C146-E312

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Technical Report

^{13}C-Metabolic Flux Analysis of Central Carbon Metabolism Using GC-MS

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Abstract:

This technical report describes the principles and procedures of ^{13}C-metabolic flux analysis using gas chromatography-mass spectrometry (GC-MS). The ^{13}C-metabolic flux analysis of the central carbon metabolism in *Escherichia coli* successfully determined the branch ratio of flux levels through glycolysis and the pentose phosphate pathway, the direction of flux in the TCA cycle, and the deactivated reactions such as glyoxylate shunt and the Entner–Doudoroff pathway. The analysis also provided useful information for identifying disease-specific metabolic disorders in human cells and evaluating the metabolism of valuable chemicals-producing microbes.

Keywords: ^{13}C-metabolic flux analysis, amino acid, GC-MS, *Escherichia coli* (*E. coli*)

1. Introduction

In recent years, quantifying the intracellular metabolic flux by ^{13}C-metabolic flux analysis (i.e., the molar amount of chemical reaction per cell and per unit time) has attracted attention in fields such as medical, pharmacology, and engineering for purposes such as identifying characteristic metabolism of cancer cells, understanding the effects of drugs on metabolism, and evaluating the productivity of valuable chemicals using biological process [1, 2, 3]. Several techniques have been proposed for understanding intracellular metabolic status including, pool sizes of metabolites (metabolome), expression levels of enzyme genes (transcriptome) and quantities of enzyme proteins (proteome). Although intracellular metabolic flux distribution has been indirectly estimated based on the above data, the estimation is often difficult since the pool sizes of metabolites and the enzyme gene expressions do not necessarily correlate with the levels of metabolic flux. Furthermore, post-translational modifications and the allosteric regulation of proteins should be considered in the flux estimation, which indicates that there are limitations for predicting *in vivo* metabolic flux based on metabolome, transcriptome, and proteome data.

^{13}C-metabolic flux analysis is a method that provides intracellular metabolic flux distribution for a direct understanding of the metabolic phenotype to identify activated and inhibited reactions from the directions, branch ratios, and conversion rates of the metabolic process. This report describes the principles and procedures of the ^{13}C-metabolic flux analysis, and its application to the analysis of the central carbon metabolism for a model microorganism, *Escherichia coli* wild-type strain, using high-performance liquid chromatography (HPLC) analysis of cultured media components, and gas chromatography-mass spectrometry (GC-MS) measurement of ^{13}C-labeling of proteinogenic amino acids.

2. Principle of ^{13}C-Metabolic Flux Analysis

^{13}C-metabolic flux analysis is performed by feeding ^{13}C-labeled carbon sources to cells (Fig. 1, step 1). For example, the split ratio of flux through glycolysis and the pentose phosphate pathway can be determined by culturing cells in a medium containing [1-^{13}C] glucose, where the glucose carbon at position 1 is replaced with ^{13}C, followed by measuring ^{13}C-labeling of pyruvate (or alanine synthesized from pyruvate keeping the same carbon backbone) using a mass spectrometer. The [1-^{13}C] glucose catabolized via glycolysis produces a 1:1 ratio of unlabeled pyruvate and labeled pyruvate containing one ^{13}C. On the other hand, when the [1-^{13}C] glucose is metabolized via the pentose phosphate pathway, the ^{13}C at position 1 is lost through decarboxylation, producing only unlabeled pyruvate. In this way, the split ratio of flux in the branch point of the metabolic pathway can be determined, since the ^{13}C-labeling of metabolites reflects the pathway-specific carbon rearrangement. Besides the analysis of ^{13}C-labeling, the balance of carbon uptake and product secretion by cells can be evaluated through time-course measurement of medium components by HPLC (Fig. 1, step 2). Finally, the data obtained in steps 1 and 2 can be integrated by computational analysis (Fig. 1, step 3). OpenMebius [4], the ^{13}C-metabolic flux analysis software we have developed, integrates the information of ^{13}C-labeling and mass balance, and then estimates the intracellular metabolic flux distribution.

