Published in final edited form as:

Curr Opin Biotechnol. 2014 August ; 28: 127–133. doi:10.1016/j.copbio.2014.02.006.

Metabolic flux analysis of *E. coli* knockouts: lessons from the Keio collection and future outlook

Christopher P. Long and Maciek R. Antoniewicz*

Department of Chemical and Biomolecular Engineering, Metabolic Engineering and Systems Biology Laboratory, University of Delaware, Newark DE 19716, USA

Abstract

Cellular metabolic and regulatory systems are of fundamental interest to biologists and engineers. Incomplete understanding of these complex systems remains an obstacle to progress in biotechnology and metabolic engineering. An established method for obtaining new information on network structure, regulation and dynamics is to study the cellular system following a perturbation such as a genetic knockout. The Keio collection of all viable *E. coli* single-gene knockouts is facilitating a systematic investigation of the regulation and metabolism of *E. coli*. Of all omics measurements available, the metabolic flux profile (the fluxome) provides the most direct and relevant representation of the cellular phenotype. Recent advances in ¹³C-metabolic flux analysis are now permitting highly precise and accurate flux measurements for investigating cellular systems and guiding metabolic engineering efforts.

Introduction

The elucidation and quantification of complex regulatory and metabolic systems is of fundamental interest to biologists and engineers. A useful method in unraveling this complexity is observation of the biological system under perturbed conditions, for example, by removal of identified components (e.g. genes). As a model prokaryotic organism, *Escherichia coli* is ideally suited for gene knockout studies, and indeed, many studies on *E. coli* knockouts have been performed. The Keio library of all viable *E. coli* single-gene knockouts is now further facilitating these studies [1]. In addition to having significant value in fundamental biological sciences, quantitative studies of cellular responses to gene knockouts provide relevant data for metabolic engineering and biotechnology applications.

Multiple omics techniques have been applied to gain insight into the systemic responses of *E. coli* to gene knockouts, including metabolomics [2–6], transcriptomics [3,4,7–9], enzymatic activity measurements [2,3,6,10,11], and metabolic flux analysis (fluxomics). Of all these omics levels, the fluxome provides the most relevant representation of the cellular phenotype for guiding metabolic engineering efforts [12]. Since early on in metabolic engineering, a lack of understanding and accurate predictability of complex perturbation responses has been identified as a major obstacle [13,14]. In this paper, we review recent

*Corresponding author: Maciek R. Antoniewicz, Department of Chemical and Biomolecular Engineering, University of Delaware, 150 Academy St, Newark, DE 19716, Tel.: 302-831-8960, Fax.: 302-831-1048, mranton@udel.edu.

progress in measuring and predicting metabolic flux phenotypes for *E. coli* knockouts using computational approaches, such as flux balance analysis (FBA), and experimental flux measurements techniques, particularly ^{13}C -metabolic flux analysis (^{13}C -MFA). We highlight limitations and challenges in using current data sets, describe important research directions in this active research area, and provide an outlook for the future.

Predicting flux responses of *E. coli* knockouts

There has been much work done in applying computational tools to predict responses of genetically perturbed biological systems. The performance limits of *E. coli* metabolic networks, subject to gene deletions and additions, have been traditionally assessed using FBA [15], where a linear optimization and an “objective function” are used to predict feasible flux distributions. The optimization serves as a proxy for evolutionary forces, e.g. a maximized biomass production flux represents evolutionary pressure under competitive growth conditions. The FBA approach and various objective functions have been evaluated for wild-type *E. coli*, and this approach has been shown to be generally successful at predicting fluxes [16]. However, for unevolved genetically perturbed strains, the evolution-based objective function is questionable. Indeed, for many knockouts, FBA has been limited to evaluating the feasibility of growth [17], and predicting growth rates of further evolved knockout strains [18].

Several alternative approaches have been proposed to predict flux distributions of *E. coli* knockouts. Minimization of Metabolic Adjustment (MOMA) [19] postulates that the perturbed metabolic state will be as close as possible (by Euclidean distance) to the FBA optimum of the wild-type; this favors solutions with many small changes to fluxes rather than smaller number of large changes. Given that this can be inconsistent with the concepts of regulatory adaptation cost and linearity of flow, the alternative Regulatory On/Off Minimization (ROOM) approach has been developed, which minimizes the number of large flux changes from the FBA solution [20]. A third algorithm, RELATCH (RELATIVE CHange), suggests using experimental flux and expression data from a reference strain as the starting point. It then uses two parameters to describe the cell’s efforts to first minimize regulatory and distribution pattern changes, and then after a short time activate previously latent pathways and increase the flux capacity of previously active pathways [21]. Yet another approach uses FBA with additional regulatory constraints in the form of predicted gene co-occurrence (co-regulation), or “flux-coupled genes” [22,23]. Finally, a cybernetic modeling approach has been described that has the additional benefit of addressing microbial productivity as well as yields [24]. All these algorithms have reported increased accuracy in predicting flux responses to knockouts when compared to FBA, judged against existing experimental flux data from ^{13}C -MFA studies. However, a thorough assessment of the various methods has been difficult because of significant limitations in the existing knockout flux data (as described next). These limitations have also slowed the development of more advanced mechanistic or kinetic models, which offer the potential for more detailed description and greater predictive power.

