Isotopomer distributions in amino acids from a highly expressed protein as a proxy for those from total protein

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ABSTRACT

^{13}C-based metabolic flux analysis provides valuable information about bacterial physiology. Though many biological processes rely on the synergistic functions of microbial communities, study of individual organisms in a mixed culture using existing flux analysis methods is difficult. Isotopomer-based flux analysis typically relies on hydrolyzed amino acids from a homogenous biomass. Thus metabolic flux analysis of a given organism in a mixed culture requires its separation from the mixed culture. Swift and efficient cell separation is difficult and a major hurdle for isotopomer-based flux analysis of mixed cultures. Here we demonstrate the use of a single highly-expressed protein to analyze the isotopomer distribution of amino acids from one organism. Using the model organism *E. coli* expressing a plasmid-borne, his-tagged Green Fluorescent Protein (GFP), we show that induction of GFP does not affect *E. coli* growth kinetics or the isotopomer distribution in nine key metabolites. Further, the isotopomer labeling patterns of amino acids derived from purified GFP and total cell protein are indistinguishable, indicating that amino acids from a purified protein can be used to infer metabolic fluxes of targeted organisms in a mixed culture. This study provides the foundation to extend isotopomer-based flux analysis to study metabolism of individual strains in microbial communities.

Key words: ^{13}C based metabolic flux analysis, *E. coli*, his-tagged GFP, central metabolic pathways, microbial communities.

INTRODUCTION

Metabolic flux analysis allows insight into a cell's overall carbon metabolism, energy production, and the relationship of the genotype to its phenotype[1]. Development of typical flux balance models requires assumption of an objective function (often maximizing the specific growth rate) and a series of physicochemical constraints (thermodynamic directionality, enzymatic capacity, and reaction stoichiometry)[2]. For a complicated metabolic network, there are not always enough constraints to find a unique solution. Thus, such an approach alone may not accurately describe the reversible reactions or reactions that may form a futile cycle, especially when the cell's metabolism is suboptimal or the annotated pathways are latent under experimental conditions[3, 4]. For determination of actual carbon fluxes, ^{13}C based flux analysis is an advanced approach whereby a ^{13}C-labeled carbon source, such as glucose, is fed to the cells, and the labeling pattern in resulting metabolites is measured by NMR or GC-MS[5-7]. Amino acids arise from central metabolic pathways and reflect the carbon backbones of their precursors (Figure 1). The labeling pattern in amino acids provides information about the ^{13}C distribution in their corresponding precursor metabolites, and thus allows the unique determination of carbon fluxes in branched pathways or metabolic cycles. In a typical isotopomer based flux analysis assay, labeled biomass provides the total protein, which is hydrolyzed to yield the amino acid analyte. Recent advances in isotopomer-based flux analysis have been applied to numerous microorganisms[3, 4, 8-10]. Results from such studies not only quantified fluxes through central metabolic pathways but also provided experimental evidence for predicted pathways in recently sequenced genomes[8, 11, 12]. Since analytes comprise of amino acids derived from total biomass of a culture, this

strategy is only applicable to defined monocultures; little is known about the cellular metabolic network of individual organisms in complicated mixed cultures.

Most naturally-occurring biological processes, ranging from the degradation of complex organics to elemental cycling, involve the synergistic action of multiple microorganisms[13-21]. Understanding how multiple organisms interact and function in a community is essential to improve their use in bioremediation of contaminated environments and production of renewable energy[22-24]. However, it is difficult to monitor metabolic fluxes of individual organisms in a community because it is not possible to assign amino acid isotopomer data to the source organism when the sample is derived from a hydrolysate of the total culture biomass. Traditional separation techniques such as cell sorting or gradient centrifugation are not efficient for enriching cell types for isotopomer analysis. Currently, flux analysis for microbial communities is limited to the study of the entire mixed culture to provide an overall view of the carbon metabolism[25]. Here we describe experiments to test the assumption that an enriched pure protein from an organism can provide the same information as that from total cell protein. While the origin of an amino acid is ambiguous and cannot be assigned to a sub-population in a mixed culture, the origins of a protein on the other hand can be fully assigned. As a result, the use of a single protein for isotopomer-based flux analysis would allow the study of a microbe in its community.

