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Adapting isotopic tracer and metabolic flux analysis approaches to study C1 metabolism

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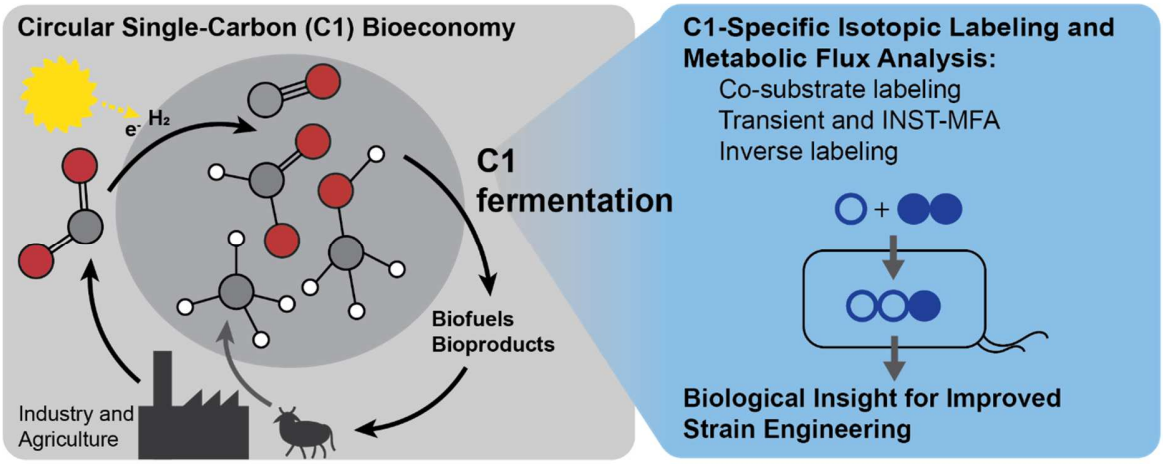
Abstract:

Single-carbon (C1, or one-carbon) substrates are promising feedstocks for sustainable biofuel and biochemical production. Crucial to the goal of engineering C1-utilizing strains for improved production is a quantitative understanding of the organization, regulation and rates of the reactions that underpin C1 metabolism. ^{13}C Metabolic flux analysis (MFA) is a well-established platform for interrogating these questions with multi-carbon substrates, and uses the differential labeling of metabolites that results from feeding a substrate with position-specific incorporation of ^{13}C in order to infer quantitative fluxes and pathway topology. Adapting isotopic tracer approaches to C1 metabolism, where position-specific substrate labeling is impossible, requires additional experimental considerations. Here we review recent studies that have developed isotopic tracer approaches to overcome the challenge of uniform metabolite labeling and provide quantitative insight into C1 metabolism.

Graphical Abstract



Highlights

- Probing C1 pathways by isotope tracers requires methods to prevent uniform labeling
- Using a labeling co-substrate allows analysis of C1 pathways at steady-state
- Transient labeling allows analysis of C1 pathways with a single C1 substrate

- Metabolic flux analysis has led to improved C1 microbial biocatalysts

Introduction

Microbial conversion of single-carbon (C1, or one-carbon) compounds to value-added products may offer sustainable solutions to a number of environmental and industrial problems. Waste industrial gases, such as the carbon monoxide-rich off gas from steel production can be converted by acetogenic microbes to ethanol as biofuel [1,2]. Methanotrophs can convert methane (CH_4), an abundant carbon resource and potent greenhouse gas, into protein (for animal feed) or polyhydroxyalkanoates [3–5]. Methanol (CH_3OH) and formic acid (CHOOH) are also emerging as attractive feedstocks, given recent progress in development of efficient catalytic processes for their production directly from CO_2 using either renewable electricity or renewable hydrogen [6–11]. A key advantage of microbial conversion of these feedstocks is the high carbon yield, low energy input and high selectivity attainable with biological catalysts.

Efficiently engineering C1 microbes to produce non-native products requires a quantitative understanding of their metabolism. Diverting flux from a native product, for example, requires knowing the steps of the metabolic pathway by which that product is synthesized, and which of those enzymatic reactions control flux, in order to provide target candidate genes to knock out or knock down. The relative absence of this knowledge in C1-utilizing microbes, compared to more traditional platform microbes, has so far hampered our ability to produce engineered strains with industrially relevant production metrics.

