Understanding metabolic adaptation by using bacterial laboratory evolution and trans-omics analysis

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Abstract

Many diseases such as metabolic syndrome, cancer, inflammatory diseases, and pathological phenomena can be understood as an adaptive reconstitution of the metabolic state (metabolic adaptation). One of the effective approaches to reveal the property of metabolic networks is using model organisms such as microorganisms that are easier to experiment with than higher organisms. Using the laboratory evolution approach, we can elucidate the evolutionary dynamics in various stress environments, which provide us an understanding of the metabolic adaptation. In addition, the integration of omics data and phenotypic data enables us to clarify the genetic and phenotypic alterations during adaptation. In this review, we describe our recent studies on bacterial laboratory evolution and the omics approach to clarify the stress adaptation process. We have also obtained high-dimensional phenotypic data using our automated culture system. By combining these genomic and transcriptomic data within high-throughput phenotypic data, we can discuss the complex trans-omics network of metabolic adaptation.

Keywords Metabolic adaptation · Adaptive laboratory evolution · Stress tolerance · Omics analysis · Laboratory automation

Introduction

Biological systems can adapt their metabolism in response to changes in the surrounding environment and maintain biological homeostasis. The result of metabolic adaptation can be observed as characteristic metabolic phenotypes in metabolic syndrome, diseases, and pathological phenomena. As the metabolic compounds are the same and the major metabolic enzymes are highly conserved among species, these individual life phenomena in different fields can be described in a unified way from the viewpoint of switching or reconstruction of the metabolic state. A deep understanding of how metabolic adaptation occurs facilitates the prediction and control of various diseases and pathological phenomena. One possible strategy to analyze metabolic adaptation is using model organisms such as microorganisms, which are easier to handle and possess conserved metabolic networks with higher organisms.

From this strategy, we can obtain a large amount of systematic data under controlled experimental conditions to understand metabolic adaptation.

Adaptive laboratory evolution (ALE) is a potent approach for observing the behavior of metabolic adaptation. In this approach, cells such as microorganisms that have a short generation time are cultured under a selective environment for many generations, leading to adaptive evolution. Then, using omics technologies, we can obtain genome-wide information about the adaptive phenotypic and genotypic changes resulting from the selective pressure. Advances in omics technologies (Bentley et al. 2008; Nagalakshmi et al. 2008; Bennett et al. 2009; Bamba et al. 2012; Matsumoto et al. 2017; Umeyama and Ito 2017; Miura et al. 2019) have made ALE a powerful approach for analyzing evolutionary biology as well as for breeding useful microorganisms (Conrad et al. 2011; Jiang et al. 2012; Tokuyama et al. 2018), and understanding stress-tolerant mechanisms (Dragosits and Mattanovich 2013; Winkler and Kao 2014; Horinouchi et al. 2017a), or analyzing antibiotic-resistant evolution (Lenski 1998; Lazar et al. 2014; Gillings et al. 2017; Levin-Reisman et al. 2017; Zampieri et al. 2017; Furusawa et al. 2018). For example, ALE of microorganisms for higher temperature (Bennett et al. 1990; Leroi et al. 1994; Bennett and Lenski 2007; Kishimoto et al. 2010; Ying et al. 2015), various

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stressors (Atsumi et al. 2010; Dragosits et al. 2013; Reyes et al. 2013; Harden et al. 2015; Matsusako et al. 2017), and alternativesubstrates (Lee and Palsson 2010) was performed previously. It is also possible to analyze metabolic adaptation by ALE for metabolic perturbation (e.g., in the presence of a metabolic inhibitor). Quantitative analysis of metabolic change during ALE provides deep insights into the adaptive reconstruction of metabolic states.

