenome analysis

Acetogen metabolism (Figure 1) is ideally characterized with fully instrumented bioreactor chemostat cultures. Measurement of gas conversion using a gas analyser (e.g. mass spectrometer) and fermentation products using standard HPLC to quantify major (acetate and ethanol) and minor (e.g. 2,3-butanediol) products, provides the boundary conditions for metabolic flux balances where a carbon balance helps to verify data. This enables unbiased quantification of condition-dependent phenotypes through steady-state data [21,22] and is more representative of industrial-scale gas fermentation, which is operated in continuous mode [2].

Phenomics captures the critical data — rates, yields and titres — necessary to design and evaluate industrial fermentation processes [2,10]. It does not, however, reveal metabolic bottlenecks or regulation that must be overcome to enhance robustness and increase productivity. It is helpful to consider the drivers for metabolic flux (J) by accounting for enzyme abundance (E), thermodynamic potential (ΔG), saturation by substrates (M) with affinity (K) and kinetic orders (a), and all other unaccounted sources of regulation (U) [23].

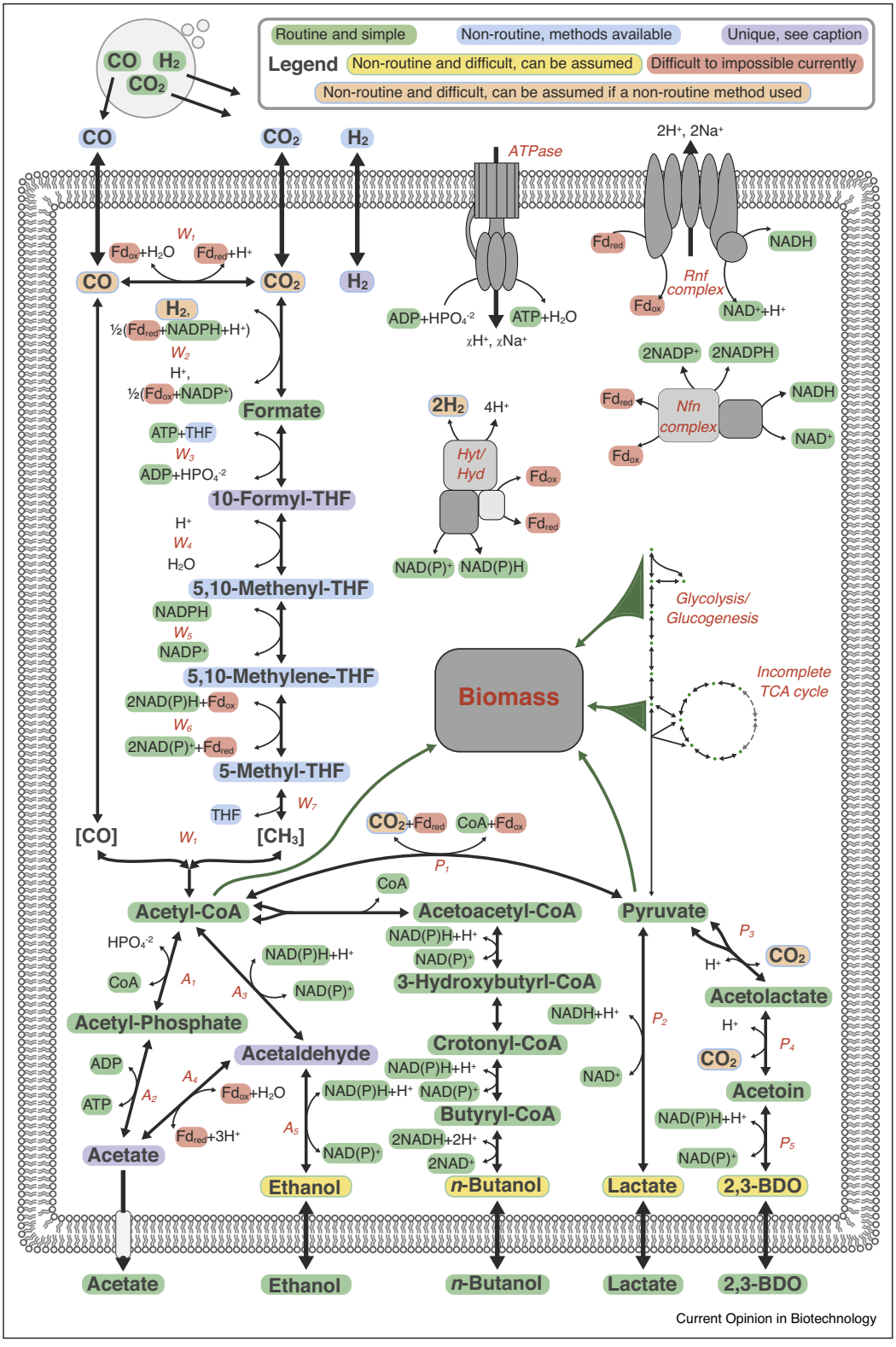
$$\underset{Flux}{J} \sim \underset{Expression}{E} \times \underset{Thermodynamics}{\left(1 - e^{\Delta G/RT}\right)} \times \underset{Saturation}{\prod_i (M_i/K_i)^{\alpha_i}} \times \underset{Kinetics}{U(M)} \quad (1)$$

