CHAPTER TWELVE

An Engineering Approach for Rewiring Microbial Metabolism

Sebastian Wenk, Oren Yishai, Steffen N. Lindner, Arren Bar-Even[1]
Max Planck Institute of Molecular Plant Physiology, Potsdam-Golm, Germany
[1]Corresponding author: e-mail address: bar-even@mpimp-golm.mpg.de

Contents

Abstract

The introduction of synthetic pathways into microbes often requires substantial modifications of the host metabolism. Here, we present and discuss key experimental aspects required for modifying microbial central metabolism. We introduce the concept of dividing pathways into metabolic modules, the activity of which can be selected for and optimized in dedicated gene-deletion strains. We provide a comprehensive methodology for systematic pathway implementation in vivo, ranging from gene-deletion methods for the creation of selection strains to cloning strategies that allow fine-tuned expression of individual pathway enzymes in synthetic operons. We further describe pathway testing and validation via high-throughput growth experiments and ^{13}C-labeling measurements. While we focus on *Escherichia coli* as bacterial host, the holistic approach we present could be easily adapted for the metabolic engineering of other microbes.

Methods in Enzymology, Volume 608
ISSN 0076-6879
https://doi.org/10.1016/bs.mie.2018.04.026

1. INTRODUCTION

As synthetic biology matures as a field, it acquires properties usually attributed to more traditional engineering fields, such as modular design, systematic construction of parts, and rigorous testing and optimization. Here, we demonstrate how these properties can be harnessed to rewire the central metabolism of the model microbe *Escherichia coli*. We specifically elaborate and exemplify four conceptual and methodological aspects: (i) selection for the activity of pathway modules within dedicated selection strains, i.e., gene-deletion strains which are auxotrophic for metabolites produced by the module, (ii) spanning gene expression levels using multiple regulatory elements, (iii) conducting high-throughput growth experiments to identify optimal pathway activity, and (iv) applying ^{13}C labeling to confirm pathway activity (Fig. 1).

To demonstrate our approach to metabolic engineering, we use a concrete project as an example: the establishment of an altered serine pathway in *E. coli*. The serine pathway operates in several methylotrophic bacteria and is responsible for the assimilation of C1 feedstocks, e.g., methanol and formate (Anthony, 2011). Establishing this pathway in *E. coli* could enable this bacterium to grow on formate as sole carbon source. Previously, we suggested several modified structures of the serine pathway that would specifically fit the endogenous metabolism of *E. coli* (Bar-Even, 2016). The PFL-threonine cycle, shown in Fig. 2, seems especially interesting as it presents two complementary entry points for formate into central metabolism. The enzyme formate-tetrahydrofolate ligase (FTL) catalyzes the assimilation of formate into 10-formyltetrahydrofolate, which is then reduced to 5,10-methylenetetrahydrofolate (5,10-CH_2-THF) and condensed with glycine to give serine. Serine is directly deaminated to pyruvate which is further metabolized to oxaloacetate using the endogenous anaplerotic pathway. Oxaloacetate is then converted to glycine and acetyl-CoA via threonine biosynthesis and degradation. Finally, acetyl-CoA is condensed with formate to produce another pyruvate molecule, as catalyzed by the oxygen-sensitive pyruvate-formate lyase (PFL), thus completing the autocatalytic bicycle.