On the contrary, metabolic adaptation causes a change in metabolic state as well as multi-layer changes in the genome, transcriptional regulation, translation, and so on. To understand the molecular mechanism underlying metabolic adaptation, analyzing the “trans-omics” network, which is defined as the interactions occurring among molecules across multi-layers in the cell (Yugi et al. 2016; Yugi and Kuroda 2018), is necessary. An advantage of ALE is that parallel evolution experiments are available. This advantage makes it possible to provide a huge amount of quantitative data of the cellular internal state and phenotype from a comparative analysis of parallel experiments. For example, we can identify the common mutation, common gene set, or compounds in which the expression or concentration was changed during metabolic adaptation. By focusing on these common changes we can obtain important information about the molecular mechanisms of metabolic adaptation.

In this article, we describe our recent study on ALE with various stress conditions in *Escherichia coli*, omics analysis, and high-throughput measurement of phenotypes (Horinouchi et al. 2017b) (Fig. 1). We performed parallel experimental evolution by adding 11 chemical compounds, which have different action mechanisms, in the medium. In addition, we

analyzed the genome and transcriptomic changes during adaptive evolution. Furthermore, we measured the stress tolerance of each evolved strain for all stress conditions as high-throughput phenotypic data. Integration of these multi-layer data enabled us to clarify the bacterial adaptive evolution process. We also introduce our custom-developed automation system (Horinouchi et al. 2014), which enables massive parallel ALEs and measurements of phenotypic data. The development of omics and automation technologies will lead to an increased data size and will support an understanding of the complex trans-omics network.

Quantitative analysis across omics data and high-throughput phenotypic data of stress adaptation

We performed ALEs of 11 stress conditions with different action mechanisms (e.g., salt, acid, alkali, surfactant, toxic metabolic intermediate), genome and transcriptome analysis, and measurement of growth performance of each strain for all stress conditions (Fig. 2a). In this study, *E. coli* MDS42 cells were cultured in microtiter plates with M9 synthetic medium with a constant concentration of stressors (set to levels that initially decreased the specific growth rate). Every 6 h, a fraction of the cells was transferred to fresh medium (called serial-transfer) containing the stress compounds. The transfer volume was adjusted to maintain the final cell concentration below a threshold to keep the cells in the exponential phase. To compare adaptive evolution pathways for each stress, five independent culture lines (55 culture lines in total) were

Fig. 1 Overview of the experimental setting of the laboratory evolution and omics approach. Laboratory evolution under various environmental stresses was performed to obtain stress-tolerant strains. High dimensional phenotypic data were obtained using the automation culture system (see Fig. 3 for details). By combining genomic and transcriptomic data within high-throughput phenotypic data, we can predict phenotypic data using transcriptomic data (see Fig. 2 for details)

Fig. 2 ALEs, omics analysis, and high-throughput measurement of phenotypes in our recent study. **a** Forty-four tolerant strains (11 stress compounds and 4 independent experiments for each compound) were analyzed. **b** Phenotypic data of each strain and conditions were predicted from the transcriptome data. **c** Contribution of the identified mutations to each stress condition. Figures are from Horinouchi et al. (2017b) and modified

maintained in parallel. After approximately 900 h, we observed an increase in the specific growth rate under all culture lines. After that, we quantified the changes in the specific growth rate of all strains relative to the parent strain under all stressors (726 measurements in total) for analyzing the links in the acquisition of fitness. From this analysis, the stress-tolerant strains often showed cross-protection (acquisition of tolerance to one stress simultaneously gave rise to tolerance against other stresses) and collateral sensitivity (increased sensitivity to other stresses) (Leyer and Johnson 1993; Greenacre and Brocklehurst 2006; Gunasekera et al. 2008; Dragosits et al. 2013) to multiple stress conditions. For example, NaCl-tolerant strains exhibited considerable KCl tolerance and vice versa (symmetric cross-protection). This result suggested that at least a part of the tolerance mechanisms was common to the NaCl- and KCl-tolerant strains. On the contrary, asymmetric cross-protection also occurred, which suggests that the mechanisms of cross-protection and sensitivity are independent or that there are hierarchical relationships between the mechanisms of tolerance or sensitivity. For example, methylglyoxal (MG)-tolerant strains showed tolerance to lactate (Lac), whereas Lac-tolerant strains did not affect MG tolerance. This asymmetric cross-protection could be