Comparing fluxomics to transcriptomics and/or proteomics between experiments will help us understand how much expression controls flux, whereas (intracellular /endo-)metabolomics is needed to understand the extent to which thermodynamics (Figure 2a) or enzyme saturation (Figure 2b) controls flux. Beyond this, other regulation such as allosteric regulation will control the flux and can be explored using kinetic models.

Transcriptome and proteome analyses

Transcriptome and proteome analyses are roughly equal in frequency (Table 1). RNA-sequencing of the transcriptome provides great sensitivity and ease of comparative studies, while proteomics is closer to the metabolic action. Each method has its advantages, especially with respect to model integration [24]. More recently the translatome has also been studied, demonstrating some changes in

Figure 1


Routine and simple
Non-routine, methods available
Unique, see caption
Legend
Non-routine and difficult, can be assumed
Difficult to impossible currently
Non-routine and difficult, can be assumed if a non-routine method used
CO
H2
CO2
ATPase
2H+, 2Na+
Rnf complex
NADH
NAD+ +H+
Nfn complex
2NADP+
2NADPH
Hyt/ Hyd
4H+
Formate
10-Formyl-THF
5,10-Methenyl-THF
5,10-Methylene-THF
5-Methyl-THF
THF
[CO]
[CH3]
Biomass
Glycolysis/ Glucogenesis
Incomplete TCA cycle
Acetyl-CoA
Acetoacetyl-CoA
Pyruvate
3-Hydroxybutyryl-CoA
Crotonyl-CoA
Butyryl-CoA
Acetaldehyde
Acetyl-Phosphate
Acetate
Ethanol
n-Butanol
Lactate
2,3-BDO
Acetolactate
Acetoin
Current Opinion in Biotechnology