One particularly powerful approach to determining pathway steps and fluxes involves the feeding of substrates with labeled isotopes incorporated at specific positions, in order to track the transition of carbon atoms between intermediates in a pathway and establish connectivity [12,13]. These techniques are particularly powerful in non-model organisms, where gene annotation is insufficiently precise to identify pathways from genome sequences alone [14]. In these experiments, isotopic labeling data is obtained by growing the organism with a ^{13}C substrate, extracting the metabolites and products, and analyzing their isotopic distribution via NMR or mass-spectrometry (GC-MS or LC-MS). Experiments can be performed under either stationary or instationary conditions, with respect to either the isotopic labeling or metabolic state [15]. Metabolic stationarity refers to the fluxes being constant over time, and is usually achieved experimentally by use of continuous culture, or assumed during batch culture in the exponential phase where cells are in balanced growth. Isotopic stationarity refers to the extent of isotope incorporation being at steady state, which is typically achieved by analyzing cells grown with the isotopic substrate for multiple

generations, in contrast to dynamic or instationary experiments in which an isotopic substrate is added midway through growth, and the incorporation is analyzed at multiple subsequent time points [15].

In addition to inferring metabolic precursors and pathway connectivity, isotopic labeling data can be used to obtain quantitative pathway fluxes, through an approach called metabolic flux analysis (MFA) [16,17]. Here, a stoichiometric metabolic model of the organism containing the relevant reactions (typically constructed from analysis of the genome and/or previous biochemical studies) is used to predict the isotopic labeling distribution for each metabolite arising from a specified flux distribution. An optimization algorithm is then employed to determine the flux distribution for which the predicted isotopologue distribution best matches the experimental data. A variety of MFA software suites have been developed, including among others Open Flux 2, METRAN, 13CFlux 2, and INCA [18–21]. Since MFA uses experimentally derived labeling patterns, the calculated fluxes are the real rates occurring in the cell. This is an advantage over flux balance analysis (FBA), in which the predicted fluxes represent a hypothetical solution optimized to meet an assumed cellular objective function, and lack experimental corroboration [17]. Similarly, while genetic and omics tools provide insights into the presence and activity of metabolic pathways, only isotopic labeling and MFA can offer direct confirmation of metabolic precursors and quantitative pathway fluxes. mRNA levels can identify relative gene transcription activity of pathway steps, but they do not always directly correlate to carbon flux [22]. As such, ^{13}C-MFA is indispensable in metabolic engineering.

Deriving useful information from an isotopic labeling experiment requires the analyzed metabolites to be differentially labeled. This presents a unique challenge in studying C1 metabolism, because if a ^{13}C substrate is the only carbon source used by the organism, then at steady state every metabolite will be uniformly comprised of ^{13}C. Thus, adapting isotopic labeling experiments and MFA to study C1 metabolism requires either dynamic (instationary) experiments, or taking advantage of cases where cells simultaneously metabolize multiple carbon sources. In this review, we discuss recent examples of approaches that have been used to address this issue, with a focus on microbes being developed for biochemical production from C1 feedstocks. The approaches used and studies referenced in this review are summarized in **Box 1**.

Differential labeling during C1 metabolism through co-substrate feeding.

The first approach to overcoming uniform labeling includes feeding a second carbon substrate, with only one of the substrates containing labelled carbon, so that the resulting metabolite labeling patterns will be non-uniform (**Fig. 1a**). Transient labeling methods (**Fig. 1b**) and inverse labeling methods (**Fig. 1c**) also provide differential

labeling for a single C1 substrate, and these are discussed in the following sections. Co-substrate experiments are particularly attractive as they can be conducted at metabolic and isotopic steady state, simplifying the experimental setup and data analysis. Fixation of CO_2 is a key consideration for biocatalyst engineering, as carbon that is lost as CO_2 from a cellular process decreases the carbon yield. In this section we describe several recent examples of using an isotopically labeled co-substrate to delineate and enhance native bacterial pathways that fix CO_2.

