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Metabolic-flux dependent regulation of microbial physiology

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According to the most prevalent notion, changes in cellular physiology primarily occur in response to altered environmental conditions. Yet, recent studies have shown that changes in metabolic fluxes can also trigger phenotypic changes even when environmental conditions are unchanged. This suggests that cells have mechanisms in place to assess the magnitude of metabolic fluxes, that is, the rate of metabolic reactions, and use this information to regulate their physiology. In this review, we describe recent evidence for metabolic flux-sensing and flux-dependent regulation. Furthermore, we discuss how such sensing and regulation can be mechanistically achieved and present a set of new candidates for flux-signaling metabolites. Similar to metabolic-flux sensing, we argue that cells can also sense protein translation flux. Finally, we elaborate on the advantages that flux-based regulation can confer to cells.

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Introduction

Microorganisms are often confronted with changes in their environment, for instance, in terms of nutrient availability. Direct assessment of the extracellular conditions, for example through two-component systems in bacteria [1], often leads to adaptations in response to environmental changes. However, there is increasing evidence showing that microbial cells can display changes in their phenotype, for example, in growth rate, gene expression and metabolism, also in response to changes in intracellular metabolic fluxes, even when the extracellular conditions are kept constant [2–5]. But does this flux-dependent regulation have a major impact on cell physiology? How can cells mechanistically sense metabolic fluxes, that is, rates of enzymatic reactions and metabolic pathways, and use this information for regulation? And why is this regulation advantageous to the cell?

Microorganisms display flux-dependent phenotypes

Accumulating evidence suggests that microbial cells can display phenotypes imposed by metabolic fluxes, and not directly by extracellular conditions. One example is the switch from respiratory to fermentative metabolism in glucose-rich conditions. When both *Escherichia coli* and yeast were grown in the same nutrient environment, but the rate of sugar uptake was controlled by inducible expression of sugar permeases or by using hexose transporter variants with different kinetics (Figure 1a) respectively, a glycolytic flux-dependence of the metabolic mode — a respiratory or fermentative metabolism — was found [4,6]. A meta-analysis of data from a number of studies that used different yeast strains grown under different conditions suggested that this switch is triggered when a specific sugar uptake rate is exceeded [7]. Because the onset of ethanol production is accompanied by a decrease in the oxygen uptake rate, this study suggested that this 'overflow metabolism' is an active response to the level of glycolytic flux, rather than a limitation in oxidative metabolism.

Intracellular flux changes under constant environmental conditions can also re-shape proteome expression. Proteome analyses carried out on bacteria grown in lactose as the sole carbon source but in which metabolic fluxes were modulated by titrating the expression of either the lactose permease or the enzyme involved in ammonia assimilation showed that as much as 50% of the proteome was altered in these conditions [8•]. Similarly, a comprehensive fluxomics and proteomics analysis in *S. cerevisiae* strains which were grown in the same environment but had different hexose uptake capacities, found that the expression of nearly half of ≈200 quantified metabolic proteins changed in a flux-dependent manner. Proteins whose expression correlated positively with glycolytic flux were found to be enriched for glycolytic proteins. On the other hand, proteins with expression levels negatively correlating with glycolytic flux were enriched for proteins involved in the TCA cycle, and in pyruvate, glyoxylate and dicarboxylate metabolism [3].

