REVIEW

Recent advances in mapping environmental microbial metabolisms through ^{13}C isotopic fingerprints

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After feeding microbes with a defined ^{13}C substrate, unique isotopic patterns (isotopic fingerprints) can be formed in their metabolic products. Such labelling information not only can provide novel insights into functional pathways but also can determine absolute carbon fluxes through the metabolic network via metabolic modelling approaches. This technique has been used for finding pathways that may have been mis-annotated in the past, elucidating new enzyme functions, and investigating cell metabolisms in microbial communities. In this review paper, we summarize the applications of ^{13}C approaches to analyse novel cell metabolisms for the past 3 years. The isotopic fingerprints (defined as unique isotopomers useful for pathway identifications) have revealed the operations of the Entner–Doudoroff pathway, the reverse tri-carboxylic acid cycle, new enzymes for biosynthesis of central metabolites, diverse respiration routes in phototrophic metabolism, co-metabolism of carbon nutrients and novel CO_2 fixation pathways. This review also discusses new isotopic methods to map carbon fluxes in global metabolisms, as well as potential factors influencing the metabolic flux quantification (e.g. metabolite channelling, the isotopic purity of ^{13}C substrates and the isotopic effect). Although ^{13}C labelling is not applicable to all biological systems (e.g. microbial communities), recent studies have shown that this method has a significant value in functional characterization of poorly understood micro-organisms, including species relevant for biotechnology and human health.

Keywords: carbon fluxes; isotopic effect; isotopomers; channelling; novel enzymes

1. INTRODUCTION

^{13}C labelling is a powerful tool for elucidating *in vivo* enzyme activities in micro-organisms. After a particular ^{13}C -labelled substrate is fed to microbes, the label is transferred to the downstream metabolites through metabolic pathways. As a consequence, some metabolic products (i.e. the analysed compound) comprise mixtures of ^{13}C isotopomers with specific labelling patterns, which are termed isotopic fingerprints. Via unique isotopologue profiles, researchers can trace the paths of reactants to products in the metabolic network and thus reveal functional enzymes and metabolic pathways. ^{13}C labelling can not only accurately define the structure of a complete pathway but also quantify global enzymatic reactions (i.e. carbon flux) in the metabolic network by

incorporating ^{13}C fingerprints into metabolic modelling (^{13}C metabolic flux analysis (^{13}C -MFA)) [1–3]. Owing to post-transcriptional/post-translational regulation mechanisms, genetic analysis sometimes cannot reflect the final metabolic outputs; however, the metabolic fluxes can provide experimental validation of the results obtained from genomics and transcriptomics studies. The ^{13}C technique is widely applied in poorly understood microbial species and has filled in gaps between genome sequencing and functional characterization. Meanwhile, the ^{13}C technique is being improved to speed up screening and exploitation of native metabolic features of non-model microbes for biotechnology applications. Figure 1 shows a protocol describing the ^{13}C technique for probing microbial metabolism, which includes two key procedures: measuring isotopic labelling patterns in metabolites and examining metabolic pathways [1,4,5].

In the past 3 years, numerous papers have reported novel metabolic insights from ^{13}C fingerprints. Other

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Figure 1. Procedures for metabolism analysis through GC-MS-based isotopic labelling.

papers have described different new methods to obtain metabolic labelling fingerprints and quantify the fluxome in the cell metabolism, such as the extraction of targeted metabolites in microbial communities, new isotopomer analysis approaches (gas chromatography–combustion–isotope ratio mass spectrometry (GC-C-IRMS) and liquid chromatography–mass spectrometry (LC-MS)) and high-performance fluxomics algorithms/software. Because ^{13}C isotopic labelling is extensively used in cell metabolism analyses, it is difficult to provide a complete and balanced view in this short review paper. Therefore, we highlight only a small portion of recent studies (after 2009) on environmental micro-organisms.

2. PATHWAY ELUCIDATION VIA ^{13}C FINGERPRINTS

For many micro-organisms, genetic analysis tools (such as genome annotation and microarrays) cannot precisely reveal the topology of the functional metabolic network. ^{14}C labelling has historically played a key role in the discovery of functional metabolic pathways. Although ^{14}C methods are sensitive owing to the radioactivity, stable ^{13}C is instrumentally safer and easier for researchers to use high-performance nuclear magnetic resonance (NMR) or mass spectrometry to detect metabolic pathways [6]. ^{13}C -labelled metabolites (isotopic fingerprints) can be used for investigating carbon metabolism. For example, by examining whether the ^{13}C fingerprints in key metabolites are consistent with the predicted labelling patterns from the annotated pathway, we can verify annotated enzymatic reactions. In parallel to the ^{13}C technique, we can elucidate the functional pathway by conducting a BLAST search for potential genes that have been reported in other micro-organisms, perform transcription analysis

of gene candidates, detect intermediate metabolites, and overexpress or knock out the target genes. Table 1 highlights several pathways that have been examined via ^{13}C fingerprints in diverse micro-organisms since 2009.

2.1. Central metabolic pathways for carbon substrate catabolism

^{13}C fingerprints have provided new insights into the central pathways in poorly characterized micro-organisms (figure 2). First, the Entner–Doudoroff pathway (ED pathway) is an alternative pathway to glycolysis that catabolizes glucose to pyruvate (PYR) via two key enzymes—6-phosphogluconate (6PG) dehydratase and 2-keto-3-deoxyphosphogluconate aldolase. This pathway has been reported in several micro-organisms, including *Pseudomonas*, *Agrobacterium*, *Rhodobacter*, *Zymomonas*, *Azotobacter* and *Sinorhizobium meliloti* [22–24]. A recent report shows that *Roseobacter denitrificans* (an aerobic marine proteobacterium) exclusively uses the ED pathway for glucose photoheterotrophic metabolism, because the Embden–Meyerhof–Parnas pathway (glycolysis) is not active owing to the lack of the enzyme 6-phosphofructokinase [7]. On the other hand, in some hyperthermophilic archaeal species (such as *Sulfolobus solfataricus*), a non-phosphorylative ED pathway metabolizes glucose to PYR and glyceraldehyde. This non-phosphorylative ED pathway is also responsible for metabolizing C5 sugars (D-xylose and L-arabinose) to PYR and glycolaldehyde (the latter can be subsequently converted to glycolate and glyoxylate) [8].

Second, the oxidative pentose phosphate pathway (PP pathway) (glucose-6-phosphate (G6P) $\rightarrow$ ribose-5-phosphate, ribulose-5-phosphate, ribulose-1,5-bisphosphate, xylulose-5-phosphate (X5P) + CO_2 + 2NADPH) is an important resource for NADPH synthesis.

Table 1. Examples of recent pathway discoveries via ^{13}C tracing.

pathway identification	microbes	labelled substrate	fingerprint metabolite	^{13}C fingerprint description	reference
ED pathway	<i>Roseobacter denitrificans</i>	$[1-^{13}\text{C}]\text{glucose}$	serine and alanine	serine labelling is significantly lower than alanine labelling	[7]
non-phosphorylative ED pathway for C5 sugar metabolism	<i>Sulfolobus solfataricus</i> and <i>acidocaldarius</i>	$[1-^{13}\text{C}]\text{xylose}$	serine, Asp and alanine	less than 4% serine and Asp are labelled, while (approx. 50%) alanine is labelled on its first position	[8]
incomplete TCA cycle	<i>Helicobacter modesticaldum</i>	$[1-^{13}\text{C}]\text{PYR}$	Asp and Glu	Asp is labelled with two carbons; Glu is labelled with one carbon	[9]
reductive branch of TCA pathway, OAA $\rightarrow$ SUC	<i>Mycobacterium tuberculosis</i>	$[1,4-^{13}\text{C}]\text{Asp}$ or $[1,4-^{13}\text{C}]\text{OAA}$	SUC	SUC retains both ^{13}C carbons (doubly labelled)	[10]
oxidative branch of TCA pathway, OAA $\rightarrow$ CIT $\rightarrow$ SUC	<i>Mycobacterium tuberculosis</i>	$[1,4-^{13}\text{C}]\text{Asp}$ or $[1,4-^{13}\text{C}]\text{OAA}$	SUC	SUC is not labelled	[10]
PEPCK activity, OAA $\rightarrow$ PEP + CO_2	<i>Mycobacterium tuberculosis</i>	$[\text{U}-^{13}\text{C}]\text{acetate}$	Asp, MAL, PYR, serine, PEP, G3P	^{13}C from OAA (reflected by Asp) is moved to glycolytic derived intermediates (PEP and serine)	[11]
co-catabolism of multiple carbon sources	<i>Mycobacterium tuberculosis</i>	only one of the substrates is labelled	central metabolites (e.g. PYR, SUC)	if the ^{13}C substrate is co-used, the ^{13}C can enter central metabolites	[12]
complete reverse TCA cycle	<i>Chlorobaculum tepidum</i>	$[3-^{13}\text{C}]\text{PYR}$ and $^{12}\text{CO}_2$	Asp, Glu and histidine	^{13}C in Asp and Glu (derived from TCA cycle) is diluted by $^{12}\text{CO}_2$, while ^{13}C in histidine (derived from R5P) is apparently higher than ^{13}C in Asp and Glu	[13]
citramalate pathway	<i>Chlorobaculum tepidum</i>	$[2-^{13}\text{C}]\text{PYR}$ or $[1-^{13}\text{C}]\text{acetate}$	leucine and isoleucine	leucine and isoleucine labelling are identical owing to same precursors	[13]
(Re)-CIT synthase (anaerobic bacteria)	<i>Dehalococcoides ethenogenes</i>	$[1-^{13}\text{C}]\text{acetate}$	Glu	glutamate is labelled in α -carboxyl group	[14]
Wood–Ljungdahl pathway (mixotrophic)	<i>Dehalococcoides ethenogenes</i>	$[1-^{13}\text{C}]\text{acetate}$ and $^{12}\text{CO}_2$	alanine and leucine	alanine and leucine labelling would be diluted by ^{12}C	[14]
utilization of exogenous amino acids	<i>Dehalococcoides ethenogenes</i>	^{13}C substrates with ^{12}C amino acids	proteinogenic amino acids	if the cells cannot de novo synthesize amino acids, but use supplemented ^{12}C amino acids, significant ^{12}C amino acids are present in proteinogenic amino acids	[15]
oxidative PP pathway	<i>Thermoanaerobacter</i>	$[1-^{13}\text{C}]\text{glucose}$	alanine	the ^{13}C is lost as CO_2 in this pathway, and thus non-labelled alanine will be more than 50%	[16]
photorespiratory: RuBP $\rightarrow$ G3P + phosphoglycolate	<i>Synechocystis</i> 6803	$^{13}\text{CO}_2$ (dynamic labelling approach)	glycerate, G3P and PEP	^{13}C INST-MFA reveals the photorespiratory flux	[17]
EMCP	<i>Methylobacterium extorquens</i>	$[1-^{13}\text{C}]\text{acetate}$ (via dynamic labelling)	CoA intermediate	^{13}C is subsequently found in acetyl-CoA, 3-hydroxybutyryl-CoA, ethylmalonyl-CoA, methylsuccinyl-CoA, mesaconyl-CoA, β -mesaconyl-CoA (C5-CoA) and propionyl-CoA (^{13}C % in the C5-CoA is similar)	[18]