explained by a hierarchical structure of the mechanisms of stress tolerance. In the pathways of the metabolic degradation of MG, one potential intermediate is Lac (Ozyamak et al. 2013), and thus, simultaneous development of Lac tolerance in MG-tolerant strains might be required. In contrast, MG is not involved in the pathway of Lac degradation; therefore, Lac tolerance would not necessarily be accompanied by MG tolerance.

To elucidate the mechanisms of tolerance to various stress conditions, we performed transcriptome analysis for all the tolerant strains obtained by ALEs. We obtained genes with changed expression levels in each tolerant strain. However, due to a huge number of obtained genes, it was difficult to discuss which genes contribute to the tolerance of each stress individually. To examine the correlation between changes in the transcriptome and stress tolerance, we used a simple mathematical model for predicting the stress tolerance using the obtained gene expression profiles. Briefly, we assumed that the changes in specific growth rates under stress conditions are represented by a linear combination of log-transformed changes in expression during the ALEs. For simplification, we neglected the non-linear effects and cross-terms of the gene expression changes. Thus, we assumed the following

simple linear model for predicting the change in growth rate based on the expression levels of N genes:

$$\Delta\text{Growth}_j^k = \sum_{i=1}^N \alpha_i^k X_{ij} + \beta^k \quad (1)$$

ΔGrowth_j^k indicates the changes in growth rate in the j th strains for the k th stress; X_{ij} is the $\log_{10}$ -transformed expression level of the i th gene in the j th strain after standardization to the zero mean and unit variance. α_i^k and β^k are fitting parameters. Then, we sought to determine the optimal number and combination of genes with the highest prediction accuracy by using cross-validation and a genetic algorithm. We found that the combination of 15 to 20 genes offered the highest prediction accuracy on an average. Figure 2 b shows the prediction accuracy by the linear model with 15 genes. This analysis enabled us to isolate the genes whose expression changes provided the most relevant information for predicting stress tolerance. For example, *thpA*, which encodes the thiamine ABC transporter, was selected as one of the most informative genes for representing the observed changes in resistance. *thpA* was specifically upregulated in Na_2CO_3 -tolerant strains, whereas it was downregulated in the strain tolerant to NaCl, KCl, and crotonic acid (Cro). Similarly, *ydiH*, which encodes a predicted protein with unknown function, was commonly upregulated in the strains resistant to CoCl_2 and Cro, whereas it was downregulated in several tolerant strains. In this study, we succeeded in the screened gene set, whose expression changes were highly correlated with the acquisition of tolerance. These screened genes could be important to provide highly accurate descriptions of the complex adaptive evolution process.

To identify the genetic basis of phenotypes and transcriptome changes, we performed genome re-sequencing analysis of tolerant strains. We identified several genes and gene functions, for which mutations were commonly fixed in the tolerant strains, suggesting that these mutations contributed to tolerance (see original paper (Horinouchi et al. 2017b) for details). To verify the possible contribution of these mutations to stress tolerance, we introduced some of the common, identified mutations into the genome of the parent strain and quantified the change in growth rate under corresponding stress conditions. The mutant strains showed a higher specific growth rate than the parent strain under corresponding stress conditions. For example, the mutation in the *proU* ABC transporter, which was identified in all NaCl-tolerant strains, conferred a part of growth recovery of the parent strain under NaCl stress conditions. Furthermore, we quantified the growth rate of each mutant strain under all the stress conditions to evaluate whether these mutations could cause cross-protection or collateral sensitivity. Figure 2 c shows the relationship between the growth rates of the mutant strains and those of the corresponding tolerant strains with mutations that

were introduced into the mutant strains. The weak correlation as shown in Fig. 2c indicated that cross-protection and collateral sensitivity could be partly explained by the examined mutation. However, upon focusing on genes whose expression changes were highly correlated with the acquisition of tolerance (showed in Fig. 2b), the link between mutation and expression change was not always clear. A similar obscured relationship between mutation and gene expression was also observed in our previous study on antibiotic-resistant evolution (Suzuki et al. 2014). These results suggest that the connection between the genome and transcriptome is rather complex.