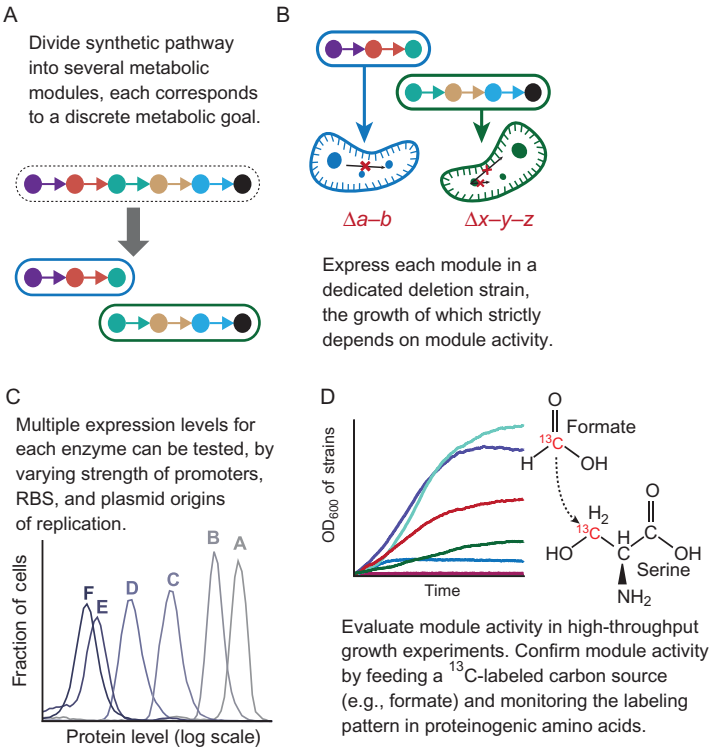

Fig. 1 Schematic representation of the metabolic engineering approach described in this manuscript. (A) To facilitate in vivo implementation, a synthetic pathway is first divided into subpathways (modules) that can be individually tested in dedicated deletion strains, the growth of which (on a given carbon source) is dependent on module activity (B). (C) Module enzymes are expressed in synthetic operons, in which the expression level of individual genes can be manipulated by a combination of ribosome-binding sites (RBSs), origins of replication and promoters. (D) High-throughput growth experiments are conducted to evaluate module activity which is further confirmed via ^{13}C-labeling experiments. *The figure is partly adapted from Yishai, O., Goldbach, L., Tenenboim, H., Lindner, S. N., & Bar-Even, A. (2017). Engineered assimilation of exogenous and endogenous formate in* Escherichia coli. ACS Synthetic Biology, 6, *1722–1731; Zelcbuch, L., Antonovsky, N., Bar-Even, A., Levin-Karp, A., Barenholz, U., Dayagi, M., et al. (2013). Spanning high-dimensional expression space using ribosome-binding site combinatorics.* Nucleic Acids Research, 41. https://doi.org/10.1093/nar/gkt151.

