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# Kinetic flux profiling of nitrogen assimilation in *Escherichia coli*

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**We present a new method for probing cellular metabolic fluxes that is based on the kinetics of assimilation of isotope-labeled nutrient into a diversity of downstream metabolites. In the case of nitrogen assimilation, half-maximal labeling of most metabolites occurs in 10–300 s. Fluxes measured on the basis of the kinetics of nitrogen assimilation in exponentially growing *E. coli* agree well with those fluxes predicted to allow optimal biomass production.**

Flux-balance analysis (FBA), a constraints-based computational method, enables estimation of metabolic fluxes in steadily growing microorganisms with clearly defined growth objectives[1,2]. Metabolic flux profiling, an experimental approach based primarily on steady-state isotope labeling patterns, is effective for determining flux distributions in central carbon metabolism[3–7]. Although these methods have proven valuable[8,9], a need remains for experimental means of probing fluxes (especially those outside of central carbon metabolism) in a dynamic manner[10]. To this end, we introduce kinetic flux profiling (KFP), a variant of metabolic flux profiling that involves monitoring the dynamics of incorporation of isotope-labeled nutrient into downstream products using LC-MS/MS[11].

The essential concept of KFP is that intracellular metabolites become labeled when cells are switched from unlabeled to isotope-labeled nutrient, and metabolites closer to the added nutrient in the metabolic network always get labeled before their downstream products. Thus, the pattern of labeling provides a check on the network structure. Within this fixed network structure, the speed of labeling provides insight into the quantitative flux through different metabolic pathways, with fast labeling of a metabolite pool resulting from large flux through the pool and/or low absolute pool size.

For the ideal case of a well-mixed system in which a nutrient is being directly converted into an intracellular metabolite, instantaneous switching of the nutrient input into its isotope-labeled form, without other modulation of the system, results over time in disappearance of the unlabeled metabolite:

$$dX^U/dt = -f_X \times X^U/X^T \quad (1)$$

where $X^T$ is the total pool of metabolite X, $X^U$ is the unlabeled form and $f_X$ is the sum of all fluxes consuming X. For $f_X$ and $X^T$ constant,

$$X^U/X^T = \exp(-f_X \times t/X^T) \quad (2)$$

$$f_X = X^T \times k_X \quad (3)$$

where $k_X$ is the apparent first-order rate constant for disappearance of the unlabeled metabolite. According to equation (3), the total flux through metabolite X can be determined on the basis of two parameters that we can measure directly: the intracellular pool size of the metabolite and the rate of disappearance of the unlabeled form. Though real cases are more complex (largely owing to the time required for nutrient switching and subsequent flow of the labeled nutrient through the metabolic network; see **Supplementary Methods** online), closely related mathematics still applies.

KFP requires the ability to switch from unlabeled to labeled nutrient rapidly without perturbing cellular physiology. To achieve this for *E. coli*, we grew them on nitrocellulose filters on top of agarose plates

