Analytical Biochemistry 465 (2014) 38–49

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Sampling of intracellular metabolites for stationary and non-stationary ^{13}C metabolic flux analysis in *Escherichia coli*

Pierre Millard [a,b,c], Stéphane Massou [a,b,c], Christoph Wittmann [d], Jean-Charles Portais [a,b,c], Fabien Létisse [a,b,c,*]

[a] Université de Toulouse, INSA, UPS, INP, LISBP, F-31077 Toulouse, France
[b] INRA, UMR 792, Ingénierie des Systèmes Biologiques et des Procédés, F-31400 Toulouse, France
[c] CNRS, UMR 5504, F-31400 Toulouse, France
[d] Universität des Saarlande, Systembiotechnologie Campus, D-66123 Saarbrücken, Germany

ARTICLE INFO

Article history:
Received 22 May 2014
Received in revised form 25 July 2014
Accepted 26 July 2014
Available online 4 August 2014

Keywords:
^{13}C-labeling experiments
Isotopes
Metabolism
Sampling procedures
Mass isotopomer
Isotopologue

ABSTRACT

The analysis of metabolic intermediates is a rich source of isotopic information for ^{13}C metabolic flux analysis (^{13}C-MFA) and extends the range of its applications. The sampling of labeled metabolic intermediates is particularly important to obtain reliable isotopic information. The assessment of the different sampling procedures commonly used to generate such data, therefore, is crucial. In this work, we thoroughly evaluated several sampling procedures for stationary and non-stationary ^{13}C-MFA using *Escherichia coli*. We first analyzed the efficiency of these procedures for quenching metabolism and found that procedures based on cold or boiling solvents are reliable, in contrast to fast filtration, which is not. We also showed that separating the cells from the broth is not necessary in isotopic stationary state conditions. On the other hand, we demonstrated that the presence of metabolic intermediates outside the cells strongly affects the transient isotopic data monitored during non-stationary ^{13}C-labeling experiments. Meaningful isotopic data can be obtained by recovering intracellular labeled metabolites from pellets of cells centrifuged in cold solvent. We showed that if the intracellular pools are not separated from the extracellular ones, accurate flux maps can be established provided that the contribution of exogenous compounds is taken into account in the metabolic flux model.

^{13}C metabolic flux analysis (^{13}C-MFA)[1] has emerged as a key strategy to quantify *in vivo* distribution of fluxes in complex metabolic networks and is currently used in a wide range of applications (for a review, see Ref. [1]), including systems biology [2,3] and biotechnology [4]. In this approach, an organism is cultivated on a ^{13}C-labeled substrate and ^{13}C atoms propagate through the metabolic network as a function of the metabolic fluxes. Metabolic fluxes are estimated from quantitative measurements of label incorporated into metabolic intermediates or products using a detailed mathematical model that describes the scrambling of ^{12}C and ^{13}C atoms through the metabolic network [5]. Because the quality of the calculated fluxes depends to a great extent on the accuracy and precision of the isotopic data [6,7], the quality of these data is a major concern in ^{13}C-MFA.

Up to now, most ^{13}C-MFA studies have relied on the measurement of label incorporation into metabolic end products (proteinogenic amino acids) [8], whereas the analysis of label incorporation directly into metabolic intermediates is of a great value because it extends the range of applications. Current advances in metabolomics make it possible to analyze metabolic intermediates beyond central metabolism and, hence, to increase the number of metabolic fluxes that can be measured. In addition, analysis of transient label incorporation into intracellular metabolites (non-stationary ^{13}C-MFA) should increase the precision of flux measurements, reduce the time and cost of labeling experiments, and make it possible to investigate transient metabolic states [9]. However, the quantification of label incorporation into intracellular metabolites is much more challenging than in metabolic end products. At the

* Corresponding author at: Laboratoire d'ingénierie des Systèmes Biologiques et des Procédés, INSA Toulouse, 135 Avenue de Rangueil, 31077 Toulouse Cedex 4, France.
E-mail address: fabien.letisse@insa-toulouse.fr (F. Létisse).

[1] *Abbreviations used:* ^{13}C-MFA, ^{13}C metabolic flux analysis; GC–MS, gas chromatography–mass spectrometry; NMR, nuclear magnetic resonance; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; 6PG, 6-phosphogluconate; P5P, xylulose-5-phosphate, ribose-5-phosphate, and ribulose-5-phosphate; S7P, sedoheptulose-7-phosphate; PEP, phosphoenolpyruvate; 2/3PG, 2- and 3-phosphoglycerate; MRM, multiple reaction monitoring; ID, isotopologue distribution; EMP, glycolysis; ED, Entner–Doudoroff; PP, pentose phosphate; IC–MS/MS, ion chromatography–tandem mass spectrometry.

http://dx.doi.org/10.1016/j.ab.2014.07.026
0003-2697/

experimental level, the sampling of metabolic intermediates is the most complex step, as shown by the vast amount of literature on this topic in metabolomics [10–13]. First, metabolic intermediates have extremely high turnover rates, and the immediate blockage of all metabolic activities (termed "quenching" of the metabolism) is required to obtain a reliable picture of metabolic activity. Although in ^{13}C-labeling experiments only relative information (i.e., labeling patterns) is required, inefficient metabolism quenching would lead to undesirable conversion of compounds and, hence, to biases in their labeling patterns. Second, as a result of active excretion of compounds or cell lysis, metabolic intermediates are found outside the cells in the culture medium [12,14]. The labeling patterns of external compounds are not necessarily the same as those of the same intracellular compounds, and isotopic data quantified using a mixture of the two pools are consequently biased. This raises the problem of separating cells from the culture medium prior to the extraction of metabolites while ensuring quenching of the metabolism. Finally, a basic difficulty is the evaluation of the sampling procedure itself. Indeed, because the labeling patterns of intracellular metabolites are not known in advance, it is impossible to determine whether the labeling data obtained with a particular sampling procedure are correct or not. The question of how to evaluate the reliability of isotopic data obtained by various sampling procedures, therefore, is critical.

Various procedures, directly transposed from metabolomics protocols, are currently used to sample metabolic intermediates in ^{13}C-MFA. The metabolism can be quenched by sampling in cold solvent (e.g., methanol) prior to the removal of the extracellular compounds by centrifugation [15–19]. Alternatively, the cells can first be separated from the broth by fast filtration and metabolism can be subsequently quenched in cold liquids such as liquid nitrogen [20,21] and cold methanol [22,23]. A derivative procedure developed by Yuan and coworkers [24] involves growing the cells directly on a membrane filter placed on top of the agarose plate and performing quenching by placing the filters in the cold organic solvent mixture. This approach makes it possible to extract labeled metabolic intermediates at the same time as ensuring separation of the extracellular metabolites. The last procedure involves rapid heating of the broth, which simultaneously blocks metabolic activity and releases intracellular metabolites following cell membrane disruption [25]. In this procedure, the cells are not separated from the extracellular medium.

Although these sampling procedures have been thoroughly investigated in the context of quantitative metabolomics, they have not been evaluated for ^{13}C-MFA. In particular, the quality of the isotopic data obtained with the different procedures and the precision and accuracy of the metabolic fluxes estimated from these data have, to our knowledge, not yet been investigated. Thus, the aim of the work reported here was to evaluate the procedures used to collect intracellular metabolites in both stationary and non-stationary ^{13}C-MFA. For stationary ^{13}C-MFA, we compared the isotopic data on intracellular metabolites collected using five different sampling procedures with the data inferred from gas chromatography–mass spectrometry (GC–MS) and nuclear magnetic resonance (NMR) analysis of proteinogenic amino acids whose low turnover rate prevents the main problems involved in sampling. For non-stationary ^{13}C-MFA, we designed the experiments to make the ^{13}C abundance of intracellular metabolites predictable, thereby providing a reliable proxy to detect potential biases that may affect the isotopic data under such conditions. The reliability of the different sampling procedures was also determined by comparing metabolic flux distributions calculated from the (stationary and non-stationary) isotopic data. This was performed on *Escherichia coli*, a model bacterium in systems biology and a platform organism in metabolic engineering and synthetic biology.

Materials and methods

Strain and cultures

E. coli strain K-12 MG1655 was cultured on minimal synthetic medium containing 5 mM KH_2PO_4, 10 mM Na_2HPO_4, 9 mM NaCl, 40 mM NH_4Cl, 0.8 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.1 g/L thiamine, and 3 g/L glucose. Glucose and thiamine were sterilized by filtration (Minisart polyamide 0.2 μm, Sartorius, Göttingen, Germany), and other solutions were autoclaved separately. All stock cultures were stored at −80 °C in LB medium containing glycerol (40%, v/v). For culture of *E. coli*, 5 ml of LB overnight cultures were used as inoculum and then subcultured in shake flasks containing 10 ml of minimum medium with 3 g/L glucose starting with OD_{600nm} = 0.1 and incubated at 37 °C and 300 rpm in an orbital shaker (Inova 4230, New Brunswick Scientific, New Brunswick, NJ, USA). Cells were harvested during the exponential growth phase ($OD_{600nm} \sim 2$) by centrifugation for 10 min at 10,000*g* at room temperature with a Sigma 3-18K centrifuge (Sigma, Seelze, Germany), washed with the same volume of fresh medium (without glucose and thiamine), and used to inoculate a 1-L baffled shake flask containing 150 ml of minimal medium with 3 g/L glucose at OD_{600nm} = 0.01 and incubated at 37 °C at 300 rpm. Cell growth was monitored by optical density at 600 nm with a Genesys 6 spectrophotometer (Thermo, Carlsbad, CA, USA). Extracellular fluxes were calculated from the concentration profiles over the time of all compounds (biomass, glucose, acetate, etc.). Glucose and acetate in the culture supernatant were quantified by high-performance liquid chromatography (HPLC) using an HPX87H column (Bio-Rad, Richmond, CA, USA) maintained at 48 °C with H_2SO_4 (5 mM) as eluant with ultraviolet (UV, 210 nm) and refractometry for detection.