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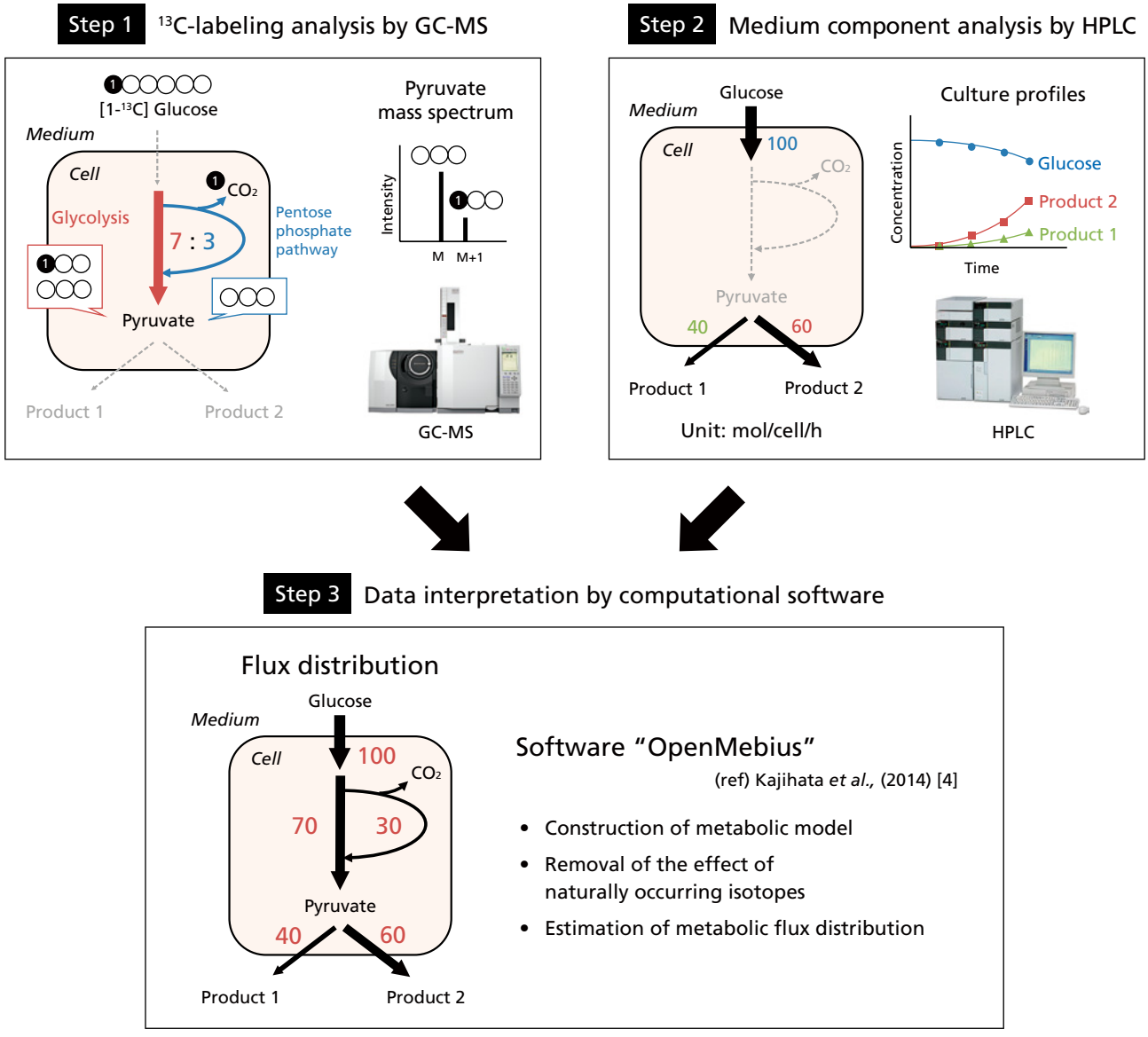