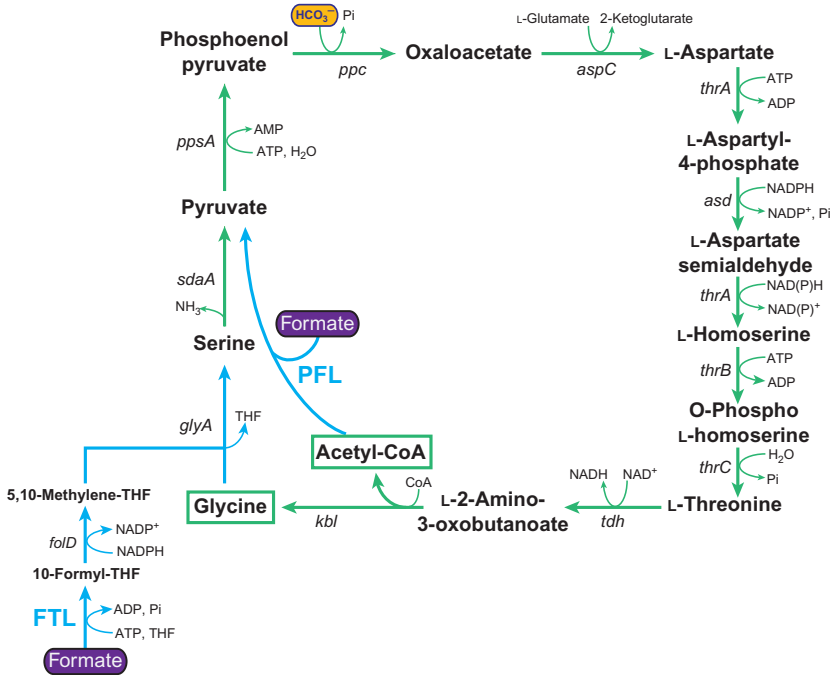

Fig. 2 The PFL-threonine cycle—a synthetic formate assimilation pathway—serves as an example for pathway implementation. The pathway overlaps with *E. coli's* central metabolism and employs only one foreign enzyme, formate-tetrahydrofolate ligase (FTL). This pathway is in fact an autocatalytic bicycle, where its products, acetyl-CoA and glycine, are reassimilated to the pathway via condensation with C1 units.

2. STRAIN ENGINEERING: IN VIVO SELECTION FOR PATHWAY MODULES

Establishing a fully operational synthetic pathway is challenging, especially if it is intended to replace a part of central metabolism (Erb, Jones, & Bar-Even, 2017). In most cases, overexpression of pathway enzymes is not sufficient to establish the required flux, which might be limited by various factors such as suboptimal activity of an enzyme, limited availability of pathway precursors, and competition with endogenous pathways (Keasling, 2010). Therefore, to facilitate the in vivo establishment of a synthetic route, it is advisable to divide it into metabolic modules, i.e., subpathways consisting of short reaction sequences (Yishai, Goldbach, Tenenboim, Lindner, & Bar-Even, 2017).

Each pathway module is expressed in a selection strain—a strain carrying multiple gene deletions such that its growth, under a specific set of conditions (e.g., specific carbon sources in minimal medium), depends on the

activity of the module. These selection strains are designed to be "biosensors" for module activity, serving as reporters with the simple readout: growth, no growth, or, occasionally, retarded growth. Multiple such selection strains can be constructed for each module, where the growth of each strain depends to a different extent on module activity. This enables to sequentially increase the selection pressure for module activity, e.g., one strain would use the module to synthesize a single amino acid, another would use it as source for an entire segment of central metabolism, while in yet another strain all biomass would be derived via the module.

If a selection for module activity fails, or results in retarded growth, different solutions can be tested, for example, fine-tuning the expression of module components and replacing module enzymes with orthologues. The selection strains could be reengineered, deleting, for example, endogenous pathways that might deleteriously compete with or disrupt module activity. Taken together, these selection strains provide a testing platform in which the activity of each module can be tinkered with and optimized separately.

Only when all modules are confirmed to be sufficiently active as separate units, should they be integrated to constitute a complete pathway. Toward this aim, further selection strains can be created, the growth of which depends on the combined activity of several modules, thus enabling stepwise implementation of the complete synthetic route.

While pathways can theoretically be broken into many different modules, we suggest several guidelines for their design: (1) module activity must result in a clear growth phenotype in a conceivable gene deletion strain, that is, relief of an auxotrophy; (2) modules should be short, enabling their expression from a single operon and allowing for fast testing of expression levels; (3) together, the modules should cover the entire pathway, where overlaps—enzymes shared between modules—are advantageous as they enable testing the required enzyme activities within different metabolic contexts; (4) preferably, modules should be easy to combine; that is, their integrated activities can be selected for within a dedicated selection strain.

We note that establishing module activity within a selection strain does not necessarily mean that the module, once expressed as part of a whole pathway, will behave in the same manner. Indeed, an enzyme that supports high flux within a particular selection strain might fail to retain its high activity within another metabolic context for many possible reasons, including changes in reactant concentrations, allosteric regulation, or the cellular energetic state. Yet, in our experience, modules tend to behave similarly across many selection regimes, and the information gained in establishing module activity facilitates the engineering of the entire pathway.