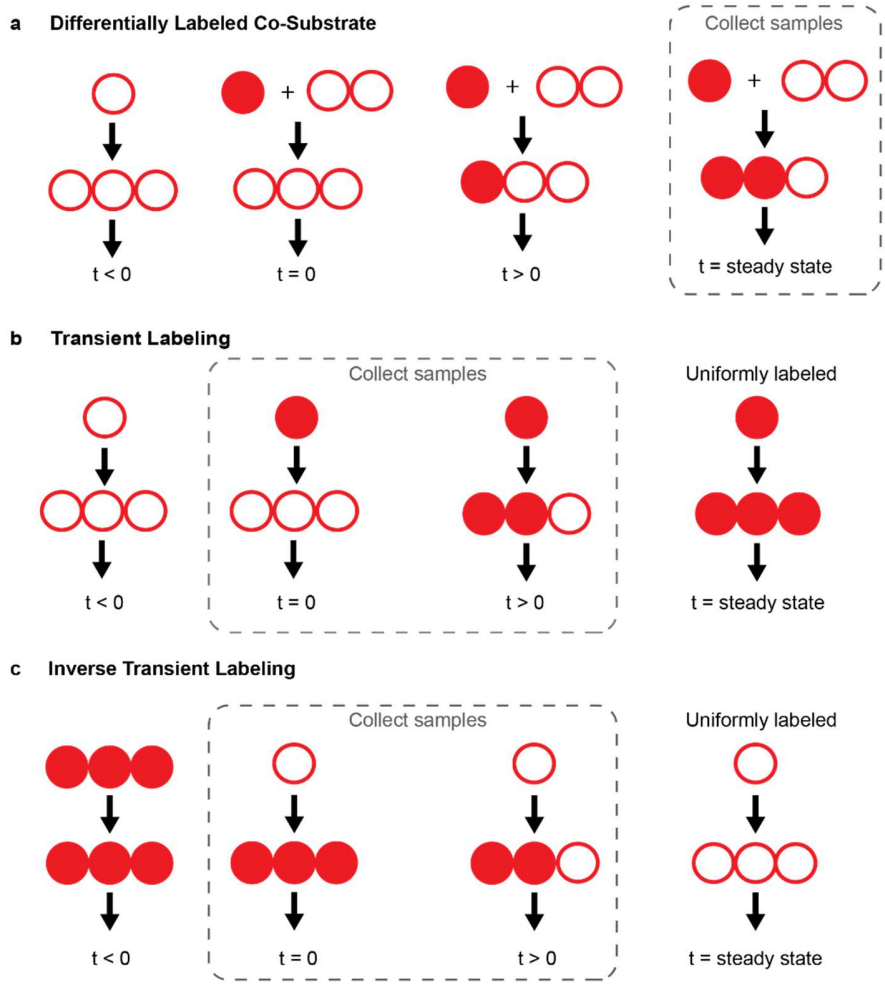


Fig. 1. General methods to achieve differential isotopic labeling during C1 metabolism. Uniform labeling must be prevented in order to use isotopic labeling to study C1 metabolism. **(a)** By providing an un-labelled co-substrate, nonuniform labeling at steady state can be achieved. **(b)** Alternatively, a labelled C1 substrate can be fed initially with samples collected over a time course before uniform labeling occurs. **(c)** By first establishing a labelled metabolome, an un-labelled C1 substrate can be fed with samples collected over a time course. Inverse labeling is useful for C1 substrates for which isotopically labeled versions are expensive or unavailable (such as carbon monoxide).

Box 1. C1 Metabolism for a variety of substrates and species has been studied using creative approaches to isotopic labeling and MFA. Recent studies highlighted in this review are summarized here. Bold text indicates studies highlighted in this review.