Fig. 1 Overview of ^{13}C-Metabolic Flux Analysis

3. ^{13}C-labeling Culture of *E. coli*

In this study, we referred to the flux analysis method shown in reference [5]. Briefly, *Escherichia coli* MG1655 was cultured aerobically in a Sakaguchi flask using 100 mL of M9 minimal medium containing 5 g/L of ^{13}C-labeled glucose ([1-^{13}C] glucose : [U-^{13}C] glucose = 1:1). The culture broth was sampled to measure the time-course of cell density (OD_{600}) and concentration of medium components. Cell density was measured using a spectrophotometer (Shimadzu UVmini-1240) based on the optical density at a wavelength of 600 nm (OD_{600}). Cell pellets for measuring the ^{13}C-labeling of proteinogenic amino acids were prepared by collecting 3 mL of the culture broth from the mid-log phase (OD_{600} ~ 1) in a Falcon tube and then centrifuging (10 min at 10,000 rpm and 4 °C). The samples were stored at –80 °C until further analysis.

4. Analysis of the ^{13}C-Labeling of Proteinogenic Amino Acids

4-1. Washing the Cells

The sample preparation workflow, from washing the *E. coli* cells to analyzing the ^{13}C-labeling of proteinogenic amino acids by GC-MS, is shown in Fig. 2. First, to wash the cells, 10 mL of saline solution was added to the cell pellets, which were then suspended by agitation with a vortex mixer. The suspension was then centrifuged (10 min at 10,000 rpm and 4 °C) and the supernatant was discarded. These steps were performed three times.

