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Research review paper

Metabolic engineering of *Bacillus subtilis* fueled by systems biology: Recent advances and future directions

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ABSTRACT

By combining advanced omics technology and computational modeling, systems biologists have identified and inferred thousands of regulatory events and system-wide interactions of the bacterium *Bacillus subtilis*, which is commonly used both in the laboratory and in industry. This dissection of the multiple layers of regulatory networks and their interactions has provided invaluable information for unraveling regulatory mechanisms and guiding metabolic engineering. In this review, we discuss recent advances in the systems biology and metabolic engineering of *B. subtilis* and highlight current gaps in our understanding of global metabolism and global pathway engineering in this organism. We also propose future perspectives in the systems biology of *B. subtilis* and suggest ways that this approach can be used to guide metabolic engineering. Specifically, although hundreds of regulatory events have been identified or inferred *via* systems biology approaches, systematic investigation of the functionality of these events *in vivo* has lagged, thereby preventing the elucidation of regulatory mechanisms and further rational pathway engineering. In metabolic engineering, ignoring the engineering of multilayer regulation hinders metabolic flux redistribution. Post-translational engineering, allosteric engineering, and dynamic pathway analyses and control will also contribute to the modulation and control of the metabolism of engineered *B. subtilis*, ultimately producing the desired cellular traits. We hope this review will aid metabolic engineers in making full use of available systems biology datasets and approaches for the design and perfection of microbial cell factories through global metabolism optimization.

Contents

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1. Introduction

Bacillus subtilis is a model gram-positive bacterium that is of great interest to fundamental and applied research (Perkins et al., 2009; Sonenshein, 2007; van Dijl and Hecker, 2013). Advances in the genetic manipulation of *B. subtilis* facilitate pathway regulatory mechanism investigation and pathway engineering, such as transcriptional regulation of gene expression and protein-protein interactions (PPIs) (Higgins and Dworkin, 2012; Westers et al., 2003; Caspi et al., 2014; Michna et al., 2014; Sierro et al., 2008). Combining random mutagenesis and the engineering of known regulatory mechanisms has been used to carry out metabolic engineering in a brute force manner for the production of industrially useful or pharmaceutically important compounds in *B. subtilis* (Liu et al., 2013a; Perkins et al., 2009). However, this brute force engineering approach is extremely labor-intensive and has limited potential applications in developing cell factories. The improvement of microbial cell factories relies strongly on system-wide analyses of regulatory networks, which are the foundation of rational engineering strategies for metabolic flux redistribution. Limitations in our understanding of systematic and dynamic regulation of *B. subtilis* metabolism—rather than limitations in genetic manipulation tools—are the main factors preventing further fine-tuning of synthetic pathways and disrupting the balance between *B. subtilis* cell growth and product synthesis.

Recent advances in systems biology have enabled analyses of multilayer omics, including transcriptome, proteome, metabolome, and promoter activities, with statistical and computational models (Buescher et al., 2012; Nicolas et al., 2012). What does systems biology tell us? What types of datasets and tools do we need to understand regulatory mechanisms and guide metabolic engineering? Furthermore, how can we guide metabolic engineering by applying a mechanistic approach that relies on the models and high-throughput data that systems biology typically uses? Because no prior review has focused on these questions, it is meaningful and timely to summarize current progress in systems biology and metabolic engineering and discuss how we can acquire omics data or use existing omics data to enhance both our understanding of metabolism and our ability to implement metabolic engineering.

This review first summarizes recent progress in the systems biology of *B. subtilis* and reveals gaps in the identification of current systematic regulatory events and understanding of their functionality. Next, we review advances in tools and strategies available for metabolic engineering and describe how ignoring combinatory optimization of gene expression, post-translational regulation, and allosteric regulation impedes metabolic flux redistribution. Finally, we discuss approaches to the systematic elucidation of metabolic regulation and the use of systems biology to guide metabolic engineering, which includes the elucidation and engineering of post-translational modification, allostery, and dynamic pathway analyses and the control of multiple layers of regulation in *B. subtilis*. We hope this review will help metabolic engineers make full use of valuable systems biology approaches and datasets to guide metabolic engineering for globally designing and optimizing microbial cell factories. We also hope that this review inspires the investigation of more industrially relevant questions in metabolic engineering through systems biology approaches with the goal of identifying rate-limiting steps and improving cellular traits.