(Figure 1 Legend) Quantifying the metabolites of acetogenic metabolism. Metabolite colouring indicates quantification ease and routineness, see legend for description of each colour. Amino acid biosynthesis pathways have not been included but have routine and simple methods available and are largely accommodated by green arrows indicating pathways to biomass. Most liquid products are free to diffuse across the cell membrane and are routinely assumed to be in equilibrium with the extracellular matrix (i.e. concentration inside the cell is the same as outside) [32••]. The number of Na^+/H^+ transported per ATP is organism dependent [59], represented as '*x*'. Unique metabolites: **10-formyl-THF** is theoretically measurable using a non-routine method for quantification of THF metabolites [41], but standards are not available for purchase; **acetaldehyde** can be quantified using gas chromatography–mass spectrometry methods [60], but has not been reported for an acetogen previously, potentially due to low concentration [61]; **intracellular acetate** concentration could be assumed based on extracellular acetate concentration (similar to ethanol and other liquid products) but is mediated by an unknown transporter [32••]. Metabolite abbreviations: CoA — coenzyme A, THF — tetrahydrofolate, BDO — butanediol. Enzymes (red): W_1 — carbon monoxide dehydrogenase (CODH, can be in complex with acetyl-CoA synthase), W_2 — formate dehydrogenase (Fdh [bottom], can be in complex with Hyt [top]), W_3 — formate:THF ligase, W_4 — 5,10-methenyl-THF 5-hydrolase, W_5 — methylene-THF dehydrogenase, W_6 — methylene-THF reductase, A_1 — phosphotransacetylase, A_2 — acetate kinase, A_3 — acetaldehyde dehydrogenase, A_4 — acetylaldehyde:ferredoxin oxidoreductase, A_5 – alcohol dehydrogenase, P_1 — pyruvate: ferredoxin oxidoreductase, P_2 — lactate dehydrogenase, P_3 — acetolactate synthase, P_4 — acetolactate decarboxylase, P_5 — (*R*)-2,3-butanediol dehydrogenase, Hyt/Hyd — hydrogenase, ATPase — F_0F_1-ATP synthase, Rnf complex — proton translocating ferredoxin:NAD^+ oxidoreductase, Nfn complex — ferredoxin-dependent transhydrogenase. Butanol synthesis pathway is shown but not labelled (e.g. from *C. carboxidivorans*).

translational efficiencies between heterotrophy and autotrophy for *A. woodii*, *C. ljungdahlii*, and *E. limosum* [25–27]. A study of *E. limosum*'s proteome and growth when cultivated in heterotrophic, autotrophic, and methylotrophic conditions (glucose, CO/CO_2, H_2/CO_2, and methanol/CO_2) suggests that methanol/CO_2 and H_2/CO_2 fermentations are more similar [28], yet the effects were not linked to metabolic changes beyond uptakes and titres.

While energy efficiency would argue for efficient use of enzymes through expression regulation, our studies with *C. autoethanogenum* [29,30,31••,32••] agree with previous studies with *C. ljungdahlii* [18•,33] showing that autotrophic metabolism is not regulated at transcriptional or translational levels (Figure 2b). For *A. woodii* however, there appeared to be modest regulation between autotrophic and formatotrophic growth, largely adjacent to the main metabolic pathway (WLP) [62]. It is possible that rapidly changing environments dictate the use of faster post-translational flux control compared to slower transcriptional control. Indeed modelling the cost-benefit dynamic of proteome regulation indicates that there are niches in which unregulated organisms have higher fitness [34], but there could be potential for optimization of cellular maintenance through synthetic engineering of a lean or reduced proteome [35].

While most proteome studies compare relative abundance of proteins between samples, absolute quantification of proteins generates a more robust dataset for comparison within and between samples (at the expense of more work). We recently completed an absolute proteomics study using 19 synthetic heavy stable isotope labelled variants of key proteins covering central metabolism to quantify more than 1000 proteins in *C. autoethanogenum* growing on three gas mixtures: CO, syngas, or CO/H_2 (Valgepea *et al.*, unpublished, available on *bioRxiv*, doi: 10.1101/2021.05.11.443690). Apart from confirming the modest changes in proteome allocation between conditions, this study showed that more than 10% of the proteome by mass has no KEGG orthology identifiers. This highlights the need for further characterisation of genotype-phenotype relationships in acetogens. As discussed by Fackler *et al.* [10], high-throughput cell engineering and characterization is difficult in strict anaerobes such as acetogens, though recent advances in automation and cell-free prototyping may facilitate this work.