For ^{13}C-labeling experiments in metabolic and isotopic steady states, the unlabeled glucose in the medium was replaced by a mixture of 80% [1-^{13}C]glucose and 20% [U-^{13}C]glucose (EurisoTop, Saint Aubin, France).

For ^{13}C-labeling experiments in isotopic non-stationary state, isotopic perturbations were performed in 20-ml syringes containing 200 μl of [U-^{13}C]glucose (150 g/L) and preheated at 37 °C. In the mid-exponential growth phase ($OD_{600nm} \sim 1.2$), 20 ml of a shake flask culture was collected and poured into the syringe under vigorous mixing. Approximately 150 μl of the whole broth was collected from the syringe during the first 90 s, every 2 s for the first 30 s and then every 15 s for the remaining 60 s, according to the sampling procedures described below. Filtrate samples were collected every 6 s, and the broth was filtered with a 0.2-μm syringe filter (Minisart polyamide 0.2 μm), which was replaced with a new filter for each individual sample.

Quantitative metabolomics

Samples were collected using the differential method described in detail in Ref. [12]. Briefly, 120 μl of broth or filtered extracellular medium (Sartolon polyamide 0.2 μm, Sartorius) was plunged with 120 μl of fully ^{13}C-labeled cellular extract (used as internal standard) in 5 ml of an ethanol/water (75:25) solution at 95 °C, incubated for 2 min, cooled on ice, and stored at −80 °C. Samples were analyzed by ion chromatography (ICS 2500 system, Dionex, Sunnyvale, CA, USA) coupled with a 4000 QTrap triple quadrupole mass spectrometer (ABSciex, Framingham, MA, USA) equipped with a Turbo V source (ABSciex) for electrospray ionization [21]. The nebulizer gas pressure was 40 psi, the desolvation gas pressure was 50 psi, the desolvation gas temperature was 650 °C, and the capillary voltage was −3.3 kV. Glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), fructose-1,6-bisphosphate (FBP),

6-phosphogluconate (6PG), combined pools of xylulose-5-phosphate, ribose-5-phosphate, and ribulose-5-phosphate (P5P), sedoheptulose-7-phosphate (S7P), phosphoenolpyruvate (PEP), and combined pools of 2- and 3-phosphoglycerate (2/3PG) were analyzed in the multiple reaction monitoring (MRM) mode, and the isotope dilution mass spectrometry (IDMS) method [26] was used to ensure accurate quantification. Fragmentation was done by collision-activated dissociation using nitrogen as the collision gas at medium pressure. The daughter ion was a phosphate group (PO_3^- $m/z = 79$ or $H_2PO_4^-$ $m/z = 97$). Three samples of broth and filtrate were collected at the mid-exponential growth phase (OD_{600nm} ~ 1.2) and analyzed. From these data, we were able to quantify the relative fractions of intra- and extracellular metabolites in the total pools; we calculated absolute concentrations of intracellular metabolites assuming a cell volume of 1.77 ml/g_{DW} [27].

Sampling procedures for ^{13}C-MFA

Sampling of intracellular metabolites for ^{13}C-MFA

Five different procedures for quenching the metabolism and extracting metabolites were tested in this study.

The total procedure (T procedure) involved the simultaneous quenching and extraction of the total amount of metabolites by spraying 120 µl of whole broth in a 10-ml glass tube containing 5 ml of an ethanol/H_2O (75:25) solution at 95 °C for 2 min. The tubes were then placed in a cooling bath of ethanol precooled at −80 °C and subsequently centrifuged at 12,000*g* and −20 °C for 5 min in a Sigma 3-18K centrifuge. Cell extracts were evaporated to dryness in a SpeedVac (SC110A SpeedVac Plus, ThermoSavant, Waltham, MA, USA) under vacuum for 4 h and then stored at −80 °C until further treatment.

In the four other procedures, quenching and extraction were performed separately. Cells were separated from the broth by centrifugation in cold quenching solutions (M, E, and G procedures) or by fast filtration prior to quenching in liquid nitrogen (F procedure). The quenching solutions were (i) 99.9% methanol (Sigma–Aldrich, cat. no. 34860) precooled at −80 °C for the M procedure, (ii) 99.8% ethanol (Sigma–Aldrich, cat. no. 02860) precooled at −80 °C for the E procedure, and (iii) glycerol/water (80:20) + 0.9% NaCl solution precooled at −23 °C for the G procedure. After quenching 120 µl of broth in 500 µl of quenching solution, the methanol and ethanol mixtures were centrifuged at 12,000*g* at −20 °C for 5 min, and the glycerol mixture was centrifuged at 16,000*g* for 20 min. The pellets were stored at −80 °C until further metabolite extraction. For the F procedure, 120 µl of broth was harvested by vacuum filtration (Sartolon polyamide 0.2 µm). Cells were washed with 1 ml of medium with reduced concentrations of phosphate and sulfate salts (0.5 mM KH_2PO_4, 1 mM Na_2HPO_4, and 0.08 mM $MgSO_4$) to avoid ion suppression effects during mass spectrometry analysis. All the other components of the medium, including labeled substrate, were unchanged in the washing solution. Filters were rapidly transferred in liquid nitrogen and stored at −80 °C until further metabolite extraction. The whole filtration procedure (including washing) took less than 5 s.

For metabolite extraction, cell pellets were incubated for 2 min in closed glass tubes containing 5 ml of an ethanol/H_2O (75:25) solution at 95 °C, which ensures highly reproducible metabolite extraction without significant degradation or interconversion of metabolites [28]. Extraction was terminated by placing the tubes in a cooling bath of ethanol precooled at −80 °C. Subsequently, the extracts were centrifuged at 12,000*g* and −20 °C for 5 min, and the supernatants were evaporated under vacuum in a SpeedVac (SC110A SpeedVac Plus) for 4 h and then stored at −80 °C until further treatment.

All of the samples were collected at the mid-exponential growth phase (OD_{600nm} ~ 1.2) of the same culture, from which the samples for GC–MS and NMR analysis were also collected. The procedures were performed in triplicate.

Sampling of proteinogenic amino acids

Culture samples were collected for GC–MS and NMR analyses at the mid-exponential growth phase (OD_{600nm} ~ 1.5) to ensure both isotopic and metabolic steady-state conditions. For GC–MS analysis, 10 mg of harvested cells was incubated in 400 µl of 6 M HCl at 100 °C for 24 h. The resulting hydrolysate was clarified (0.2 µm, Ultrafree MC, Millipore, Bedford, MA, USA) and freeze-dried. Proteinogenic amino acids were then converted into *t*-butyldimethylsilyl derivatives before analysis [29]. For NMR analysis, 200 mg of harvested cells was incubated in 5 ml of 6 M HCl at 105 °C for 24 h. The acid was removed by evaporation (SC110A SpeedVac Plus), labile protons were exchanged three times with deuterium by successive resuspension in 2 ml of D_2O 99.8% (Eur-iso-Top), and the hydrolysate was finally resuspended in 600 µl of D_2O before analysis.

Quantitative isotopic analyses

Analysis of extracellular metabolites by one-dimensional ^1H NMR

Aliquots (500 µl) of filtered broth (0.2 µm, Sartorius) were mixed with 100 µl of D_2O and then analyzed with an Avance 500-MHz spectrometer (Bruker, Rheinstetten, Germany) equipped with a 5-mm *z*-gradient BBI probe at a temperature of 286 K. A sequence using presaturation (ZGPR) was used for water signal suppression, with a 30° pulse and a relaxation delay between scans of 20 s to ensure full signal recovery. A total of 64 scans were accumulated (64k data points with a spectral width of 10 ppm) after 8 dummy scans. From each spectrum, we quantified the four isotopomers of acetate and the ^{13}C enrichment of the anomeric carbon of glucose. In the non-stationary experiment, ^{13}C enrichment was quantified from samples collected just after the end of the experiment.

Analyses of proteinogenic amino acids by two-dimensional NMR

NMR spectra of samples of proteinogenic amino acids were recorded with an Avance 500-MHz spectrometer (Bruker) equipped with a 5-mm *z*-gradient BBI probe at a temperature of 286 K. The specific enrichments were quantified using a ZQF–TOCSY (zero quantum filter–total correlation spectroscopy) sequence as described in Ref. [30]. For each 512 increments in the F1 dimension, 16k data points were acquired in the F2 dimension (8 dummy scans and 16 scans with a delay of 5 s between scans), with a spectral width of 10 ppm in each dimension. The positional isotopomers were quantified using an HSQC (heteronuclear single quantum correlation) sequence as described in Ref. [31]. For each 8k increments in the F1 (^{13}C) dimension, 4k data points were acquired in the F2 (^1H) dimension (32 dummy scans and 8 scans with a delay of 2 s between scans), with a spectral width of 150 and 10 ppm in the F1 and F2 dimensions, respectively. Spectra were processed using TopSpin 2 (Bruker) as described in Refs. [30,31].