C1-Specific Technique	Species	C1 Metabolism Insights	Reference
Co-feeding of a second substrate along with the C1 substrate, where one is labelled (^{13}C) and the other is unlabelled (^{12}C). Samples are collected when the system is at metabolic and isotopic steady state.	***C. necator***	**CBB fluxes observed under heterotrophic conditions; results used to increase CO_2 fixation**	**Alagesan, et al., 2018**
	M. thermoacetica	**Flux distribution through glycolysis and WLP used to prompt CO_2 fixation**	**Park and Liu, et al., 2019**
	C thermocellum	**Labeling result indicated new CO_2 fixation pathway**	**Xiong, et al., 2016**
	M. buryatense	**Fluxes differed between growth on methanol relative to methane**	**Fu, et al. 2020**
	M. capsulatus	Validated CO_2 fixation under methanotrophy	Henard, et al., 2021
	C. drakei	Validated CO_2 fixation under heterotrophy	Song, et al., 2020
	C. ljungdahlii	Validated CO_2 fixation under nitrate supplemented hydrogen growth	Emerson and Woolston, et al., 2019
Transient labeling and INST-MFA with labelled C1 substrate.	***M. buryatense***	**Fluxes differed between growth on methanol relative to methane**	**He, et al. 2019**
	B. methanolicus	**On methanol, higher RuMP flux relative to heterotrophic growth**	**Delépine, et al., 2020**
	E. coli	**Mdh enzyme kinetics were a key limiting factor**	**Woolston, et al., 2018**
	K. stuttgartiensis	Direct formate assimilation through WLP and confirmation of central carbon metabolism	Lawson, et al., 2020
	A. gossypii	Partial MFA informed timely supplementation of formate, increasing product titer	Schwechheimer, et al., 2018
	Synechococcuss PCC 7002	Carbon flux changed after removal of glycogen synthase gene	Hendry, et al., 2017
	Synechococcuss PCC 7002	Carbon flux diverted after removal of CO_2 storage compartment	Abernathy, et al., 2019
Inverse transient labeling	***C. carboxidovorans***	**WLP was active during growth on syngas**	**Wan, et al. 2017**
	C ljungdahlii	**Reducing equivalents were recycled through product uptake**	**Liu, et al. 2020**

*Cupriavidus necator (*f. *Ralstonia eutropha)* is an aerobic hydrogen-oxidizing or Knallgas bacterium capable of autotrophic growth on CO_2 or formate using the Calvin Benson Bassham (CCB) cycle, and is an attractive candidate for C1-based bioprocesses [23]. In the first stable isotope carbon labeling experiment in this organism, Alagesan and coworkers grew cells heterotrophically with 1,2-^{13}C-glycerol, and determined fluxes via MFA [24]. Interestingly, a small but significant flux through the CBB in *C. necator* was observed during growth on glycerol (endogenous CO_2, **Fig. 2a**), suggesting growth was not solely heterotrophic. The authors then fed a mixture of 1,2-^{13}C-glycerol and unlabeled CO_2 (exogenous CO_2 **Fig. 2b**), and observed an 8-fold increase in CBB flux, indicating active CO_2 fixation and mixotrophic growth. This was accompanied by a dramatically enhanced flux through the citric acid (TCA) cycle. While prior proteomic and transcriptomic experiments had suggested activity of the CBB during heterotrophic conditions, the MFA results confirmed and quantified this activity. These results suggest the potential for CO_2 fixation during sugar fermentation by *C. necator*, and the potential to increase flux through the TCA by providing CO_2 as a co-substrate with glycerol, which could be beneficial for production of targets derived from TCA intermediates.