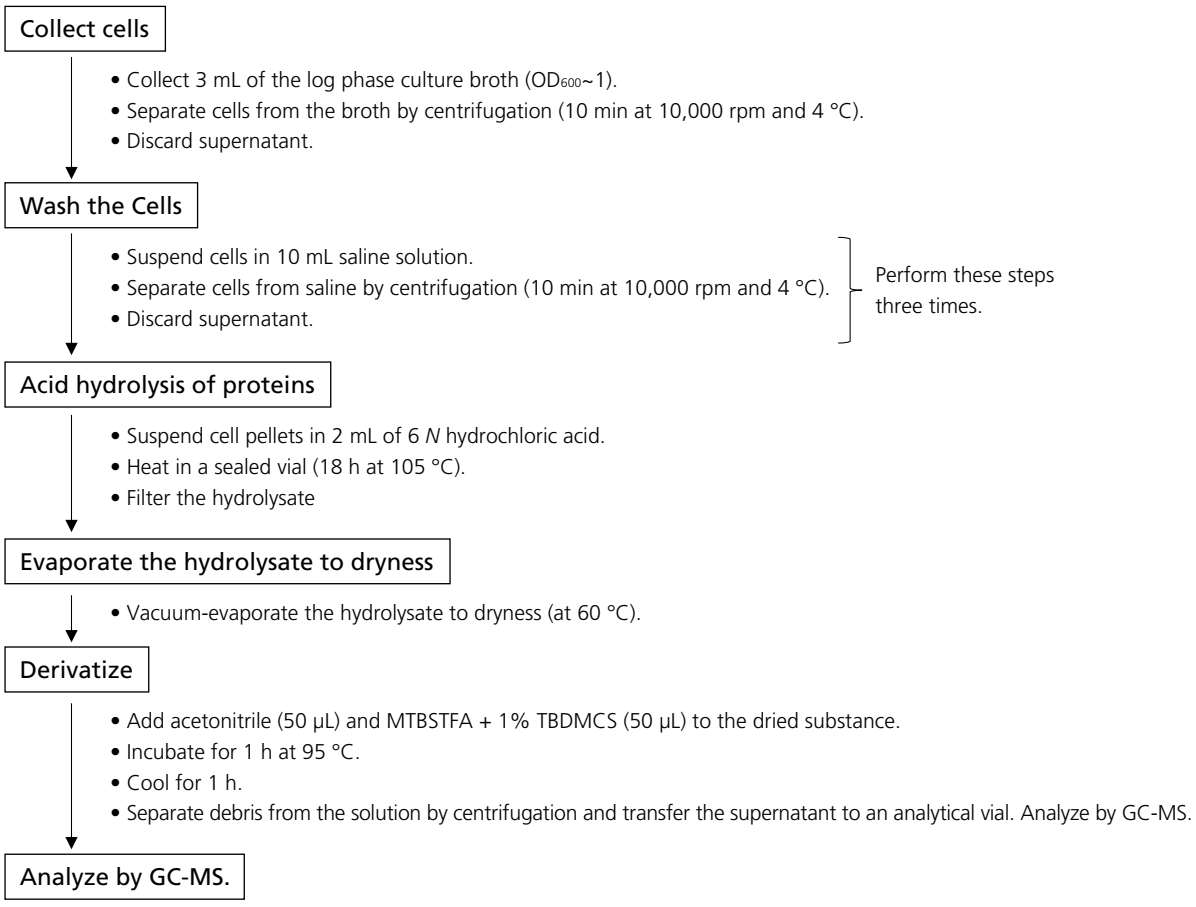

Fig. 2 Sample Pretreatment Workflow

4-2. Acid Hydrolysis of Proteins in Cells

A Cell suspension was prepared in 2 mL of 6 *N* hydrochloric acid. After transferring the suspension to a frozen ampoule vial and vacuum- sealing the vial, it was heated for 18 h at 105 °C in a thermostatic heat block (TAITEC TU-1C dry thermo unit) to hydrolyze the cell proteins. Then, insoluble debris were removed by filtering the hydrolysis solution. Then, 100 µL of the hydrolysis solution was placed in an Eppendorf tube and vacuum-evaporated to dryness at 60 °C in a vacuum evaporator.

4-3. TBDMS-Derivatization of Proteinogenic Amino Acids

N-Methyl-*N*-(*tert*-butyldimethylsilyl) trifluoroacetamide + 1% *tert*-butyldimethyl-chlorosilane (MTBSTFA + 1% TBDMCS) was used for *tert*-butyldimethylsilyl (tBDMCS) derivatization of proteinogenic amino acids. First, 50 µL acetonitrile (Thermo Scientific TS-20062) and 50 µL MTBSTFA + 1% TBDMCS (Thermo Scientific TS-48927) were added to the dried sample and heated at 105 °C for 1 h. After cleaning for 1 h, the solution was separated by centrifugation (5 min at 15,000 rpm) and the supernatant was transferred to an analytical vial for GC-MS analysis.

4-4. Analytical Conditions

The GC-MS analytical conditions are shown in Table 1. To obtain information about the position of ^{13}C atoms in the carbon backbones of metabolites, flux analysis requires measurements of the fragment ions generated by electron ionization (EI). Figure 3 shows the mass spectrum of tBDMS-derivatized aspartate. The signals at $[M-57]^+$ and $[M-85]^+$ indicate fragment ions generated by the cleavage of the molecular ions by ionization. The precision of the flux estimation can be improved by measuring each of these fragments. In addition, isotopes M, M+1, M+2, M+3, and so on, must be measured to determine the number of ^{13}C in the fragment ions. Consequently, many more channels are required for selected ion monitoring (SIM) measurement for each target metabolite. For the given measurement, the Smart SIM automatic method creation function was used to optimize the dwell time and develop the SIM method.

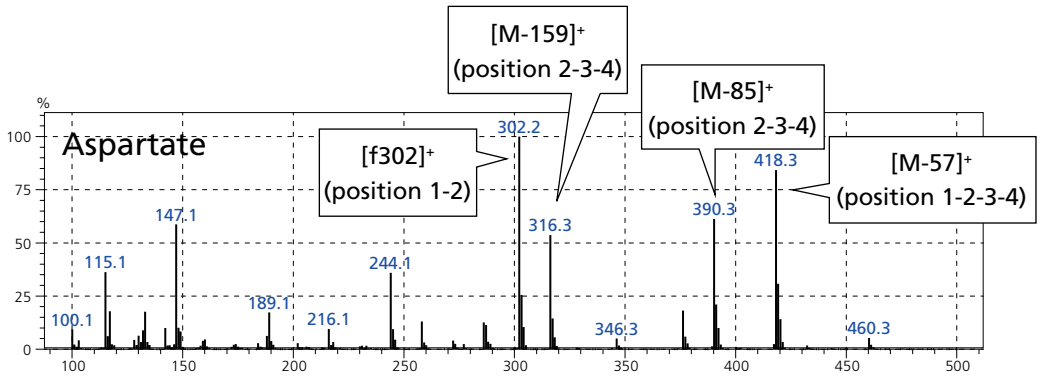

Fig. 3 Mass Spectrum of tBDMS-Derivatized Aspartate and Specific Fragments with Backbone Carbons