2. Systems biology advances in *B. subtilis*

2.1. Dynamic adaptation to environmental change and the contributions of various layers of regulation to metabolic flux control

Under natural conditions, *B. subtilis* is exposed to soil. To survive during environmental changes, the bacterium must adapt to fluctuating conditions. The ideal approach to the elucidation of regulatory mechanisms is to investigate multilayer regulation and its interactions, such as gene expression, allosteric regulation, and post-translational regulation, during environmental changes. Buescher et al. (2012) pioneered the integration analysis of multilevel omics data aided by statistical and model-based data analyses to infer the regulatory interactions of metabolism, including transcriptome, proteome, metabolome, and promoter activities (Fig. 1 and Table 1). This research provides a paradigm for multilayered data integration analysis in *B. subtilis* to facilitate our understanding of the interactions between gene expression regulation and the metabolic state during environmental changes, which are generally applicable to other microorganisms as well. Dynamic data are invaluable for modeling the metabolism of *B. subtilis* during environment shifts. The results of integration analysis have uncovered hundreds of novel potential interactions—including transcriptional factor binding targets, post-transcriptional regulatory events, and other processes—that require further investigation using genetic and biochemical methods (Buescher et al., 2012).

Applying more advanced omics data-acquiring methods or optimizing omics data detection methods can increase the coverage and accuracy of data acquisition, which is helpful for further investigation of systematic mechanisms. To this end, high-quality or high-coverage transcriptome, proteome, and intra- and exometabolomes of *B. subtilis* have been investigated with RNA sequencing, liquid chromatography/mass spectrometry-based proteomics, and targeted metabolomics (Brinsmade et al., 2014; Maaß et al., 2014; Meyer et al., 2014; Muntel et al., 2014). Specifically, analysis of the transcriptome of *B. subtilis*, which contains CodY mutants detected by RNA sequencing, revealed that the global transcriptional factor CodY is involved in hierarchical gene expression control (Brinsmade et al., 2014). Furthermore, *B. subtilis* proteomics with higher coverage and accuracy revealed that large amounts of cellular resources are allocated to chaperones and proteases depending on stress conditions (up to 30% of the total biomass) (Maaß et al., 2014). Metabolomics data may be useful for thermodynamic and pathway kinetic modeling under relevant conditions (Meyer et al., 2014). Deeper investigations and integration of experimental data with advanced approaches are needed to identify novel interactions *via* statistical analysis and kinetic modeling.

Metabolic flux is an outcome regulated by multilayer regulatory events and directly reflects the metabolic status, providing informative evidence that can be used to infer regulatory mechanisms. Investigation of the coordination of various regulatory layers and their contributions to metabolic flux control help elucidate the responses used by *B. subtilis* to survive environmental fluctuation (Chubukov et al., 2014; Schilling et al., 2007). ^{13}C metabolic flux analysis and transcriptional level analysis of central carbon metabolism have been integrated to investigate the dynamic responses of *B. subtilis* grown in medium with glucose as the sole carbon source to the addition of organic acid. Notably, the transcription level does not correlate with changes in metabolic flux, implying that post-translational regulation is a critical process (Schilling et al., 2007). In steady-state cultures of *B. subtilis* grown on various carbon sources, multilayer omics data were obtained based on ^{13}C metabolic flux analysis, DNA microarray-based transcriptomics, and targeted metabolomics. Changes in neither enzyme abundance nor metabolite concentration sufficiently explained the variations in metabolic flux under the different growth conditions. This result demonstrated that regulatory mechanisms—such as post-transcriptional regulation (*e.g.*, small regulatory RNA [sRNA]- or antisense RNA-based regulation), post-translational regulation (*e.g.*, phosphorylation, acetylation, and succinylation), and allosteric interactions—that affect pathway enzyme activities are key contributors to flux control (Chubukov et al., 2014; Gerosa and Sauer, 2011). This conclusion explains why metabolic engineers cannot always change the metabolic flux of targeted pathways merely by changing gene expression at the transcriptional level. Therefore, metabolic mechanisms and pathway engineering should focus not only on transcriptional regulation but also on mechanisms beyond that regulation, such as post-transcriptional and post-translational regulation and allosteric interactions.