Figure 1
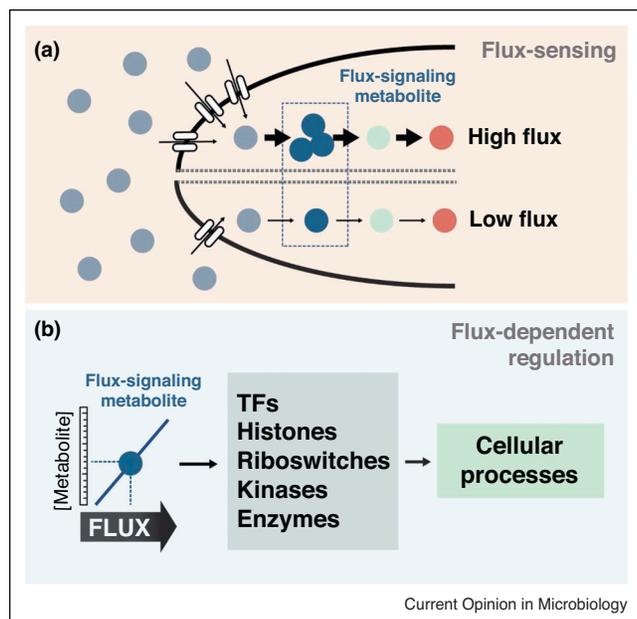
Schematic illustration of flux-sensing and flux-dependent regulation. **(a)** Kinetics of enzymes and their regulation is such that certain intermediates become flux-signaling metabolites, that is, their levels either correlate (or anticorrelate) with metabolic flux. At constant nutrient conditions, differences in metabolic fluxes can be achieved through variations in nutrient transporter levels (illustrated in the scheme), or through variations of flux-limiting enzymes. **(b)** Information about metabolic flux is imprinted into the concentration of a flux-signaling metabolite, which then interacts with regulatory factors and enzymes (grey box) to control other cellular processes (including metabolism). *Abbreviation*: TFs, transcription factors.

Apart from determining the metabolic mode and dictating protein expression, metabolic fluxes control growth. Glucose influx determines growth rate in *S. cerevisiae* [2,4], and in *E. coli* [9]. In addition, the fraction of *E. coli* cells that enters persistence, a state of no or slow-growth characterized by antibiotic tolerance, was shown to anticorrelate with glucose influx when the ratio between glucose and a non-metabolizable analogue was modulated [10]. Similarly, after a nutrient shift of *E. coli* from glucose to fumarate, persister cells are formed, and the rate of persister formation correlates negatively with fumarate uptake rate [11,12•]. While the most prevalent notion has been that persistence is triggered by toxin–antitoxin systems, recent work demonstrated that previous findings considering toxin–antitoxin systems contained artifacts [13]. Thus, as suggested by the above-mentioned findings, persistence entry is likely metabolic flux-dependent.

How do cells measure and use fluxes for regulation?

The important question that arises is how cells are capable of assessing the level of metabolic flux, and use this information for regulation. Changes in flux, induced by environmental changes or stochastic expression of transporters or enzymes, could be assessed by changes in the concentration of pathway intermediates (Figure 1a). However, the concentrations of metabolites are determined by the combination of the kinetics of the consuming and producing reactions. Metabolite concentrations do not necessarily change when fluxes are altered [14•], nor do they necessarily scale with flux [15]. Therefore, to accomplish flux-sensing via the concentration of certain metabolites, specific kinetics of the involved enzymes and specific regulation of these enzymes are required, such that the strict correlation (or alternatively, anti-correlation) between the metabolite concentration and metabolic flux is an emerging behavior. We refer to metabolites with such a behavior as *flux-signaling metabolites*.

The glycolytic intermediate fructose-1,6-bisphosphate (FBP) has been identified as a flux-signaling metabolite [16•]. FBP levels correlate with glycolytic flux across a broad range of microbial species and conditions [3,7,14•,17•,18–20], and even in dynamic perturbations of glycolysis [21]. It has been found recently that the molecular system translating the glycolytic flux into the FBP level encompasses all enzymes of lower glycolysis including the feedforward activation of pyruvate kinase by FBP, which ensures that FBP concentration correlates linearly with glycolytic flux over a broad range of fluxes [17•].

To transduce the flux information 'stored' in the concentration of a flux-signaling metabolite (e.g. FBP) into a response, a concentration-dependent interaction between the flux-signaling metabolite and other cellular components is required. In fact, it is well documented that metabolites interact with and regulate metabolic enzymes [22], transcription factors [23,24], protein kinases [25,26], and *cis*-regulatory RNA sequences (*riboswitches*) (Figure 1b). Additionally, some metabolites (e.g. acetyl-CoA) can have a critical role in the expression of specific genes because they are utilized as substrate for covalent modifications of histones [27,28]. However, the physiological relevance of such interactions in most cases is still unclear. Most available information stems from *in vitro* studies focusing on purified individual proteins or RNA species [29], in part because direct perturbation of metabolite levels in living cells without off-target effects is still impossible. However, by systematically investigating metabolites that affect transcriptional regulation *in vivo*, Kochanowski and co-workers showed that indeed (flux-signaling) metabolites (cyclic AMP, FBP, and fructose-1-phosphate) interacting with two major transcription factors (Crp and Cra) are responsible for the majority of the transcriptional regulation observed across 23 diverse growth conditions in *E. coli* [30].

