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Network Biology: Biology as a Complex System

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As complex biological systems are very robust to genetic and/or environmental changes on all levels of organization, their inherent robustness has been of great interest in biology as well as in engineering theory [1]. The biological function of *E. coli* metabolism can be sustained against single-gene or even multiple-gene mutation possibly by utilizing the redundant pathways [2, 3]. While the investigations on the topological and functional/phenotypic properties of metabolic networks have been increasingly populated [2, 4-7], they still provide a limited understanding of the metabolic robustness despite its biological significance. In this work, we focus on the interplay between such robustness and the underlying metabolism, and how the robustness can be accomplished at the level of the metabolites which are the fundamental entities [8, 9] integrated/dissipated by the metabolic processes. To this end, we constructed the computational models at a system level, and simulated them with a constraints-based flux analysis [10].

To explore the robustness of *E. coli* metabolism from the metabolite perspective, we should identify the metabolites which are substantial in cellular functions. In this regard, all intracellular metabolites are classified into two categories, essential and non-essential metabolites according to the phenotypic effects on cell survival when the consumption rate of the given metabolite is suppressed to zero. The resultant list of essential metabolites is identified under nineteen different environments which are specified by combinations of several C, P, N, and S sources, and aerobic/anaerobic conditions. By disrupting multiple genes around essential/non-essential metabolites *in vivo*, we could validate the predicted effects of the metabolite essentiality on cell survival.

As an example of the experimental validation in the pipeline, the associated genes of an essential metabolite, tetrahydrofolate, were selected for the multiple-gene disruption. Each single and double gene deletion mutant (Δ*purN*, Δ*lpdA*, Δ*glyA*, and Δ*purN*Δ*lpdA*) could still survive albeit with some growth rate changes, but simultaneous deletions of the triple genes (Δ*purN*Δ*lpdA*Δ*glyA*) did not allow the cell to grow at all, reflecting that the combinatory suppression of the tetrahydrofolate pool is indeed fatal to the cell.

On the contrary, 1-deoxy-D-xylulose 5-phosphate had been identified as a non-essential metabolite *in silico*, and experimental removals of all the reactions producing the metabolite by constructing Δ*dxs*Δ*xylB* caused the only slight change and even increase of growth rate compared with wild type. Throughout these experiments, the measured growth rates of the gene deletion mutants relative to that of the wild type were found to be consistent with the *in silico* predictions. These results indicate that deletion strains for essential metabolites can suffer from the deleterious impact on cellular functions, while those for non-essential metabolites show the negligible influence on the actual growth.

We also investigated the inherent network property of essential metabolites to elucidate the correlation between the structural property and functional behavior from the metabolite perspective. We found that essential metabolites are likely to be connected with more reactions than non-essential ones. Furthermore, the metabolic networks of 227 organisms with fully sequenced genomes disclose that the metabolites essential for various growth conditions are commonly distributed across the organisms, showing the high degree of phylogenetic conservation.

To better understand the robustness of the cellular metabolism from the metabolite perspective, it is necessary to quantify the usage of all relevant fluxes to a single metabolite. In this sense, we introduce the flux-sum (Φ) of the metabolite, which is defined as the summation of all incoming or outgoing fluxes for given metabolite i as follows:

$$\Phi_i = \sum_{j \in P_i} S_{ij} \nu_j = -\sum_{j \in C_i} S_{ij} \nu_j = \frac{1}{2} \sum_j \left| S_{ij} \nu_j \right|,$$

where S_{ij} is the stoichiometric coefficient of metabolite i in reaction j, and ν_j is the flux of reaction j. P_i denotes the set of reactions producing metabolite i, C_i the set of reactions consuming metabolite i. Under the stationary assumption, Φ_i is the mass flow contributed by all fluxes producing (consuming) metabolite i.