Metabolic flux analysis studies of *E. coli* knockouts

In the past decade, numerous ^{13}C -MFA studies have been performed to measure fluxes in *E. coli* knockouts. The goals of these studies have been to explore specific parts of the metabolic and regulatory network, and to assess the general perturbation response. Examples of the former are studies which combine genetic and environmental perturbations to study oxygen sensing and aerobic regulatory response [25–27]. Another example is a study by Nakahigashi et al., in which a novel hidden reaction in the pentose phosphate pathway was discovered by studying sets of double knockouts [28]. In addition to helping to resolve network structure, knockout studies can also help elucidate regulation and dynamics. For example, Siddiquee et al. studied flux distributions, enzyme activities, expression levels, and metabolite concentrations of a *pykF* knockout mutant to quantitatively describe the regulation of PEP carboxylase, malic enzyme, phosphofructokinase, acetate formation, and the oxidative pentose phosphate pathway [7].

There has also been interest in identifying challenges induced by knockouts, and the general and specific adaptive responses to them. For example, Canonaco and Sauer showed that due to overproduction of NADPH in the *pgi* knockout, transhydrogenase activity was kinetically limiting cell growth [29]. Fong et al. showed that the initial response of *E. coli* to many gene knockouts was to activate latent pathways, such as the glyoxylate shunt and Entner-Doudoroff pathway; however, when the responses were monitored over many generations it was observed that the sub-optimal latent pathways were subsequently re-repressed and the capacities of more efficient pathways were expanded to reach new optima [8]. Other examinations of the range of adaptive responses include the immediate flux response, based on a plasmid-based inducible knockout approach [30], and changes over the course of an extended batch culture [5]. These studies inform our understanding of bacterial evolution, and have practical consequences for the industrial use of genetically modified strains. However, as a window to viewing the native metabolic and regulatory structure of *E. coli*, the studies of evolved strains are probably less informative than those studies immediately following a perturbation.

The most widely studied knockouts to date have been those of central carbon metabolism and global regulation. A summary of genes whose knockout mutant fluxes have been reported in literature are shown in Figure 1 [2–11,26–43]. While knockout flux studies are clearly an area of strong interest, bias in coverage and poor comparability of results have prevented these results from being utilized to their full potential. Understandably, much attention has been paid to phosphoglucose isomerase (*pgi*), glucose-6-phosphate dehydrogenase (*zwf*), 6-phosphogluconate dehydrogenase (*gnd*), and pyruvate kinase (*pykA* and *pykF*) genes, as these represent important nodes in metabolism: *pgi* initiates glycolysis, *zwf* and *gnd* initiate and propagate the oxidative pentose phosphate pathway, and *pykA/F* provides the main route to pyruvate and acetyl-CoA, from which common fermentation products are produced. Regulatory gene knockouts, particularly of global regulators, have also been studied to some extent. The best studied is the *ArcA/B* system, which controls the aerobic metabolic response. All of these studies describe the flux responses to knockouts, and often use well-understood metabolic features (e.g. the role of the oxidative pentose phosphate pathway in NADPH production) to provide some basic rational interpretation of

the systems. Some further elucidate specific regulatory relationships using transcriptomic data and use these to provide additional interpretation. While it is understandable that these few genes were targeted for initial studies, the overall limited coverage is limiting potential biological discoveries and broader analyses such as for understanding the general perturbation response and advancing systems knowledge and modeling.

Most studies to date have been performed under either substrate-rich conditions (e.g. batch), or substrate-limited conditions (e.g. continuous cultures). Cellular responses under these two conditions are likely to be significantly different. For example, remarkably robust flux profiles (i.e. relatively small flux changes) for 24 knockout strains grown under chemostat growth conditions were reported by Ishii et al. [4]. In contrast, much more pronounced metabolic responses were observed for similar strains grown under batch conditions. As an example of the difference, in batch culture the *zwf*-KO strain was reported to secrete acetate with a normalized flux of 44 and have a citrate synthase flux of 51 [31], while in a continuous culture the acetate flux was 0 and citrate synthase flux was 103 [4]. The fact that various continuous culture studies used different dilution rates further complicates a direct comparison of these studies. Thus, with current data it is often difficult to deconvolute the effects of the knockout mutation and environmental changes.