Table 1 Analytical Conditions of GC-MS for Measuring the ^{13}C-labeling of Proteinogenic Amino Acids

System	: GCMS-TQ8040, AOC-20i (autoinjector)
GC Conditions	
Column	: DB-5MS+DG (Agilent Technologies) (30 m long × 0.25 mm I.D., with 0.25 µm thick film)
Injection Port Temp.	: 250 °C
Column Temp.	: 150 °C (2 min) → 3 °C/min → 270 °C → 10 °C/min → 300 °C (5 min)
Injection Mode	: Split (1:10)
Carrier Gas	: He
Linear Velocity	: 38.1 cm/sec
Injection Volume	: 1 µL
MS Conditions	
Ion Source Temp.	: 200 °C
Interface Temp.	: 250 °C
Event Time	: 0.3 sec
Measurement Mode	: Selected Ion Monitoring (471 channels for 15 amino acids)

5. Analysis of Culture Medium Components

The collected culture broth was separated in a centrifuge (5 min at 15,000 rpm and 4 °C) and the supernatant was filtered through a filter cartridge with a 0.45 µm pore size to remove the cells. A mixture of 100 µL of culture solution and 100 µL of pimelate solution (internal standard) was analyzed by HPLC based on the conditions shown in Table 2.

Table 2 Analytical Conditions of HPLC for Cultured Medium Supernatant

System	: HPLC Prominence
Detector	: RID-20A (35 °C)
Column	: Aminex HPX-87H Column (BIO-RAD)
Temp.	: 65 °C
Eluent	: 1.5 mM H_2SO_4
Flowrate	: 0.5 mL/min
Injection Volume	: 20 µL

6. Estimation of Metabolic Flux

OpenMebius, ^{13}C-metabolic flux analysis software (http://www-shimizu.ist.osaka-u.ac.jp/hp/en/software/) was used to interpret the ^{13}C-labeling of proteinogenic amino acids [4]. This software includes functionality for generating metabolic models required for the analysis, eliminating the effects of naturally-occurring stable isotopes, and estimating metabolic flux distributions.

7. Results

To estimate the flux distribution of the central carbon metabolism for *Escherichia coli* MG1655, GC-MS was used to analyze the ^{13}C-labeling of proteinogenic amino acids. Figure 4 shows the total ion chromatogram of tBDMS-derivatized proteinogenic amino acids. The ^{13}C-labeling of amino acids was obtained for 15 standard amino acids. Figure 5 shows the ^{13}C-labeling of alanine, where alanine without ^{13}C (M) accounts for the largest proportion before ^{13}C-labeling, but the proportion of alanine that includes ^{13}C atoms (such as M+1, M+2, and M+3) increases after ^{13}C-labeling. The intensity values from this spectrum were entered in the specified Excel spreadsheet in the OpenMebius ^{13}C-metabolic flux analysis software (Fig. 6).

