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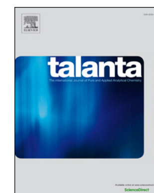

# Determination of intracellular metabolites concentrations in *Escherichia coli* under nutrition stress using liquid chromatography-tandem mass spectrometry

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ABSTRACT

Cells show a timely and appropriate physiological adjustment on all levels of cellular activities in response to nutrient stress. However, the regulations for cells under different carbon/nitrogen influxes are poorly understood. To unveil a fully metabolic regulatory profile, we applied a mass spectrometry based "bottom-up" approach to investigate the metabolic response of *Escherichia coli* to nutrient stress. A novel cell sample preparation procedure was developed to decrease the variation and leakage of intracellular metabolites. Volatile ion-pair reagent tributylamine was used to improve the retention and selectivity of charged metabolites on a C18 reversed-phase column. The growth rate and intracellular concentrations of 12 central carbon metabolites were measured systematically under various carbon/nitrogen influxes by manipulating titratable promoters. Fructose-1,6-biphosphate (FBP) concentration as a sensor of carbon influx was positively correlated with the growth rate, whereas α-ketoglutarate (αkg), served as a coordinator of carbon and nitrogen flux showed different dependence on growth rate between carbon limitation and nitrogen limitation. By integrating different behaviors of the metabolites with knowledge from previous reports, a scenario of feedback control under carbon and nitrogen limitations was proposed. Our findings revealed the key role of αkg in the coordination of carbon and nitrogen utilization under nutrition stress and highlighted the great potential of mass spectrometry based approach in deciphering the complex metabolic network.

## 1. Introduction

Cells survive under different kinds of nutrient stress by adjusting their cellular metabolism. The growth of a cell would come to a halt under a variety of stress conditions, in particular, the depletion of a critical nutrient in the medium [1]. However, the timing of regulatory steps including shut down growth, adjust the influx and the way cells coordinate its protein composition to enter into a different growth mode or the stationary phase, are poorly understood up to now. Thus, characterization of the cellular response of microbial systems to environmental perturbations especially nutrient stress has attracted increasing interests in recent years. Many efforts have been made to investigate the correlation between the type of stress and the response in *Escherichia coli* (*E. coli*) at the gene expression level [2–4]. However, transcriptional regulation alone is not sufficient to completely determine the metabolite flow pattern on the complex metabolic network. It is known that metabolites and proteins can actually regulate themselves to some extent and many downstream mechanisms can play important roles in cellular responses through self-adjustment. For example, allosteric regulation by small molecules can change the conformation of proteins so that rapid regulation can be achieved, with a much shorter time scale as compared to transcriptional regulation [5]. It is thus important to take into account the changes in metabolite concentrations in downstream in addition to gene and protein expression changes in order to obtain a more complete picture of cellular stress response.

On the other hand, central carbon metabolism, as the main frame of metabolic network is considered crucial in the investigation on the regulation response to the environment in model organism *E. coli* [6]. The intracellular concentrations of the relevant metabolites carry information that can lead to quantitative understanding of in vivo enzyme kinetic parameters or control of metabolic flux with the aid of mathematical tools and theoretical models [7]. For the determination of central carbon metabolites in *E. coli*, liquid chromatogram-mass

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spectrometry (LC-MS) based methods have been applied for most studies, with different sample preparation procedures [8–15]. Mass spectrometry (MS) is preferred for the aims of high throughput, resolution, and sensitivity to detect central carbon metabolites because most of them have no or weak UV absorption or florescence [16]. Most of the MS methods, however, focused on one step of sample preparation with miscellaneous results [17–19]. Therefore, evaluation and optimization of the comprehensive sample preparation procedures for central carbon metabolism is necessary to retain the original cell state.

In this work, LC-MS based "bottom-up" approach was employed to investigate the cellular response of *E. coli* to nutrient stress. Firstly, different strains of *E. coli* were constructed for titrating carbon/nitrogen (C/N) sources. A novel and efficient sample preparation method with the subsequent LC-MS/MS analysis for quantifying 12 central carbon metabolites in *E. coli* was established. Then the growth rate and concentrations of the metabolites were monitored under various carbon or nitrogen influxes. Based on the metabolites changes, a scenario of feedback control coordinating the carbon and nitrogen utilization by α-ketoglutarate (αkg) was proposed.

## 2. Materials and methods

### 2.1. Strains construction for carbon/nitrogen source titration

Construction of titratable lacY (NQ381) and titratable glpFK (NQ399) strains for C source titration: DNA fragment containing the Pu promoter (−1 bp to −178 bp relative to the transcriptional start site) was amplified by PCR from a Pu promoter containing plasmid pEZ9, then inserted into the *Sal*I and *Bam*HI sites of plasmid pKD13, producing plasmid pKDPu. Using this plasmid as a template, the region containing the km gene and Pu promoter was PCR amplified and integrated into the chromosome of *E. coli* strain NQ351 between the lacZ and lacY (from lacZ stop codon to lacY start codon), and in front of glpF (−1 bp to −252 bp relative to the translational start point of glpF) respectively, by using the λ Red system [20]. As the activation of Pu promoter needs the XylR protein, a strain NQ386 was constructed in which a synthetic lac promoter PLlac-O1 [21] (a promoter that is repressed by LacI but does not need Crp-cAMP for activation) driving xylR (xylR gene was cloned from pEZ6 [22]) was inserted at the attB site. The km-Pu-lacY and km-Pu-glpFK constructs in NQ351 were transferred into strain NQ386 containing PLlac-O1-xylR by P1 transduction, resulting in strains NQ381 and NQ399, respectively.