Based on this measure pertaining to the behavioral characteristic of metabolites, we can analyze the robustness of *E. coli* metabolism to maintain the cellular functions against the genetic mutations. The sensitivity to genetic perturbation for a given metabolite can be quantified by evaluating the relative fluctuation of Φ_i in response to each deletion of non-lethal reactions: $\sqrt{\langle \Phi_i^2 \rangle - \langle \Phi_i \rangle^2} / \langle \Phi_i \rangle$ where $\langle \Lambda \rangle$ denotes the average over the reaction deletions. It turns out that the essential metabolites are more likely to have small relative fluctuations. This indicates that flux-sums of essential metabolites are relatively insensitive to genetic perturbation compared with those of non-essential ones. Indeed, 94.3% of total metabolites found in the fluctuation range of less than 0.0875 are essential, while there are only non-essential

metabolites in the twenty highest ranks in relative fluctuations. Thus, essential metabolites are resistant to the internal variation compared with non-essential ones by maintaining the basal mass flow of the corresponding metabolite, thereby leading to the robustness of the cellular metabolism.

To clarify such resistance of essential metabolites against the internal disturbance, the severe perturbation was conducted by deleting the most contributing reaction to the flux-sum for a given essential metabolite. Remarkably, for many essential metabolites, the resultant flux loss is mostly recovered by the fluxes of other remaining reactions, thereby leading to very small change of the flux-sum, in spite of removing the dominant reaction[5] with the largest flux value. For instance, the flux-sum of an essential metabolite, carbamoyl phosphate, is reproducible by other fluxes even when the largest flux from carbamate kinase is eliminated; other reaction, carbamoyl-phosphate synthase can compensate such flux loss fully, thus resulting in the recovery of 98.9% of the basal flux-sum. For many essential metabolites, the flux-sum is only changed much less than the reduced flux corresponding to the deleted reaction. Accordingly, even though the reaction with relatively high flux is eliminated, the flux-sum can be compensated by other fluxes around the essential metabolite, recovering such flux loss. Moreover, using the stoichio-similarity, we develop the method to predict the most probable reaction which would recover the flux-sum after disruption. Hence, we believe that cellular robustness can be elucidated by such functional property of metabolic network manifesting the resilience of essential metabolites against the disturbed flux configuration.

Essential metabolites play a pivotal role in the cell survival, steadily maintaining the mass flow to produce or consume the metabolites against any internal disturbance within the cell. In other sense, this metabolite perspective on the robustness of *E. coli* provides us the cellular-level fragility: the failure of maintaining the flux-sum of a single essential metabolite can suppress the whole cellular growth drastically. Especially, for most essential metabolites (85%), reducing the flux-sum by half below the basal level intentionally leads to the growth rate down to half or even less, while only 28.9% of active non-essential metabolites have the same effect on the cell growth for such flux-sum perturbation.

The effect of the reduced flux-sum on cell growth is manifested when the amount of the flux-sum becomes gradually depleted. Subjected to the continuous attenuation of the flux-sum, each essential metabolite exhibits the characteristic profile of the cell growth rate, mostly classified into three types - A, B, and C. The growth rate is sensitive to the reduced amount of the flux-sum for types A and C, but not so much to that for type B. We found that 212 essential metabolites belong to type A, of which a metabolite tunes the whole cellular growth proportionally to the attenuated flux-sum, like a 'acclimator' metabolite acting on the cell growth. On the other hand, 32 essential metabolites correspond to type B, and 6 essential metabolites to type C. We conclude that the acclimator metabolites with type A allow

us to adjust the cell growth rate as finely as the level of the flux-sum, thereby providing the effective control scheme on the cellular growth, eventually applicable for therapeutic purpose.

The functional robustness of metabolic networks reflects the resistance towards internal defects and environmental fluctuations as an end product of a long evolutionary process. Such fault-tolerance or robustness may be a key to cell survival against environmental or genetic change. In this regard, a metabolite-based perspective could provide us a new guideline to interpret the cellular robustness. Essential metabolites substantial to the cell survival are capable of rerouting metabolic fluxes while sustaining their usage level. This capability of the essential metabolites leads to the quite dramatic tolerance to a wide range of internal disturbances. From a therapeutic point of view, disrupting (knock-out) the multiple non-lethal genes around an essential metabolite can lead to fatal cell damage; even attenuating (knock-down) the relevant genes may cause the same effect. Thus, synthetic lethal mutations [11, 12] can be systematically identified in conjunction with experimental screening techniques available [13, 14], thereby facilitating the discovery of drug targets for the genetic therapy [15].

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