EXPERIMENTAL SECTION

E. coli growth and metabolite assays for isotopomer studies. Difco M9 minimal salts (5X) were purchased from Becton, Dickinson and Company (Franklin Lakes, NJ). Unlabeled glucose was obtained from Fisher Scientific (Fair Lawn, NJ) and labeled glucose (1-^{13}C, 99%) from Cambridge Isotope Laboratories (Andover, MA). Isopropyl β-D-1-thiogalactopyranoside was procured from Sigma-Aldrich (St. Louis, MO). Chelating sepharose fast flow resin was purchased from GE Healthcare Bio-Sciences (Piscataway, NJ).

The gene encoding green fluorescence protein (*gfp*) was cloned in the *Nco*I and *EcoR*I restriction sites in a pET-30 vector (Novagen, Madison, WI) to create the expression vector pET30-GFP. *E. coli* DH10B was used for cloning and plasmid maintenance while *E. coli* BLR(DE3) was used as the host for protein production. Total protein and purified GFP in this study were isolated from the wild type (*E. coli* BLR(DE3)) or the GFP expression strain (*E. coli* BLR(DE3) pET30-GFP). All cultures were grown in M9 minimal medium supplemented with 1% glucose, 0.01 mM $FeSO_4$, and a trace metal solution[26]. Shake flasks (250 mL) were used to grow these cultures at 37°C with shaking at 200 rpm. Kanamycin (50 µg/mL) was used for strains containing pET30-GFP.

To avoid excessive lag time during adaptation to minimal medium, cultures were subjected to the following procedure. A single colony was used to inoculate 10 mL of Luria Broth and this culture was grown overnight. An aliquot (500 µL) of this culture was used to inoculate 20 mL of M9 minimal medium, and this culture was incubated overnight at 37°C. If the optical density at a wavelength of 600 nm (OD_{600}) of this culture

was greater than 1.0/mL, the cells were subcultured (2%) into fresh M9 minimal medium and allowed to grow for 24 hours. This procedure was repeated until the OD_{600} of overnight cultures was greater than 3.5/mL for at least two subcultures to adapt cells to defined medium. One-mL aliquots were stored at -80°C for future use. A thawed aliquot was used to inoculate overnight cultures in M9 minimal medium, and this culture (0.1%) was used to inoculate 50 mL of M9 minimal medium for subsequent studies. When the OD_{600} of the cultures containing the plasmid reached 0.7/mL, IPTG was added to a final concentration of 1 mM to induce production of recombinant his-tagged GFP. Cells were incubated at 37°C for three hours and cell pellets were harvested by centrifugation (10,000 × g for 20 minutes). Cell growth was monitored by measuring the absorbance at OD_{600}. The concentrations of glucose and acetate in the culture medium were measured using enzyme kits as per the manufacturer's instructions (r-Biopharm, Darmstadt, Germany).

Protein purification. The cell pellets were resuspended in ice-cold, 50 mM HEPES buffer, pH 8. The cells were lysed by sonication and the total soluble protein was separated from the insoluble debris by centrifugation (10,000 × g for 30 minutes). The his-tagged recombinant GFP was purified from the total soluble protein using immobilized nickel ion affinity chromatography. Briefly, 0.5 mL resin was used in each case and the resin was prepared according to the manufacturer's instructions (GE Healthcare, Piscataway, NJ). Total soluble protein was added to the resin. The resin was washed with increasing concentrations of imidazole; the protein was eluted with 250 mM imidazole. Protein samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE). To confirm the presence of tagged GFP, a Western blot was carried out using the S-tag alkaline phosphatase Western blot kit as per the manufacturer's instructions (Novagen, Madison, WI).

Sample preparation for GC-MS. One-mL purified protein (containing about 3 mg his-tagged GFP) was precipitated using 0.3 mL 60 wt % trichloroacetic acid (TCA). After adding TCA, samples were incubated at 4°C for 10 min and then centrifuged at 20,000 × g for 5 min at 4°C. The supernatant was removed, and the protein pellet was washed three times using 1 mL deionized water followed by three washes with 0.5 mL cold acetone. These washes minimized carry forward of any residual phosphate salts from the growth medium and eliminate imidazole and other organic compounds that may interfere with GC-MS signals. To hydrolyze proteins to amino acids, the protein pellet was dried at 100°C for 2 min, resuspended in 1.5 mL 6 M HCl, and transferred to a clear glass, screw-top GC vial. Vials were capped and incubated at 100°C for 24 hours. After hydrolysis, the caps were removed and the samples were dried overnight under a stream of air.