Analyses of proteinogenic amino acids by GC–MS

The isotopic pattern of *t*-butyldimethylsilyl derivatives of proteinogenic amino acids was quantified by GC–MS (HP 7890, inert MSD 57979C, Agilent, Waldbronn, Germany) as described previously [32]. All samples were first measured in scan mode [33]. The relative fractions of the isotopologues of interest were then determined in duplicate in selective ion monitoring (SIM) mode.

Analysis of intracellular metabolites by ion chromatography–tandem mass spectrometry

After resuspension of cell extracts in 200 µl of MilliQ water, cell debris was removed by centrifugation at 10,000*g* for 10 min. Samples were analyzed with a Dionex ICS 2500 system coupled to a 4000 QTrap mass spectrometer as described in the "Quantitative metabolomics" section above. Isotopic clusters of molecular ions $[M-H]^-$ were quantified in the MRM mode where phosphate fragments and fragments with a loss of carboxylic group $[M-H-CO_2]^-$ were the daughter ions of phosphorylated metabolites (see above) and organic acids (citrate [CIT] and malate [MAL]), respectively. MRM transitions were chosen according to Kiefer and coworkers [21]. The injection volume was 15 µl, originating from approximately 3 µg of biomass.

Computational part

Correction of mass spectrometry isotopic data for naturally occurring isotopes

The isotopologue distributions (IDs) of intracellular metabolites and proteinogenic amino acids were calculated from their isotope clusters after correction for naturally occurring isotopes of elements other than carbon. This was performed with the software IsoCor [34].

Calculation of molecular ^{13}C abundance of metabolites

The molecular ^{13}C abundance (A_M) of each metabolite was calculated from its ID according to the following equation:

$$A_M = \sum_{i=1}^{n} \frac{i.M_i}{n}, \quad (1)$$

where M_i is the proportion of isotopologues with i ^{13}C atoms for a metabolite containing n carbon atoms.

Flux calculations in stationary state

Flux calculations were performed with influx_s [35]. The metabolic network implemented in the FTBL model included all major reactions of the central carbon metabolism: glucose uptake, glycolysis (EMP), Entner–Doudoroff (ED), and pentose phosphate (PP) pathways, tricarboxylic acid (TCA) cycle, and acetate production and amino acid biosynthesis pathways. In total, the model was made up of 88 reactions for central carbon metabolism and 63 biosynthetic reactions for a total of 103 fluxes (73 unidirectional and 15 reversible reactions). Precursor requirements for biomass formation were determined according to the molecular composition of *E. coli* [36] and the measured growth rate. Metabolic fluxes were estimated by minimizing the variance-weighted sum of square residuals between the experimental and simulated isotopic data using the NLSIC algorithm implemented in influx_s.

Flux calculations in non-stationary state

Non-stationary flux calculations were performed as described by Schaub and coworkers [37]. We focused this analysis on reactions upstream from the pyruvate. The metabolic network investigated here included reactions of EMP, ED, and PP pathways and output reactions of the central metabolic intermediaries toward biomass synthesis. This model contained a total of 37 fluxes (15 unidirectional and 11 reversible reactions). An iterative procedure was used to estimate metabolic fluxes. The propagation of ^{13}C atoms through the network was simulated by solving a system of 692 differential isotopomer balance equations (implemented in Fortran) using the LSODA method in the R "deSolve" package. The presence of extracellular pools at natural abundance in the samples had a strong impact on the transient isotopic data monitored. We took label dilution into account in the calculation by summing the IDs of the extracellular metabolites (at natural abundance) and the simulated IDs of intracellular metabolites, both weighted by the fraction of each pool. Consequently, the fractions of extracellular metabolites relative to the total pools were additional parameters. Finally, the model contained 41 free parameters: the fraction of [^{13}C]glucose in the substrate, 14 free fluxes, the concentration of 19 intracellular metabolites, and the fractions of 7 extracellular metabolites for which transient isotopic data were available. These parameters were estimated using the NLSIC algorithm by fitting simulated data to (i) time-course isotopic data of G6P, F6P, FBP, PEP, P5P, S7P, and 2/3PG, (ii) intracellular concentrations of G6P, F6P, FBP, 6PG, PEP, P5P, and 2/3PG, and (iii) extracellular fluxes. Concentrations of intracellular metabolites were constrained in a physiological range (0.01–10 mM), fractions of extracellular pools were constrained between 0 and 1, and exchange coefficients were constrained between 10^{-4} and 0.99.

Statistical methods

To compare the datasets, hierarchical clustering was performed with the Warp method in the R "hclust" package. To investigate the sensitivity of metabolic fluxes, their 95% confidence intervals were estimated using a Monte Carlo procedure. A total of 100 iterations were performed for each dataset, with artificial noise added according to the experimental standard deviations of IDs (with a threshold of 1% [15]). For non-stationary flux calculations, we also took into account the experimental standard deviations of concentrations of intracellular metabolites and extracellular fluxes.

Results

General strategy for evaluation of sampling procedures in isotopic stationary state

In this study, we evaluated the ability of procedures for sampling metabolite intermediates to provide reliable isotopic data. Intracellular metabolites were collected using five sampling procedures (described in Fig. 1) from a batch culture of *E. coli* grown on a mixture of 80% [1-^{13}C]glucose and 20% [U-^{13}C]glucose as sole carbon source. The sampling procedures were evaluated by comparing the labeling patterns of intracellular metabolites, as well as the fluxes calculated from these data, with those obtained using a well-established ^{13}C-MFA approach with no sampling problems. To this end, a flux map of the central metabolism of *E. coli* was first established by analyzing the labeling patterns of proteinogenic amino acids [38] (Fig. 1), a highly stable material with a low turnover rate. Proteinogenic amino acids were collected from the same culture as the one used for the evaluation of the sampling procedures. To obtain a highly accurate flux map, complementary isotopic information on the proteinogenic amino acids was measured on different analytical platforms. The positional isotopomers and specific enrichments were quantified by NMR, and the IDs were quantified by GC–MS. In total, we used a substantial dataset of 184 isotopic measurements (see Tables S1 and S2 in online Supplementary data) to calculate the reference flux map. Flux calculation is an iterative process in which the labeling patterns of metabolites are simulated from an arbitrary initial set of fluxes and the fluxes are iteratively adjusted to minimize differences between experimental and simulated isotopic data. These differences, therefore, reflect the quality and self-consistency of the measurements. For the reference flux map, the correlation factor (R^2) between the experimental and simulated isotopic data was 0.998 (see Fig. S1 in Supplementary material), indicating that the isotopic data were highly consistent with the metabolic model. The flux distribution