Even among studies using the same gene knockout and growth condition, significant variability in reported fluxes has been observed. This can result from differences in the genetic background of the wild-type, or differences in ^{13}C -MFA methodology employed. For example, for *pgi* knockouts grown under batch conditions, estimates of the normalized citrate synthase flux range from 20 [32] to 62 [8]. In continuous studies of the *pykF* knockout at dilution rates 0.1 hr^{-1} and 0.2 hr^{-1} major differences were reported for *pgi*, *pyk* and *ppc* fluxes [4,6].

A complete knockout flux data set would be valuable

Due to a lack of coverage and discrepancies in experimental conditions and methodology, knockout flux results are currently difficult to compare and generalize. To facilitate fundamental analysis of *E. coli* metabolic and regulatory processes, an unbiased and high-resolution data set consisting of methodologically self-consistent ^{13}C -flux results for a large number of knockout mutants would be ideal. This is now increasingly feasible given the significant improvements in ^{13}C -flux methodology in recent years [44–47], miniaturization of experiments [32], as well as the availability of a large number of viable *E. coli* knockout mutants from the Keio collection [1]. A complete, systematic data set would provide an unbiased basis to assess the general *E. coli* perturbation response. Such a data set would also provide highly valuable inputs for systems biology approaches, e.g. the data could be integrated with other omics levels allowing discovery of novel network connectivities, regulatory relationships and metabolic activities [8,48,49]. It would also be enabling for metabolic and regulatory model development.

A well-developed quantitative and mechanistic model of metabolism would be an invaluable tool for testing current knowledge and a significant step forward as a predictive design tool for metabolic engineering. Several approaches to this kind of global metabolic modeling

have been proposed and attempted. These include joint regulatory and metabolic models [50], models that incorporate enzyme activity [51], cybernetic models [52], and differential equation based models [53]. Ensemble modeling is one of these approaches where the goal is to obtain global metabolic mass-action kinetic parameters by iteratively constraining the parameter space with experimental metabolite and flux data, an application for which a large-scale ^{13}C -MFA knockout data set would be ideal [54]. Many other modeling approaches have been equally limited by the available data for training and testing. Advanced mechanistic models which seek to estimate parameters and map regulatory interactions for individual enzymes would particularly benefit from single-gene knockout data, rather than requiring data from multiple knockouts of isozymes or parallel pathways. A comprehensive single-gene knockout flux data set would therefore be a valuable resource for the community and enable significant refinements of current models leading to improved accuracy and predictive power.

An improved quantitative understanding would then also facilitate improved interpretation of metabolism. Many existing theoretical frameworks could be applied to, tested, and enriched by comprehensive knockout flux data. Examples include graph theoretic assessments of metabolic robustness [55–58] and flux coupling [23,59], quantifications of the influences of thermodynamics on network function [60], classifications of transcriptionally vs. metabolically limited fluxes [61], modular cost-benefit analysis [62], Bayesian analysis of modularized networks [63], evaluation of FBA optimality [64], and the logic of pathway latency/silencing [8,48]. Interpretation of knockout flux data through these frameworks would greatly improve our understanding of the evolved “logic” of cellular life.

Future outlook: towards a large-scale standardized flux data set

Prioritization will be important in future efforts to produce a large set of flux data for *E. coli* knockouts. With almost four thousand viable single gene knockouts, and the relatively laborious nature of ^{13}C -MFA, a truly complete set would be a massive undertaking. A few key sets of metabolic genes would be of highest interest and practical value, and should therefore be prioritized. The first and most important knockouts are those of central carbon metabolism. This involves the high-traffic pathways that generate energy, cofactors, and precursors for all other cellular processes. Understanding bottlenecks and kinetics in central carbon metabolism will therefore be critical to many metabolic engineering efforts.

The next gene set of interest is that of regulators of central carbon metabolism. This regulatory network layer is essential to include in future comprehensive models, and quantitative understanding at this level is still not fully developed. For example, improved understanding of the regulatory system is essential for replacing stand-in modeling concepts such as “objective function” and other heuristics with more fundamental mechanistic models. Also essential to study are genes related to energy and redox metabolism, given that energetics and co-factor availability are fundamental to increasing metabolic yields. Finally, it would be valuable to investigate genes related to the aerobic/anaerobic response, stress response, and catabolism of carbon sources other than glucose, including xylose, glycerol and acetate. Multiple perturbation experiments (double, triple, etc. knockouts) would also

provide valuable information, but at a much higher cost of experimental effort than using the Keio library.