While previously interactions between metabolites and other cellular molecules were mostly found by

serendipity or intuition, recently, significant advances were made towards unbiased and global identification methods. For instance, Li *et al.* used mass spectrometry to identify hydrophobic metabolites bound to protein kinases and enzymes involved in the biosynthesis of ergosterol in yeast [31]. This study revealed novel interactions among intermediates of the ergosterol pathway and 17 different enzymes (70% of the ergosterol biosynthesis enzymes). In another study, a method was developed based on limited digestion of proteomes extracted under non-denaturing *in vivo* conditions coupled to targeted proteomics. This allowed for screening of conformational rearrangements of proteins upon metabolite binding [32•]. Besides confirming previously described metabolite-protein interactions, the authors also suggested many novel allosteric interactions [32•].

Apart from proteins, metabolites can also interact with riboswitches — RNA elements that conditionally regulate gene expression (transcriptional regulation, in most cases) depending on the presence of a small compound. Recently, Dar *et al.* mapped the 3' and 5' ends and global transcript levels in different model microorganisms and in a complex microbial consortium from oral microbiota and discovered a plethora of unknown potential riboswitches. Interestingly, by comparing the levels of conditional transcription termination after depleting lysine from the medium or adding an antibiotic, they identified riboswitches that specifically respond to a given metabolite. This work has the potential to be developed into a pipeline for high throughput screening of metabolite-sensitive RNA regulators [33•].

Collectively, the interaction of flux-signaling metabolites with other macromolecules can exert flux-dependent regulation at different levels (e.g. gene expression and enzyme activity regulation). Through the recent development in techniques to identify metabolite–macromolecule interactions we are getting closer to a full picture on how and which processes might be regulated in a flux-dependent manner.

On the quest for flux-signaling metabolites

Although several metabolites may exert control over cellular functions, how can we identify flux-signaling metabolites? As mentioned, flux-signaling metabolites (i) exhibit changes in their concentration in response to changes in metabolic flux, and (ii) interact with other macromolecules in order to translate the flux-information into a cellular response. With the goal to identify metabolites that fulfill these criteria, and are thus candidates for mediating flux-signaling, we first gathered concentration data of glycolytic, tricarboxylic acid (TCA) cycle, and pentose phosphate pathway (PPP) metabolites which were generated in quantitative metabolomics experiments, from seven independent studies performed on three microbes (*E. coli*, *B. subtilis*, and *S. aureus*) [21,34–

39]. To maximize the chances to identify flux-signaling metabolites, the data set contained 30 different nutrient regimes at steady-state or during dynamic perturbations. We performed a statistical analysis on these data (using a linear mixed effects model) to identify those metabolites whose concentrations vary most across the conditions. Here, we found that different metabolites have largely different variances (Figure 2a, top row). The metabolites with the highest variance across conditions are FBP, citrate, succinate, 6-phosphogluconate (6PG), ribulose-5-phosphate (Ru5P), and sedoheptulose-7-phosphate (S7P).

Secondly, we gathered information about interactions of the same metabolites with enzymes (https://metacyc.org/, [40]), regulatory proteins, as well as transcriptional and translational regulators (www.rcsb.org, [41]; http://regulondb.ccg.unam.mx/, [42]). Here, we found that certain metabolites had many more interactions with enzymes and regulatory proteins than others of the same pathway. Particularly, citrate and alpha-ketoglutarate (A-KG) from the TCA cycle, and FBP, phosphoenolpyruvate (PEP) and pyruvate from glycolysis stood out (Figure 2a, central and bottom rows).