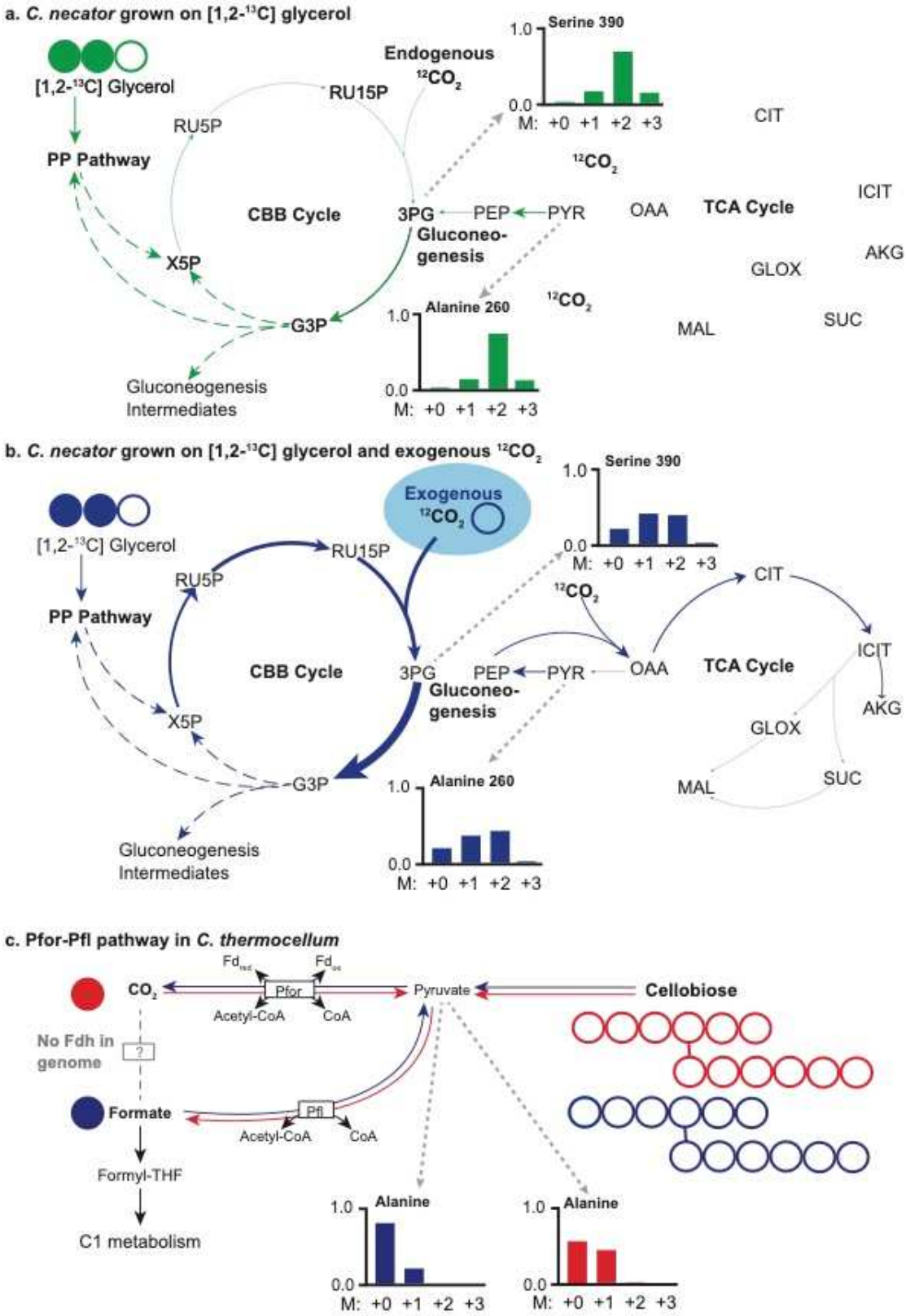

a. C. necator grown on [1,2-13C] glycerol
[1,2-13C] Glycerol
PP Pathway
RU5P
RU15P
Endogenous 12CO2
CBB Cycle
X5P
G3P
3PG
PEP
PYR
OAA
TCA Cycle
CIT
ICIT
AKG
GLOX
MAL
SUC
Gluconeo-genesis
Gluconeogenesis Intermediates
Serine 390
Alanine 260
M: +0 +1 +2 +3
b. C. necator grown on [1,2-13C] glycerol and exogenous 12CO2
Exogenous 12CO2
c. Pfor-Pfl pathway in C. thermocellum
CO2
Pfor
Pyruvate
Cellobiose
Acetyl-CoA
CoA
No Fdh in genome
Formate
Pfl
Formyl-THF
C1 metabolism
Alanine