(Continued.)

Table 1. (Continued.)

pathway identification	microbes	labelled substrate	fingerprint metabolite	¹³ C fingerprint description	reference
serine cycle associated with EMCP	<i>Methylobacterium extorquens</i>	[¹³ C]methanol and ¹² CO ₂	glycine and serine	3rd carbon of serine is labelled, 2nd carbon of glycine is highly labelled	[18]
glyoxylate shunt	<i>Methylobacterium extorquens</i>	[1,2- ¹³ C]glyoxylate and ¹² C ethylamine	MAL	glyoxylate shunt through the malyl-CoA route produces doubly labelled MAL, if acetyl CoA is not labelled	[19]
alternative glycine and serine synthesis glyoxylate → glycine glycine + C1 → serine quantify mixotrophic CO ₂ fixation (Calvin–Benson cycle)	<i>Methylobacterium extorquens</i>	[1,2- ¹³ C]glyoxylate and ¹² C ethylamine	glycine and serine	glycine is doubly labelled, serine can be doubly labelled (with ¹² C1) or triply labelled (with ¹³ C1)	[19]
quantify mixotrophic CO ₂ fixation (Calvin–Benson cycle)	<i>Cyanothece</i> 51142	¹² CO ₂ with labelled carbon substrates	histidine (precursor R5P) and serine (precursor G3P)	serine and histidine labelling are significantly diluted owing to ¹² CO ₂ fixation	[20]
anaplerotic pathway: PEP + CO ₂ → OAA	<i>Cyanothece</i> 51142	¹³ CO ₂ and ¹² C glycerol	Asp	Asp labelling will be enriched in its fourth position	[20]
3-hydroxypropionate bi-cycle for glyoxylate assimilation	<i>Chloroflexus aurantiacus</i>	[1,2,3- ¹³ C]propionyl-CoA (from labelled propionate)	PYR and C5 CoA- intermediate (e.g. mesaconyl-CoA and citramalyl-CoA)	PYR will be fully labelled, while C5 CoA- intermediates are labelled with three carbons	[21]

Thermoanaerobacter sp. strain X514 (a thermophilic ethanol producer) has been studied via ¹³C experiments with [1-¹³C]glucose. On the basis of an isotopic analysis of PYR (reflected by alanine) labelling, the oxidative PP pathway for glucose metabolism shows low activity in strain X514. This result indicates that X514 may contain an alternative route for NADPH production [16].

Third, phosphoenolpyruvate carboxykinase (PEPCK) redirects the carbon flux from the tricarboxylic acid (TCA) cycle metabolites to gluconeogenesis (oxaloacetate (OAA) + GTP (or ATP) → phosphoenolpyruvate (PEP) + CO₂ + GDP (or ADP)). This reaction offers microbes an alternative route (PEP–glyoxylate cycle: OAA → PEP → acetyl-coenzyme A (AcCoA) → citrate (CIT) → glyoxylate → OAA) to oxidize intermediates in the TCA cycle to CO₂ [25]. This enzyme is also essential for *Mycobacterium tuberculosis* growth on fatty acids [11].

Fourth, the TCA cycle in most microbes is often branched under oxygen-limited conditions. In *M. tuberculosis*, Watanabe *et al.* [10] have proved that the branched TCA cycle is active for succinate (SUC) accumulation under hypoxia. Their finding is based on the observation that SUC has a labelling pattern similar to that of aspartate (Asp) after feeding the cells with [1, 4-¹³C]Asp. As another example, a study of *Plasmodium falciparum* [26] determined the major carbon source contributing to TCA intermediates by culturing parasite-infected red blood cells in a ¹³C- and ¹⁵N-labelled medium ([U-¹³C]glucose, [U-¹³C-¹⁵N]Asp or [U-¹³C-¹⁵N]glutamine). After separation of *P. falciparum* from red blood cells, an LC-MS analysis of its central metabolites showed that this parasite relies on glucose fermentation for energy, but its branched TCA cycle is disconnected from glycolysis (i.e. its TCA cycle metabolites are synthesized from glutamate/glutamine). Furthermore, heliobacteria (a phylum of anaerobic anoxygenic phototrophic bacteria) were considered to have an incomplete reverse TCA cycle owing to their lack of a conventional CIT synthase. Recently, Tang *et al.* [9] reported that the oxidative TCA route (OAA → CIT → α-ketoglutarate) is operative via a novel (*Re*)-CIT synthase in contrast to the conventional (*Si*)-CIT synthase. The stereo-specific enzyme reaction of (*Re*)-CIT synthase has been revealed by tracing ¹³C transitions from OAA (reflected by Asp) to α-ketoglutarate (reflected by glutamate). The *in vivo* activity of (*Re*)-CIT synthase has also been found in other anaerobic bacteria via ¹³C tracing along with the heterologous gene expression method. The (*Re*)-CIT synthases from *Clostridium* and *Desulfovibrio* are phylogenetically related to isopropylmalate synthase [27–29]. Meanwhile, a new (*Re*)-CIT synthase (previously annotated as homoCIT synthase) is reported to be responsible for the TCA pathway function in *Dehalococcoides* [30].

2.2. CO₂ fixation pathways

The Calvin–Benson cycle is the common autotrophic CO₂ assimilation pathway in microalgae. Using labelled carbon substrates and unlabelled CO₂, Feng *et al.* [20] have studied the mixotrophic metabolism in a hydrogen-producing cyanobacterium *Cyanothece* 51142. They

Figure 2. Metabolic pathways revealed by ^{13}C fingerprints. DHAP, dihydroxyacetone phosphate; E4P, erythrose-4-phosphate; F6P, fructose-6-phosphate; S7P, sedoheptulose-7-phosphate; ICT, isocitrate.

found that the CO_2 fixation rate is dependent on both the nitrogen source and the carbon substrate. Under favourable conditions, with glycerol and nitrate, *Cyanothece* 51142 can switch its mixotrophic metabolism to photoheterotrophic metabolism to promote biomass growth. Under such conditions, *Cyanothece* still uses the non-autotrophic CO_2 fixation pathway via PEP carboxylase.

Furthermore, AcCoA-related carbon fixation pathways have been recently reported in different micro-organisms. These pathways include the reverse TCA cycle, the Wood-Ljungdahl pathway, dicarboxylate/4-hydroxybutyrate cycle, 3-hydroxypropionate/4-hydroxybutyrate cycle, the 3-hydroxypropionate bi-cycle and the ethylmalonyl-CoA pathway (EMCP). These alternative autotrophic pathways may lead to new insights into the global carbon cycle, the early evolution of metabolism and interpretations of geological records [31]. Feng *et al.* [13] have studied a green sulphur bacterium, *Chlorobaculum tepidum*, for acetate and PYR metabolism, in which they reported three routes for CO_2 fixation: the reverse TCA cycle, $\text{AcCoA} + \text{CO}_2 \rightarrow \text{PYR}$ and $\text{PEP} + \text{CO}_2 \rightarrow \text{OAA}$. Moreover, *Dehalococcoides ethenogenes* 195 has been speculated to use the Wood-Ljungdahl pathway because its genome contains several key enzymes in this pathway. However, the isotopomer data disproved the complete function of this pathway when strain 195 was

grown with H_2 , trichloroethylene (TCE) and acetate [14]. The EMCP has been proposed in the photosynthetic proteobacterium *Rhodobacter sphaeroides* [32]. This pathway starts from AcCoA, and forms intermediate metabolites, including ethylmalonyl-CoA, methylsuccinyl-CoA, methylmalonyl-CoA and propionyl-CoA. The propionyl-CoA can then be converted to malate (MAL) in the TCA cycle. The net reaction of EMCP is: $\text{AcCoA} + 2\text{CO}_2 \rightarrow 2 \text{ glyoxylate} + \text{CoA}$. Peyraud *et al.* [18] applied $[1-^{13}\text{C}]$ acetate and ^{13}C metabolomics to determine the order of biochemical reactions starting from AcCoA during growth of *Methylobacterium extorquens* AM1 on methanol. During short-term ^{13}C -labelling experiments, an LC-MS analysis of CoA-based intermediate metabolites tracked ^{13}C percolating through cell metabolism. Meanwhile, they used ^{13}C -labelled methanol in combination with NMR so as to provide final demonstration of the methanol utilization pathway by measurement of proteinogenic glycine labelling. This study not only discovered the operation of the EMCP, but also identified a serine cycle to assimilate methanol and convert the EMCP product glyoxylate to central metabolites. In the serine cycle, glyoxylate produces glycine, and glycine assimilates methanol through a 5,10-methylene-THF (C1) unit (N^5 , N^{10} -methylene-tetrahydrofolate) to form serine. Serine can be converted to 2-phosphoglycerate that enters

glycolysis and the TCA cycle. The complete serine cycle fixes both CO₂ and methanol (or methane). Furthermore, Okubo *et al.* [19] reported that *M. extorquens* AM1 is able to condense glyoxylate and AcCoA to MAL (the glyoxylate shunt) via a novel maly-CoA/ β -methylmaly-CoA lyase. This finding indicates an alternative route responsible for glyoxylate consumption, besides the serine cycle. Later on, Schneider *et al.* [33] used LC-MS-based ¹³C-MFA to profile the carbon fluxes through three metabolic cycles for acetate metabolism in *M. extorquens* (metabolic activities: the TCA cycle > the EMCP > the serine cycle).