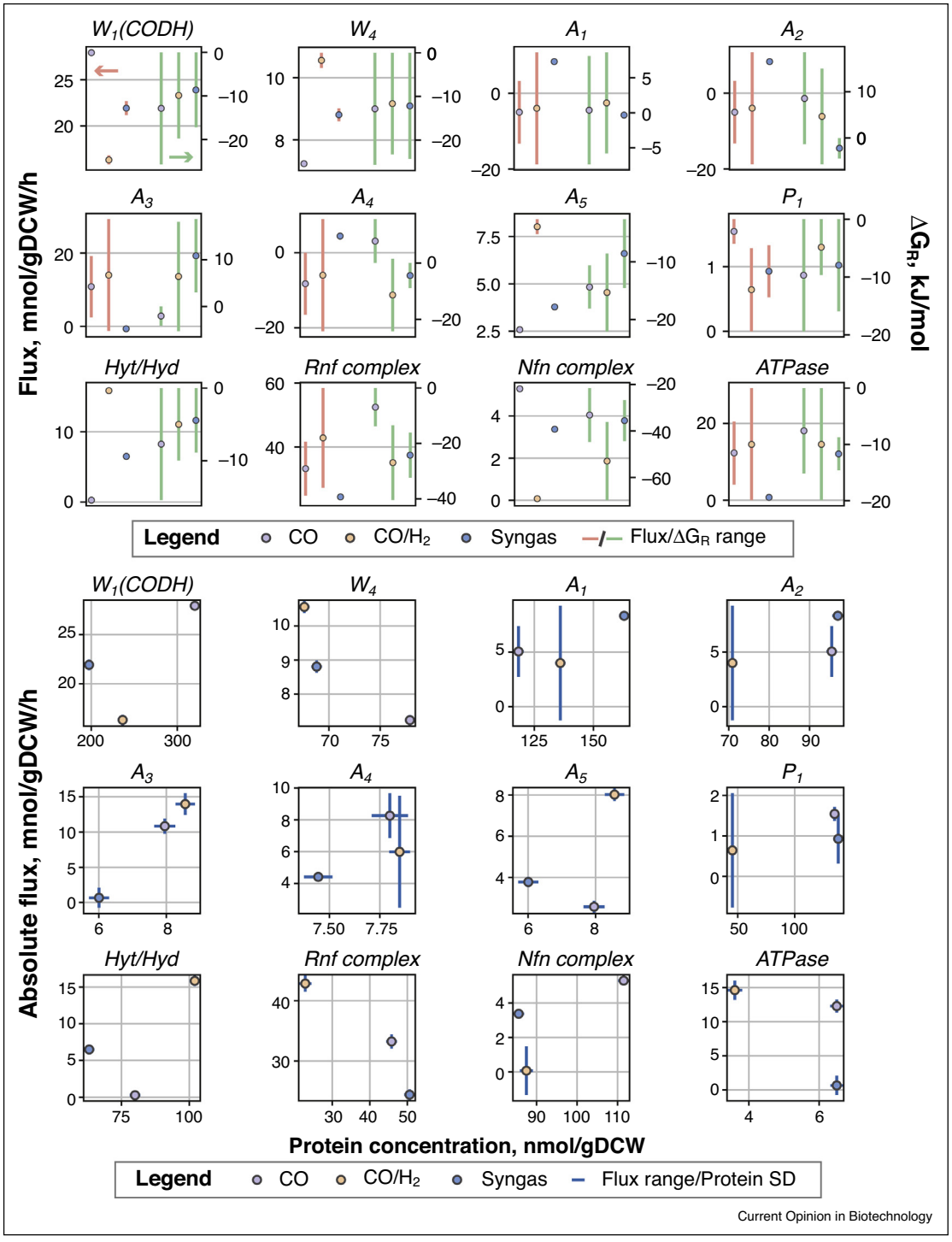
Metabolome analysis

The modest changes observed at expression level emphasise the need for metabolomics to understand acetogen metabolism. Ideally, all metabolites and co-factors in primary gas metabolism (Figure 1) should be quantified. However, there are several challenges making this very difficult.

The first challenge is how to quench metabolism to avoid changes in the metabolome before metabolite extraction. We routinely pellet cells in a centrifuge (cold/4°C to quench metabolism) and extract the pellet immediately with acetonitrile. On paper, this approach suffers many inadequacies: cells are without gas substrate for minutes, while being exposed to oxygen. In practise, however, this approach produces clean mass spectra, and robust data similar to those observed in direct extraction [13]. A similar approach has been demonstrated to work reliably for other Clostridia [36,37•]. Filtering and quenching/extracting with cold solvents (typically mixtures of acetonitrile and/or methanol with water) is also commonly used either anaerobically [38,39] or aerobically [33,40•]. This relies on precisely depositing cell mass onto a filter using a syringe, while anaerobic versions of this method are also restricted to bottle cultivations as the samples are performed in an anaerobic glove box.