Fig. 2. Carbon fixation pathways delineated through steady-state labeling experiments using co-substrates. (a) Quantitative fluxes for the CBB cycle in glycerol-grown *C. necator* [24]. **(b)** Quantitative fluxes in the CBB cycle in glycerol-grown *C. necator* with exogenous CO_2. Line thicknesses generally present the relative flux magnitude. Dashed lines represent steps where the quantitative flux was not explicitly presented in the original work. Experimentally determined amino acid isotopologue distributions are connected by gray lines to their direct metabolite precursor. **(c)** Analysis of formate production in *C. thermocellum* via the Pfor-Pfl pathway. In red, ^{13}C bicarbonate and un-labelled cellobiose were fed to wild type *C. thermocellum*, revealing incorporation of CO_2 into pyruvate, and production of ^{13}C-formate via an unknown pathway as identified via ^{13}C-NMR. In blue, ^{13}C-formate and unlabeled cellobiose were fed, with pyruvate labeling showing reversible activity of Pfl. Adapted from [25]. Abbreviations: CBB Calvin Benson Bassham, PP Pentose Phosphate, RU5P ribulose-5-phosphate, RU15P ribulose-1,5-bisphosphate, 3PG 3-phosphoglycerate, G3P glyceraldehyde-3-phosphate, X5P xylulose 5-phosphate, TCA Tricarboxylic Acid, ED Entner-Doudoroff, EMP Embden-Meyerhof-Parnas, Fdh Formate dehydrogenase, Pfor pyruvate:ferredoxin oxidoreductase, OAA oxaloacetate, PEP phosphoenolpyruvate, MAL malate, AKG alpha-ketoglutarate

Moorella thermoacetica is a thermophilic acetogen capable of growing on a wide variety of C1 feedstocks and converting them at high efficiency to acetate [26]. In an effort to enhance autotrophic growth, Park and co-workers recently investigated the co-metabolism of glucose and CO_2 in this organism. ^{13}C MFA during co-feeding using U-^{13}C-glucose and unlabeled CO_2 revealed that the glycolytic intermediates except pyruvate were >90% labeled, and therefore derived from glucose, whereas CO_2 and glucose contributed jointly to TCA cycle intermediates and pyruvate [27]. The mixed labeling of pyruvate indicated activity of the pyruvate:ferredoxin oxidoreductase (Pfor) in the CO_2-fixing direction, and the high degree of labeling in phosphoenolpyruvate (the glycolytic precursor to pyruvate) indicated forward-driven activity of the pyruvate kinase to produce ATP. Using these insights, the researchers designed a co-feeding scheme with glucose, H_2 and CO_2, in which a controlled glucose feed was used to provide the cell's ATP needs, while H_2 provided electrons for CO_2 fixation. This resulted in a 4-fold increase in specific CO_2 uptake rate compared to the no-glucose control, and a synergistic enhancement in acetate productivity. These results highlight how an understanding of the metabolic network derived from ^{13}C experiments can lead to significant strain improvement without the need for genetic modification.

Co-substrate analysis can also identify novel metabolic pathways. *Clostridium thermocellum* is a thermophilic cellulose-degrading microbe under development for consolidated bioprocessing applications. Through steady-state isotopic labeling, a new CO_2 fixation pathway active during heterotrophic growth, was recently identified (**Fig. 2c**) [25]. Despite the absence of a formate dehydrogenase in the genome, co-feeding ^{13}C-bicarbonate (a source of $^{13}CO_2$) and unlabeled cellobiose resulted in production of ^{13}C-formate, indicating an alternative pathway for CO_2 fixation. The authors hypothesized that CO_2 could be incorporated into pyruvate through Pfor, and

subsequently converted to formate by the action of pyruvate-formate lyase. A series of elegant mutational experiments and tracer experiments with 2-^{13}C-acetate confirmed this hypothesis, and bioinformatic analysis in a range of *Clostridia* suggested this pathway may be more widespread. Though the reason for the operation of this pathway was not fully elucidated, the authors speculate that the provision of oxidized ferredoxin by operation of Pfor in the CO_2-fixing direction may keep the driving force high for cellobiose consumption. If borne out, this would have ramifications for the engineering of *C. thermocellum* for production of biofuel products that may affect cellular redox balance.

The co-substrate steady-state isotopic labeling approach has been used in several other recent studies to validate insights into metabolism related to CO_2 fixation, specifically methanotrophy in *Methylococcus capsulatus* [28], heterotrophy in *Clostridium drakei* [29], and nitrate supplemented hydrogen growth of *Clostridium ljungdahlii* [30]. Isotopic tracer approaches in clostridial metabolism beyond C1 substrates have been reviewed recently [31]. Similarly, steady-state isotopic labeling with CO_2 as a co-substrate confirmed synthetic formatotrophy and methylotrophy recently accomplished in *Escheria coli* by verifying flux through the heterologous pathways [32].