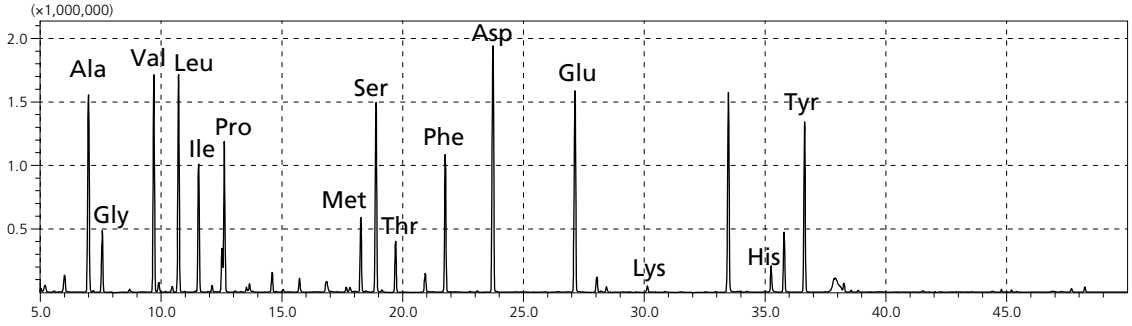

Fig. 4 GC-MS Analysis of tBDMS-Derivatized Proteinogenic Amino Acids (Total Ion Chromatogram)

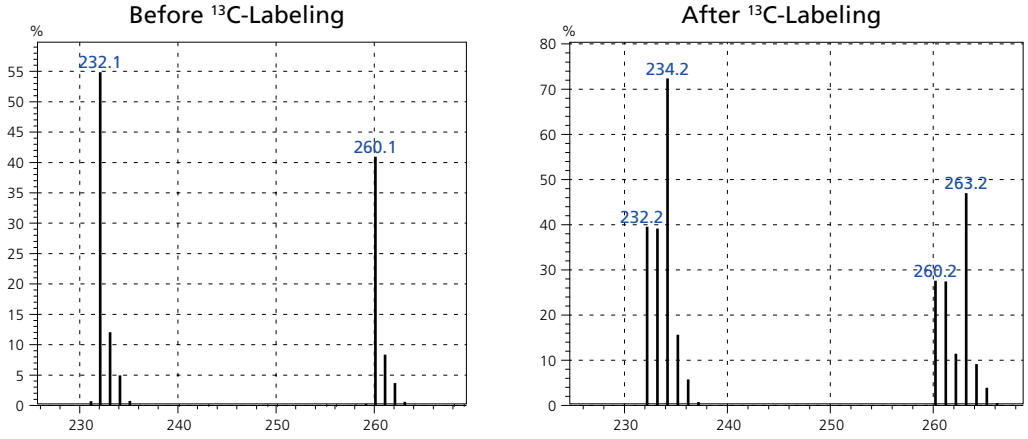

Fig. 5 Mass Spectra of Alanine Before and After ^{13}C-Labeling

The above are spectra of fragments $[M-57]^+$ (m/z = 260) and $[M-85]^+$ (m/z = 232) of tBDMS-derivatized alanine measured by GC-MS. Before ^{13}C-labeling (left), the largest proportion of alanine contained no ^{13}C carbons. In contrast, after ^{13}C-labeling (right), alanine with increased mass numbers was observed according to the number of ^{13}C atoms included in the fragment.

Fragment name

Number of spectra entered

Isotope peak area values obtained in GC-MS analysis are entered in order from the top for M, M+1, and so on.

	A	B	C	D	
1	Ala_57	Ala_85	Asp_57	Asp_85	⋯
2	7	6	8	7	⋯
M	3	349908	519610	235390	233119	
M+1	4	349415	515574	449382	403483	
M+2	5	144005	960851	494934	506681	
M+3	6	600341	210249	632532	561052	⋯
M+4	7	117453	77344	568444	190945	
M+5	8	49844	10611	197138	73778	
M+6	9	6506		73774	15928	
⋮	10			15984		
	11					

Fig. 6 Entering Isotope Intensity in OpenMebius

Next, the culture medium components were analyzed by HPLC. The acetate peak observed in the chromatogram indicates that the *Escherichia coli* on the mid-log phase releases acetate into the culture medium (Figs. 7 and 8). The specific consumption rate of the carbon source and the specific secretion rate of the products obtained by time-course observations of the concentrations for respective culture components were entered in the specified Excel spreadsheet in OpenMebius (Fig. 9). The efflux for cell growth (BIOMASS in Fig. 9) was calculated on the basis of the specific growth rate (0.70 h^{-1}) and cellular components shown in reference [6].