We use the PFL-threonine cycle to demonstrate how a pathway can be divided into modules. As shown in Fig. 3, we can divide this synthetic pathway into several modules. One basic module is acetyl-CoA condensation with formate to give pyruvate, as catalyzed by PFL (Fig. 3A). A previous study showed that we can select for the activity of this module in an *E. coli* strain in which the glyoxylate shunt is deleted (*ΔaceAB*) such that acetate can be assimilated only via its condensation with formate (Zelcbuch et al., 2016). Another basic module is the production of glycine and serine via threonine biosynthesis and degradation, which can be selected for in a strain deleted in the endogenous serine biosynthesis route (*ΔserA*) (Yishai et al., 2017). Fig. 3B shows a selection scheme for the activity of both modules, in which acetate and formate serve as carbon sources and glycine and serine are produced via threonine metabolism. An additional module is the assimilation of formate into the THF system, providing 5,10-CH_2-THF which is condensed with glycine to provide serine. The activity of this module was previously tested and confirmed in a strain deleted in the endogenous serine biosynthesis route (*ΔserA*) as well as in the glycine cleavage system (*ΔgcvTHP*) which normally cleaves glycine for the generation of 5,10-CH_2-THF (Yishai et al., 2017). As shown in Fig. 3C, selecting for the activity of the three basic modules together would support the activity of an almost complete pathway, in which formate is required for both pyruvate and serine biosynthesis.

The ultimate step would be the removal of acetate and selection for growth on formate as a sole carbon source where acetyl-CoA is regenerated from threonine cleavage. Clearly, this would be highly challenging. Hence, it is helpful to first engineer a strain which would depend on the activity of the complete autocatalytic pathway for the production of only a fraction of its biomass. As shown in Fig. 4A, it is possible to separate *E. coli*'s central metabolism into segments: deletion of enolase (*Δeno*) would metabolically separate "upper" metabolism, i.e., glycolysis, from "middle" metabolism, while deletion of citrate synthase (*ΔgltA ΔprpC*) and 2-ketoglutarate dehydrogenase (*ΔsucAB*) would separate "lower" metabolism, i.e., glutamate-derived metabolism, from "middle" metabolism. In this strain, glycerol and 2-ketoglutarate would be added to the medium to provide biomass precursors for "upper" and "lower" metabolism, while the PFL-threonine cycle would use formate to provide all biomass precursors for "middle" metabolism, e.g., acetyl-CoA, pyruvate, and oxaloacetate. Only once the synthetic cycle is shown to support sustainable growth by feeding "middle" metabolism, could it be tested to provide biomass precursors for "upper" and "lower" metabolism as well, as shown in Fig. 4B.