The filamentous anoxygenic phototrophic bacterium *Chloroflexus aurantiacus* uses the 3-hydroxypropionate bi-cycle for CO₂ fixation [21]. Similar to the EMCP, the first cycle starts from AcCoA and forms malonyl-CoA by fixing one CO₂. The malonyl-CoA is the precursor of 3-hydroxypropionate. Via multiple enzymatic steps, 3-hydroxypropionate is converted to propionyl-CoA, and subsequently to maly-CoA by fixing another CO₂. The maly-CoA splits to glyoxylate and AcCoA as the end products from the first cycle of the 3-hydroxypropionate bi-cycle. The second cycle consumes the glyoxylate from the first cycle of the 3-hydroxypropionate bi-cycle. Glyoxylate and propionyl-CoA form β -methylmaly-CoA, followed by three reactions to generate citramaly-CoA; the citramaly-CoA is then cleaved into AcCoA and PYR to form the second cycle.

Finally, two additional CO₂ fixation cycles (dicarboxylate/4-hydroxybutyrate cycle and 3-hydroxypropionate/4-hydroxybutyrate cycle) have been discovered via ¹³C and ¹⁴C isotopic labelling. Both cycles contain a key metabolic route (i.e. 4-hydroxybutyrate pathway: succinyl-CoA (SucCoA) $\rightarrow$ 4-hydroxybutyrate $\rightarrow$ acetoacetyl-CoA $\rightarrow$ AcCoA). A recent review has a thorough discussion of the two CO₂ fixation cycles in various environmental micro-organisms, including enzyme steps, energy (cofactor) demand and thermodynamic equilibrium of each reaction [31].

2.3. Unconventional biosynthesis pathways

¹³C experiments are useful tools to check annotated biosynthesis pathways. A recent report confirms diverse amino acid synthesis routes in the pathogen *Streptococcus pneumoniae*, using labelled glucose and glycine [34]. It is the first ¹³C study of this pathogen that revealed multiple carbon usage (i.e. identifying, that some amino acids were unlabelled and taken from the medium, whereas others were made de novo). In some non-model micro-organisms, ¹³C experiments may find unconventional biosynthesis pathways. The citramalate pathway is an alternate pathway for isoleucine synthesis in some anaerobic bacteria (such as *Geobacter* species and *Thermoanaerobacter* species) and methanogenic Archaea [16,35–37]. Recently, this threonine-independent pathway (using PYR and AcCoA as the precursors) has also been discovered in photosynthetic bacteria, including *Rsb. denitrificans* [7], *Hbt. modesticaldum* [9], *Cba. tepidum* [13] and *Cyanothece* 51142 [38]. The citramalate synthase can be used to engineer the carbon flux to 2-ketobutyrate (a precursor of butanol), and thus promote alcohol biosynthesis [39]. Moreover, glyoxylate can be an alternative precursor

instead of glycerate 3-phosphate (G3P) for synthesis of glycine and serine. For example, *Mycobacterium smegmatis* may employ glycine dehydrogenase to convert glyoxylate to glycine and consume excess NADH under oxygen-limited growth conditions [40]. This amino acid synthesis route forms an initial step to remove glyoxylate from the EMCP in *M. extorquens*, generating serine and subsequently metabolites for glycolysis [18].

The ¹³C technique has been applied to investigate the biosynthesis of the building blocks of products. Tao *et al.* [41] applied ¹³C-NMR to study *Aspergillus glaucus* (a filamentous fungus) for the biosynthesis of aspergiolide A (a novel anti-tumour compound). Through tracer experiments using singly or fully labelled ¹³C-labelled sodium acetate in a nutrient-rich medium followed by ¹³C-NMR spectroscopic investigation, they found that acetate is the only carbon source for the polyketide pathway for aspergiolide A synthesis, and 12 carbon atoms of the final product are derived from the carboxylic group of acetate. Another example is the investigation of the carbon sources for ergosterol biosynthesis in *Trypanosoma brucei* (a parasite that causes African trypanosomiasis) [42]. Via ¹³C-labelled lanosterol, acetate, leucine and glucose labelling, this study analysed the incorporation of ¹³C into sterols and investigated the operation of the sterol metabolic network in the parasite. The results demonstrate sets of coordinated pathways to yield distinct sterol profiles, including glucose metabolism in the glycosome, AcCoA synthesis and leucine catabolism in the mitochondria, the acetate mevalonate pathway and the isopentenyl pyrophosphate pathway (regulated in the cytosol), and ergosterol synthesis reactions.

2.4. Carbon nutrient utilization

¹³C-labelled experiments are used extensively to study the co-utilization of carbon nutrients by micro-organisms. In such experiments, one of the carbon substrates is ¹³C labelled, while other substrates are naturally labelled. By measuring the labelling enrichment in central metabolites, we can quantify the co-catabolism of carbon nutrients. For example, Feng *et al.* [20] have examined the uptake of ¹³C-labelled glucose by *Cyanothece* 51142 under CO₂ photoautotrophic growth conditions. The results showed that *Cyanothece* 51142 cannot use glucose efficiently owing to the lack of a specific glucose transporter. Furthermore, by tracing ¹³C enrichment of the TCA cycle metabolites using a bis(trimethylsilyl)trifluoroacetamide-based GC-MS method, Tang *et al.* [40] have reported that *M. smegmatis* upregulates Tween 80 utilization once it enters hypoxia. Meanwhile, ¹³C-based metabolomics [12] revealed that *M. tuberculosis* catabolizes multiple carbon sources (glucose, acetate and glycerol) simultaneously to achieve optimal biomass growth. Finally, a ¹³C experiment can optimize nutritional conditions for slow-growing micro-organisms. To differentiate selective utilization of exogenous amino acids by *D. ethenogenes* 195, ¹³C-labelled acetate and non-labelled amino acids have been used in the culture medium [15]. After five subcultures in the rich medium with labelled acetate, strain 195 showed significant improvement of biomass growth and TCE degradation. The analysis of ¹³C enrichment in proteinogenic amino

acids from those subcultures implied that phenylalanine, isoleucine, leucine and methionine are most highly imported from the culture medium, while strain 195 cannot efficiently uptake other amino acids to promote biomass synthesis. The cultivation experiments showed that the four highly used amino acids can enhance biomass growth and dechlorination activities to levels similar to those observed in a rich medium.

3. METABOLIC FLUX PROFILING IN NON-MODEL MICRO-ORGANISMS VIA ^{13}C FINGERPRINTS

^{13}C -MFA uses ^{13}C fingerprints to calculate intracellular metabolic fluxes. ^{13}C -MFA has been extensively applied to study model micro-organisms and their mutants, such as *Escherichia coli*, *Bacillus subtilis* and *Saccharomyces cerevisiae*. Here, ^{13}C -MFA has also been shown to be a powerful tool to answer unresolved metabolic questions in non-model microbial species, including cofactor generation/consumption, regulation of pathways under different growth conditions, and operation of complicated metabolic cycles.

The Calvin–Benson cycle is considered to be indispensable for photoheterotrophic metabolisms. McKinlay *et al.* [43] studied a photoheterotrophic bacterium (*Rhodospseudomonas palustris*). They found that cells oxidize ^{13}C -labelled acetate to CO_2 via the glyoxylate shunt and the partially functioning TCA cycle, and then fix 68 per cent of this CO_2 into biomass via the Calvin–Benson cycle. The Calvin–Benson cycle in *R. palustris* actually plays a major role in cofactor recycling, which can reoxidize nearly half of the reduced cofactors (NADH) generated during conversion of acetate to biomass. Meanwhile, in a nitrogenase active mutant, CO_2 fixation by the Calvin–Benson cycle is downregulated, because *R. palustris* channels NADH to produce H_2 . By further analysing *R. palustris* growth with different ^{13}C substrates (fumarate, SUC, acetate or butyrate), the same authors [44] determined the factors that influence the H_2 yield of *R. palustris*. Their ^{13}C -MFA results indicate that the Calvin–Benson cycle and H_2 production are two key pathways to compete for the reducing equivalents generated during the oxidation of organic carbon sources. Thus, inhibition of the Calvin–Benson cycle will promote H_2 production.

^{13}C -MFA can be used to examine the mixotrophic metabolisms. For example, it is well known that the green sulphur bacteria use the reverse TCA cycle for CO_2 fixation, but not much was known about its electron-balancing function [45]. ^{13}C -MFA has been used in quantitative analysis of reducing equivalents in *Cba. tepidum* under mixotrophic growth conditions [13]. The fluxomics not only estimated the absolute CO_2 fixation by the reverse TCA cycle during assimilation of acetate or PYR, but also revealed the reducing equivalents harvested from phototrophic reactions.