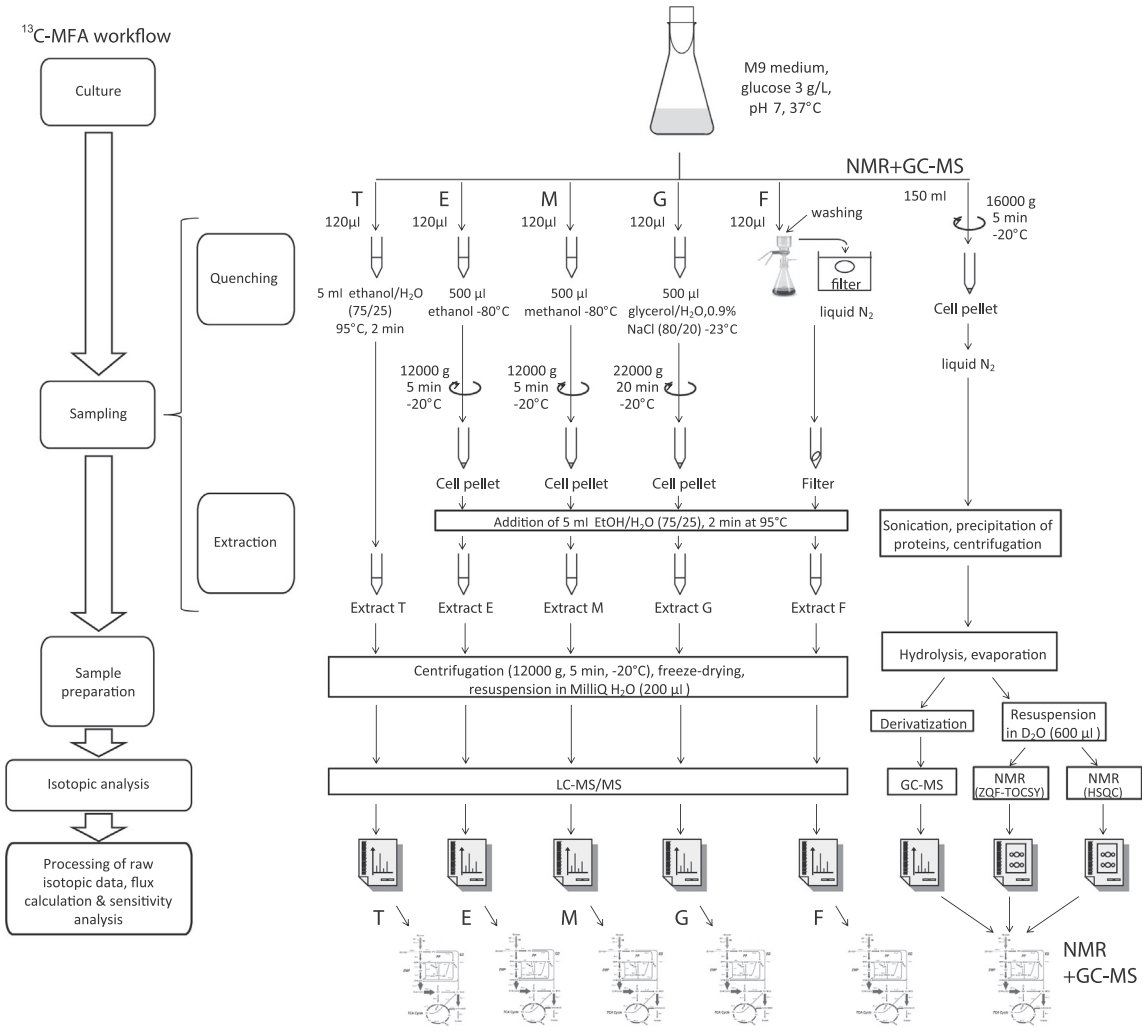

Fig.1. Schematic overview of the sampling procedures evaluated in this work. T, simultaneous quenching and extraction of the total pools of metabolites; E, M, and G, separation of cells from extracellular medium by centrifugation in cold ethanol, cold methanol, and cold glycerol, respectively; F, separation of cells from extracellular medium by fast filtration; NMR+GC–MS, procedure based on analysis of proteinogenic amino acids; ZQF–TOCSY, zero quantum filter–total correlation spectroscopy; HSQC, heteronuclear single quantum correlation.

(Fig. 2) was also in good agreement with previously published data [39,40].

We predicted the IDs of all the intracellular metabolites from the reference flux map by reverse simulation. The predicted isotopic dataset was then compared with the isotopic data obtained from samples of intracellular metabolites collected using each sampling procedure. The aim here was to determine whether the predicted and measured isotopic data were in agreement or not and to judge whether the sampling procedures provided consistently reliable isotopic data.

Comparison of labeling data obtained from ^{13}C metabolites

For each sampling procedure evaluated, three independent samples were collected and analyzed. We quantified the IDs of intracellular metabolites covering the central metabolic pathways of *E. coli* using a highly sensitive ion chromatography–tandem mass spectrometry (IC–MS/MS) method [21]. For each sample, 54 isotopic data were measured in 9 metabolites located at several key points in the central metabolism of *E. coli*. The complete dataset is given in Table S3 of the Supplementary material. Standard deviations on IDs obtained with each sampling procedure were approximately 1%, indicating that all of the procedures are highly reproducible. Statistical analysis of the isotopic datasets by hierarchical clustering showed that the data obtained with procedures M, G, E, and T clustered together and with the data predicted from the reference flux map (Fig. 3A). In contrast, the dataset obtained by fast filtration was clearly separated from the others. A *Z*-score analysis (Fig. 3B) revealed that the main differences between fast filtration and the four other sampling procedures were related to the metabolites FBP, 2/3PG, P5P, and S7P. The sampling procedure in which the cells were not separated from the culture medium (T) provided labeling patterns close to those obtained with procedures M, E, and G.

Comparison of metabolic flux distributions

To further evaluate the different sampling procedures, metabolic flux maps were established from each isotopic dataset. For procedures T, E, G, and M, the correlation between measured and simulated IDs was high ($R^2 \geqslant 0.99$; see Fig. S1). This shows that these procedures provided self-consistent isotopic data from which fluxes can be estimated. In contrast, significant differences were observed between measured and simulated IDs ($R^2 = 0.88$) when metabolites were sampled by fast filtration (procedure F), meaning that this procedure provides inconsistent isotopic infor-

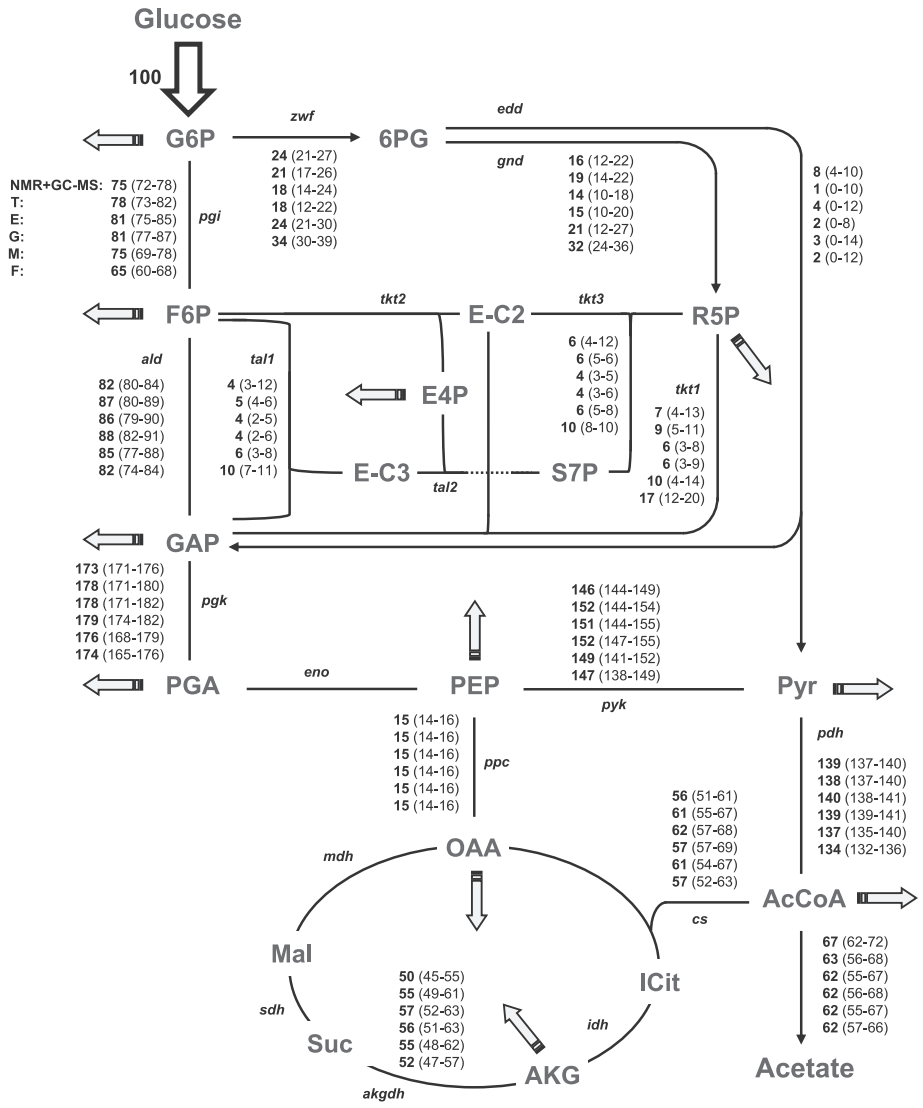

Fig.2. Flux distribution in the central carbon metabolism of *E. coli* K-12 MG1655 grown on glucose calculated from the isotopic data obtained by the different sampling procedures evaluated in this study. T, simultaneous quenching and extraction of the whole broth; E, M, and G, separation of cells from the extracellular medium by centrifugation in cold ethanol, cold methanol, and cold glycerol, respectively; F, separation of cells from the extracellular medium by fast filtration; NMR+GC–MS, procedure based on analysis of proteinogenic amino acids.

mation with regard to the metabolites and the metabolic network considered here.

We assessed the overall reliability of each sampling procedure by comparing the flux distributions with the reference flux map (Fig. 2; see also Table S4 of the Supplementary material). The flux values obtained by fast filtration (F) differed significantly from the reference flux map and from the flux maps obtained using the other procedures. The main discrepancies were found at the G6P node, with underestimation of fluxes through the glycolysis (65 vs. 75% in phosphoglucose isomerase [*pgi*], corresponding to a relative difference of 15%) and with overestimation of fluxes toward the pentose phosphate pathway (32 vs. 16% in glucose-6-phosphate dehydrogenase [*zwf*] corresponding to a relative difference of 100%). This clearly demonstrated that the inconsistency of isotopic data of metabolites collected by fast filtration biased the estimated flux distribution, likely because this procedure does not allow immediate quenching of metabolic activity. In contrast, flux values estimated from the labeling patterns of metabolites collected with procedures T, E, M, and G were similar to those of the reference flux map. This confirms the consistency of the isotopic data obtained with these four procedures from which accurate flux distribution can be calculated. In general, the precision of fluxes calculated from the IDs of metabolic intermediates was similar to that of the reference flux map, whereas the number of isotopic data measured on intracellular metabolites was much lower than that on proteinogenic amino acids (54 vs. 184). Isotopic data measured on intracellular metabolites were even slightly more precise for some fluxes (e.g., for one of the transketolase reactions [*tkt3*] of the non-oxidative part of PP pathway). This is due to the richer isotopic information provided by direct analysis of PP pathway intermediates (i.e., P5P and S7P) compared with the indirect partial information measured in the end products (aromatic amino acids) synthesized from these intermediates. This underlines the advantage of analyzing the labeling patterns of intracellular metabolites instead of—or in addition to—those of proteinogenic amino acids.