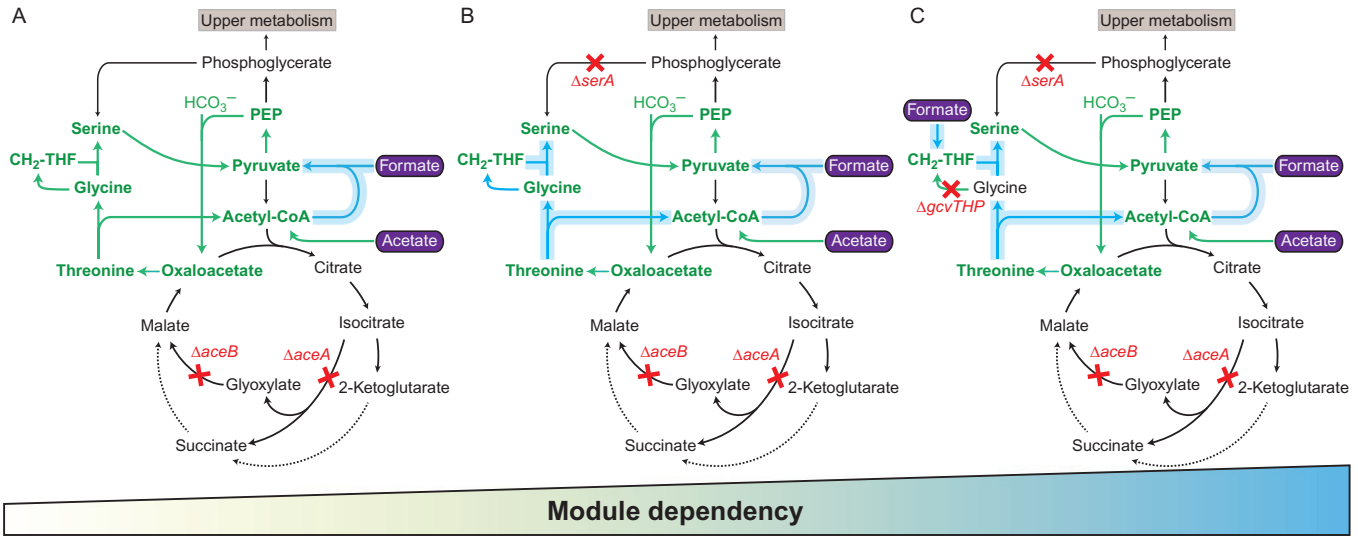

Fig. 3 Selection schemes for the stepwise implementation of the modules of the PFL-threonine cycle in *E. coli*. (A) The first module—assimilatory activity of PFL—is tested in a strain whose growth depends on the condensation of acetyl-CoA and formate as it cannot assimilate acetate via the glyoxylate shunt (*ΔaceAB*). (B) The activity of two pathway modules is tested together: PFL-dependent assimilation of acetate and formate and production of glycine and serine from threonine metabolism. (C) Selection for the activity of three modules: PFL activity, glycine production from threonine metabolism, and serine production via formate assimilation via the tetrahydrofolate system.