Taking the data on the metabolites' concentration variance and interactions with enzymes and regulators together (Figure 2b), we confirmed FBP as a flux-signaling metabolite [17•]. Furthermore, we identified new candidates for flux-signaling metabolites. For instance, citrate, phosphoenolpyruvate (PEP) and A-KG score high on both criteria in Figure 2b, and are thus excellent candidates, as well as succinate (SUC) and pyruvate (PYR). In fact, it was recently shown in *S. cerevisiae* that citrate concentration increases when nitrogen is limited, and that its concentration correlates well with the degree of nitrogen limitation [14•], suggesting that citrate could report on the magnitude of nitrogen influx. Citrate could exert flux-dependent regulation as an inhibitor of pyruvate kinase [14•]; nitrogen influx would be sensed via citrate and then lead to regulation of the flux through glycolysis. Interestingly, α-ketoglutarate, the other TCA metabolite that we identified as a potential flux-signaling metabolite, was also reported to coordinate glycolytic flux with nitrogen uptake, but in *E. coli* [43]. Such regulatory cross-talk between metabolic pathways (some of which possibly mediated in a flux-dependent manner) seems to be rather common in central metabolism: metabolites from one metabolic pathway to regulate enzymes in a different pathway (Figure 2c).

Unstable proteins as reporters of translation flux

While flux-signaling metabolites can report on metabolic flux through specific pathways, important cellular decisions, as for example the entry to cell division, possibly requires the assessment of the cellular metabolic activity on a global level. The rate of protein synthesis can be

Figure 2
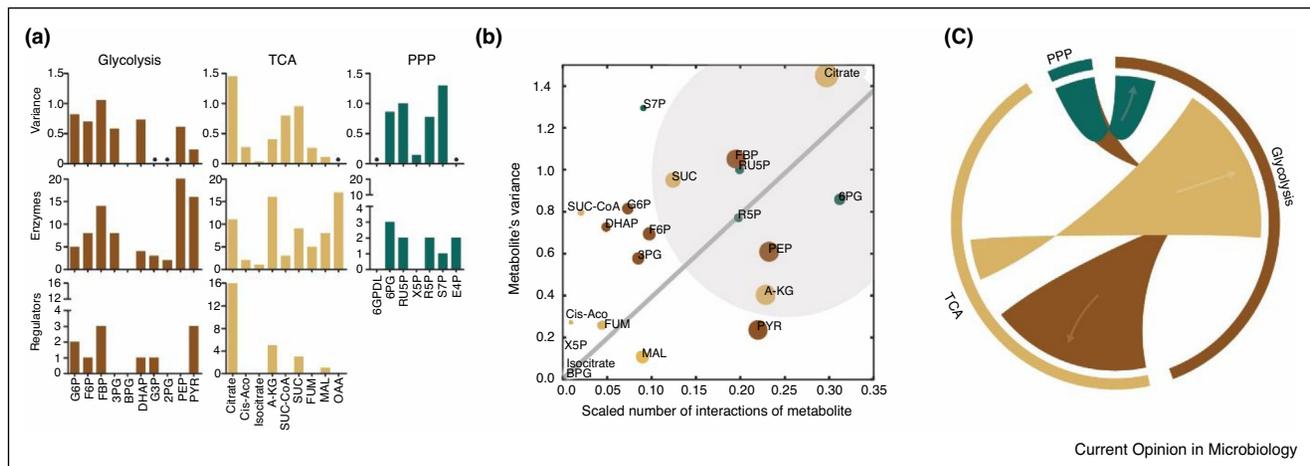
Identification of flux-signaling metabolites and metabolite-mediated regulatory crosstalk between different metabolic pathways. **(a)** From top to bottom, variance in concentration of metabolites across nutrient conditions (black dot indicates lack of data), number of unique metabolite–enzyme regulatory interactions, and number of unique metabolite interactions with proteins involved in the regulation of gene expression. *Abbreviations*: G6P, glucose-6phosphate; F6P, fructose-6-phosphate; FBP, fructose 1,6-bisphosphate; 3PG, glycerate 3-phosphate; BPG, 1,3-bisphosphoglycerate; DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde 3 phosphate; 2PG, glycerate 2-phosphate; PEP, phosphoenol pyruvate; PYR, pyruvate; Cis-Aco, cis-aconitate; A-KG, α-ketoglutarate; SUC-CoA, succinyl-CoA; SUC, succinate; FUM, fumarate; MAL, malate; OAA, oxaloacetate; 6GPDL, 6-phosphogluconolactone; 6PG, 6-phosphogluconate; RU5P, ribulose-5-phosphate; X5P, xylulose-5-phosphate; R5P, ribose-5-phosphate; S7P, sedoheptulose-7-phosphate; E4P, erythrose-4-phosphate. For the estimation of the variance components, a linear mixed effects model was fit to the metabolite concentrations, whereby the interest centered on how much the various metabolite concentrations varied across the different conditions, controlling for the state (dynamic/steady) and the various studies. For each metabolite, a random intercept was estimated across the conditions and the variance component associated with that random intercept expressed how much each metabolite varied across the conditions. A few metabolites (RU5P, X5P, S7P, isocitrate) were only measured within one study and therefore did not need to be controlled for study and a few other metabolites (3PG, PYR, citrate, Cis-Aco, SUC-CoA) were only measured at one condition and did not require control for that variable either. To obtain putative interactions between metabolites and regulatory proteins, a search in the Protein Data Bank was performed using the name of each metabolite together with the word 'transcription' as keywords, and the hits were manually examined. **(b)** Metabolite variance versus the number of a metabolite's interactions with enzymes and regulators relative to the total number of interactions of all metabolites in the pathway. To estimate the uncertainty in normalized interactions, we bootstrapped (i.e. recalculated these values from reduced datasets where we each time left out 10% of the interactions) and then determined the coefficient of variance. Marker sizes reflect the inverse of the coefficient of variations. For regression line: $R^2 = 0.69$, *p*-value 1.58e−6. Metabolites with large markers within the grey circles are strong candidates for flux-signaling metabolites. **(c)** Circos plot [44] showing the cross-regulation between pathways through metabolite–enzyme interactions. The full length of each ideogram is proportional to the total number of enzymes in the pathway that were found to be regulated by metabolites in one of the other shown pathways (10, 10, and 1 enzymes for glycolysis, TCA, and PPP respectively). The ribbons indicate which fraction of these enzymes (end-point of ribbon) are regulated by metabolites of another pathway (ribbon color). For example, the yellow ribbon indicates that approximately one-third of the metabolite-regulated glycolytic enzymes, are regulated by TCA metabolites.