Young *et al.* [17] applied dynamic isotopic labelling analysis to decipher the autotrophic metabolism of *Synechocystis* sp. PCC6803. They built an isotopically non-stationary MFA (INST-MFA) to quantify the

carbon flux from CO_2 to the overall central metabolism. Their results indicated that a significant amount of the fixed carbon is lost via the oxidative PP pathway, which had been previously considered to be minimal in phototrophic metabolisms. Their INST-MFA also indicated that the glyoxylate shunt is absent, the measurable glyoxylate flux is redirected to the photorespiratory pathway and the malic enzyme flux ($\text{MAL} \rightarrow \text{PYR}$) is the rate-limiting step of biomass synthesis. A recent paper [46] reported two new enzymes that perform the function of the missing α -ketoglutarate dehydrogenase in cyanobacteria to close the TCA cycle. However, INST-MFA indicated no measurable flux from α -ketoglutarate to SUC.

^{13}C -MFA has wide applications in microbes important for bioremediation, disease control and bioenergy. Chavarría *et al.* [47] used ^{13}C -MFA to analyse the phosphoenolpyruvate phosphotransferase system (PTS) in *Pseudomonas putida*, a soil bacterium capable of degrading polycyclic aromatic hydrocarbons. By comparing glucose and fructose metabolisms in both wild-type and PTS mutant strains, they showed that a PTS mutant strain lacking PTS^{Ntr} (N-related PTS that controls cellular functions unrelated to the transport of carbohydrates) displays significantly higher fluxes in the PYR shunt ($\text{MAL} \rightarrow \text{PYR}$), which indicates that PTS^{Ntr} regulates *P. putida* sugar metabolism by mediating its TCA cycle. ^{13}C -MFA was also used to probe the metabolism of the pathogen *Mycobacterium bovis*. The results showed that carbon fluxes through a complete TCA cycle are minimal, while the PYR is metabolized via the glyoxylate shunt coupled with the anaplerotic reactions [48]. In the bioenergy field, ^{13}C -MFA has recently been applied to study oleaginous *Chlorella protothecoides* for oil production [49]. Under heterotrophic conditions, the isotopic fingerprints in proteinogenic amino acids were used to study the fluxes through glycolysis, the PP pathway and the TCA cycle. The results indicated that the PP pathway is upregulated, while global flux distribution remains relatively stable after the culture was switched from a nitrogen-sufficient condition to a nitrogen-limited condition.

4. RECENT ADVANCES IN ^{13}C TECHNIQUES

Paralleling the application of ^{13}C techniques to identify functional pathways and quantify metabolic fluxes, good progress has been recently made in improving ^{13}C techniques.

4.1. Dynamic flux analysis

Traditionally, ^{13}C -MFA is inferred from steady-state distributions of carbon–carbon bonds of metabolites from different metabolic pathways. However, INST-MFA can speed up the experimentation for industrially relevant processes (e.g. non-growing cells or fed batch cultures) with moderately changing process conditions [50]. Such non-stationary ^{13}C -MFA depends on dynamic carbon-labelling experiments for metabolic steady-state cultures (after medium replacement with ^{13}C substrate, measuring multiple labelling samples

during the isotopically transient time course) [51]. Proof-of-concept studies have developed algorithms to decipher fluxes based on measured metabolite pool sizes and isotopomer concentrations [51–53], and to speed up INST-MFA calculation [54]. A recent milestone INST-MFA paper on environmental microbes resolved photoautotrophic metabolism in cyanobacteria [17]. A steady-state culture using single carbon CO₂ as the sole carbon source cannot provide any constraints for solving the flux distribution [55]. To overcome this problem, this pioneering study investigated the photoautotrophic metabolism by examining the change of isotopomer distributions in fast-turnover metabolites immediately after step-switching the carbon source from ¹²CO₂ to ¹³CO₂ [17]. Such INST-MFA estimates both intracellular metabolic fluxes and metabolite pool sizes, on the basis of the measured isotope labelling dynamics of 16 metabolites. The tracking of free metabolites uses both LC-MS and GC-MS. As expected, INST-MFA requires solving nonlinear differential equations to track the labelling of metabolites as a function of time. To reduce the computational complexity of INST-MFA, the model employs an elementary metabolite unit (EMU) approach to simplify simulation of isotopomer distributions throughout the metabolic network [56].

Exploratory studies have also been performed to analyse metabolic unsteady-state bioprocesses to obtain global enzyme kinetic parameters [57]. The Sauer group reported the use of dynamic amino acid labelling to obtain time-dependent fluxomics results from increasing glucose limitation in a *B. subtilis* culture overproducing riboflavin [58]. Because metabolic transition in the culture is slow, dynamic ¹³C-MFA decomposes the culture process into numerous quasi-steady states (time interval = 30 ~ 60 min). Then steady-state ¹³C-MFA can be used to resolve the fluxomics and estimate the cofactor balance at different quasi-steady time intervals (mini-¹³C-MFA). Through snapshots of the metabolism at various time points, the dynamic metabolism of *B. subtilis* was revealed. Another milestone dynamic MFA paper reported a new approach to profile *E. coli* metabolism in a fed-batch process [59]. Via a rigorous statistical analysis, inflection time points can be determined so that the MFA model divides the time domain of the entire fed batch culture into several intervals where the fluxes have linear change in each interval. Then the algebraic solutions to time-dependent metabolite mass balance equations can be obtained to facilitate the calculation of dynamic fluxes in each time interval.

4.2. Microbial community studies

In microbial communities, species evolve cross-feeding interactions (one species obtains nutrients from another). Microbial communities play important roles in ecology, bioremediation, biofuel production and biomedical research [60]. However, these communities present great modelling challenges because flux models for communities have to simulate both the metabolism in individual species and the exchange of metabolites among species. In the past, an isotope-

based microbial community study usually treated the community as a single metaorganism without considering the metabolic interactions between individual subspecies. For example, ¹³C-MFA was applied for gut microflora to review the overall nutrient metabolism in intestinal bacteria [61]. By linking MFA with the other systems-biology technologies, researchers can have insights into the metabolic regulations involved in microbe–host mutualism and their relevance for health. Besides, communities are also characterized in terms of a subcompartment-based flux balance analysis [60,62], where the intracellular flux in subspecies and the metabolite interactions between species are predicted using objective functions (e.g. optimal biomass growth). Such an analysis (without using isotopic labelling) is not precise because true metabolisms in the communities are often suboptimal. Therefore, it is beneficial to employ novel ¹³C-MFA to facilitate understanding the links between the physiology of individual species and their community characteristics. However, traditional ¹³C-MFA has not been readily applicable to mixed cultures because it is difficult to obtain ¹³C-labelling patterns of metabolites from a given population within a mixed culture. The targeted species have to be separated using centrifugation or fluorescence-assisted cell sorting. Unfortunately, centrifugation separates only species with sufficient density differences, while fluorescence-assisted sorting takes too long to obtain enough cells for a reliable ¹³C-labelling pattern analysis. To overcome this problem, the Sauer group [63] invented a proof-of-concept ¹³C-MFA of a microbial community. In their approach, a reporter protein is used to assist the subpopulation-specific MFA of *E. coli* mutants in a mixed culture of *E. coli* strains. Specifically, a reporter protein that has been synthesized in only one species of the consortium can be separated via protein purification, and thus ¹³C patterns of proteinogenic amino acids from the reporter protein can be used to resolve the fluxes in this single species. This method (i.e. labelling patterns of amino acids from purified proteins can be used to infer metabolic fluxes of targeted organisms in a mixed culture) may eventually extend ¹³C-MFA to study more complex community systems [64].

4.3. Investigation on host–pathogen systems

¹³C-labelling approaches (such as MS-based amino acid analysis) have been used to examine the metabolic interactions between a microbial pathogen and its host [65]. The investigation of host–pathogen systems not only provides understanding of the key central pathways for survival of pathogens (as described in §2.1, [26]), but also reveals host responses to pathogen infections. Combined with other experimental techniques, ¹³C isotopic labelling gives important medical insights into the treatment of pathogens. For example, *Listeria monocytogenes* is a food-borne pathogen that can cause invasive infection in susceptible humans. For proliferation within hosts, this facultative intracellular pathogen uses specific metabolic pathways (e.g. an incomplete TCA cycle owing to a lack of 2-ketoglutarate dehydrogenase) for nutrient utilization during infection [66].

A $^{13}\text{C}/^{14}\text{C}$ -labelling study on extracellular *L. monocytogenes* cultures demonstrated that the ATP-dependent PYR carboxylase (*pycA*) plays an important role in its central carbon metabolism. Later on, the virulence of the *pycA* defective mutant was shown to be highly attenuated in mouse infection experiments [67]. To decipher the metabolism of pathogens inside host cells during the infection phase, Götz *et al.* [68] supplied $[\text{U-}^{13}\text{C}]$ glucose to the enteroinvasive *E. coli* and *Salmonella enterica* replicating in epithelial colorectal adenocarcinoma cells. To separate the pathogens and infected host cells after ^{13}C -labelling cultivation, they lysed host cells to release bacteria first; then removed the host cell debris from bacteria by repeated centrifugations. Such a separation protocol minimized the contamination of the host cell protein fraction with bacterial proteins (less than 10%), and thus the proteo-genic amino acids from host cells or bacteria could be isolated for GC-MS measurements. By comparing the labelling patterns in bacterial amino acids from wild-type and mutant strains (e.g. devoid of sugar uptake systems), they revealed the carbon sources (e.g. glucose, glucose phosphate, glycerol, lactate, PYR, amino acids) used by pathogens as well as bacterial metabolic pathways (e.g. anaplerotic reactions and the ED pathway) during the infections. However, the pathogen infections seem not to affect the carbon metabolism of the used Caco-2 host cells, as judged by ^{13}C incorporation into host cell amino acids.