GC-MS samples were prepared as previously described[8, 11]. Briefly, samples were dissolved in 100 µL tetrahydrofuran (THF) and 100 µL N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide (Sigma-Aldrich, St. Louis, MO). All samples were derivatized in a water bath at 65-80°C for 1 hour, resulting in tert-butyldimethlysilyl (TBDMS) derivatives. One µL of the derivatized sample was injected into a gas chromatograph (Agilent model 6890, Wilmington, DE) equipped with a DB5-MS column (J&W Scientific, Folsom, CA) and analyzed using a quadrupole mass selective detector (EI) operated at 70 eV (Agilent 5973, Wilmington, DE). The MS was operated in scan mode

(started after 4 min, mass range 60-550 a.m.u. at 2.94 s/scan). The sample injection volume was 1 μL at a carrier gas flow of 2 mL/min helium with a split ratio of 1:20. The GC operation conditions were as follows: the GC column was held at 150°C for 2 min, heated at 3°C per minute to 280°C, heated at 20°C per minute to 300°C, and held for 5 min at that temperature. Two types of positively charged ions were clearly observed by GC-MS: the derivatized amino acids after the loss of a tert-butyl group $[M-57]^+$, and the ion after fragmentation at the α-carboxyl group, $[M-159]^+$. The natural abundance of isotopes was corrected using a published algorithm before using the data for calculating the label distribution[12].

RESULTS AND DISCUSSION

To test the hypothesis that a pure protein could be used to obtain the isotopomer data to compute flux for the source organism, two important requirements had to be fulfilled. First, in using a recombinant protein it was important to assess if production of GFP affects *E. coli* central metabolism. Second, it was important to determine if the isotopomer labeling pattern in amino acids from GFP reflected the labeling pattern in amino acids from total protein.

The experimental strategy is shown in Figure 2: sample 1 is *E. coli* BLR(DE3) cultured with un-labeled glucose; sample 2 is *E. coli* BLR(DE3) cultured with 99% 1-^{13}C glucose, samples 3, 4 and 5 are *E. coli* BLR(DE3) pET30-GFP cultured with 99% 1-^{13}C glucose and induced for his-tagged GFP production. For isotopomer analysis, amino acids derived from either hydrolyzed total protein or hydrolyzed purified GFP were used. Samples 2 and 3 were used for analyzing amino acids from total protein in un-induced

and induced cultures, respectively. Sample 4 was used for analyzing amino acids from pure, his-tagged, GFP. Sample 6 was a 1:1 mixture of sample 1 (un-labeled wild-type biomass) and sample 5 (labeled induced biomass) and represented an artificial mixed culture from which his-tagged GFP was purified. To prove that protein induction does not affect central metabolism, the isotopomer data obtained from samples 2 and 3 were required to be comparable. To establish that a purified protein can be used for an isotopomer-based flux analysis of the source organism, data from the his-tagged GFP from sample 6 were required to be equivalent to the data from sample 2.

Under aerobic conditions, all cultures outlined above had a doubling time of ~1 hr. In order to determine the isotopomer patterns in amino acids, samples were taken at mid-log phase (OD_{600} ~ 2), which represents a (quasi) steady state that is often used for flux analysis[21]. At the time of sampling, glucose consumption and acetate production were measured (Table 1). No significant difference was observed between the various cultures, indicating that GFP production did not significantly affect *E. coli* BLR(DE3) growth kinetics. Total soluble protein was obtained from the cells as described in the Materials and Methods section. His-tagged GFP was obtained from total soluble protein of samples 4 and 6. Total protein was loaded on Ni-charged resin, and bound GFP was eluted with 250 mM imidazole. All the samples were analyzed by SDS-PAGE. The elution fractions corresponding to samples 4 and 6 indicated that GFP was more than 99% pure (Figure 3A). A Western blot was carried out to confirm the presence of recombinant GFP (Figure 3B).