As discussed, the experimental conditions used for culturing has a dramatic effect on flux results. For such a large effort, this choice must be considered carefully. Both aerobic and anaerobic conditions represent unique and important modes of metabolism, and there is value in studying both with knockout flux analysis. Substrate limited (continuous) metabolism is also significantly different from substrate rich (batch) metabolism. Batch conditions remove external limitations to growth and thereby are able to reveal more internal metabolic kinetic limitations and result in more extreme metabolic and regulatory responses. Therefore we propose that batch growth studies are of higher priority initially. Finally, studies of the evolution of knockout (and other genetically perturbed) strains are valuable and complimentary to this proposed work.

While significant sections of this proposed work (one or two sets of gene targets and conditions) can be reasonably performed by a single lab, a more complete effort will require contributions from multiple research labs. To facilitate collaboration and the collection and dissemination of methods, data, and results, an openly accessible database for this type of work will be greatly valuable.

An effort to produce a large-scale data set of *E. coli* knockout fluxes should utilize best practices in ^{13}C -MFA. Within reasonable limits of effort and cost, the goal should be to produce the highest possible flux resolution. For example, optimal tracer selection and the use of parallel labeling experiments have been shown to dramatically improve flux measurement precision [65–69]. Consistent and proper reporting of flux data, error analysis, and transparency of methods, are also of utmost importance to enable reproducibility and comparability of results [70]. A recent review has proposed minimum data standards for ^{13}C -MFA studies that will facilitate sharing of flux results in the future [71]. With these guidelines and conditions, an experimental effort to obtain a large body of *E. coli* knockout flux data is warranted and promises to deliver significant value.

Acknowledgments

This work was supported by NSF CAREER award (CBET-1054120).

References

Papers of special interest (*)

Papers of outstanding interest (**)

1. Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Syst. Biol.* 2006; 2:1–11.
2. Zhu J, Shimizu K. Effect of a single-gene knockout on the metabolic regulation in *Escherichia coli* for D-lactate production under microaerobic condition. *Metab. Eng.* 2005; 7:104–115. [PubMed: 15781419]
3. Li M, Ho PY, Yao S, Shimizu K. Effect of *sucA* or *sucC* gene knockout on the metabolism in *Escherichia coli* based on gene expressions, enzyme activities, intracellular metabolite

concentrations and metabolic fluxes by ^{13}C -labeling experiments. *Biochem. Eng. J.* 2006; 30:286–296.