4.4. Metabolism profiling using integrated approaches

The ^{13}C technique is often applied with other approaches, including genome-scale reconstruction, enzyme assay, metabolite profiling, genetic manipulation (i.e. knockout of key enzymes) and transcription expression measurements. First, mis-annotations can be easily generated from genome sequences and propagated through databases. ^{13}C metabolism analysis is useful for the development and validation of new methods for accurate gene predictions. For example, ^{13}C -labelled leucine experiments have identified the leucine degradation pathway in *B. subtilis*, and facilitated the development of a new computational algorithm (an automatic policing method) to detect biochemical mis-annotations [69]. Second, unknown genes may be revealed by integrating the ^{13}C technique with genomic analyses. For example, Veit *et al.* [70] analysed co-metabolism of acetate and glucose in *Corynebacterium glutamicum*. Via ^{13}C labelling, genetic mutation, enzyme and microarray analysis, they not only profiled the fluxomics during co-utilization of acetate and glucose, but also discovered a redundant gene encoding CoA transferase that can activate acetate and propionate in the presence of glucose. Third, for genome-scale metabolic reconstruction, integration of metabolic models with ^{13}C -MFA can unravel the topology of cell metabolism. For example, recent work combined *in silico* metabolic network reconstruction with ^{13}C fluxomics and found that methanol assimilation pathways in *M. extorquens* operate as a unique process, which is tightly connected by several major metabolic cycles (serine cycle, EMCP, TCA cycle and anaplerotic

processes) [71]. Moreover, ^{13}C -MFA can measure gene regulation by correlating fluxomics (functional output) with multiple-level genomics data (such as transcription and protein profiles). In a global investigation of *B. subtilis*, it has determined the influence of the ferric uptake regulator (Fur) on the TCA cycle fluxes [72]. The Sauer group has also examined metabolic adaptation across multiple levels of regulation in *B. subtilis*, including transcriptomics, proteomics, promoter activities, metabolomics, fluxomics and biomass growth. Such analyses reveal controlling genes responsible for metabolic adaptation and post-transcriptional regulatory mechanisms [73].

4.5. New analytical and computational tools for ^{13}C -metabolic flux analysis

The scope and the resolution of ^{13}C -MFA can be improved by metabolomics tools (GC-MS and LC-MS) to obtain extensive ^{13}C fingerprints. Rühl *et al.* [74] applied LC-MS/MS to measure both intact and fragmented central metabolites, obtaining complete ^{13}C fingerprints in each metabolite. Because collisional fragmentation of metabolites may partially give ^{13}C positional information for certain isotopomers, LC-MS/MS-based ^{13}C -MFA improved estimations of fluxes in the PP pathway, glycolysis and the TCA cycle. Moreover, GC-C-IRMS is used for analysis of isotopomers when ^{13}C enrichment in the metabolites is low (well below 10%). The GC-C-IRMS isotopomer data allow ^{13}C -MFA to provide good flux resolution even when the tracer experiments are performed using very low ^{13}C concentration substrates in order to reduce the cost of ^{13}C substrates [75].

NMR is a powerful tool to probe ^{13}C in organic chemicals [76]. NMR has been extensively used for collecting isotopic data and has provided valuable metabolic insights into a broad range of biological systems [23]. Although recent studies on environmental microbes (after 2009) have relied more on MS techniques because of low cost/high sensitivity of metabolite measurement, NMR still plays an important role in metabolism studies. For example, by measuring time courses for ^{13}C enrichment in metabolites using NMR, a kinetic model quantified the fluxes through human gut bacterial metabolism [61]. In general, NMR identifies the positional labelling information in metabolites, while MS measures the total labelling enrichment in the metabolites. So combining NMR with MS techniques can provide complementary isotopic labelling information to reveal novel metabolic insights [18,77].

Computational tools for high-performance ^{13}C -MFA have been continuously published in the past 3 years. User-friendly software (such as ^{13}C -FLUX [78]) allows scientists with little programming knowledge to perform ^{13}C fluxomics studies. A ^{13}C -MFA toolbox, OpenFLUX, is a computationally efficient software package [79]. The software incorporates a high-performance EMU framework (a decomposition method identifying the minimum amount of information needed to simulate isotopic labelling) for calculation of isotopomer balances within a reaction network [56]. For a more detailed introduction to computational tools, a recent review paper summarized major computational models and

Figure 3. Example of glycolysis channelling. It provides an alternative route for the labelling of PYR. The filled boxes represent labelled carbon. When the first position glucose is used as the carbon source, the oxidative PP pathway (green line) generates only non-labelled G3P and PYR. Both the ED pathway (red line) and glycolysis channelling (orange line) increase the labelled PYR fractions (measured from alanine), but reduce labelled G3P fractions (measured from serine).

software [80], covering areas of both metabolic reconstruction tools and ^{13}C flux analysis platforms. Finally, new mathematical algorithms are proposed to reformulate ^{13}C -MFA problems and simplify the flux calculation. Recently, a new set of variables (called ‘fluxomers’) has been invented by combining both fluxes and isotopomer abundances [81]. The fluxomers represent the rate of a metabolic reaction transferring labelling from substrate isotopomers to product isotopomers. Such variables provide efficient computation of metabolic fluxes and show robustness to measurement noise and initial conditions.

5. INFLUENTIAL FACTORS IN ^{13}C METABOLISM ANALYSIS

Several factors that may affect ^{13}C metabolism analysis have been reported recently. First, metabolite channelling resulting from the formation of multi-enzyme complexes is common in some eukaryotes [82]. In this process, metabolites in metabolic pathways are transferred from enzyme to enzyme without being released into the bulk phase of the cell. The channelling can change isotopomer fingerprints because it may prevent the randomization of orientation for symmetric metabolites [83]. For example, the orientations of the TCA cycle metabolites (SUC and MAL) can be conserved in *S. cerevisiae* in the pathway from $[4-^{13}\text{C}]\alpha$ -ketoglutarate to OAA [84]. Because of the channelling of SUC/fumarate formation enzymes (SucCoA synthetase, SUC dehydrogenase and fumarase), the ^{13}C fractions in the second and third carbon in OAA resulting from $[4-^{13}\text{C}]\alpha$ -ketoglutarate are not completely randomized (i.e. $^{13}\text{C}_2/^{13}\text{C}_3 \neq 1$). Because ^{13}C -MFA assumes a homogeneous condition for all intracellular metabolites, channelling may affect ^{13}C -MFA-based flux results. In mammalian cells, isotopic experiments (^3H and ^{13}C) have been used to investigate metabolite channelling and compartmentation in cytosolic and mitochondrial metabolite pools [82,85]. In plant cells, channelled

fluxes can be detected in glycolysis and the TCA cycle. Therefore, it is suggested that ^{13}C -MFA of eukaryotes may require correction coefficients to account for the impact of channelling on the redistribution of ^{13}C in key metabolites [83].

Moreover, metabolic channelling has also been discovered in bacterial species. For example, the channelling phenomenon in *E. coli* has been investigated by feeding cells with ^{14}C -labelled glucose and ^{14}C -labelled glycolytic intermediates [86]. By measuring the evolved $^{14}\text{CO}_2$ production, the authors concluded that fructose 1,6-bisphosphate (FBP) was strongly channelled all the way to CO_2 , whereas fructose-6-phosphate (F6P) was not. Metabolic channelling may provide an alternative route for the labelling of pathway intermediates, leading to a change in the isotopic fingerprints (figure 3). Similarly, a ^{13}C INST-MFA of autotrophic metabolism finds metabolic channelling in *Synechocystis* 6803 [17]. Specifically, PEP showed higher ^{13}C enrichments than other metabolites right after the cells were fed with $^{13}\text{CO}_2$, while the Calvin–Benson cycle intermediates (e.g. F6P, glyceraldehyde-3-phosphate (GAP) and ribose-5-phosphate (R5P)) were significantly less labelled than their downstream products, such as ribulose-5-phosphate (Ru5P) and ribulose-1,5-bisphosphate (RuBP). To correct for this effect, the flux model has to include ‘dilution parameters’ for these Calvin–Benson cycle intermediates. In other words, ^{13}C INST-MFA becomes a new tool for investigating the occurrence and extent of enzymatic channelling in microbial species.

Isotopic fractionation is present if ^{13}C and ^{12}C substrates are both in the medium. Enzymes show subtle selectivity in the uptake of their ^{12}C and ^{13}C substrates. It has been well established that, by using the CO_2 -fixing enzyme RuBisCO, photosynthetic micro-organisms favour $^{12}\text{CO}_2$ over $^{13}\text{CO}_2$. The reported isotopic fractionation, $\delta^{13}\text{C}$ (a measure of the relative ratio of $^{13}\text{C}:^{12}\text{C}$, reported in parts per thousand), of biological processes (Cyanobacteria or Calvin–Benson cycle plants) can be over -20 [87]. Feng and Tang [88]

examined the effect of isotopic fractionation on flux analysis using 20 per cent fully labelled glucose and 80 per cent naturally labelled glucose. The measured $\delta^{13}\text{C}$ (based on several key amino acids) is around -40 , which is just above the GC-MS measurement errors (instrumental bias may also contribute to $\delta^{13}\text{C} \approx -20$ or higher). The study also pointed out that commercial fully labelled glucose from the market often contains 3–4% of five-carbon labelled glucose, and such isotopic impurity in ^{13}C substrates causes a much greater impact on flux calculation than does isotopic fractionation. Therefore, isotopic fractionation, though evident, has a smaller effect on conventional ^{13}C -MFA than other experimental noise sources. If tracer experiments employ a very low labelling percentage of ^{13}C substrates (well below 10%) to save experimental cost, the isotope effect on ^{13}C -MFA is reported to be higher [75]. In such tracer experiments, ^{13}C enrichment data for metabolites (measured by sensitive GC-C-IRMS) can be corrected using a mathematical approach before using the isotopomer data for flux calculations.