GC-MS analysis is a high-throughput and sensitive (≤ 2% error) method widely used for ^{13}C isotopomer analysis. Application of GC-MS to resolve derivatized protein

hydrolysate gave chromatographic peaks corresponding to 16 amino acids (asparagine, cysteine, glutamine, and tryptophan could not be determined, possibly due to sample degradation) (Figure 4A). Several amino acid pairs derived from the same precursor, such as proline and glutamate (from precursor oxoglutarate), threonine and aspartate (from precursor oxaloacetate), tyrosine and phenylalanine (from precursors phosphoenolpyruvate and erythrose-4-phosphate), had similar isotopomer distribution patterns. Nine key amino acids, representing their precursor's labeling that are often used for isotopomer analysis are listed in Table 2, and the remaining amino acids provide redundant isotopomer information[3]. Two types of ions were used (Table 2 and Figure 4B): fragment [M-57]+ is the *tert*-butyldimethylsilyl (TBDMS)-derivatized amino acid with a loss of the *tert*-butyl group; fragment [M-159]+ is the TBDMS-derivatized amino acid with a loss of the COO-TBDMS portion due to a break between the α- and β-carbon atoms of the amino acid[12].

The GC-MS spectra for key amino acids from total protein of wild-type and induced *E. coli*, as well as purified GFP (samples 2, 3 and 4) had identical isotopomer distributions (Table S-1). Isotopomer data of amino acids derived from GFP isolated from the artificial mixed culture (sample 6) was also equivalent to data from the control sample (sample 2) (Table 2 and Figure S-1). The difference in isotopomer distribution is <2%, well within the measurement noise. These results indicate that this method can be used to investigate the metabolic fluxes in an individual microorganism in a microbial community using a purified protein. The same strategy may be extrapolated to any protein that can be isolated in adequate levels and then used to quantify the isotopomer distribution in the corresponding amino acids.

While the method outlined in this study is fairly universally applicable and uses generally accessible reagents and instrumentation, certain precautions were necessary prior to obtaining accurate spectra for isotopomer analysis. Media used for culturing bacteria contains phosphate that may remain in the total protein samples. Residual phosphate can be derivatized and its products interfere with other compounds of interest during GC-MS separation[27]. Moreover, reagents such as urea and imidazole that are part of typical cell lysis and protein purification buffers may also be derivatized by TBDMS and complicate the GC-MS data acquisition and spectral quality. Examples of urea, imidazole and phosphate contaminated spectra are provided in Figure S-2. To overcome this problem, TCA precipitation of proteins followed by extensive washing with water and acetone proved effective. Another factor to be considered is the quantity of pure protein required. Despite the sensitivity of GC-MS to detect low sample levels, due to the multiple clean up steps, efficiency of protein hydrolysis and efficiency of derivatization, approximately 1 mg of GFP was required to enable detection of all key amino acids essential for flux analysis. Lower amounts of protein may cause the loss of signal of rarer amino acids like methionine and histidine.

CONCLUSIONS

Flux analysis provides key information about cellular metabolism. This information is central to many studies: physiological characterization of stress responses, impact of metabolic engineering, to name a few. However, isotopomer-based flux analysis methods cannot be currently applied to assess the core physiology of an organism unless it is present in a mono-culture. In order to address this hurdle, we show

here that the isotopic labeling in key amino acids derived from purified over-expressed protein in an organism serves as a proxy for total protein of that organism. As it is the isotopomer data from these key amino acids that are used to determine flux through central metabolic pathways, the flux distribution information of a target organism in a mixed culture can be obtained from a single purified protein.

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FIGURE CAPTIONS

Figure 1. Central metabolic and amino acid biosynthetic pathways. E4P, erythrose-4-phosphate; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; PEP, phosphoenolpyruvate; PGA, 3-phosphoglycerate; C5P, ribose-5-phosphate; T3P, Triose-3-phosphate; and S7P, sedoheptulose 7-phosphate.

Figure 2. Experimental strategy outlining the various samples used for proof of concept isotopomer analyses. Samples were numbered as shown. Sample 6 is a mixture of un-labeled culture (sample 1) and labeled induced culture (sample 5).

Figure 3. Total protein and pure recombinant GFP used in isotopomer analysis. (A) Coomassie stained SDS-PAGE analysis of protein samples. Lane 1 contains the ProSieve color protein marker (Cambrex, Rockland, ME). Lanes 2 and 3 show total soluble protein, while lanes 4 and 5 show pure, recombinant GFP from samples 4 and 6 respectively. **(B)** Anti S-tag Western blot to confirm the presence of GFP. Lane 1 contains the ProSieve color protein marker. Lanes 2 and 4 show total soluble protein while lanes 3 and 5 show pure recombinant GFP obtained from samples 4 and 6, respectively.