- 4**. Ishii N, Nakahigashi K, Baba T, Robert M, Soga T, Kanai A, Hirasawa T, Naba M, Hirai K, Hoque A, et al. Multiple high-throughput analyses monitor the response of *E. coli* to perturbations. *Science*. 2007; 316:593–597. Responses of *E. coli* to genetic and environmental perturbations were studied using multiple high-throughput measurements. Analysis of knockout mutants of central carbon metabolism, grown in continuous cultures, revealed unexpectedly small changes in metabolome, transcriptome and proteome, for most knockout strains. Overall, the results revealed that the metabolic network of *E. coli* is remarkably stable and robust in the face of various types of disturbances. [PubMed: 17379776]
5. Toya Y, Ishii N, Nakahigashi K, Hirasawa T, Soga T, Tomita M, Shimizu K. ^{13}C -metabolic flux analysis for batch culture of *Escherichia coli* and its *Pyk* and *Pgi* gene knockout mutants based on mass isotopomer distribution of intracellular metabolites. *Biotechnol. Prog.* 2010; 26:975–992. [PubMed: 20730757]
6. Siddiquee KAZ, Arauzo-Bravo MJ, Shimizu K. Metabolic flux analysis of *pykF* gene knockout *Escherichia coli* based on ^{13}C -labeling experiments together with measurements of enzyme activities and intracellular metabolite concentrations. *Appl. Microbiol. Biotechnol.* 2004; 63:407–417. [PubMed: 12802531]
7. Siddiquee KAZ, Arauzo-Bravo MJ, Shimizu K. Effect of a pyruvate kinase (*pykF*-gene) knockout mutation on the control of gene expression and metabolic fluxes in *Escherichia coli*. *FEMS Microbiol. Lett.* 2004; 235:25–33. [PubMed: 15158258]
8. Fong SS, Nanchen A, Palsson BO, Sauer U. Latent pathway activation and increased pathway capacity enable *Escherichia coli* adaptation to loss of key metabolic enzymes. *J. Biol. Chem.* 2006; 281:8024–8033. [PubMed: 16319065]
9. Waegeman H, Beauprez J, Moens H, Maertens J, De Mey M, Foulquié-Moreno MR, Heijnen JJ, Charlier D, Soetaert W. Effect of *iclR* and *arcA* knockouts on biomass formation and metabolic fluxes in *Escherichia coli* K12 and its implications on understanding the metabolism of *Escherichia coli* BL21 (DE3). *BMC Microbiol.* 2011; 11
10. Zhao J, Baba T, Mori H, Shimizu K. Global metabolic response of *Escherichia coli* to *gnd* or *zwf* gene-knockout, based on ^{13}C -labeling experiments and the measurement of enzyme activities. *Appl. Microbiol. Biotechnol.* 2004; 64:91–98. [PubMed: 14661115]
11. Li M, Ho PY, Yao S, Shimizu K. Effect of *lpdA* gene knockout on the metabolism in *Escherichia coli* based on enzyme activities, intracellular metabolite concentrations and metabolic flux analysis by ^{13}C -labeling experiments. *J. Biotechnol.* 2006; 122:254–266. [PubMed: 16310273]
12. He L, Xiao Y, Gebreselassie N, Zhang F, Antoniewicz MR, Tang YJ, Peng L. Central metabolic responses to the overproduction of fatty acids in *Escherichia coli* based on ^{13}C -metabolic flux analysis. *Biotechnol. Bioeng.* 2013; doi: 10.1002/bit.25124
13. Bailey JE. Toward a Science of Metabolic Engineering. *Science*. 1991; 252:1668–1675. [PubMed: 2047876]
14. Stephanopoulos G. Metabolic fluxes and metabolic engineering. *Metab. Eng.* 1999; 1:1–11. [PubMed: 10935750]
15. Burgard AP, Maranas CD. Probing the Performance Limits of the *Escherichia coli* Metabolic Network Subject to Gene Additions or Deletions. *Biotechnol. Bioeng.* 2001; 74:364–375. [PubMed: 11427938]
16. Schuetz R, Kuepfer L, Sauer U. Systematic evaluation of objective functions for predicting intracellular fluxes in *Escherichia coli*. *Mol. Syst. Biol.* 2007; 3:119. [PubMed: 17625511]
17. Edwards JS, Palsson BO. The *Escherichia coli* MG1655 in silico metabolic genotype: its definition, characteristics, and capabilities. *Proc. Natl. Acad. Sci. U. S. A.* 2000; 97:5528–5533. [PubMed: 10805808]
18. Fong SS, Palsson BO. Metabolic gene-deletion strains of *Escherichia coli* evolve to computationally predicted growth phenotypes. *Nat. Genet.* 2004; 36:1056–1058. [PubMed: 15448692]
19. Segre D, Vitkup D, Church GM. Analysis of optimality in natural and perturbed metabolic networks. *Proc. Natl. Acad. Sci. U. S. A.* 2002; 99:15112–15117. [PubMed: 12415116]