The extracted sample can be analysed using ion-pairing chromatography and triple quad mass spectrometry to quantify most species from acetyl-CoA down (Figure 1) and most of the co-factors (ATP, NADPH, NADH). To improve quantitative accuracy, there is a trend towards utilising ^{13}C-labelled internal standards in combination

Figure 2



An example of data interpretation and visualisation for a metabolic analysis of gas fermenting Clostridia. **(a)** Flux and Gibbs free energy of reaction (ΔG_R) ranges predicted by TFA for three *C. autoethanogenum* bioreactor steady state conditions: (left to right) purple — CO, orange — CO/H_2, blue — syngas (data from Ref. [30]). Fluxes are represented by mean values with red ranges (left axis, mmol/gDCW/h), Gibbs free energies are represented as mean values and green ranges (right axis, kJ/mol). Positive flux for AOR (A_4) corresponds to ethanol production and typical forward reactions for other enzymes (i.e. downwards in Figure 1) **(b)** Correlation between enzyme concentration and flux through corresponding reactions at steady state – same steady states as (a). Absolute protein concentrations (nmol/gDCW) were quantified in a recent preprint by Valgepea *et al.* (unpublished). Data points represent means, where error bars represent standard deviation for protein concentration and range estimated using TFA for flux (mmol/gDCW/h). See Figure 1 for reaction/flux stoichiometries and enzyme abbreviations. *W_1(CODH)* values are for CO oxidation only, not acetyl-CoA synthase.

with ^{12}C authentic standards, where the internal standard is made by growing an organisms on uniformly ^{13}C-glucose and extracting the metabolites [32••,33].

Oxygen-sensitive and reactive metabolites are a particularly difficult part of analysing the metabolome of acetogens. We used an anaerobic, direct extraction method for the quantification of WLP tetrahydrofolate species in chemostat cultivations of *C. autoethanogenum* [41], and this may be extendable to other oxygen-sensitive compounds. Involved in several critical steps, oxidised and reduced ferredoxin is an especially difficult pair to quantify, both relatively and absolutely. This is due to ferredoxins auto-oxidizing, the presence of multiple types of ferredoxins, and the complexity of quantifying a protein and/or (depending on absolute/relative quantification respectively) its charged metal ligands using mass spectrometry (compared to small molecules; e.g. NAD^+/NADH). Through estimations based on thermodynamic flux analysis (TFA) a consistent mean (Fd_{ox}/Fd_{red} ratio and total ferredoxin) was found for broad conditions [32••], however, this relies on adequate quantification of other metabolites and is inferior to direct experimental quantification.

Determining the intracellular concentration of exchange metabolites is also challenging due to the high absolute amount in the supernatant. Extracellular concentrations can be used to constrain intracellular concentration using TFA [32••]. For the sparsely soluble gas substrates (CO, H_2), we generally do not have the dissolved concentrations, though broad constraints can be developed from inlet and outlet gas concentrations and Henry's Law. However, a recent study measuring online dissolved CO concentration using a tubing method indicates that the dissolved gas concentration is far below the equilibrium gas concentration inferred from Henry's Law [42••].

Routine measurement of dissolved gas tension has the potential to enhance reactor operation. Increased dissolved CO tension (DCOT) was linked to increased solventogenesis in CO only cultures, before the ultimate metabolic crash in *C. ljungdahlii* [42••]. Co-feeding of H_2 is known to increase solventogenesis in applicable Clostridia [30], but only when DCOT remains low enough to prevent its inhibition of hydrogenases [43]. Use of the method by other research groups would ensure its validity, while also providing more detailed and comparable data on bioreactor/culture performance.