Construction of titratable GOGAT strain (NQ393) for N source titration: Using the λ Red system [20], the promoter (+123 bp to −176 bp) of gltBDF operon was replaced by the synthetic lac promoter PLlac-O1 [21] (a promoter that is repressed by LacI but does not need Crp-cAMP for activation) together with selection maker Km gene. The resulting Km-PLlac-O1-gltBDF construct was transferred to strain NCM3722 by P1 transduction [23]. The Km gene was then eliminated by using plasmid PCP20 [24]. A sp-lacIQ-tetR cassette providing constitutive expression of lacI to tightly repress PLlac-O1 activity was inserted at the attB site by P1 transduction. Lactose permease encoded by lacY can concentrate intracellular Isopropyl β-D-1-thiogalactopyranoside (IPTG) and will narrow the titration range, we inactivated lacY by P1 transduction using strain JW0334-1from CGSC (*E. coli* Genetic Stock Center, Yale University) as lacY donor following by Km gene elimination. The gdhA gene was knocked out by P1 transduction using strain JW1750-2 from CGSC as gdhA donor following by Km gene elimination to obtain the final strain NQ393.

### 2.2. Mediums and growth measurements

All growth media used in this study were based on M9 minimal medium and MOPS base medium with slight modifications. The lactose minimal medium and the glucose minimal medium had 0.2% (w/v) lactose and 0.2% (w/v) glucose added to the M9 minimal medium, respectively. The glycerol minimal medium had 0.2% (w/v) glycerol added to the MOPS base medium. For the C-limitation growth, 1 mM IPTG and various concentrations (0–500 μM) of the inducer 3-Methylbenzyl alcohol (3MBA) were added to the lactose minimal medium or glycerol minimal medium. For the N-limitation growth, concentrations of IPTG in the range of 30–100 μM and 25–75 μM were added to the glucose minimal medium and glycerol minimal medium, respectively.

All batch culture growth was performed in a 37 °C water bath shaker shaking at 250 rpm. The culture volume was 10 ml in 25 mm × 150 mm test tubes. Each growth experiment was carried out in three steps: "seed culture" in LB broth, "pre-culture" and "experimental culture" in identical minimal medium. For seed culture, one colony from fresh LB agar plate was inoculated into liquid LB and cultured at 37 °C with shaking. After 4–5 h, cells were transferred into the minimal medium and cultured overnight (pre-culture), which allowed cells to grow for at least 3 doublings. Pre-culture cells was then diluted to $OD_{600}$ = 0.005–0.025 in identical pre-warmed minimal medium, and cultured in 37 °C water bath shaker (experimental culture). 200 μL of cell culture was collected in a Starna Sub-Micro Cuvette (Starna Cells, Atascadero, CA) for $OD_{600}$ measurement using a Thermo GENESYSTM 20 Spectrophotometer around every half doubling of growth. About 5–7 $OD_{600}$ data points within the range of ~0.05 and ~0.5 (above $OD_{600}$ = ~0.6 the spectrophotometer was determined to be slightly nonlinear) were used for calculating growth rate.

### 2.3. Sample preparation for quantification of intracellular metabolites

The *E. coli* cell samples were collected during the exponential growth. The filter membrane (25 mm diameter with 0.45 μm pore size, Millipore) was pre-rinsed by pre-warmed Milli-Q water and culture medium, then 10 ml of culture with known OD was quickly filtrated under vacuum. 2 ml of wash medium was pipetted onto the membrane twice. Then the membrane was immediately immersed in 3 ml of acidic methanol/acetonitrile/water mixture solution (40:40:20 in volume, with 0.1 M formic acid) in a plastic tube. These two steps were finished within 30 s. After 30 s vortexing, the tube was frozen by liquid nitrogen for 1 min and vortexed for another 30 s after its thawing. After centrifugation, the supernatant was taken out and frozen by liquid nitrogen once again. The resulted solutions were then centrifuged through a Millipore Amicon Ultra Filter at 3000 g under 4 °C for 20 min for deproteinization. Finally 2 ml of supernatant was dried and re-dissolved in 200 μL of acidic acetonitrile/methanol/water for LC-MS/MS analysis.

### 2.4. LC-MS/MS analysis of intracellular metabolites

Analysis was performed on a ultra-performance liquid chromatography (UPLC) coupled with a triple-quadrupole mass spectrometry equipped with an ESI source (Waters Corporation, Milford, MA, USA) with a reversed-phase C18 column (2.1 mm × 100 mm, 1.7 μm) running in ion-pair mode. The detailed method was developed based on a previous study with some modifications [25]. Briefly, the mobile phase consisted of two components: A (10 mM tributylamine aqueous solution adjusted pH to 4.95 with 15 mM acetic acid) and B (methanol). Linear gradient elution was as follows: 100% A at 0 mins, 80% A at 5 mins, 5% A at 20 mins, and 100% A at 22 mins. An injection volume of 10 μL was selected with a flow rate of 0.3 ml/min. MS analysis was operated in negative ion multiple reaction monitoring (MRM) mode. The ion transition, collision energy and cone voltage were optimized by relative standards from Sigma to increase the instrument response (Table S1). The optimized MS parameters were described as follows: the capillary voltage was 3000 V; the dwell time was 0.05 s; the extractor voltage was 2.5 V; the temperatures of the negative ESI source and desolvation gas were 118 and 500 °C, respectively; the cone gas and the desolvation gas flows were 40 and 650 L/h, respectively. Instrument operation and data acquisition were processed by using the Waters MassLynx V4.1

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