6. CONCLUSIONS

Metabolism analysis through ^{13}C isotopic fingerprints is a powerful tool to map microbial ultimate phenotypic outcomes, both qualitatively and quantitatively. New methods of ^{13}C metabolism analysis are being developed and greatly facilitate the analytic resolution and scope, and provide complementary knowledge to results obtained from other ‘omics’ approaches. ^{13}C -based metabolism analyses have often been used to profile industrial microbial metabolisms in order to improve their product yields, but few successful cases for significant improvements of product yields through ^{13}C -MFA have been demonstrated so far. On the other hand, recent studies show the significant value of ^{13}C metabolism analyses of poorly characterized environmental micro-organisms as well as human pathogens. Many non-model microbes play important roles in the processes involved in ecology, disease, biofuels and bioremediation, and therefore the ^{13}C technique can be widely applied to speed up their screening and provide us with novel metabolic insights and benefits there from.

This study was supported in part by an NSF career grant (MCB0954016) to Y.J.T. and a NASA exobiology grant (NNX12AD85G) to R.E.B. J.K.H.T. was supported by start-up funds from Clark University. We are thankful for Dr Danny Kohl’s useful discussion on channelling, and Dr Xueyang Feng’s useful comments on ^{13}C -MFA.

REFERENCES

- Zamboni, N., Fendt, S. M., Rühl, M. & Sauer, U. 2009 ^{13}C -based metabolic flux analysis. *Nat. Protoc.* **4**, 878–892. (doi:10.1038/nprot.2009.58)
- Zamboni, N. & Sauer, U. 2009 Novel biological insights through metabolomics and ^{13}C -flux analysis. *Curr. Opin. Microbiol.* **12**, 553–558. (doi:10.1016/j.mib.2009.08.003)
- Wiechert, W. 2001 ^{13}C metabolic flux analysis. *Metab. Eng.* **3**, 195–206. (doi:10.1006/mben.2001.0187)
- Wittmann, C. 2007 Fluxome analysis using GC-MS. *Microb. Cell Factories* **6**, 6. (doi:10.1186/1475-2859-6-6)
- Wittmann, C. 2002 Metabolic flux analysis using mass spectrometry. *Adv. Biochem. Eng./Biotechnol.* **74**, 39–64. (doi:10.1007/3-540-45736-4_3)
- Tang, Y. J., Martin, H. G., Myers, S., Rodriguez, S., Baidoo, E. K. & Keasling, J. D. 2009 Advances in analysis of microbial metabolic fluxes via ^{13}C isotopic labeling. *Mass Spectrom. Rev.* **28**, 362–375. (doi:10.1002/mas.20191)
- Tang, K.-H., Feng, X., Tang, Y. J. & Blankenship, R. E. 2009 Carbohydrate metabolism and carbon fixation in *Roseobacter denitrificans* OCh114. *PLoS ONE* **4**, e7233. (doi:10.1371/journal.pone.0007233)
- Nunn, C. E., Johnsen, U., Schönheit, P., Fuhrer, T., Sauer, U., Hough, D. W. & Danson, M. J. 2010 Metabolism of pentose sugars in the hyperthermophilic archaea *Sulfolobus solfataricus* and *Sulfolobus acidocaldarius*. *J. Biol. Chem.* **285**, 33 701–33 709. (doi:10.1074/jbc.M110.146332)
- Tang, K.-H., Feng, X., Zhuang, W.-Q., Alvarez-Cohen, L., Blankenship, R. E. & Tang, Y. J. 2010 Carbon flow of *Hellobacterium modesticaldum* is more related to *Clostridia* than to the green sulfur bacteria. *J. Biol. Chem.* **285**, 35 104–35 112. (doi:10.1074/jbc.M110.163303)
- Watanabe, S., Zimmermann, M., Goodwin, M. B., Sauer, U., Barry III, C. E. & Boshoff, H. I. 2011 Fumarate reductase activity maintains an energized membrane in anaerobic *Mycobacterium tuberculosis*. *PLoS Pathog.* **7**, e1002287. (doi:10.1371/journal.ppat.1002287)
- Marrero, J., Rhee, K. Y., Schnappinger, D., Pethe, K. & Ehrt, S. 2010 Gluconeogenic carbon flow of tricarboxylic acid cycle intermediates is critical for *Mycobacterium tuberculosis* to establish and maintain infection. *Proc. Natl Acad. Sci. USA* **107**, 9819–9824. (doi:10.1073/pnas.1000715107)
- de Carvalho, L. P., Fischer, S. M., Marrero, J., Nathan, C., Ehrt, S. & Rhee, K. Y. 2010 Metabolomics of *Mycobacterium tuberculosis* reveals compartmentalized co-catabolism of carbon substrates. *Chem. Biol.* **17**, 1122–1131. (doi:10.1016/j.chembiol.2010.08.009)
- Feng, X., Tang, K.-H., Blankenship, R. E. & Tang, Y. J. 2010 Metabolic flux analysis of the mixotrophic metabolisms in the green sulfur bacterium *Chlorobaculum tepidum*. *J. Biol. Chem.* **285**, 39 544–39 550.
- Tang, Y. J., Yi, S., Zhuang, W., Zinder, S. H., Keasling, J. D. & Alvarez-Cohen, L. 2009 Investigation of carbon metabolism in ‘*Dehalococcoides ethenogenes*’ strain 195 via isotopic and transcriptomic analysis. *J. Bacteriol.* **191**, 5224–5231. (doi:10.1128/JB.00085-09)
- Zhuang, W. Q., Yi, S., Feng, X., Zinder, S. H., Tang, Y. J. & Alvarez-Cohen, L. 2011 Selective utilization of exogenous amino acids by *Dehalococcoides ethenogenes* strain 195 and its effects on growth and dechlorination activity. *Appl. Environ. Microbiol.* **77**, 7797–7803. (doi:10.1128/AEM.05676-11)
- Feng, X. et al. 2009 Characterization of the central metabolic pathways in *Thermoanaerobacter* sp. x514 via isotopomer-assisted metabolite analysis. *Appl. Environ. Microbiol.* **75**, 5001–5008. (doi:10.1128/AEM.00715-09)
- Young, J. D., Shastri, A. A., Stephanopoulos, G. & Morgan, J. A. 2011 Mapping photoautotrophic metabolism with isotopically nonstationary ^{13}C flux analysis. *Metab. Eng.* **13**, 656–665. (doi:10.1016/j.ymben.2011.08.002)
- Peyraud, R., Kiefer, P., Christen, P., Massou, S., Portais, J.-C. & Vorholt, J. A. 2009 Demonstration of the ethylmalonyl-CoA pathway by using ^{13}C metabolomics. *Proc. Natl Acad. Sci. USA* **106**, 4846–4851. (doi:10.1073/pnas.0810932106)