20. Shlomi T, Berkman O, Ruppin E. Regulatory on/off minimization of metabolic flux changes after genetic perturbations. *Proc. Natl. Acad. Sci. U. S. A.* 2005; 102:7695–7700. [PubMed: 15897462]
- 21*. Kim J, Reed JL. RELATCH: relative optimality in metabolic networks explains robust metabolic and regulatory responses to perturbations. *Genome Biol.* 2012; 13:R78. A new framework was developed to predict cellular responses to perturbations. The RELATCH (RELATIVE CHange) approach uses flux and gene expression data from a reference state to predict metabolic responses in a genetically, or environmentally perturbed state. Improved prediction accuracy was demonstrated compared to the alternative approaches FBA and ROOM. [PubMed: 23013597]
22. Park JM, Kim TY, Lee SY. Prediction of metabolic fluxes by incorporating genomic context and flux-converging pattern analyses. *Proc. Natl. Acad. Sci. U. S. A.* 2010; 107:14931–14936. [PubMed: 20679215]
23. Kim HU, Kim WJ, Lee SY. Flux-coupled genes and their use in metabolic flux analysis. *Biotechnol. J.* 2013; 8:1035–42. [PubMed: 23420780]
24. Song H-S, Ramkrishna D. Prediction of dynamic behavior of mutant strains from limited wild-type data. *Metab. Eng.* 2012; 14:69–80. [PubMed: 22500302]
25. Nizam SA, Shimizu K. Effects of *arcA* and *arcB* genes knockout on the metabolism in *Escherichia coli* under anaerobic and microaerobic conditions. *Biochem. Eng. J.* 2008; 42:229–236.
26. Nizam SA, Zhu J, Ho PY, Shimizu K. Effects of *arcA* and *arcB* genes knockout on the metabolism in *Escherichia coli* under aerobic condition. *Biochem. Eng. J.* 2009; 44:240–250.
27. Portnoy VA, Scott DA, Lewis NE, Tarasova Y, Osterman AL, Palsson BO. Deletion of genes encoding cytochrome oxidases and quinol monooxygenase blocks the aerobic-anaerobic shift in *Escherichia coli* K-12 MG1655. *Appl. Environ. Microbiol.* 2010; 76:6529–6540. [PubMed: 20709841]
- 28**. Nakahigashi K, Toya Y, Ishii N, Soga T, Hasegawa M, Watanabe H, Takai Y, Honma M, Mori H, Tomita M. Systematic phenome analysis of *Escherichia coli* multiple-knockout mutants reveals hidden reactions in central carbon metabolism. *Mol. Syst. Biol.* 2009; 5:306. Systematic double-knockout mutants of central carbon catabolism genes were constructed for *E. coli*. Investigation of growth on several carbon sources revealed a new bypass in the pentose phosphate pathway. Surprisingly, the bypass reactions emerged without any significant changes in the gene expression of the associated enzymes. [PubMed: 19756045]
29. Canonaco F, Hess Ta, Heri S, Wang T, Szyperski T, Sauer U. Metabolic flux response to phosphoglucose isomerase knock-out in *Escherichia coli* and impact of overexpression of the soluble transhydrogenase *UdhA*. *FEMS Microbiol. Lett.* 2001; 204:247–252. [PubMed: 11731130]
30. Usui Y, Hirasawa T, Furusawa C, Shirai T, Yamamoto N, Mori H, Shimizu H. Investigating the effects of perturbations to *pgi* and *eno* gene expression on central carbon metabolism in *Escherichia coli* using ¹³C metabolic flux analysis. *Microb. Cell Fact.* 2012; 11:87. [PubMed: 22721472]
31. Nicolas C, Kiefer P, Letisse F, Krömer J, Massou S, Soucaille P, Wittmann C, Lindley ND, Portais J-C. Response of the central metabolism of *Escherichia coli* to modified expression of the gene encoding the glucose-6-phosphate dehydrogenase. *FEBS Lett.* 2007; 581:3771–3776. [PubMed: 17631881]
- 32*. Haverkorn van Rijsewijk BRB, Nanchen A, Nallet S, Kleijn RJ, Sauer U. Large-scale ¹³C-flux analysis reveals distinct transcriptional control of respiratory and fermentative metabolism in *Escherichia coli*. *Mol. Syst. Biol.* 2011; 7:477. ¹³C-Flux analysis was performed on 91 transcriptional regulator *E. coli* knockouts, grown on glucose and galactose, to unravel condition-dependent transcriptional control of metabolism. More than half of the investigated regulators were controlling absolute fluxes in *E. coli*. [PubMed: 21451587]
33. Emmerling M, Dauner M, Ponti A, Fiaux J, Hochuli M, Szyperski T, Wu K, Bailey JE, Sauer U. Metabolic Flux Responses to Pyruvate Kinase Knockout in *Escherichia coli*. 2002; 184:152–164.
34. Fischer E, Sauer U. Metabolic flux profiling of *Escherichia coli* mutants in central carbon metabolism using GC-MS. *Eur. J. Biochem.* 2003; 270:880–891. [PubMed: 12603321]
35. Hua Q, Yang C, Baba T, Mori H, Shimizu K. Responses of the Central Metabolism in *Escherichia coli* to Phosphoglucose Isomerase and Glucose-6-Phosphate Dehydrogenase Knockouts. *J. Bacteriol.* 2003; 185:7053–7067. [PubMed: 14645264]