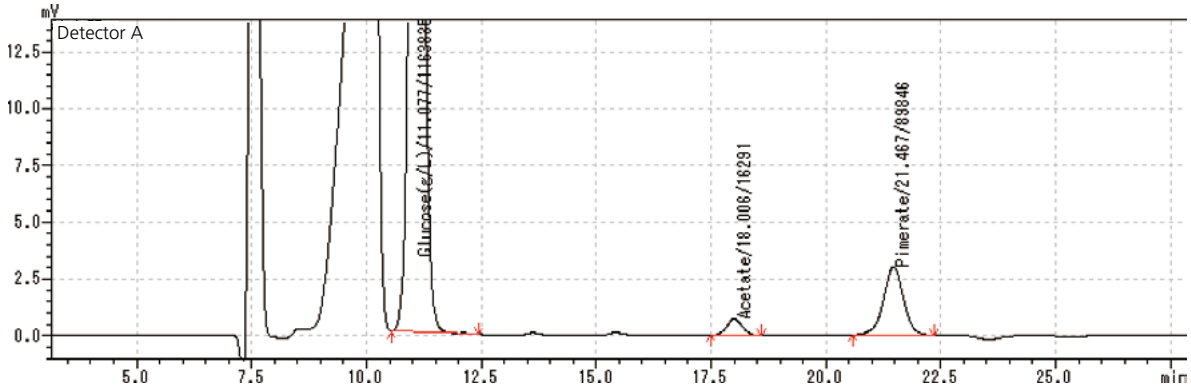

Fig. 7 Chromatogram of Cultured Medium by HPLC Analysis

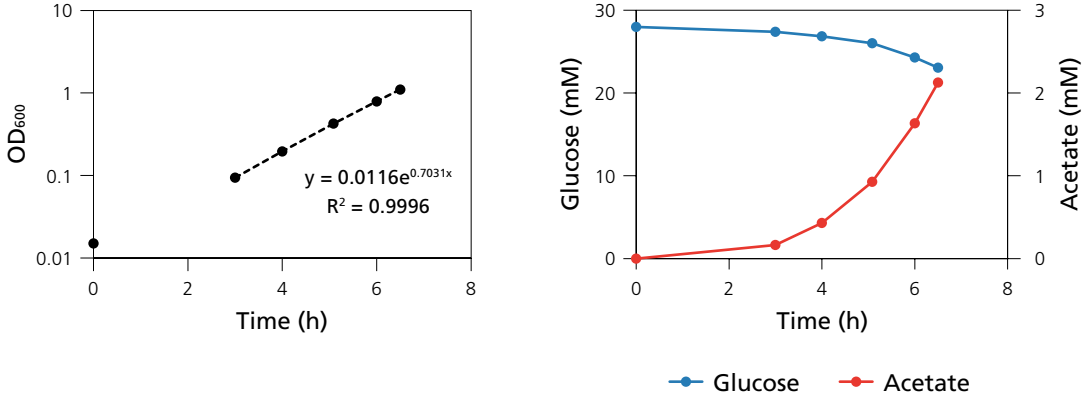

Fig. 8 Cell Growth (left) and Changes in Glucose and Acetate Concentration in the Culture Medium (right)

Flux values
In this case, relative values were entered,
normalized to a value of 100 for the uptake of glucose.
(Absolute values would also be acceptable.)

Reaction names

	A	B
1	FluxID	Flux
2	r1_in_gluc	100
3	r59_ex_ace	40.55173
4	r44_BIOMASS_g6p	1.685339
5	r45_BIOMASS_f6p	0.582881
	⋮	⋮

Fig. 9 Entering Specific Consumption and Production Rates

The flux distribution of the central carbon metabolism for *Escherichia coli* was successfully determined by integrating the information of ^{13}C-labeling of proteinogenic amino acids measured by GC-MS, and the specific rates measured by HPLC via OpenMebius [4] (Fig. 10). Of the total glucose uptake, 75% was metabolized by glycolysis and 23% was metabolized by the pentose phosphate pathway. Consequently, the split ratio at the branch point of metabolic pathway could be determined.