Fully automated culture system for ALEs using a laboratory automation workstation

ALEs by serial-transfer require massive human resources for daily and long-term experimental periods during evolution, which limits the number of culture series for an evolution experiment. To overcome the limitation of manual experiments, we developed an automated culture system using a laboratory automation workstation (Horinouchi et al. 2014). Figure 3 shows an overview of the automated culture system in our laboratory. A Biomek® NX^P span8 laboratory automation workstation (Backman Coulter, Tokyo, Japan) is installed in a sterile booth and combined with a microplate reader (FilterMax F3/F5; Molecular Devices, CA, USA), a shaker incubator (STX44, LiCONiC, Mauren, Liechtenstein), and a microplate hotel (LPX220, LiCONiC). All instruments are connected to the communication ports of a computer and controlled appropriately. The capacity of the incubator is 44 microplates, which enables us to maintain a maximum of 16,896 culture lines simultaneously using 384-well microplates. During the ALEs using this system, a cultured plate is transferred from the incubator. Cell density is measured by the microplate reader. The culture volume to be transferred into

Fig. 3 The automated culture system for laboratory evolution. A shaker incubator, microplate hotel, and microplate reader are connected with an automation workstation

the new microplate is calculated. Cells are diluted with fresh medium and serial-transfer is performed. The consumption of tips, medium, and other necessary tools are managed by variables in the computer and are supplied from the plate hotel.

We constructed several protocols for various types of long-term serial-transfer experiments. The first protocol is to maintain the microorganisms in the exponential-growth phase, used for ALEs under various stress compounds as introduced in the previous section (Horinouchi et al. 2017b). For this purpose, we set an appropriate transfer volume of serial-transfer according to the cell density measured by a microplate reader. The second protocol is for stable serial-transfer experiments using drugs such as antibiotics which show a sharp dose-response curve. In this protocol, we prepared a series of media with different concentrations of drugs. We selected the highest drug concentration possible, in which cells were able to sustain growth. Using the latter protocol, we have performed several studies of antibiotic-resistant evolution so far. For example, we evaluated the effect of knock-out of transcriptional factors (173 transcriptional factors in total) for antibiotic-resistant evolution in *E. coli* (Horinouchi et al. 2020). We also determined the sensitivities of SOS response regulation gene deletion to various chemical compounds (217 compounds totally) in *E. coli* (Maeda et al. 2019). As shown above, a huge amount of phenotypic data obtained by these automated experiments supports the understanding of complex trans-omics networks.

Concluding remarks

The development of omics technologies has enabled us to quantify the comprehensive cellular state. Combining these technologies with an experimental evolution approach, we can facilitate new insights into the mechanisms by which biological systems adapt to environmental change. We can apply this approach for evolutionary biology as well as medical science and bioengineering. The development of laboratory automation technologies also accelerates the research process of these fields. The benefit of laboratory automation technologies is varied and includes reduction of experimental loads, avoidance of human error, normalization of experimental procedure, and ensuring reproducibility. The advances and commodification of automation technologies are highly important for the accumulation and usability of omics data. Our recent studies suggest that the association between the genome, transcriptome, and phenotype is complex and that it is difficult to combine them. One strategy to overcome this problem is to quantify an additional omics-layer (e.g., proteome, metabolome). The development of omics technologies and automation technologies will thus allow us to further understand the complex trans-omics network of metabolic adaptation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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