These results showed that sampling procedures based on cold solvents provided reliable isotopic information on intracellular metabolites. Interestingly, the flux distribution estimated from the labeling patterns of total metabolites (i.e., from cells + medium, procedure T) was similar to that obtained after cells were separated from the medium (procedures E, M, and G). Therefore, under

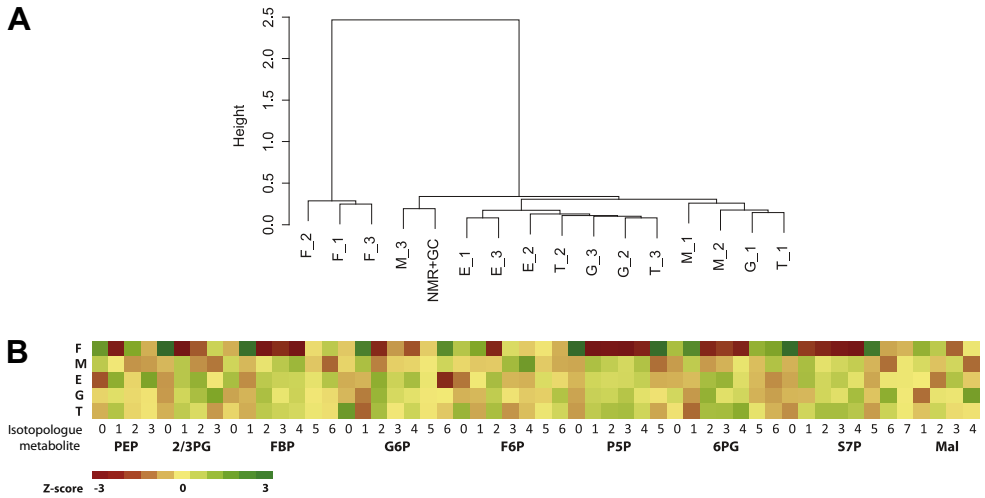

Fig.3. (A) Hierarchical clustering of the isotopic dataset (in triplicate) obtained with each sampling procedure (F, M, E, G, or T) and of the dataset simulated from the flux distribution established from the labeling pattern of proteinogenic amino acids measured by NMR+GC–MS. (B) *Z*-score comparison of IDs obtained with the F, M, E, G, and T procedure. T, simultaneous quenching and extraction of the whole broth; E, M, and G, separation of cells from the extracellular medium by centrifugation in cold ethanol, cold methanol, and cold glycerol, respectively; F, separation of cells from the extracellular medium by fast filtration; NMR+GC–MS, procedure based on analysis of proteinogenic amino acids.

isotopic and metabolic steady-state conditions, internal and external metabolites do not need to be separated, thereby significantly reducing the experimental effort.

General strategy for evaluation of sampling procedures for ^{13}C metabolites in non-stationary state

To determine whether any of the sampling procedures can be applied to isotopic non-stationary ^{13}C-MFA, we first identified the potential issues associated with non-stationary conditions. Non-stationary ^{13}C-MFA exploits the kinetics of incorporating label into metabolic intermediates after the sudden switch of the substrate from unlabeled to ^{13}C labeled. ^{13}C atoms are first incorporated into the intracellular metabolites, which are then excreted into the extracellular medium or used for biosynthesis of metabolic end products. Until the isotopic stationary state is reached, the labeling patterns of intracellular metabolites differ from those of the same extracellular metabolites, meaning that the labeling patterns measured for the former compounds can be biased if the samples are contaminated by the latter. To estimate this potential bias, we measured the IDs of the central metabolites collected using the different sampling procedures during non-stationary ^{13}C-labeling experiments and calculated the molecular ^{13}C abundance (A_M) of the metabolites (Eq. (1)), which represents the mean content of ^{13}C atoms in the molecule.

Because, in contrast to the IDs, A_M is independent of the total number of carbon atoms, this parameter can advantageously be used to compare the labeling patterns of metabolites containing a different number of carbon atoms. Moreover, because glucose was the only source of carbon whose carbon positions have the same ^{13}C content, all of the carbon positions of all metabolites should have the same molecular ^{13}C abundances once isotopic steady state is reached (independent of the origin of the carbon atoms and, thus, of the flux values). Therefore, the difference between predicted and experimental A_M values can easily be used to evaluate label dilution, meaning that this parameter is appropriate for the purpose of this study. Finally, to evaluate the impact of isotopic dilution on metabolic fluxes, the fluxes were calculated from the transient IDs of the metabolites collected using the different sampling procedures.

Difference in labeling kinetics between intra- and extracellular metabolites

First, we measured the ^{13}C molecular abundances of metabolites in the culture broth (cells + medium) using sampling procedure T (simultaneous quenching and extraction of metabolites in boiling ethanol). The ^{13}C abundances of all the metabolites increased immediately after the addition of labeled glucose and, in approximately 20 s, reached a value that did not subsequently vary significantly (Fig. 4A). The values of the plateau varied with the metabolite (from 20 to 59% for PEP and FBP, respectively), and most were significantly lower than the ^{13}C molecular abundance of the input glucose (the final proportion of [U-^{13}C]glucose measured by NMR was 60 ± 1%). Therefore, it can be assumed that the label dilution observed in the total pool of metabolites results from the different kinetics of label incorporation between the intra- and extracellular metabolites. To test this hypothesis, we measured the labeling patterns of metabolites present in filtrates of the culture medium (Fig. 4B). The A_M values of all the metabolites in the medium remained stable during the first 75 s of the experiments (Fig. 4B) and were barely higher than the natural abundance of ^{13}C isotope (1.07%). A slight incorporation of label was observed after 75 s, indicating the release of labeled molecules from the cells. Nevertheless, the A_M values of most metabolites were still less than 6% after 90 s (i.e., much lower than the A_M values measured in the broth). Sampling the broth and the filtrate over a wider time window (dozens of minutes) revealed a continuous increase in the ^{13}C abundance of the metabolites accumulated in the culture medium, whereas the ^{13}C abundance of the metabolites measured in the whole broth tended toward the theoretical values (see Fig. S2 of Supplementary material). These results illustrate the striking differences in the labeling kinetics between intra- and extracellular metabolites within very short labeling periods, stressing that proper separation of cells and cultivation medium is critical in non-stationary ^{13}C-labeling experiments.

Therefore, it can be expected that sampling procedures that separate the cells from the culture medium may reduce label dilution by extracellular metabolites. The kinetics of label incorporation into metabolic intermediates collected by the two procedures E and G (Fig. 4C and D) was consistently similar to that

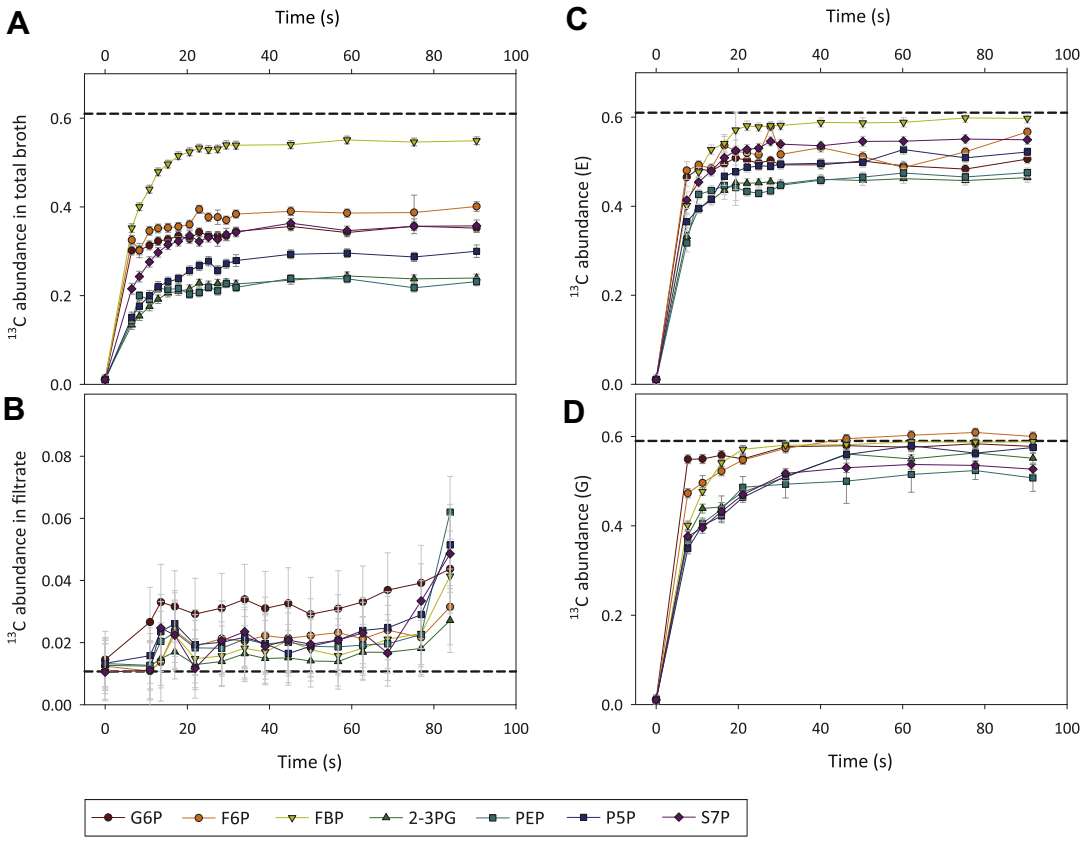

Fig.4. Kinetics of ^{13}C-label incorporation into metabolites during short-term labeling experiments performed with [U-^{13}C]glucose: (A) in total metabolites (simultaneous quenching and extraction of the whole broth); (B) in extracellular metabolites (obtained after filtration of the whole broth); (C) in metabolites sampled by procedure G (separation of cells from the extracellular medium by centrifugation in cold glycerol); (D) in metabolites sampled by procedure E (separation of cells from extracellular medium by centrifugation in cold ethanol). The dotted line denotes the ^{13}C abundance of the labeled glucose measured by NMR (A,C,D) or the natural abundance of the ^{13}C isotope (0.0107) (B).