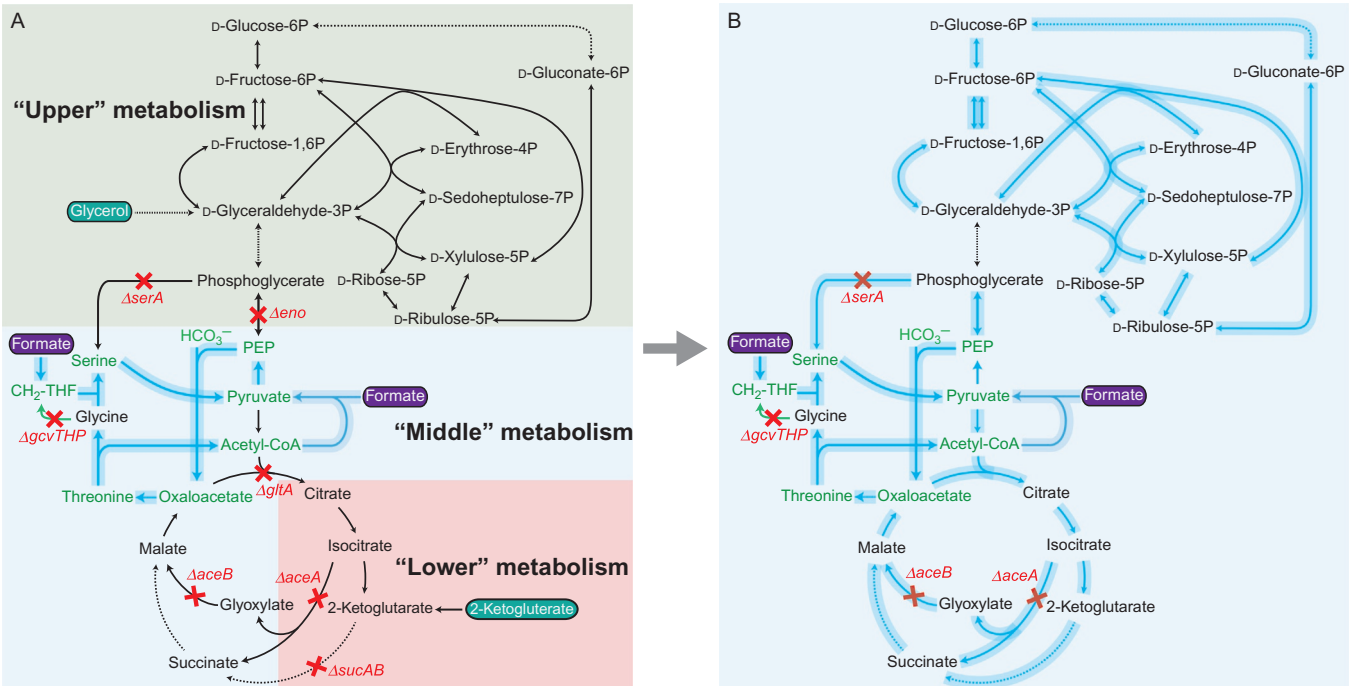

Fig. 4 Stepwise establishment of the complete PFL-threonine cycle in *E. coli*. (A) Deletion of key central metabolism enzymes, enolase (*eno*) and citrate synthase (*gltA*), separate cellular metabolism into several segments. The synthetic pathway provides biomass precursors only to the middle metabolism segment, such that low pathway flux is sufficient. (B) Next, by reconnecting all segments of cellular metabolism, it would be would be possible to select for pathway activity to provide all biomass precursors for cellular growth.

2.1 Gene Deletion Methods

The construction of *E. coli* selection strains requires the deletion of multiple genes. A common gene-deletion approach relies on homologous recombination (HRC) to replace a genomic region with a selection marker, e.g., an antibiotic resistance cassette (Zhang, Buchholz, Muyrers, & Francis Stewart, 1998). Gene-deletion systems based on bacteriophage λ Red recombinase facilitate HRC-based gene deletion using a PCR-amplified dsDNA fragment with relatively short homology arms (Datsenko & Wanner, 2000). To enable HRC, the target strain must be first transformed with a temperature-sensitive λ Red helper plasmid which harbors the recombineering genes (Murphy, 1998). After deletion is completed, cells are plasmid-cured at elevated temperature. As multiple gene deletions are usually required, the selection marker must be recycled, i.e., removed from the genome. To facilitate this, the antibiotic cassette is flanked by FRT (Flippase Recognition Target) sites (Senecoff, Bruckner, & Cox, 1985). The cell is transformed with a flippase (FLP) recombinase helper plasmid, which carries a gene encoding FLP which recombines at the FRT sites and removes the antibiotic cassette (Senecoff et al., 1985). Elevated temperature is subsequently used to cure the cell from the FLP plasmid. Taken together, the deletion of multiple genes using this method requires numerous cycles of plasmid transformation and curing, making the entire process labor intensive and time consuming.

To overcome the need for cotransformation with helper plasmids and speed up the procedure, Nielsen et al. integrated the λ Red recombineering genes and the FLP gene into the genome of *E. coli* K-12 MG1655, where the former are regulated by an L-arabinose-induced promoter and the latter by an L-rhamnose-induced promoter (Jensen, Lennen, Herrgård, & Nielsen, 2015). As the resulting SIJ488 strain supports a considerably faster gene-deletion procedure, we use it as a basis for constructing all selection strains. However, we found that some genes could not be easily deleted using this method. In these cases we commonly get false-positive colonies in which the cassette is integrated in a wrong genomic locus. Furthermore, it is often the case that gene deletion becomes more challenging if a strain already carries multiple deletions, where the genomic FRT "scars" may recombine with the introduced deletion template or react in the flippase reaction to hamper marker recycling (Datsenko & Wanner, 2000).

When gene deletion via λ Red recombination fails, we employ an alternative method which relies on the transfer of a genetic marker from a donor

strain to a recipient strain by P1 phage transduction (Lennox, 1955). P1 phage is a commonly used transducing agent because it can package and transfer random sections of the host chromosome. This enables the transfer of a genomic region in which a gene is replaced with an antibiotic marker (Ikeda & Tomizawa, 1965). As a source of donor strains, the Keio collection provides single-gene deletion strains of all nonessential *E. coli* K-12 genes (when grown on LB), where coding regions were replaced with a kanamycin (Kan) cassette flanked by FRT sites (Baba et al., 2006). If a gene deletion of interest is not available in the Keio collection, which means it is essential under LB culturing conditions, we can delete the gene in the SIJ488 strain using dedicated growth conditions and use the resulting strain as donor for P1 phage transduction.