considered as an excellent reporter of overall metabolic activity of a cell, for the following reasons: (i) synthesizing ribosomes requires most of the cellular biosynthetic capacity [45], (ii) protein translation is by far the most expensive biosynthetic process in the cell [46], and (iii) a high rate of protein synthesis also reflects a well-coordinated activity of central metabolism, as the production of the different amino acids required for protein synthesis demands a well-coordinated operation of several pathways in central metabolism [47,48].

Also here, to assess translation flux, flux is translated into a measurable quantity, and in specific a protein concentration (Figure 3). Information about translation flux can be imprinted into the levels of a protein if this protein is constitutively expressed and has a very short half-life (i.e. there is a high protein degradation flux). In this case, the

level of this protein reflects the instantaneous translation rate. In fact, Bell and colleagues measured the half-life of 3751 proteins in exponentially growing *S. cerevisiae*, and found a number of very unstable proteins (161 proteins with a half-life of <4 minutes) that were enriched in proteins involved in cell regulation [49•]. Similar conclusions were also drawn from another proteome-wide study, in which it was shown that the classes of short-lived proteins are enriched in cellular regulators [50], although in a more recent study also proteins involved in ribosomes and amino acid biosynthesis had high turnover rates [51]. Through such short-lived proteins, reporting translation flux [52], a cell could exert regulation on the basis of its overall metabolic activity (Figure 3).

An example of translation-flux based regulation involves the budding yeast cyclin Cln3, which is a remarkably

Figure 3
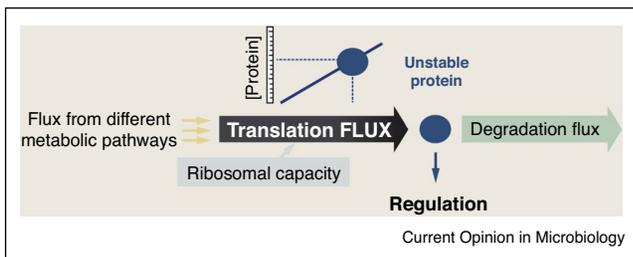
Inferring global metabolic activity from translation flux via unstable proteins. The rate of translation reflects the coordinated activity of central metabolism. The concentration of constitutively expressed, highly unstable proteins, reports the rate of translation (i.e. the translation flux). Such constitutively expressed, unstable proteins can be exploited by cells for regulation based on their overall metabolic activity.

short-lived protein [53], and whose synthesis is thought to depend on the translation capacity of the cell [54]. Because Cln3 is a potent activator of the cell division program in *S. cerevisiae* [55], Cln3 transmits information about translation flux to the cell cycle machinery [52], thus using information about the overall metabolic activity of the cell to make an important cell fate decision.