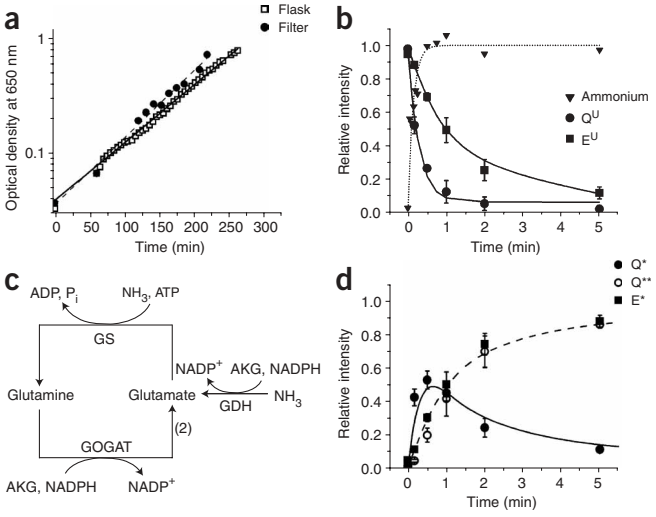


**Figure 1** Kinetics of isotope labeling of the central intermediates in nitrogen assimilation. (**a**) Growth curves of *E. coli* under conditions that enable rapid switching from unlabeled to [$^{15}$N]ammonia (filter) versus typical liquid culture (flask). (**b**) Kinetics of diffusion of ammonia onto a cell-free filter compared to kinetics of disappearance of unlabeled glutamine ($Q^U$) and unlabeled glutamate ($E^U$) on switching from unlabeled to [$^{15}$N]ammonia. (**c**) Pathways of ammonia assimilation in *E. coli*; (2) indicates the production of two glutamates per glutamine by glutamate synthase (GOGAT). AKG, α-ketoglutarate (**3**); GDH, glutamate dehydrogenase; GS, glutamine synthetase. (**d**) Kinetics of formation of $^{15}$N-labeled glutamine (Q*, amide-nitrogen–labeled; Q**, double-labeled) and glutamate (E*). In **b** and **d**, the *x* axis represents minutes after isotope switching, the *y* axis represents the fraction of the observed compound signal attributable to the indicated isotopic form, and the error bars show ±2 s.d. ($n = 3$ independent experiments). Curves represent a fit of the data to equation (8) in **Supplementary Methods**.

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**Table 1 Measured and predicted parameters for *E. coli* growing on minimal medium**

| Metabolite | Pool size ($\mu$mol $g_{CDW}^{-1}$)[a] | $k$ ($min^{-1}$)[a,b] | Measured flux (KFP) (mmol $g_{CDW}^{-1}$ $hr^{-1}$)[c] | Predicted flux (FBA) (mmol $g_{CDW}^{-1}$ $hr^{-1}$)[c] |
|---|---|---|---|---|
| Glutamine (**1**) | 3.92 ± 0.17 | 14.29 ± 6.28 | 3.36 | 0.88 |
| Glutamate (**2**) | 100.55 ± 17.54 | 0.79 ± 0.04 | 4.77 | 4.61 |
| Alanine (**4**) | 6.81 ± 1.70 | ≥1.6 ± 0.38 | ≥0.65 | 0.26 |
| Asparagine (**5**) | 2.02 ± 0.46 | 3.06 ± 0.71 | 0.37 | 0.11 |
| Aspartate (**6**) | 6.45 ± 3.54 | 2.88 ± 0.25 | 1.12 | 1.08 |
| Methionine (**7**) | 0.29 ± 0.07 | ≥1.47 ± 0.33 | ≥0.025 | 0.071 |
| Phenylalanine (**8**) | 0.20 ± 0.03 | 5.12 ± 1.62 | 0.063 | 0.081 |
| Proline (**9**) | 1.10 ± 0.15 | ≥3.02 ± 0.66 | ≥0.20 | 0.097 |
| Threonine (**10**) | 1.34 ± 0.16 | 7.52 ± 2.71 | 0.61 | 0.24 |
| Tyrosine (**11**) | 0.41 ± 0.25 | 9.51 ± 7.73 | 0.23 | 0.061 |
| Valine (**12**) | 2.41 ± 0.27 | 3.88 ± 1.14 | 0.56 | 0.19 |
| Carbamoyl aspartate (**13**) | 0.84 ± 0.28 | 4.11 ± 0.98 | 0.21 | 0.15 |
| IMP (**14**) | 0.38 ± 0.01 | ≥2.83 ± 0.27 | ≥0.064 | 0.26 |

[a]Error estimates for pool size are s.d. of quadruplicate measurements; error estimates for $k$ are standard errors from the curve fit. [b]$k$ = apparent first-order rate constant for $^{15}N$ labeling; see **Supplementary Methods**, equation (8). [c]KFP measures gross fluxes, whereas FBA predicts net fluxes. $g_{CDW}$ = grams of cell dry weight. IMP, inosine monophosphate.

loaded with minimal essential medium. Nutrients diffused readily up through the agarose and filters to the *E. coli*, which grew exponentially with a doubling time similar to that obtained in comparable liquid medium (75 min versus 87 min; **Fig. 1a**). The *E. coli*–laden filters can be transferred from one agarose plate to another, thereby enabling nondisruptive, fast modification of the nutrient environment.