Dynamic labeling experiments and instationary MFA

In cases where a second substrate is not applicable, dynamic isotopic labeling experiments and instationary MFA (INST-MFA) can be used. In these experiments, an organism is grown on a ^{12}C substrate, then the labeled substrate is fed to the organism and samples are collected over a time course before uniform labeling occurs [33]. INST-MFA makes it possible to study metabolism for an organism grown solely on a C1 substrate, which is particularly useful for methane and methanol, as described below. We focus here on the use of reduced C1 feedstocks, noting that use of the INST-MFA for photoautotrophic growth is well established and has been recently reviewed. [34–36].

Methylomicrobium buryatense 5GB1 is a promising alkaliphilic methanotroph capable of robust growth on natural gas (primarily methane) and methanol. INST-MFA has recently been used to provide insight into the metabolic differences between growth of *M. buryatense* 5GB1C on methanol and methane [39]. This study was the first effort to fully quantitate central metabolism of a methanotroph using the transient approach. Although the methanol uptake rate was slower than the methane uptake rate, a larger fraction of methanol compared to methane was incorporated into central metabolism through the ribulose monophosphate (RuMP) cycle, implying more efficient growth on methanol. In a related study from the same group, RNA-seq results showed that differences in the relevant transcripts between the two growth modes were generally

less than 2-fold, highlighting the importance of MFA for making this comparison [22]. INST-MFA also revealed a bifurcated TCA cycle, as was also found for *Bacillus methanolicus,* another promising methylotroph [40].

Another transient isotopic labeling method that can be used for C1 substrates involves use of the kinetic isotope effect (KIE), a phenomenon where the presence of a heavy isotope slows an enzymatic reaction in which the bond to the heavy atom is broken. The labeled substrate is introduced to perturb the rate of a specific reaction in the metabolic network, and simultaneously allow determination of the rate at which the substrate is incorporated into metabolic intermediates. This approach was recently used to identify bottlenecks in a strain of *E. coli* engineered to metabolize methanol [41]. Here the researchers fed deuterated methanol (CD_3OD), whose oxidation to formaldehyde via methanol dehydroganse (Mdh) has a strong KIE, to identify conditions and strain variants where Mdh was rate-limiting.

The INST-MFA approach is useful for C1 substrates other than methanol and methane, and has led to recent insights about formate metabolism in *Candidatus Kuenenia stuttgartiensis* [42] and partial MFA has helped address formate feeding bottlenecks in *A. gossypii* [43]**.**

Inverse Isotopic Labeling

Acetogenic bacteria are promising microbial biocatalysts for syngas fermentation [44]. Compared to other C1 utilizers, acetogens can be particularly difficult to study using isotopic approaches because isotopically labelled gaseous substrates (e.g. ^{13}CO, $^{2}H_2$) can be difficult or expensive to obtain. One approach is to employ a variation of dynamic labeling in which cells are grown first on an accessible ^{13}C substrate, then switched to the unlabeled gaseous substrate of interest. In recent work, the ethanol producing acetogen *Clostridium carboxidivorans* P7 was studied using this inverse dynamic labeling approach [45]. Cells were initially grown on ^{13}C glucose with nitrogen gas in the head space, then an unlabeled mixture of $CO/CO_2/H_2$ was supplied and metabolite labeling was analyzed over time. The presence of yeast extract in the media prevented a full MFA, but isotopic labeling results and metabolite turnover rates provided insight into core carbon metabolism. Based on the dilution rates of ^{13}C from intracellular metabolites over time, qualitative insights about flux were drawn, including high activity of the WLP, low activity of gluconeogenesis compared to the TCA cycle [45], and incorporation of CO_2 through Pfor.