Table 1
^{13}C abundances of central intermediates (A_M) calculated from the transient isotopic data obtained with the T procedure (simultaneous quenching and extraction of the whole broth), E procedure (separation of cells from the extracellular medium by centrifugation in cold ethanol), and G procedure (separation of cells from the extracellular medium by centrifugation in cold glycerol) and fractions (f_{out}) of extracellular pools measured in whole broth by quantitative metabolomics or in the samples collected using each sampling procedure.

	Metabolomics	T		E		G	
	f_{out}	A_M	f_{out}	A_M	f_{out}	A_M	f_{out}
G6P	0.49 ± 0.05	0.34 ± 0.01	0.45 ± 0.02	0.50 ± 0.01	0.19 ± 0.01	0.57 ± 0.01	0.03 ± 0.02
F6P	0.45 ± 0.03	0.38 ± 0.02	0.39 ± 0.03	0.52 ± 0.03	0.14 ± 0.05	0.58 ± 0.04	0.02 ± 0.06
FBP	0.14 ± 0.08	0.54 ± 0.01	0.12 ± 0.01	0.59 ± 0.01	0.04 ± 0.01	0.59 ± 0.01	0.01 ± 0.01
2/3PG	0.62 ± 0.03	0.23 ± 0.01	0.63 ± 0.01	0.46 ± 0.01	0.26 ± 0.01	0.55 ± 0.02	0.07 ± 0.04
PEP	0.66 ± 0.06	0.22 ± 0.01	0.65 ± 0.02	0.45 ± 0.02	0.27 ± 0.03	0.50 ± 0.03	0.11 ± 0.03
S7P	0.49 ± 0.05	0.34 ± 0.01	0.45 ± 0.02	0.54 ± 0.01	0.11 ± 0.01	0.53 ± 0.01	0.10 ± 0.01
P5P	0.39 ± 0.07	0.29 ± 0.01	0.52 ± 0.01	0.50 ± 0.01	0.17 ± 0.02	0.57 ± 0.01	0.05 ± 0.02

obtained with procedure T, but the plateau values were closer to the predicted values (60 ± 1%), ranging from 45 to 59% for procedure E and from 50 to 59% for procedure G (Table 1). In samples collected using procedure E, only the FBP showed ^{13}C abundances close to the predicted value (Fig. 4C). In contrast, when metabolites were sampled with procedure G, the ^{13}C abundances of F6P, G6P, and FBP reached the predicted value (Fig. 4D). Nevertheless, these data revealed that a small pool of extracellular metabolites still resulted in label dilution whatever sampling procedure was used.

Estimation of contamination by extracellular metabolites

Using the transient isotopic data, we quantified the fraction of residual extracellular metabolites present in the different samples. Assuming that the isotopic dilution is mainly caused by external unlabeled metabolites, the ^{13}C abundance measured in the total pool of a metabolite in a sample corresponds to the sum of the ^{13}C abundances of extra- and intracellular pools weighted by their relative proportions according to the following equation:

$$A_{total} = f_{out}.A_{out} + (1 - f_{out}).A_{in}, \quad (2)$$

where A_{total} is the ^{13}C molecular abundance in the total metabolite pool, f_{in} is the fraction of extracellular metabolites, A_{out} is the ^{13}C molecular abundance of the extracellular pool, and A_{in} is the ^{13}C molecular abundance of the intracellular pool. Consequently, the term $1 - f_{out}$ denotes the fraction of intracellular metabolites.

We used the data shown in Fig. 4A to calculate the fractions of extracellular metabolites (f_{out}) in whole broth with Eq. (2). Because

we detected no significant label incorporation in extracellular metabolites during the first 75 s following the addition of [U-^{13}C]glucose, A_{out} was assumed to be natural ^{13}C abundance. A_{total} was the mean value of the plateaus reached during the ^{13}C-labeling experiments. The f_{out} values were compared with the fractions of extracellular metabolites in whole broth quantified by metabolomics. The values were in good agreement, with differences ranging between 1 and 12% with an average value of 3% (Table 1). This demonstrated the reliability of the proposed methodology to quantify the fraction of extracellular metabolites and confirmed that the presence of unlabeled extracellular metabolites is the major cause of label dilution.

We then applied Eq. (2) to the isotopic data obtained from cell extracts prepared with procedures E and G to calculate the fraction of extracellular metabolites that contaminated the cell pellets after the centrifugation step (Table 1). These fractions reached a mean value of 15% when centrifugation was performed in cold ethanol. The average level of contamination was lower (5%) when centrifugation was performed in cold glycerol. These results quantitatively confirmed that cells were better separated from the extracellular medium by procedure G than by procedure E. Because the results obtained in steady-state conditions showed that the two procedures had comparable quenching efficiency, the higher concentrations of extracellular metabolites in the cell pellets obtained with procedure E may be explained by their coprecipitation with salts as a consequence of the high ethanol content.

Isotopic non-stationary flux calculations

Finally, to assess the impact of label dilution in intracellular pools by extracellular metabolites on the calculated fluxes, we inferred metabolic fluxes from the transient isotopic data obtained using each sampling procedure. Given the metabolites examined in this work, we focused this analysis on metabolic fluxes upstream from pyruvate (EMP, PP, and ED). The computational workflow is detailed in Fig. S3 of the Supplementary material. Label propagation was simulated by solving a system of ordinary differential equations (ODEs) describing the detailed isotopomer balance for each metabolite [37,41]. The metabolic fluxes were estimated by an iterative process involving finding the parameter values (fluxes and metabolite concentrations) that fitted the experimental data best. The latter included (i) the rates of glucose uptake and acetate production, (ii) intracellular metabolite concentrations determined by quantitative metabolomics, and (iii) non-stationary isotopic data (731, 645, and 430 isotopologues for the T, E, and G procedures, respectively).

Consistent with the results described in this section above, the data could not be satisfactorily fitted without accounting for label dilution by external metabolites ($R^2 < 0.90$). This was achieved by adding—at each iteration—the contribution of the extracellular metabolites at natural abundance (ID_{out}) to the (simulated) transient IDs of intracellular metabolites (ID_{in}) according to the following equation:

$$ID_{total}(t) = f_{out}.ID_{out}(t) + (1 - f_{out}).ID_{in}(t). \quad (3)$$

As with Eq. (2), the IDs of each metabolite pool were weighted by the fraction of the corresponding pool. Experimental data were fitted to ID_{total}, and the fractions of extracellular pools (f_{out}) were additional free parameters. With these changes, we were able to obtain good fits for the transient isotopic data obtained with each procedure (Fig. 5A, $R^2 \geqslant 0.98$). The experimental glucose uptake flux and the concentrations of intracellular metabolites were also accurately fitted (see Tables S5 and S6 of Supplementary material). Finally, we applied a non-linear statistical method (Monte Carlo procedure) to determine the 95% confidence intervals on the estimated parameters.

Calculated fluxes and their confidence intervals are shown in Fig. 5B and Table S5. The distribution of fluxes between the main pathways was in good agreement with that obtained by stationary ^{13}C-MFA. The relative fluxes through glycolysis ranged from 0.77 to 0.81, toward the PP pathway from 0.15 to 0.19, and toward the ED pathway from 0.03 to 0.05. Contrary to our expectations, the precision of the fluxes obtained in the non-stationary condition was slightly lower than that obtained with the stationary approach [41]. This can be explained by the label input used in the non-stationary labeling experiment (a mixture of unlabeled glucose and [U-^{13}C]glucose), which is not the most appropriate for flux determination. It may be recalled that this mixture was chosen because it makes the molecular enrichment of metabolites predictable and, therefore, was extremely valuable to evaluate the consistency of isotopic data in the current study. Thus, flux precision could be improved by optimizing the labeling of the glucose used as input [6] and/or by increasing sampling frequency. Be that as it may, we clearly showed that the significant differences between the transient isotopic datasets obtained with each procedure are due to contamination of intracellular pools by unlabeled material and not by different metabolic states. Whatever the sampling procedure used, fluxes estimated from the data were not affected by the differences as long as the variable amounts of extracellular metabolites in the samples were explicitly accounted for in the model.

Discussion

In this work, we thoroughly evaluated the reliability of different procedures for sampling metabolic intermediates to provide high-quality isotopic data for metabolic flux calculation in stationary and non-stationary ^{13}C-MFA. We showed that fast filtration did not immediately block the metabolism despite all of the precautions we took when performing the experiment. This sampling procedure caused significant biases both in the isotopic data and in the fluxes inferred from these data. In contrast, cold or boiling solvents ensured efficient quenching of the metabolism and provided a reliable isotopic picture from which fluxes were accurately calculated. These results show that isotopic approaches are the most appropriate to evaluate the quenching efficiency of sampling procedures and that they should also be used to evaluate sampling procedures in the framework of metabolomics.