The kinetics of ammonia diffusion from an agarose plate onto a blank filter are shown in **Figure 1b**. Also shown in **Figure 1b** are the labeling kinetics of the central-nitrogen-metabolism compounds glutamine (**1**) and glutamate (**2**), following transfer of an *E. coli*–laden filter from normal minimal medium to minimal medium containing [$^{15}N$]ammonia. Labeling of the amide nitrogen of glutamine, which results from addition of ammonia to glutamate at the expense of ATP (**Fig. 1c**), occurred slightly less rapidly than the limit implied by the ammonia diffusion rate (**Fig. 1b**), resulting in a large rate constant $k_Q$ for turnover of the glutamine amide nitrogen (**Table 1**).

Glutamate is present in enteric bacteria in amounts ~25-fold larger than those of glutamine (**Table 1**)[12]. Glutamate can be synthesized either directly from ammonia via the glutamate dehydrogenase system or indirectly via glutamine and glutamate synthetase (**Fig. 1c**). We found that glutamate becomes labeled less rapidly than glutamine ($k_E << k_Q$, where $k_E$ is the rate constant for turnover of glutamate's nitrogen), but the glutamate flux is nevertheless slightly greater than that of glutamine owing to the much larger glutamate pool size (**Table 1**). Production of double-labeled glutamine precisely mirrored labeling of glutamate, which provides glutamine's amino nitrogen (**Fig. 1d**). This observation is consistent with the notion that glutamate serves as the parent of doubly labeled glutamine, with $k_Q >> k_E$.

Nitrogen is funneled from glutamine and glutamate into nucleotides and amino acids[13] (**Supplementary Fig. 1** online). We found that nucleotide biosynthetic intermediates become labeled very rapidly, consistent with their being products of glutamine that are present in relatively low amounts but sit on high-flux pathways. Because most amino acids receive nitrogen from glutamate, they became labeled very shortly after glutamate, consistent with the large pool size of glutamate relative to its amino acid products.

The flux measurements obtained here can be compared to the predictions of FBA, assuming that *E. coli* grown under the present conditions are optimizing their growth rate per molecule of glucose consumed (**Table 1**). The most important conceptual difference between the FBA predictions and our results is that FBA predicts net flux (that is, for reversible reactions, forward flux – reverse flux), whereas the present observations are of gross flux (that is, forward flux). With this caveat in mind, the overall agreement between the measured and predicted values is reasonable, especially for irreversible reactions (for example, glutamate synthesis and pyrimidine biosynthesis). The most notable discrepancy between the measured fluxes and those predicted by FBA occurs for glutamine and arises from assuming, in the optimization step of FBA, maximization of growth yield per glucose molecule. Our data show that when adequate glucose is available, even with ample extracellular ammonia present, ATP is spent to synthesize glutamate largely via glutamine, despite the presence of the alternative, lower-energy-cost pathway of glutamate dehydrogenase[14]. The observed glutamine flux is ~400% of that expected if no glutamate were made via glutamine, and ~60% of that expected if all glutamate were.

The experiments conducted here provide support for both the predictive power of FBA and the measurement capabilities of KFP. They support a picture of nitrogen assimilation[12] in *E. coli* in which glutamine is a short-lived intermediate in the synthesis of glutamate, the main warehouse of free nitrogen in enteric bacteria. Because the time between isotope switching and collection of critical nitrogen flux data is short (~5 min), KFP should be able to provide insight into the changes in flux patterns that occur in response to environmental perturbations. Preliminary data supporting this, obtained using the example of fast turnoff of biosynthetic fluxes during carbon starvation, are presented in **Supplementary Figure 2** online. Future studies exploring the applicability of KFP to other nutrients, organisms and experimental conditions are warranted.

*Note: Supplementary information is available on the Nature Chemical Biology website.*

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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