In addition to inverting the labeled isotope *substrate*, for cells in which product formation is partially reversible, adding isotopically labeled *products* can provide insight into metabolism. *Clostridium ljungdahlii* is an acetogen that encodes two pathways for

production of ethanol, one from acetyl-CoA via the AdhE pathway, and the second from acetate via the Aor pathway [46]. Previous genetic studies have provided some insight into how ethanol is produced, but these represent a significant perturbation to metabolism [47,48]. To further probe the relative contribution of the two pathways in wild-type cells, stationary phase *C. ljungdahlii* cells were provided either ^{13}C acetate or ^{13}C ethanol, without CO. When provided ^{13}C-acetate, very little ^{13}C-ethanol was detected, implying low activity of the Aor pathway under these conditions. Interestingly, when provided ^{13}C-ethanol, ^{13}C-acetate was detected, showing that ethanol was oxidized, which the authors speculated allows production of reducing equivalents (NADH) needed after growth slows in the stationary phase. Unfortunately, ^{13}C product uptake was not examined in the presence of CO, so firm conclusions could not be made about pathway contribution under growth conditions. However, by feeding labelled products instead of labelled substrate, the researchers provided insights into the reversibility of product formation and redox balance during syngas fermentation.

Conclusion

To use isotopic labeling and ^{13}C MFA to study C1 metabolism, the issue of uniform isotopic labeling must be addressed. As discussed in this review, two clear options arise: First, to co-feed another carbon substrate with the C1 substrate of interest with only one labeled; and second, to perform the experiment transiently. As interest grows in microbial C1 conversion, these approaches will continue to provide insight into energy conservation, substrate utilization and growth. Additional isotopic labeling strategies, such as non-carbon tracers, which have been reviewed recently [49], could be leveraged alongside the methods discussed here for C1 metabolism. By using these approaches, metabolic engineers will continue to make key insights for rational pathway design, improved carbon yield, product titer, and enhanced CO_2 fixation.

Acknowledgements

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A partial MFA showed quantitative differences in flux distribution between growth on methanol relative to methane, even though both substrates feed the same central carbon pathways.

* **Ref. [24]** Alagesan S, Minton NP, Malys N: **13 C-assisted metabolic flux analysis to investigate heterotrophic and mixotrophic metabolism in Cupriavidus necator H16**. *Metabolomics* 2018, **14**:1–10.
Growth on heterotrophic substrate was found to be mixotrophic by fixing endogenous CO_2. This is the first carbon labeling and MFA study performed for this organism.

** **Ref. [25]** Xiong W, Lin PP, Magnusson L, Warner L, Liao JC, Maness PC, Chou KJ: **CO2-fixing one-carbon metabolism in a cellulose-degrading bacterium Clostridium thermocellum**. *Proc Natl Acad Sci U S A* 2016, **113**:13180–13185.
Novel CO_2 fixation pathway was identified in *C. thermocellum* through isotopic labeling and was confirmed through clever gene knockout experiments.

* **Ref. [27]** Park JO, Liu N, Holinski KM, Emerson DF, Qiao K, Woolston BM, Xu J, Lazar Z, Islam MA, Vidoudez C, et al.: **Synergistic substrate cofeeding stimulates reductive metabolism**. *Nat Metab* 2019, **1**:643–651.
Flux distribution insights allowed researchers to significantly enhance CO_2 fixation and improve product generation by adjusting substrate feeding, without gene modications.

* **Ref. [39]** He L, Fu Y, Lidstrom ME: **Quantifying Methane and Methanol Metabolism of Methylotuvimicrobium buryatense 5GB1C under Substrate Limitation** . *mSystems* 2019, **4**:1–14.
This is the first time that full central carbon metabolism for a methanotroph, grown on methane alone, was quantified using MFA.

* **Ref. [40]** Delépine B, López MG, Carnicer M, Vicente CM, Wendisch VF, Heux S: **Charting the Metabolic Landscape of the Facultative Methylotroph Bacillus methanolicus**. *mSystems* 2020, **5**.
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This study devised a clever inverse labeling approach to study gas fermentation, by feeding labelled glucose first, and following with unlabeled carbon monoxide to achieve differential labeling.

*** Ref. [46]** Liu ZY, Jia DC, Zhang K Di, Zhu HF, Zhang Q, Jiang WH, Gu Y, Li FL: **Ethanol Metabolism Dynamics in Clostridium ljungdahlii Grown on Carbon Monoxide**. *Appl Environ Microbiol* 2020, **86**:1–13.
This study used inverse labeling by feeding labeled product, rather than labeled substrate, to study gas fermentation, gaining into recycling of reducing equivalents.

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