Flux balance analysis

In order to explore the flux-flow relationship (1), the fluxes in the network must be estimated. Direct measurement of fluxes is impractical and fluxes are inferred from flux balance analysis (FBA) using the GSMR together with phenomics data. In acetogen metabolism, the major fluxes downstream of acetyl-CoA are almost completely defined by the product rates (acetate, ethanol, 2,3-butanediol) and biomass production (Figure 1). There is a degree of freedom around ethanol production — production via acetate or directly from acetyl CoA — and a degree of freedom around the anaplerotic reactions from pyruvate to phosphoenolpyruvic acid (PEP), which is less important given the low level of biomass production (~5% in carbon balance). Upstream of acetyl CoA, there are several degrees of freedom around redox metabolism particularly on complex gas mixes. With off gas analysis, this can be reduced to a single degree of freedom even on syngas.

The free fluxes cannot be resolved by standard steady-state ^{13}C-metabolic flux analysis as there is no carbon rearrangement when fixing C1-substrates. TFA is more promising due to differences in co-factor use as well as potential difference in concentrations. For example, ethanol production via acetate involves ATP generation and ferredoxin oxidation, while direct production involves NADH or NADPH oxidation.

We recently used our state of the art TFA method [44] to explore the feasibility of intracellular flux based on culture conditions (Figure 2a), using thermodynamics in order to better understand oscillations observed in high density *C. autoethanogenum* cultures grown on syngas [32••]. TFA incorporates intracellular and extracellular metabolite measurements or broad feasible ranges for these, if not measured. Using available measurements, we established that acetate is transported using anionic transporters with the balancing proton transported using the Rnf complex; the commonly assumed proton symport reaction was not thermodynamically feasible, while an ABC transporter would exhaust all ATP. TFA further verified that under steady state syngas conditions, acetaldehyde:ferredoxin oxidoreductase (AOR) is responsible for ethanol production via acetate [32••]. This is consistent with a range of experimental data correlating AOR expression with ethanol production [17,30,31••,33] as well as KO experiments [45].

TFA could not rule out direct production of ethanol from acetyl CoA under all conditions. To do so, metabolite concentrations must be further constrained, in particular the ratios of relevant redox species: ferredoxin, NADPH and NADH. Considering the challenge of measuring these accurately [32••], advances in measuring dissolved gas tensions [42••] is a promising approach as the dissolved tensions are believed to be much lower than the range inferred from gas equilibrium concentrations with the exhaust. For instance, although typical off-gas analysis can accurately determine the flux of carbon monoxide dehydrogenases and hydrogenases, estimating dissolved carbon monoxide and hydrogen concentration and their thermodynamic feasibility of reaction have large ranges and thus poor constraint of ferredoxin concentration and

redox ratio (Figure 2a — W_1*(CODH)* and *Hyt/Hyd* reactions). Combined with other measurement limitations, this leads to loose bounds around acetyl-CoA-acetate-acetaldehyde reactions and unspecified directionality for CO and CO/H_2 fermentations (Figure 2a — A_1 to A_4 reactions). Tightening these boundary conditions reduces the solution space of the model significantly, increasing the accuracy and solvability.

Flux balance modelling

The M-models used for FBA are readily applied for design-focused flux balance modelling and harnessed for rational engineering of metabolism in design-build-test cycles [3,10,20]. For example, we recently used FBA on data from CO_2/H_2 fermentation to identify the potential for supplementing CO — doing so led to an increase in net CO_2 uptake [46]. FBA can be used to explore more complex scenarios, for example, Benito-Vaquerizo *et al.* [47•] modelled a co-culture of *C. autoethanogenum* and *Clostridium kluyveri* using FBA and highlighted potential methods for metabolic optimisation using substrate supplementation and synthetic biology. GSMR construction and FBA also predicted the glycine synthase-reductase pathway (GSRP) cooperating with the WLP during *C. drakei* autotrophic growth [16••], then thoroughly validated by metabolomics of culture grown on ^{13}C-labelled CO_2 and introduction of the GSRP into *E. limosum*.