- 19 Okubo, Y., Yang, S., Chistoserdova, L. & Lidstrom, M. E. 2010 Alternative route for glyoxylate consumption during growth on two-carbon compounds by *Methylobacterium extorquens* AM1. *J. Bacteriol.* **192**, 1813–1823. (doi:10.1128/JB.01166-09)
- 20 Feng, X., Banerjee, A., Berla, B., Page, L., Wu, B., Pakrasi, H. B. & Tang, Y. J. 2010 Mixotrophic and photoheterotrophic metabolisms in *Cyanothece* sp. ATCC 51142 under continuous light. *Microbiology* **156**, 2566–2574. (doi:10.1099/mic.0.038232-0)
- 21 Zarzycki, J., Brecht, V., Müller, M. & Fuchs, G. 2009 Identifying the missing steps of the autotrophic 3-hydroxypropionate CO₂ fixation cycle in *Chloroflexus aurantiacus*. *Proc. Natl Acad. Sci. USA* **106**, 21 317–21 322. (doi:10.1073/pnas.0908356106)
- 22 Fuhrer, T., Fischer, E. & Sauer, U. 2005 Experimental identification and quantification of glucose metabolism in seven bacterial species. *J. Bacteriol.* **187**, 1581–1590. (doi:10.1128/JB.187.5.1581-1590.2005)
- 23 Beale, J. M. & Foster, J. L. 1996 Carbohydrate fluxes into alginate biosynthesis in *Azotobacter vinelandii* NCIB 8789: NMR investigations of the triose pools. *Biochemistry* **35**, 4492–4501. (doi:10.1021/bi951922v)
- 24 Portais, J.-C., Tavernier, P., Gosselin, I. & Barbotin, J.-N. 1999 Cyclic organization of the carbohydrate metabolism in *Sinorhizobium meliloti*. *Eur. J. Biochem.* **265**, 473–480. (doi:10.1046/j.1432-1327.1999.00778.x)
- 25 Fischer, E. & Sauer, U. 2003 A novel metabolic cycle catalyzes glucose oxidation and anaplerosis in hungry *Escherichia coli*. *J. Biol. Chem.* **278**, 46 446–46 451. (doi:10.1074/jbc.M307968200)
- 26 Olszewski, K. L., Mather, M. W., Morrissey, J. M., Garcia, B. A., Vaidya, A. B., Rabinowitz, J. D. & Llinas, M. 2010 Branched tricarboxylic acid metabolism in *Plasmodium falciparum*. *Nature* **466**, 774–778. (doi:10.1038/nature09301)
- 27 Tang, Y., Pingitore, F., Mukhopadhyay, A., Phan, R., Hazen, T. C. & Keasling, J. D. 2007 Pathway confirmation and flux analysis of central metabolic pathways in *Desulfovibrio vulgaris hildenborough* using gas chromatography-mass spectrometry and Fourier transform-ion cyclotron resonance mass spectrometry. *J. Bacteriol.* **189**, 940–949. (doi:10.1128/JB.00948-06)
- 28 Crown, S. B., Indurthi, D. C., Ahn, W. S., Choi, J., Papoutsakis, E. T. & Antoniewicz, M. R. 2011 Resolving the TCA cycle and pentose-phosphate pathway of *Clostridium acetobutylicum* ATCC 824: isotopomer analysis, *in vitro* activities and expression analysis. *Biotechnol. J.* **6**, 300–305. (doi:10.1002/biot.201000282)
- 29 Li, F., Hagemeyer, C. H., Seedorf, H., Gottschalk, G. & Thauer, R. K. 2007 *Re*-Citrate synthase from *Clostridium kluyveri* is phylogenetically related to homocitrate synthase and isopropylmalate synthase rather than to *Si*-citrate synthase. *J. Bacteriol.* **189**, 4299–4304. (doi:10.1128/JB.00198-07)
- 30 Marco-Urrea, E., Paul, S., Khodaverdi, V., Seifert, J., von Bergen, M., Kretschmar, U. & Adrian, L. 2011 Identification and characterization of a *Re*-citrate synthase in *Dehalococcoides* strain CBDB1. *J. Bacteriol.* **193**, 5171–5178. (doi:10.1128/JB.05120-11)
- 31 Fuchs, G. 2011 Alternative pathways of carbon dioxide fixation: insights into the early evolution of life? *Annu. Rev. Microbiol.* **65**, 631–658. (doi:10.1146/annurev-micro-090110-102801)
- 32 Erb, T. J., Berg, I. A., Brecht, V., Müller, M., Fuchs, G. & Alber, B. E. 2007 Synthesis of C5-dicarboxylic acids from C2-units involving crotonyl-CoA carboxylase/reductase: the ethylmalonyl-CoA pathway. *Proc. Natl Acad. Sci. USA* **104**, 10 631–10 636. (doi:10.1073/pnas.0702791104)
- 33 Schneider, K., Peyraud, R., Kiefer, P., Christen, P., Delmotte, N., Massou, S., Portais, J.-C. & Vorholt, J. A. 2012 The ethylmalonyl-CoA pathway is used in place of the glyoxylate cycle by *Methylobacterium extorquens* AM1 during growth on acetate. *J. Biol. Chem.* **287**, 757–766. (doi:10.1074/jbc.M111.305219)
- 34 Härtel, T., Eylert, E., Schulz, C., Petruschka, L., Gierok, P., Grubmüller, S., Lalk, M., Eisenreich, W. & Hammerschmidt, S. 2012 Characterization of central carbon metabolism of *Streptococcus pneumoniae* by isotopologue profiling. *J. Biol. Chem.* **287**, 4260–4274. (doi:10.1074/jbc.M111.304311)
- 35 Risso, C., Van Dien, S. J., Orloff, A., Lovley, D. R. & Coppi, M. V. 2008 Elucidation of an alternate isoleucine biosynthesis pathway in *Geobacter sulfurreducens*. *J. Bacteriol.* **190**, 2266–2274. (doi:10.1128/JB.01841-07)
- 36 Xu, H., Zhang, Y. Z., Guo, X. K., Ren, S. X., Staempfli, A. A., Chiao, J. S., Jiang, W. H. & Zhao, G. P. 2004 Isoleucine biosynthesis in *Leptospira interrogans* serotype lai strain 56601 proceeds via a threonine-independent pathway. *J. Bacteriol.* **186**, 5400–5409. (doi:10.1128/JB.186.16.5400-5409.2004)
- 37 Howell, D. M., Xu, H. & White, R. H. 1999 (*Re*)-citramalate synthase in methanogenic archaea. *J. Bacteriol.* **181**, 331–333.
- 38 Wu, B., Zhang, B., Feng, X., Rubens, J. R., Huang, R., Hicks, L. M., Pakrasi, H. B. & Tang, Y. J. 2010 Alternative isoleucine synthesis pathway in cyanobacterial species. *Microbiology* **156**, 596–602. (doi:10.1099/mic.0.031799-0)
- 39 Atsumi, S. & Liao, J. C. 2008 Directed evolution of *Methanococcus jannaschii* citramalate synthase for biosynthesis of 1-propanol and 1-butanol by *Escherichia coli*. *Appl. Environ. Microbiol.* **74**, 7802–7808. (doi:10.1128/AEM.02046-08)
- 40 Tang, Y. J., Shui, W. Q., Myers, S., Feng, X., Bertozzi, C. & Keasling, J. D. 2009 Central metabolism in *Mycobacterium smegmatis* during the transition from O₂-rich to O₂-poor conditions as studied by isotopomer-assisted metabolite analysis. *Biotechnol. Lett.* **31**, 1233–1240. (doi:10.1007/s10529-009-9991-7)
- 41 Tao, K., Du, L., Sun, X., Cai, M., Zhu, T., Zhou, X., Gu, Q. & Zhang, Y. 2009 Biosynthesis of aspergillide A, a novel antitumor compound by a marine-derived fungus *Aspergillus glaucus* via the polyketide pathway. *Tetrahedron Lett.* **50**, 1082–1085. (doi:10.1016/j.tetlet.2008.12.094)
- 42 Nes, C. R., Singha, U. K., Liu, J., Ganapathy, K., Villalta, F., Waterman, M. R., Lepesheva, G. I., Chaudhuri, M. & Nes, W. D. 2012 Novel sterol metabolic network of *Trypanosoma brucei* procyclic and bloodstream forms. *Biochem. J.* **443**, 267–277. (doi:10.1042/BJ20111849)
- 43 McKinlay, J. B. & Harwood, C. S. 2010 Carbon dioxide fixation as a central redox cofactor recycling mechanism in bacteria. *Proc. Natl Acad. Sci. USA* **107**, 11 669–11 675. (doi:10.1073/pnas.1006175107)
- 44 McKinlay, J. B. & Harwood, C. S. 2011 Calvin cycle flux, pathway constraints, and substrate oxidation state together determine the H₂ biofuel yield in photoheterotrophic bacteria. *MBio* **2**, e00323-10.
- 45 Tang, K.-H., Tang, Y. J. & Blankenship, R. E. 2011 Carbon metabolic pathways in phototrophic bacteria and their broader evolutionary implications. *Front. Microbiol.* **2**, 165. (doi:10.3389/fmicb.2011.00165)
- 46 Zhang, S. & Bryant, D. A. 2011 The tricarboxylic acid cycle in cyanobacteria. *Science* **334**, 1551–1553. (doi:10.1126/science.1210858)
- 47 Chavarria, M., Kleijn, R. J., Sauer, U., Pflüger-Grau, K. & de Lorenzo, V. 2012 Regulatory tasks of the