36. Yang C, Hua Q, Baba T, Mori H, Shimizu K. Analysis of *Escherichia coli* anaplerotic metabolism and its regulation mechanisms from the metabolic responses to altered dilution rates and phosphoenolpyruvate carboxykinase knockout. *Biotechnol. Bioeng.* 2003; 84:129–144. [PubMed: 12966569]
37. Jiao Z, Baba T, Mori H, Shimizu K. Analysis of metabolic and physiological responses to *gnd* knockout in *Escherichia coli* by using C-13 tracer experiment and enzyme activity measurement. *FEMS Microbiol. Lett.* 2003; 220:295–301. [PubMed: 12670695]
38. Zhao J, Baba T, Mori H, Shimizu K. Effect of *zwf* gene knockout on the metabolism of *Escherichia coli* grown on glucose or acetate. *Metab. Eng.* 2004; 6:164–174. [PubMed: 15113569]
39. Perrenoud A, Sauer U. Impact of Global Transcriptional Regulation by ArcA, ArcB, Cra, Crp, Cya, Fnr, and Mlc on Glucose Catabolism in *Escherichia coli*. *J. Bacteriol.* 2005; 187:3171–3179. [PubMed: 15838044]
40. Zhu J, Shalel-Levanon S, Bennett G, San K-Y. Effect of the global redox sensing/regulation networks on *Escherichia coli* and metabolic flux distribution based on C-13 labeling experiments. *Metab. Eng.* 2006; 8:619–627. [PubMed: 16962353]
41. Yao R, Hirose Y, Sarkar D, Nakahigashi K, Ye Q, Shimizu K. Catabolic regulation analysis of *Escherichia coli* and its *crp*, *mlc*, *mgsA*, *pgi* and *ptsG* mutants. *Microb. Cell Fact.* 2011; 10:67. [PubMed: 21831320]
42. Waegeman H, Maertens J, Beauprez J, De Mey M, Soetaert W. Effect of *iclR* and *arcA* deletions on physiology and metabolic fluxes in *Escherichia coli* BL21 (DE3). *Biotechnol. Lett.* 2012; 34:329–337. [PubMed: 22009573]
43. Toya Y, Nakahigashi K, Tomita M, Shimizu K. Metabolic regulation analysis of wild-type and *arcA* mutant *Escherichia coli* under nitrate conditions using different levels of omics data. *Mol. Biosyst.* 2012; 8:2593–2604. [PubMed: 22790675]
- 44*. Leighty RW, Antoniewicz MR. COMPLETE-MFA: Complementary parallel labeling experiments technique for metabolic flux analysis. *Metab. Eng.* 2013; 20:49–55. A new approach was developed for high-resolution ¹³C metabolic flux analysis based on parallel labeling experiments. The COMPLETE-MFA approach represents current state-of-the-art in flux measurement technology. It will be valuable tool in future studies focused on measuring highly precise and accurate metabolic fluxes for *E. coli* knockouts. [PubMed: 24021936]
45. Antoniewicz MR. Tandem mass spectrometry for measuring stable-isotope labeling. *Curr. Opin. Biotechnol.* 2013; 24:48–53. [PubMed: 23142542]
46. Antoniewicz MR. Dynamic metabolic flux analysis-tools for probing transient states of metabolic networks. *Curr. Opin. Biotechnol.* 2013; 24
- 47*. Antoniewicz MR. ¹³C metabolic flux analysis: optimal design of isotopic labeling experiments. *Curr. Opin. Biotechnol.* 2013; 24 An up-to-date review article that describes current state-of-the-art methods for optimal design of ¹³C-labeling experiments and the importance of this step in ¹³C metabolic flux analysis studies.
48. Nishikawa T, Gulbahce N, Motter AE. Spontaneous reaction silencing in metabolic optimization. *PLoS Comput. Biol.* 2008; 4:e1000236. [PubMed: 19057639]
- 49*. Cornelius SP, Sang J, Motter AE. Dispensability of *Escherichia coli*'s latent pathways. *Proc. Natl. Acad. Sci. U. S. A.* 2011; 108:3124–3129. Computational analysis of the *E. coli* metabolic network was performed to reveal that latent metabolic pathways offer, in many cases, no growth advantages after a genetic perturbation and in fact may have a negative effect on the growth rate. This unexpected result raises the possibility that latent pathway activation is a potentially suboptimal adaptive response of *E. coli*. [PubMed: 21300895]
50. Yeang C-H, Vingron M. A joint model of regulatory and metabolic networks. *BMC Bioinformatics.* 2006; 7:332. [PubMed: 16820044]
51. Kurata H, Zhao Q, Okuda R, Shimizu K. Integration of enzyme activities into metabolic flux distributions by elementary mode analysis. *BMC Syst. Biol.* 2007; 1:31. [PubMed: 17640350]
52. Young JD, Henne KL, Morgan JA, Konopka AE, Ramkrishna D. Integrating cybernetic modeling with pathway analysis provides a dynamic, systems-level description of metabolic control. *Biotechnol. Bioeng.* 2008; 100:542–559. [PubMed: 18438875]