We also thoroughly assessed the impact of the presence of metabolic intermediates outside the cells on the quality of both isotopic data and fluxes. In agreement with the literature [12,14], we observed significant amounts of metabolic intermediates outside the cells. The fractions of extracellular pools varied across the different metabolites, indicating that the presence of these metabolic intermediates cannot be explained only by cell lysis. To identify the best way to deal with the presence of metabolic intermediates outside the cells, we distinguished the isotopic stationary state from the non-stationary state.

Under isotopic stationary conditions, we demonstrated that separating the cells from the broth is not necessary. This means that quenching and extraction can be performed simultaneously with boiling ethanol, as in the current study, or with cold solvents such as acidic acetonitrile/methanol/water mix [42]. Such procedures can be easily automated and, therefore, are of great interest for high-throughput ^{13}C-MFA.

Under non-stationary ^{13}C-labeling conditions, we demonstrated that the kinetics of label incorporation into extracellular metabolites are much slower than those of intracellular metabolites so that the two pools exhibit different labeling patterns. Thus, a separation step is needed to avoid any biases in the determination of their labeling patterns. We showed that cold glycerol appears to be

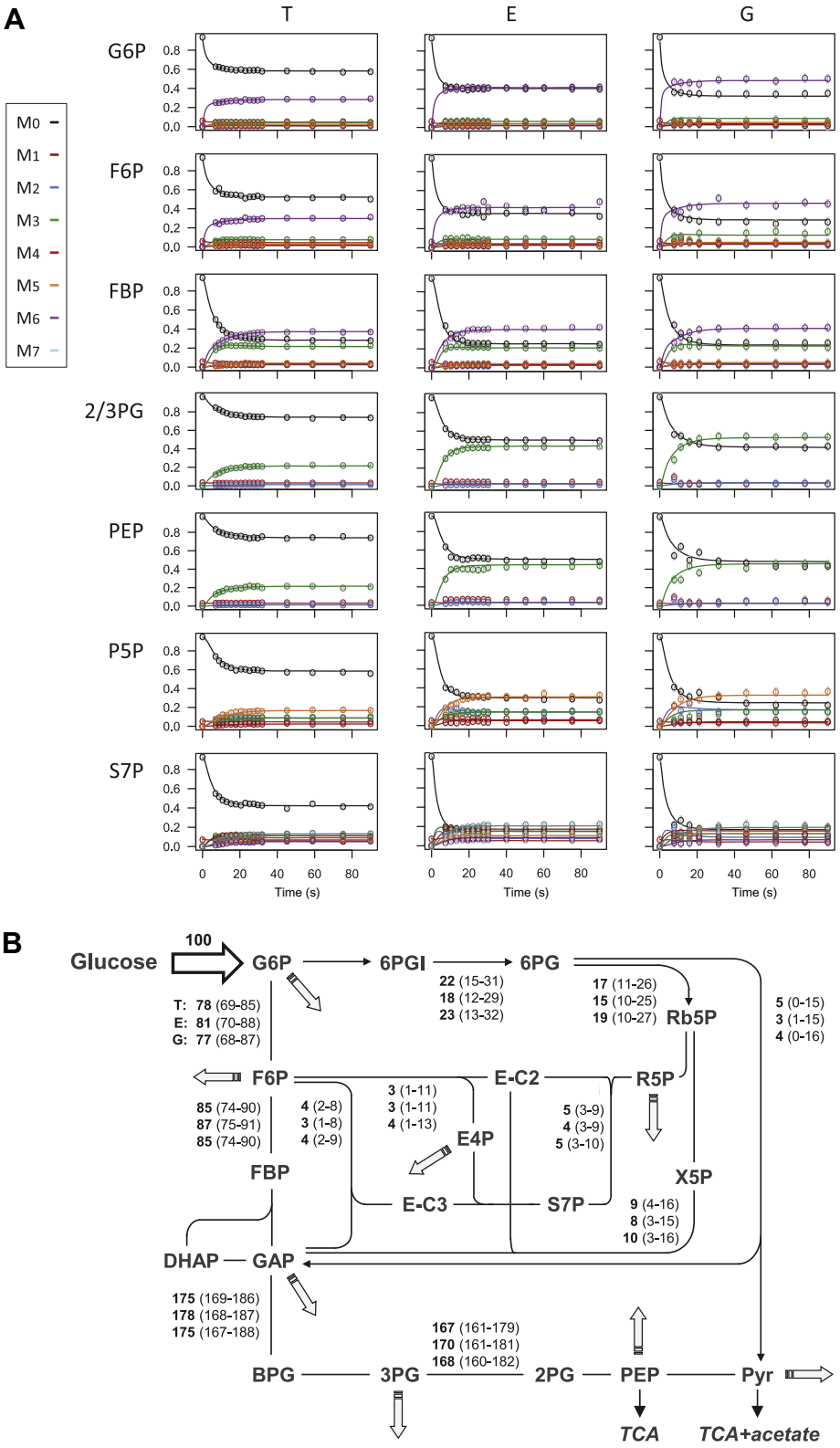

Fig.5. Measured and fitted IDs obtained using the T procedure (simultaneous quenching and extraction of the whole broth), E procedure (separation of cells from the extracellular medium by centrifugation in cold ethanol), and G procedure (separation of cells from the extracellular medium by centrifugation in cold glycerol) (A) and estimated fluxes with their 95% confidence intervals (B).

a satisfactory solvent for separating cells from the medium while providing consistent data for ^{13}C-MFA. However, small amounts (~5%) of extracellular metabolites remained in the pellet after a single centrifugation step. Additional washes of the cell pellets should reduce contamination. To capture the dynamics of label incorporation into metabolic intermediates, the sampling of

metabolites must be performed at sufficiently high frequency [9]. When the bacterium *E. coli* is cultivated in batch culture on glucose as it was done in this work, the frequency of sampling can be estimated to be on the order of 1 s. Although this remains challenging, sampling the labeled metabolites using the cold glycerol procedure is manageable within such a short time. The problem of the presence of metabolic intermediates outside the cells can be circumvented by considering the contribution of the extracellular pools in the modeling step itself. This significantly simplifies the sampling effort that can be challenging when samples need to be collected in 1 s—if not in subseconds. Because the cells do not need to be separated from the medium, quenching and extraction can be performed in the stationary isotopic state as described above.

We believe that further application of these procedures and respecting the guidelines provided in this work will avoid experimental biases and help to generate reliable flux distributions that are indispensable in systems biology. Isotope-based approaches have a vast range of applications in metabolism studies other than ^{13}C-MFA, including identifying new pathways or *in vivo* network topology, gaining insights into the location of biochemical reactions among specific cell compartments, and obtaining information on the chemical structure and composition of metabolites.

Acknowledgments

We thank MetaToul (Toulouse Metabolomics & Fluxomics facilities, Toulouse, France, http://www.metatoul.fr), Stéphanie Durand and Jane Hubert for providing technical expertise and assistance for IC–MS/MS and NMR analyses, and Daphne Goodfellow for careful reading of the manuscript. Pierre Millard received support from INRA (CJS Programme) and from the European Union in the framework of the Marie Curie FP7 COFUND People Programme through the award of an AgreenSkills fellowship (under grant agreement 267196).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2014.07.026.