- phosphoenolpyruvate-phosphotransferase system of *Pseudomonas putida* in central carbon metabolism. *MBio* **3**, e00028.
- 48 Beste, D. J. V. et al. 2011 ^{13}C metabolic flux analysis identifies an unusual route for pyruvate dissimilation in mycobacteria which requires isocitrate lyase and carbon dioxide fixation. *PLoS Pathog.* **7**, e1002091. (doi:10.1371/journal.ppat.1002091)
 - 49 Xiong, W., Liu, L., Wu, C., Yang, C. & Wu, Q. 2010 ^{13}C -tracer and gas chromatography-mass spectrometry analyses reveal metabolic flux distribution in the oleaginous microalga *Chlorella protothecoides*. *Plant Physiol.* **154**, 1001–1011. (doi:10.1104/pp.110.158956)
 - 50 Wiechert, W. & Nöh, K. 2005 From stationary to instationary metabolic flux analysis. *Adv. Biochem. Eng. Biotechnol.* **92**, 145–172.
 - 51 Nöh, K., Wahl, A. & Wiechert, W. 2006 Computational tools for isotopically instationary ^{13}C labeling experiments under metabolic steady state conditions. *Metab. Eng.* **8**, 554–577. (doi:10.1016/j.ymben.2006.05.006)
 - 52 Schaub, J., Mauch, K. & Reuss, M. 2008 Metabolic flux analysis in *Escherichia coli* by integrating isotopic dynamic and isotopic stationary ^{13}C labeling data. *Biotechnol. Bioeng.* **99**, 1170–1185. (doi:10.1002/bit.21675)
 - 53 Nöh, K., Grönke, K., Luo, B., Takors, R., Oldiges, M. & Wiechert, W. 2007 Metabolic flux analysis at ultra short time scale: isotopically non-stationary ^{13}C labeling experiments. *J. Biotechnol.* **129**, 249–267. (doi:10.1016/j.jbiotec.2006.11.015)
 - 54 Young, J. D., Walther, J. L., Antoniewicz, M. R., Yoo, H. & Stephanopoulos, G. 2008 An elementary metabolite unit (EMU) based method of isotopically nonstationary flux analysis. *Biotechnol. Bioeng.* **99**, 686–699. (doi:10.1002/bit.21632)
 - 55 Shastri, A. A. & Morgan, J. A. 2007 A transient isotopic labeling methodology for ^{13}C metabolic flux analysis of photoautotrophic microorganisms. *Phytochemistry* **68**, 2302–2312. (doi:10.1016/j.phytochem.2007.03.042)
 - 56 Antoniewicz, M. R., Kelleher, J. K. & Stephanopoulos, G. 2007 Elementary metabolite units (EMU): a novel framework for modeling isotopic distributions. *Metab. Eng.* **9**, 68–86. (doi:10.1016/j.ymben.2006.09.001)
 - 57 Wahl, S., Noh, K. & Wiechert, W. 2008 ^{13}C labeling experiments at metabolic nonstationary conditions: an exploratory study. *BMC Bioinform.* **9**, 152. (doi:10.1186/1471-2105-9-152)
 - 58 Rühl, M., Zamboni, N. & Sauer, U. 2010 Dynamic flux responses in riboflavin overproducing *Bacillus subtilis* to increasing glucose limitation in fed-batch culture. *Biotechnol. Bioeng.* **105**, 795–804.
 - 59 Leighty, R. W. & Antoniewicz, M. R. 2011 Dynamic metabolic flux analysis (DMFA): a framework for determining fluxes at metabolic non-steady state. *Metab. Eng.* **13**, 745–755. (doi:10.1016/j.ymben.2011.09.010)
 - 60 Stolyar, S., Van Dien, S., Hillesland, K. L., Pinel, N., Lie, T. J., Leigh, J. A. & Stahl, D. A. 2007 Metabolic modeling of a mutualistic microbial community. *Mol. Syst. Biol.* **3**, 92.
 - 61 de Graaf, A. A., Maathuis, A., de Waard, P., Deutz, N. E. P., Dijkema, C., de Vos, W. M. & Venema, K. 2010 Profiling human gut bacterial metabolism and its kinetics using $[\text{U-}^{13}\text{C}]$ glucose and NMR. *NMR Biomed.* **23**, 2–12. (doi:10.1002/nbm.1418)
 - 62 Salimi, F., Zhuang, K. & Mahadevan, R. 2010 Genome-scale metabolic modeling of a clostridial co-culture for consolidated bioprocessing. *Biotechnol. J.* **5**, 726–738. (doi:10.1002/biot.201000159)
 - 63 Rühl, M., Hardt, W. D. & Sauer, U. 2011 Subpopulation-specific metabolic pathway usage in mixed cultures as revealed by reporter protein-based ^{13}C analysis. *Appl. Environ. Microbiol.* **77**, 1816–1821. (doi:10.1128/AEM.02696-10)
 - 64 Shaikh, A. S., Tang, Y. J., Mukhopadhyay, A. & Keasling, J. D. 2008 Isotopomer distributions in amino acids from a highly expressed protein as a proxy for those from total protein. *Anal. Chem.* **80**, 886–890. (doi:10.1021/ac071445+)
 - 65 Eisenreich, W., Dandekar, T., Heesemann, J. & Goebel, W. 2010 Carbon metabolism of intracellular bacterial pathogens and possible links to virulence. *Nat. Rev. Microbiol.* **8**, 401–412. (doi:10.1038/nrmicro2351)
 - 66 Fuchs, T. M., Eisenreich, W., Kern, T. & Dandekar, T. 2012 Towards a systemic understanding of *Listeria monocytogenes* metabolism during infection. *Front. Microbiol.* **3**, 23. (doi:10.3389/fmicb.2012.00023)
 - 67 Schär, J., Stoll, R., Schauer, K., Loeffler, D. I. M., Eylert, E., Joseph, B., Eisenreich, W., Fuchs, T. M. & Goebel, W. 2010 Pyruvate carboxylase plays a crucial role in carbon metabolism of extra- and intracellularly replicating *Listeria monocytogenes*. *J. Bacteriol.* **192**, 1774–1784. (doi:10.1128/JB.01132-09)
 - 68 Götz, A., Eylert, E., Eisenreich, W. & Goebel, W. 2010 Carbon metabolism of enterobacterial human pathogens growing in epithelial colorectal adenocarcinoma (Caco-2) cells. *PLoS ONE* **5**, e10586. (doi:10.1371/journal.pone.0010586)
 - 69 Hsiao, T.-L., Revelles, O., Chen, L., Sauer, U. & Vitkup, D. 2010 Automatic policing of biochemical annotations using genomic correlations. *Nat. Chem. Biol.* **6**, 34–40. (doi:10.1038/nchembio.266)
 - 70 Veit, A., Rittmann, D., Georgi, T., Youn, J.-W., Eikmanns, B. J. & Wendisch, V. F. 2009 Pathway identification combining metabolic flux and functional genomics analyses: acetate and propionate activation by *Corynebacterium glutamicum*. *J. Biotechnol.* **140**, 75–83. (doi:10.1016/j.jbiotec.2008.12.014)
 - 71 Peyraud, R., Schneider, K., Kiefer, P., Massou, S., Vorholt, J. & Portais, J.-C. 2011 Genome-scale reconstruction and system level investigation of the metabolic network of *Methylobacterium extorquens* AM1. *BMC Syst. Biol.* **5**, 189. (doi:10.1186/1752-0509-5-189)
 - 72 Smaldone, G. T., Revelles, O., Gaballa, A., Sauer, U., Antelmann, H. & Helmman, J. D. 2012 A global investigation of the *Bacillus subtilis* iron-sparing response identifies major changes in metabolism. *J. Bacteriol.* **194**, 2594–605. (doi:10.1128/JB.05990-11)
 - 73 Buescher, J. M. et al. 2012 Global network reorganization during dynamic adaptations of *Bacillus subtilis* metabolism. *Science* **335**, 1099–1103. (doi:10.1126/science.1206871)
 - 74 Rühl, M., Rupp, B., Nöh, K., Wiechert, W., Sauer, U. & Zamboni, N. 2012 Collisional fragmentation of central carbon metabolites in LC-MS/MS increases precision of ^{13}C metabolic flux analysis. *Biotechnol. Bioeng.* **109**, 763–771. (doi:10.1002/bit.24344)
 - 75 Yuan, Y., Yang, T. H. & Heinzle, E. 2010 ^{13}C metabolic flux analysis for larger scale cultivation using gas chromatography-combustion-isotope ratio mass spectrometry. *Metab. Eng.* **12**, 392–400. (doi:10.1016/j.ymben.2010.02.001)
 - 76 Caytan, E., Remaud, G. S., Tenailleau, E. & Akoka, S. 2007 Precise and accurate quantitative ^{13}C NMR with reduced experimental time. *Talanta* **71**, 1016–1021. (doi:10.1016/j.talanta.2006.05.075)
 - 77 Tang, Y. J., Hwang, J. S., Wemmer, D. & Keasling, J. D. 2007 The *Shewanella oneidensis* MR-1 fluxome under various oxygen conditions. *Appl. Environ. Microbiol.* **73**, 718–729. (doi:10.1128/AEM.01532-06)

- 78 Wiechert, W., Möllney, M., Petersen, S. & de Graaf, A. A. 2001 A universal framework for ^{13}C metabolic flux analysis. *Metab. Eng.* **3**, 265–283. (doi:10.1006/mben.2001.0188)
- 79 Quek, L.-E., Wittmann, C., Nielsen, L. K. & Krömer, J. O. 2009 OpenFLUX: efficient modelling software for ^{13}C -based metabolic flux analysis. *Microb. Cell Factories* **8**, 25. (doi:10.1186/1475-2859-8-25)
- 80 Copeland, W. B., Bartley, B. A., Chandran, D., Galdzicki, M., Kim, K. H., Sleight, S. C., Maranas, C. D. & Sauro, H. M. 2012 Computational tools for metabolic engineering. *Metab. Eng.* **14**, 270–280. (doi:10.1016/j.ymben.2012.03.001)
- 81 Srour, O., Young, J. D. & Eldar, Y. C. 2011 Fluxomers: a new approach for ^{13}C metabolic flux analysis. *BMC Syst. Biol.* **5**, 129. (doi:10.1186/1752-0509-5-129)
- 82 Malaisse, W. J., Zhang, Y., Jijakli, H., Courtois, P. & Sener, A. 2004 Enzyme-to-enzyme channelling in the early steps of glycolysis in rat pancreatic islets. *Int. J. Biochem. Cell Biol.* **36**, 1510–1520. (doi:10.1016/j.biocel.2003.12.013)
- 83 Williams, T. C. R., Sweetlove, L. J. & Ratcliffe, R. G. 2011 Capturing metabolite channeling in metabolic flux phenotypes. *Plant Physiol.* **157**, 981–984. (doi:10.1104/pp.111.184887)
- 84 Sumegi, B., Sherry, A. D., Malloy, C. R. & Srere, P. A. 1993 Evidence for orientation-conserved transfer in the TCA cycle in *Saccharomyces cerevisiae*: carbon-13 NMR studies. *Biochemistry* **32**, 12 725–12 729. (doi:10.1021/bi00210a022)
- 85 Niklas, J., Sandig, V. & Heinzle, E. 2011 Metabolite channeling and compartmentation in the human cell line AGE1.HN determined by ^{13}C labeling experiments and ^{13}C metabolic flux analysis. *J. Biosci. Bioeng.* **112**, 616–623. (doi:10.1016/j.jbiosc.2011.07.021)
- 86 Shearer, G., Lee, J. C., Koo, J. A. & Kohl, D. H. 2005 Quantitative estimation of channeling from early glycolytic intermediates to CO_2 in intact *Escherichia coli*. *FEBS J.* **272**, 3260–3269. (doi:10.1111/j.1742-4658.2005.04712.x)
- 87 Madsen, E. L. 2008 *Environmental microbiology: from genomes to biogeochemistry*. Malden, MA: Blackwell Publishing.
- 88 Feng, X. & Tang, Y. J. 2011 Evaluation of isotope discrimination in ^{13}C -based metabolic flux analysis. *Anal. Biochem.* **417**, 295–297. (doi:10.1016/j.ab.2011.06.022)