Why sensing intracellular fluxes?

Why should cells exert regulatory activity on the basis of metabolic flux, rather than, for example, solely on the basis of extracellular nutrient concentration? Microbial cells can grow on many different carbon sources (up to 180 for *E. coli* [56]). The simultaneous expression of so many sensors probing the extracellular environment would present a significant burden to cells. Furthermore, signals from different sensors would need to be 'integrated' to ultimately lead to a coherent cellular response. Instead, with intracellular flux-sensing, measuring flux at different points in metabolism (for instance, where different inflowing nutrients converge) through flux-signaling metabolites, requires fewer sensing mechanisms [16•]. However, this gain in expenditure comes at the cost of only roughly reporting the nature of the inflowing nutrients. In fact, it appears that cells display sub-optimal control of gene expression in response to environmental conditions (reviewed in [57]), suggesting that they are prepared to face a range of environmental regimes, rather than a specific one. Thus, we can consider flux-sensing an economic way to regulate metabolism, and as an elegant way to handle the problem of 'integrating signals'.

Furthermore, flux-dependent regulation could also be a robust way to regulate metabolism and cellular processes. First, flux-sensing allows cells to determine the *actual* metabolic rates. Thus, regulation can be exerted on the basis of what is actually happening inside cells (in terms of

metabolic activity) instead of what substrate would be available in the extracellular environment. Maybe because of this, important cellular decisions, for instance the entry into bacterial persistence, are made on the basis of metabolic flux [11,12•]. Second, flux-sensing is integrated in global feedback loops (*flux controls flux*) [11,16•], which allows for corrections of stochastically induced alterations in gene expression, which recently was shown to also affect metabolism [58].

Conclusion

Flux-sensing and flux-based regulation constitutes possibly a common, previously underappreciated, phenomenon in microorganisms. Nevertheless, even identifying which elements comprise a flux-signaling system is a far from trivial task. Although quantitative metabolomics is nowadays at a stage where the levels of many metabolites can be quantitatively determined, measuring metabolic fluxes across different conditions in a truly quantitative manner is still a challenge. However, significant advances have recently been accomplished towards this direction [59]. Moreover, the systematic identification of interactions between metabolites and proteins or RNAs has been so far relatively limited. In fact, the recent developments on high-throughput identification of such interactions [31,32•,33•,60] suggest that the limited number of currently known interactions is due to methodological limitations, rather than due to their limited presence in biological systems. Finally, in order to experimentally prove the functioning of flux-sensing systems in living cells, complex metabolic perturbations are required (i.e. perturbing metabolic flux, perturbing metabolite levels) which are typically very difficult to achieve, or only with off-target effects. Therefore, combining mathematical modelling with elegant targeted perturbation methods, as for example the recently developed optogenetics-based method for controlling enzyme activity [61], will be essential towards elucidating and proving flux-sensing and flux-dependent regulation.

Understanding how metabolic fluxes are sensed and translated to physiological responses will be highly valuable for metabolic engineering, as for example in the construction cell factories, which involves the redirection of metabolic fluxes for the synthesis of commercially interesting chemicals. Also, knowing which metabolites are flux-signaling will allow the construction of biosensors, whereby the concentration of the flux-signaling metabolite is translated into a measurable output, such as the expression of a fluorescent protein [62]. Such flux-reporting biosensors could possibly also be used as a research tool for screening of drugs targeting metabolic diseases and cancer. Overall, the elucidation of the architecture and function of flux-sensing systems will provide an important, currently missing, perspective on metabolic regulation with potentially powerful applications.

Conflict of interests

The authors declare no conflict of interests.

Author contributions

AL and ADO contributed equally to this work. AL, ADO, and MH conceived the study and wrote manuscript. AL and ADO collected and analyzed data. EW performed the analysis on the variance of the metabolite concentrations.

Acknowledgements

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