Figure 4. Gas chromatograph-mass spectrum of amino acids from purified GFP (sample 6). (**A**) 16 of the 20 amino acids can be clearly observed in the spectrogram (glutamine, asparagine, tryptophan, and cysteine were not observed). Standard abbreviations of amino acids are used to label the corresponding peaks. **(B)** Mass

spectrum of a TBDMS-derivatized amino acid. An amino acid is usually derivatized at both the carboxylic acid and amino group. The spectrum shown is for glutamic acid, where ions with m/z = 432 – 437 are the isotopomers of [M-57]+ fragments and the ions with m/z = 330 – 334 are the isotopomers of [M-159]+ fragments.

Table 1. Growth kinetics of *E. coli* after IPTG induction for GFP production.

	Sample 2	Samples 3, 4 and 5
Glucose remaining, g/L	7.4 ± 0.3	7.6 ± 0.1
OD_{600}	~2	~2
Acetate, g/L	0.15 ± 0.02	0.17 ± 0.05
Doubling time	~1 hr	~1 hr

Table 2. Comparison of GC-MS data, total protein and his-tagged GFP.

Amino acid	Precursor/Pathway	Ion[a]	Total protein from BLR(DE3) (sample 2)		Purified GFP (sample 6)	
			[-57][a]	[-159]	[-57]	[-159]
Alanine	Pyruvate	M0	0.56±0.01	0.56±0.02	0.55±0.02	0.55±0.01
		M1	0.43±0.02	0.41±0.01	0.44±0.01	0.42±0.02
		M2	0.01±0.0	0.03±0.01	0.01±0.0	0.03±0.01
Glycine	Serine	M0	0.97±0.03	0.99±0.01	0.99±0.0	1.0±0.0
		M1	0.02±0.01	0.01±0.0	0.01±0.0	0.0±0.0
Valine	Pyruvate	M0	0.34±0.01	0.33±0.02	0.33±0.01	0.32±0.02
		M1	0.48±0.01	0.47±0.02	0.48±0.01	0.48±0.01
		M2	0.17±0.01	0.19±0.01	0.18±0.01	0.19±0.01
Leucine	Pyruvate & Acetyl-CoA	M0	Peak overlap	0.20±0.02	Peak overlap	0.19±0.02
		M1		0.41±0.02		0.41±0.02
		M2		0.30±0.01		0.31±0.03
Isoleucine	Pyruvate & oxaloacetate	M0	Peak overlap	0.23±0.02	Peak overlap	0.22±0.01
		M1		0.42±0.02		0.43±0.0
		M2		0.27±0.01		0.27±0.0
Serine	3-P-Glycerate	M0	0.57±0.03	0.61±0.02	0.56±0.02	0.60±0.01
		M1	0.42±0.02	0.38±0.02	0.42±0.01	0.39±0.02
		M2	0.01±0.0	0.01±0.01	0.01±0.0	0.01±0.0
Phenylalanine	P-enolpyruvate &Erythrose-4-P	M0	0.25±0.01	0.24±0.02	0.25±0.02	0.24±0.02
		M1	0.44±0.02	0.45±0.01	0.43±0.02	0.45±0.01
		M2	0.25±0.01	0.23±0.02	0.26±0.01	0.24±0.02
Aspartic acid	Oxaloacetate	M0	0.37±0.02	0.43±0.01	0.37±0.01	0.43±0.02
		M1	0.47±0.02	0.44±0.0	0.47±0.02	0.44±0.01
		M2	0.10±0.02	0.10±0.01	0.10±0.01	0.10±0.02
Glutamic acid	2-oxo-glutarate	M0	0.26±0.03	0.29±0.02	0.25±0.01	0.29±0.02
		M1	0.42±0.03	0.46±0.02	0.43±0.02	0.45±0.03
		M2	0.23±0.01	0.21±0.02	0.23±0.02	0.22±0.01
Histidine	Ribose-5-P	M0	0.29±0.03	0.37±0.02	0.28±0.02	0.36±0.02
		M1	0.39±0.02	0.45±0.03	0.39±0.02	0.46±0.02
		M2	0.24±0.01	0.15±0.01	0.24±0.01	0.16±0.01

[a]M0, M1, M2 represent unlabeled, singly ^{13}C and doubly ^{13}C labeled ions, respectively, of a given fragment.

Note: the standard error of each measurement was 0~3% (n=2). Ions [-57] of leucine and isoleucine could not be resolved because their peaks were overlapped by other peaks, and thus their isotopomer data are not clear.