The result also shows the activation of oxidative TCA cycle metabolism, which provided information about the direction of the reaction. Finally, zero flux was estimated for the glyoxylate shunt and the Entner–Doudoroff pathway, which enabled the identification of reactions with no activity for the given culture condition.

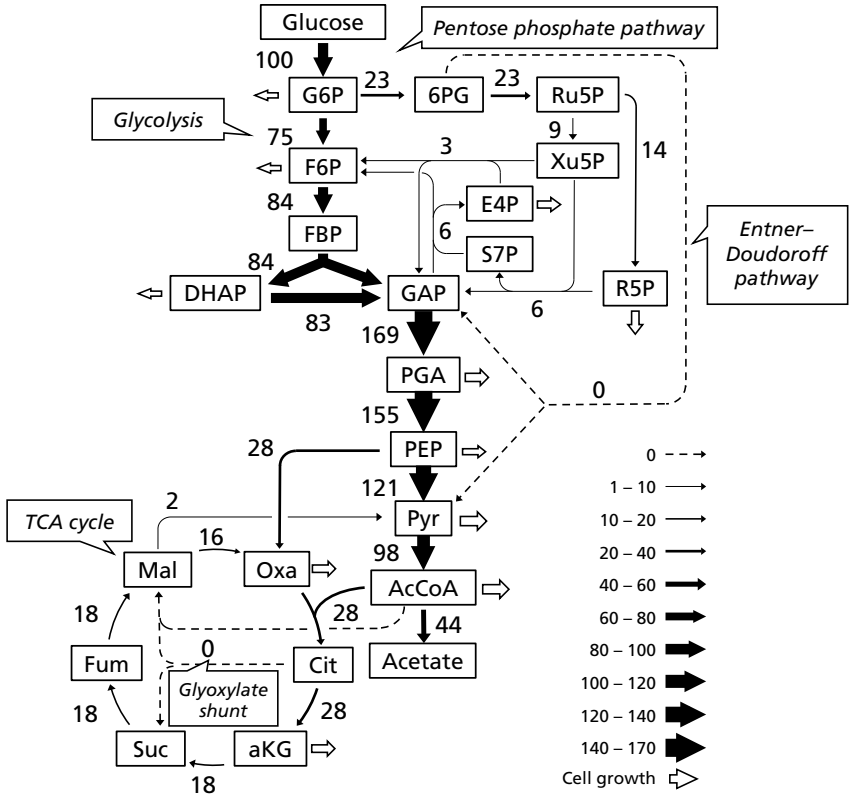

Fig. 10 Metabolic Flux Distribution of Growing *Escherichia Coli*

Each figure beside an arrow indicates the level of flux for each reaction (molar per unit time and per unit weight of the dried cells). The values were normalized based on a glucose uptake rate of 100 (8.76 mmol/g-DCW/h).

8. Summary

^{13}C-metabolic flux analysis was used to determine the metabolic flux distribution of the central carbon metabolism of *Escherichia coli*. The described method is beneficial for evaluating the metabolism of microorganisms that produce useful chemicals and diseased cells, such as cancer [1, 2, 3].

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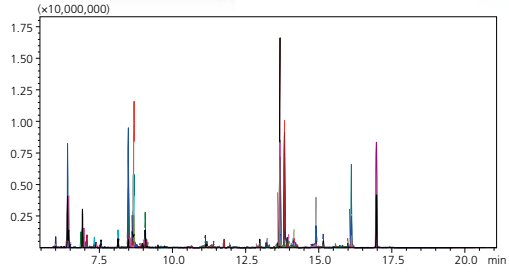
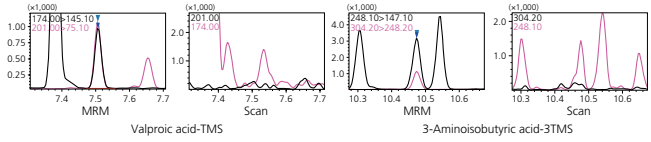


Total Ion Current Chromatogram (TIC) from MRM Analysis of Metabolic Components in Standard Human Plasma



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Printed in Japan 3655-05618-500NS