References

[1] U. Sauer, Metabolic networks in motion: ^{13}C-based flux analysis, Mol. Syst. Biol. 2 (2006) 62.
[2] O. Revelles, P. Millard, J.-P. Nougayrède, U. Dobrindt, E. Oswald, F. Létisse, J.-C. Portais, The carbon storage regulator (Csr) system exerts a nutrient-specific control over central metabolism in *Escherichia coli* strain Nissle 1917, PLoS ONE 8 (2013) e66386.
[3] N. Ishii, K. Nakahigashi, T. Baba, M. Robert, T. Soga, A. Kanai, T. Hirasawa, M. Naba, K. Hirai, A. Hoque, P.Y. Ho, Y. Kakazu, K. Sugawara, S. Igarashi, S. Harada, T. Masuda, N. Sugiyama, T. Togashi, M. Hasegawa, Y. Takai, K. Yugi, K. Arakawa, N. Iwata, Y. Toya, Y. Nakayama, T. Nishioka, K. Shimizu, H. Mori, M. Tomita, Multiple high-throughput analyses monitor the response of *E. coli* to perturbations, Science 316 (2007) 593–597.
[4] M. Kohlstedt, J. Becker, C. Wittmann, Metabolic fluxes and beyond—Systems biology understanding and engineering of microbial metabolism, Appl. Microbiol. Biotechnol. 88 (2010) 1065–1075.
[5] W. Wiechert, ^{13}C-metabolic flux analysis, Metab. Eng. 3 (2001) 195–206.
[6] P. Millard, S. Sokol, F. Letisse, J.-C. Portais, IsoDesign: A software for optimizing the design of ^{13}C-metabolic flux analysis experiments, Biotechnol. Bioeng. 111 (2014) 202–208.
[7] M. Möllney, W. Wiechert, D. Kownatzki, A.A. de Graaf, Bidirectional reaction steps in metabolic networks: IV. Optimal design of isotopomer labeling experiments, Biotechnol. Bioeng. 66 (1999) 86–103.
[8] N. Zamboni, S.-M. Fendt, M. Rühl, U. Sauer, ^{13}C-based metabolic flux analysis, Nat. Protoc. 4 (2009) 878–892.
[9] K. Nöh, W. Wiechert, The benefits of being transient: Isotope-based metabolic flux analysis at the short time scale, Appl. Microbiol. Biotechnol. 91 (2011) 1247–1265.
[10] M. Oldiges, S. Lütz, S. Pflug, K. Schroer, N. Stein, C. Wiendahl, Metabolomics: Current state and evolving methodologies and tools, Appl. Microbiol. Biotechnol. 76 (2007) 495–511.
[11] C.J. Bolten, P. Kiefer, F. Letisse, J.-C. Portais, C. Wittmann, Sampling for metabolome analysis of microorganisms, Anal. Chem. 79 (2007) 3843–3849.
[12] H. Taymaz-Nikerel, M. de Mey, C. Ras, A. ten Pierick, R.M. Seifar, J.C. van Dam, J.J. Heijnen, W.M. van Gulik, Development and application of a differential method for reliable metabolome analysis in *Escherichia coli*, Anal. Biochem. 386 (2009) 9–19.
[13] W.M. van Gulik, Fast sampling for quantitative microbial metabolomics, Curr. Opin. Biotechnol. 21 (2010) 27–34.
[14] N. Paczia, A. Nilgen, T. Lehmann, J. Gätgens, W. Wiechert, S. Noack, Extensive exometabolome analysis reveals extended overflow metabolism in various microorganisms, Microb. Cell Fact. 11 (2012) 122.
[15] M. Rühl, B. Rupp, K. Nöh, W. Wiechert, U. Sauer, N. Zamboni, Collisional fragmentation of central carbon metabolites in LC–MS/MS increases precision of ^{13}C-metabolic flux analysis, Biotechnol. Bioeng. 109 (2012) 763–771.
[16] W.A. van Winden, J.C. van Dam, C. Ras, R.J. Kleijn, J.L. Vinke, W.M. van Gulik, J.J. Heijnen, Metabolic-flux analysis of *Saccharomyces cerevisiae* CEN.PK113-7D based on mass isotopomer measurements of ^{13}C-labeled primary metabolites, FEMS Yeast Res. 5 (2005) 559–568.
[17] S.G. Villas-Bôas, P. Bruheim, Cold glycerol–saline: The promising quenching solution for accurate intracellular metabolite analysis of microbial cells, Anal. Biochem. 370 (2007) 87–97.
[18] K. Nöh, K. Grönke, B. Luo, R. Takors, M. Oldiges, W. Wiechert, Metabolic flux analysis at ultra short time scale: Isotopically non-stationary ^{13}C labeling experiments, J. Biotechnol. 129 (2007) 249–267.
[19] R.J. Kleijn, W.A. van Winden, C. Ras, W.M. van Gulik, D. Schipper, J.J. Heijnen, ^{13}C-labeled gluconate tracing as a direct and accurate method for determining the pentose phosphate pathway split ratio in *Penicillium chrysogenum*, Appl. Environ. Microbiol. 72 (2006) 4743–4754.
[20] X. Chen, A.P. Alonso, D.K. Allen, J.L. Reed, Y. Shachar-Hill, Synergy between ^{13}C-metabolic flux analysis and flux balance analysis for understanding metabolic adaptation to anaerobiosis in *E. coli*, Metab. Eng. 13 (2011) 38–48.
[21] P. Kiefer, C. Nicolas, F. Letisse, J.-C. Portais, Determination of carbon labeling distribution of intracellular metabolites from single fragment ions by ion chromatography tandem mass spectrometry, Anal. Biochem. 360 (2007) 182–188.
[22] Y. Toya, N. Ishii, T. Hirasawa, M. Naba, K. Hirai, K. Sugawara, S. Igarashi, K. Shimizu, M. Tomita, T. Soga, Direct measurement of isotopomer of intracellular metabolites using capillary electrophoresis time-of-flight mass spectrometry for efficient metabolic flux analysis, J. Chromatogr. A 1159 (2007) 134–141.
[23] D. Amador-Noguez, I.A. Brasg, X.-J. Feng, N. Roquet, J.D. Rabinowitz, Metabolome remodeling during the acidogenic–solventogenic transition in *Clostridium acetobutylicum*, Appl. Environ. Microbiol. 77 (2011) 7984–7997.
[24] J. Yuan, B.D. Bennett, J.D. Rabinowitz, Kinetic flux profiling for quantitation of cellular metabolic fluxes, Nat. Protoc. 3 (2008) 1328–1340.
[25] J. Schaub, C. Schiesling, M. Reuss, M. Dauner, Integrated sampling procedure for metabolome analysis, Biotechnol. Prog. 22 (2006) 1434–1442.
[26] L. Wu, M.R. Mashego, J.C. van Dam, A.M. Proell, J.L. Vinke, C. Ras, W.A. van Windenn, W.M. van Gulik, J.J. Heijnen, Quantitative analysis of the microbial metabolome by isotope dilution mass spectrometry using uniformly ^{13}C-labeled cell extracts as internal standards, Anal. Biochem. 336 (2005) 164–171.
[27] C. Chassagnole, N. Noisommit-Rizzi, J.W. Schmid, K. Mauch, M. Reuss, Dynamic modeling of the central carbon metabolism of *Escherichia coli*, Biotechnol. Bioeng. 79 (2002) 53–73.
[28] A.B. Canelas, A. ten Pierick, C. Ras, R.M. Seifar, J.C. van Dam, W.M. van Gulik, J.J. Heijnen, Quantitative evaluation of intracellular metabolite extraction techniques for yeast metabolomics, Anal. Chem. 81 (2009) 7379–7389.
[29] C. Wittmann, M. Hans, E. Heinzle, In vivo analysis of intracellular amino acid labelings by GC/MS, Anal. Biochem. 307 (2002) 379–382.
[30] S. Massou, C. Nicolas, F. Letisse, J.-C. Portais, Application of 2D–TOCSY NMR to the measurement of specific ^{13}C-enrichments in complex mixtures of ^{13}C-labeled metabolites, Metab. Eng. 9 (2007) 252–257.
[31] S. Massou, C. Nicolas, F. Letisse, J.-C. Portais, NMR-based fluxomics: Quantitative 2D NMR methods for isotopomers analysis, Phytochemistry 68 (2007) 2330–2340.
[32] P. Kiefer, E. Heinzle, O. Zelder, C. Wittmann, Comparative metabolic flux analysis of lysine-producing *Corynebacterium glutamicum* cultured on glucose or fructose, Appl. Environ. Microbiol. 70 (2004) 229–239.
[33] C. Wittmann, Fluxome analysis using GC–MS, Microb. Cell Fact. 6 (2007) 6.
[34] P. Millard, F. Letisse, S. Sokol, J.-C. Portais, IsoCor: Correcting MS data in isotope labeling experiments, Bioinformatics 28 (2012) 1294–1296.
[35] S. Sokol, P. Millard, J.-C. Portais, Influx_s: Increasing numerical stability and precision for metabolic flux analysis in isotope labeling experiments, Bioinformatics 28 (2012) 687–693.
[36] F. Neidhardt, Chemical composition of *Escherichia coli*, in: F. Neidhardt, R. Curtiss, J.L. Ingraham, E.C.C. Lin, K.B. Low, B. Magasanik, W.S. Reznikoff, M. Riley, M. Schaechter, H.E. Umbarger (Eds.), *Escherichia coli* and *Salmonella typhimurium:* Cellular and Molecular Biology, second ed., American Society for Microbiology, Washington, DC, 1996, pp. 13–16.
[37] J. Schaub, K. Mauch, M. Reuss, Metabolic flux analysis in *Escherichia coli* by integrating isotopic dynamic and isotopic stationary ^{13}C-labeling data, Biotechnol. Bioeng. 99 (2008) 1170–1185.

[38] C. Wittmann, J.-C. Portais, Metabolic Flux Analysis, in: M. Lämmerhofer, W. Weckwerth (Eds.), Metabolomics in Practice: Successful Strategies to Generate and Analyze Metabolic Data, John Wiley, Weinheim, Germany, 2013, pp. 285–312.
[39] T. Fuhrer, E. Fischer, U. Sauer, Experimental identification and quantification of glucose metabolism in seven bacterial species, J. Bacteriol. 187 (2005) 1581–1590.
[40] C. Nicolas, P. Kiefer, F. Letisse, J. Krömer, S. Massou, P. Soucaille, C. Wittmann, N.D. Lindley, J.-C. Portais, Response of the central metabolism of *Escherichia coli* to modified expression of the gene encoding the glucose-6-phosphate dehydrogenase, FEBS Lett. 581 (2007) 3771–3776.
[41] S. Noack, K. Nöh, M. Moch, M. Oldiges, W. Wiechert, Stationary versus non-stationary ^{13}C-MFA: A comparison using a consistent dataset, J. Biotechnol. 154 (2011) 179–190.
[42] J.D. Rabinowitz, E. Kimball, Acidic acetonitrile for cellular metabolome extraction from *Escherichia coli*, Anal. Chem. 79 (2007) 6167–6173.