As P1 phage transfers very big genomic segments (up to 100 kb), high efficiency of native homologous recombination in *E. coli* can be achieved and false-positive colonies are very rare (Thomason, Costantino, & Court, 2007). However, the P1 phage also transfers sequences that flank the deleted gene, which can introduce undesired mutations present in the donor genome to the accepting genome. This is especially problematic when trying to delete a gene that is adjacent to the locus of a previously deleted gene: the P1 phage may reintroduce the WT copy of the deleted gene into the genome (Thomason et al., 2007). Consequently, we limit the use the P1 phage method to genes which we fail to delete using the genomic λ Red recombineering enzymes. With the combination of the two gene deletion methods we can delete almost any gene in a fast and accurate manner.

2.2 Protocol for Gene Deletion in the SIJ488 Strain

As deletion of genes is expected to affect the host's physiology, the cultivation medium should be adapted to support the growth of the gene-deleted strain, that is, if special carbon sources or supplements are necessary for its growth, they should be added to the medium. This is especially relevant when deleting central metabolism enzymes like enolase that effectively divide cellular metabolism into disconnected segments, each requiring a dedicated carbon source (as shown in Fig. 4). Another example is the deletion of phosphoglycerate mutase which was shown to require supplementation with a carbon source for "upper" metabolism (e.g., glycerol) and a carbon source for "lower" metabolism (e.g., pyruvate) (Zelcbuch at al., 2015).

Equipment

- Standard PCR equipment
- Standard gel electrophoresis equipment, 1% agarose gels
- Cooling benchtop centrifuge
- Electroporation device for bacterial transformation (e.g., Biorad Gene Pulser)
- Electroporation cuvettes
- Thermoshaker (e.g., Eppendorf Thermomixer)
- 37°C shaking incubator for bacteria cultivation
- 37°C plate incubator for bacteria cultivation

Buffers and reagents

- Standard PCR reagents
- High-fidelity polymerase (e.g., PrimeStar, TaKaRa; Phusion, Thermo Fisher Scientific)
- PCR purification kit
- Gel purification kit
- 1 *M* L-arabinose solution
- 1 *M* L-rhamnose solution
- 10% glycerol solution
- Nonselective and selective medium (e.g., LB liquid medium and LB agar plates) supplemented with antibiotics, depending on the introduced antibiotic cassette: 50 μg/mL kanamycin; 30 μg/mL chloramphenicol (Cam)

Strains

- *E. coli* SIJ488

Preparations

- Design primers to amplify an FRT-flanked antibiotic cassette and add homology arms—corresponding to the sequences flanking the desired deletion region—on each side of the cassette. Use the same procedure for the homology arms as described in Baba et al. (2006).
- Amplify FRT-flanked antibiotic cassette from a plasmid using the above primers. Use a high-fidelity polymerase to avoid introduction of point mutations.
- Apply 5 μL of the PCR on a 1% agarose gel and verify the size of the PCR product. If the correct band size is obtained, purify the PCR products. If unspecific bands are visible, purify the deletion cassette by gel purification.

- Design gene-specific primers for the verification of the desired deletion, binding upstream and downstream of the gene of interest (approximately within 200 bp on either side of the deleted region).
- Design forward and reverse primers that can bind to the introduced antibiotic cassette. This is especially important when resistance marker and target gene have the same size.