53. Kadir TAA, Mannan AA, Kierzek AM, McFadden J, Shimizu K. Modeling and simulation of the main metabolism in *Escherichia coli* and its several single-gene knockout mutants with experimental verification. *Microb. Cell Fact.* 2010; 9:88. [PubMed: 21092096]
54. Tran LM, Rizk ML, Liao JC. Ensemble modeling of metabolic networks. *Biophys. J.* 2008; 95:5606–5617. [PubMed: 18820235]
55. Ghim C-M, Goh K-I, Kahng B. Lethality and synthetic lethality in the genome-wide metabolic network of *Escherichia coli*. *J. Theor. Biol.* 2005; 237:401–411. [PubMed: 15975601]
56. Wunderlich Z, Mirny L. Using the topology of metabolic networks to predict viability of mutant strains. *Biophys. J.* 2006; 91:2304–2311. [PubMed: 16782788]
57. Wilhelm T, Behre J, Schuster S. Analysis of structural robustness of metabolic networks. *Syst. Biol. (Stevenage).* 2004; 1:114–120. [PubMed: 17052121]
58. Behre J, Wilhelm T, von Kamp A, Ruppin E, Schuster S. Structural robustness of metabolic networks with respect to multiple knockouts. *J. Theor. Biol.* 2008; 252:433–441. [PubMed: 18023456]
59. Burgard AP, Nikolaev EV, Schilling CH, Maranas CD. Flux coupling analysis of genome-scale metabolic network reconstructions. *Genome Res.* 2004; 14:301–312. [PubMed: 14718379]
60. Henry CS, Jankowski MD, Broadbelt LJ, Hatzimanikatis V. Genome-scale thermodynamic analysis of *Escherichia coli* metabolism. *Biophys. J.* 2006; 90:1453–1461. [PubMed: 16299075]
61. Shlomi T, Eisenberg Y, Sharan R, Ruppin E. A genome-scale computational study of the interplay between transcriptional regulation and metabolism. *Mol. Syst. Biol.* 2007; 3:101. [PubMed: 17437026]
62. Carlson RP. Metabolic systems cost-benefit analysis for interpreting network structure and regulation. *Bioinformatics.* 2007; 23:1258–1264. [PubMed: 17344237]
63. Kim HU, Kim TY, Lee SY. Framework for network modularization and Bayesian network analysis to investigate the perturbed metabolic network. *BMC Syst. Biol.* 2011; 5(Suppl 2):S14.
64. Chen X, Alonso AP, Allen DK, Reed JL, Shachar-Hill Y. Synergy between ^{13}C -metabolic flux analysis and flux balance analysis for understanding metabolic adaptation to anaerobiosis in *E. coli*. *Metab. Eng.* 2011; 13:38–48. [PubMed: 21129495]
65. Crown SB, Antoniewicz MR. Selection of tracers for ^{13}C -Metabolic Flux Analysis using Elementary Metabolite Units (EMU) basis vector methodology. *Metab. Eng.* 2012; 14:150–161. [PubMed: 22209989]
66. Crown SB, Antoniewicz MR. Parallel labeling experiments and metabolic flux analysis: Past, present and future methodologies. *Metab. Eng.* 2013; 16:21–32. [PubMed: 23246523]
67. Ahn WS, Antoniewicz MR. Parallel labeling experiments with $[1,2-^{13}\text{C}]$ glucose and $[U-^{13}\text{C}]$ glutamine provide new insights into CHO cell metabolism. *Metab. Eng.* 2013; 15:34–47. [PubMed: 23111062]
68. Leighty RW, Antoniewicz MR. Parallel labeling experiments with $[U-^{13}\text{C}]$ glucose validate *E. coli* metabolic network model for ^{13}C metabolic flux analysis. *Metab. Eng.* 2012; 14:533–541. [PubMed: 22771935]
69. Crown SB, Ahn WS, Antoniewicz MR. Rational design of ^{13}C -labeling experiments for metabolic flux analysis in mammalian cells. *BMC Syst. Biol.* 2012; 6:43. [PubMed: 22591686]
70. Antoniewicz MR, Kelleher JK, Stephanopoulos G. Determination of confidence intervals of metabolic fluxes estimated from stable isotope measurements. *Metab. Eng.* 2006; 8:324–337. [PubMed: 16631402]
- 71**. Crown SB, Antoniewicz MR. Publishing ^{13}C metabolic flux analysis studies: A review and future perspectives. *Metab. Eng.* 2013; 20:42–48. In this comprehensive review paper, practical guidelines are provided for performing ^{13}C metabolic flux analysis studies. For the first time, minimum data standards are established for publishing ^{13}C -MFA results. These guidelines will be valuable in future to increase clarity, reproducibility, and sharing of fluxome results. [PubMed: 24025367]