Models were also implemented to investigate dynamic bioreactors [48] and large-scale reactors [49,50], with incorporations of thermodynamics [51], and energy efficiency and economics [52] furthering analysis in unique ways. In these applications the metabolic models are not dynamic, but rather M-models [49,50] or simplified M-models [48,51,52] are used to capture stoichiometric conversion with physicochemical differential equations solved for estimation of environmental conditions and gross kinetic parameters. As identified by the authors, these models fail to capture metabolic regulation in response to environmental changes (e.g. predicting effect of fluctuations in feed gas composition) [48,51].

ME-models

Genome-scale models of *m*etabolism and gene *e*xpression (ME-model) cast metabolism as a growth optimisation problem recognizing that the cost of maintaining a given flux is not only supplying substrates to reactions (OPEX), but equally maintaining sufficient enzyme activity (CAPEX). Whereas M-models must be explicitly constrained in carbon uptake, ME-models are naturally constrained by membrane availability for transporters and their turnover numbers. The only ME-model for an acetogen (*C. ljungdahlii*) used a constant turnover number for all enzymes and the metabolic predictions were only slightly more accurate than the corresponding M-model assuming a fixed maximum uptake rate [18•]. As more experimental data combining absolute proteomics and fluxomics becomes available, it will be possible to estimate turnover numbers more accurately (e.g. using the maximum k_{app} observed across all data [53]). For now, the true advantage of a ME-model is interrogation of aspects such as transcription-flux relationships and impact of metal availability. For example, Liu *et al.* [18•] suggest that Ni^{2+} uptake boosts growth by increasing carbon monoxide dehydrogenase/acetyl-CoA synthase activity.

Even with better estimates of turnover numbers, ME-models will very likely struggle to explain flux changes under different gas fermentation conditions as flux regulation occurs post transcription and translation (Figure 2b) [18•,29,30,31••,32••,33]. Even the metabolic oscillations observed in high density syngas fermentation display no variation in protein expression across the cycles [32••]. This suggests that kinetic modelling is an important model framework for gas fermentation.

Kinetic models

Acetogenic metabolism is well suited for kinetic modelling with more than 95% of the total flux captured in only 32 reactions [32••]. Kinetic models can incorporate various degrees of detail based on available model structures, omics data, and kinetic parameters — reviewed by Saa and Nielsen [54]. Ensemble modelling (EM) is a recent approach suitable for working with a modest number of multi-omics datasets (fluxomics, metabolomics, and proteomics), in which thermodynamically feasible kinetic parameter sets are randomly generated and screened for fitness, including stability and agreement with observed data. The surviving parameter sets describe the EM and are used for predictions. The first acetogen model accurately predicted intracellular metabolite concentrations for steady-state datasets at different biomass concentration [31••]. The model also validated the directionality of the AOR reaction and suggested overexpression/underexpression targets for optimisation of ethanol production. At 70 reactions and 62 metabolites, the model captured more than 97% of total flux.

A key next step would be development of a kinetic model for prediction of dynamic environmental conditions, as broader information can be obtained when testing metabolic robustness [32••]. Machine learning is a computational tool that could be employed in future improvements to kinetic models, potentially reducing data requirements and need for regulatory information [55]. However, this tool is likely to require datasets with numerous different conditions or time points, not just large amounts of omics data.

Conclusions

Analytical tools have proven invaluable in the design of industrial gas fermentation. Continued improvements are necessary to enable kinetic modelling and a more complete understanding of metabolic regulation. Kinetic

modelling is critical given the limited role of gene expression in controlling metabolism and tighter measurements are needed to improve modelling. Routine measurement of dissolved gasses will help constrain concentrations through TFA, though direct measurement of tetrahydrofolates and ferredoxin would be preferable.

Conflict of interest statement

Nothing declared.

CRediT authorship contribution statement

James K Heffernan: Writing – original draft, Writing – review & editing, Visualization. **Vishnu Mahamkali:** Writing – review & editing, Visualization. **Kaspar Valgepea:** Conceptualization, Writing – review & editing. **Esteban Marcellin:** Conceptualization, Writing – review & editing. **Lars K Nielsen:** Conceptualization, Writing – review & editing.

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- • of special interest
- •• of outstanding interest

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