Day 1: Delete the gene of interest

1. Inoculate SIJ488 in 4 mL LB medium and culture at 37°C, shaking the culture tubes at 240 rpm in a shaking incubator until OD_{600} has reached ~0.4.
2. Induce expression of λ Red recombineering genes with 15 m*M* L-arabinose and culture for 1 h at 37°C, shaking at 240 rpm.
3. Centrifuge 2 mL of culture for 30 s at 11,000 rcf in a cooled benchtop centrifuge (at 2°C). Discard the supernatant and place the pellet on ice.
4. Resuspend the pellet in 1 mL chilled 10% glycerol and mix the suspension. Centrifuge for 30 s at 11,000 rcf in a cooled benchtop centrifuge (at 2°C).
5. Repeat the washing step (discard supernatant, resuspension in glycerol, and centrifugation).
6. Discard the supernatant, leaving 100–150 μL in the tube with the pellet.
7. Add 3–5 μL (~250 ng DNA) of the deletion cassette (purified PCR product) to the cell suspension and mix by pipetting.
8. Transfer the cell suspension from the tube to a chilled electroporation cuvette. If the volume does not reach the top of the electrode, fill up with 10% glycerol.
9. Electroporate cells in an electroporation device (e.g., Biorad Gene Pulser) with the following settings:
   Voltage: 1780 V for 1 mm cuvettes and 2500 V for 2 mm cuvettes
   Capacitance: 25 μF
   Resistance: 200 Ω
10. Resuspend the electroporated cells in 1 mL medium (e.g., LB) without antibiotics and transfer them to a new microfuge tube.
11. Incubate at 37°C in a thermoshaker, shaking at 1000 rpm for 1–2 h.
12. Plate the cells on agar plates (e.g., LB) supplemented with the introduced antibiotic and incubate overnight at 37°C in a plate incubator.

Day 2: Reduce WT background

1. Pick and restreak single colonies (~10–50) to a new selective plate (e.g., LB) supplemented with the introduced antibiotic to get rid of WT background.
2. Incubate at 37°C in a plate incubator overnight.

Day 3: Verify gene deletion

1. Do a colony PCR to confirm the desired gene deletion. Include a WT control for comparison.
2. Analyze the PCR products on an agarose gel. Positive knockouts carrying the antibiotic marker should lead to PCR products that differ in size from the WT band.
3. If the PCR shows ambiguous results, i.e., both WT and deletion band are present for the same sample, streak out several colonies on an new agar plate (e.g., LB) supplemented with antibiotics to remove the WT. Verify removal of WT background with another colony PCR on the following day.

Please note: If the antibiotic marker is of similar length to the deleted gene, design a primer which binds to a sequence within the marker and combine it with one of the flanking verification primers (e.g., primers k1 and k2 from the Keio collection that bind within the kanamycin cassette).

Day 4: Removal of antibiotic resistance cassette

1. Inoculate a colony that carries the desired gene deletion in 4 mL nonselective medium (e.g., LB) and culture at 30°C, shaking the culture tubes at 240 rpm in a shaking incubator until OD_{600} has reached ~0.4.
2. Induce FLP expression with 50 m*M* L-rhamnose and incubate at 30°C, shaking at 240 rpm.
3. After 4–12 h streak out a small amount of culture (<5 μL) with an inoculation loop on a nonselective plate (e.g., LB) to obtain single colonies.

Day 5: Verify removal of antibiotic resistance cassette

1. Pick single colonies and restreak them on two different plates in parallel, one supplemented with the introduced antibiotic, and the other one without addition of antibiotics.
2. Incubate at 37°C in a plate incubator overnight.

Day 6: Verify removal of antibiotic resistance cassette

1. Clones growing on the nonselective plate, but not on the plate supplemented with antibiotics, have successfully removed the selection marker by FLP–FRT recombination.

2. To verify the removal of the antibiotic cassette, perform a colony PCR on the candidate colonies, including a WT as control.

When the antibiotic marker is successfully removed, another gene can be deleted by restarting the protocol. It is also possible to delete two or three genes one after another without removing the antibiotic cassette when working with different antibiotics as selection markers.

2.3 Protocol for Gene Deletion Using P1 Phage Transduction

As P1 phages can infect all *E. coli* strains, it is advisable to use a separate bench and equipment for all work related to phages. The virulent P1 phage mutant, P1vir (Ikeda & Tomizawa, 1965), should be acquired from a supplier (e.g., CGSC, The Coli Genetic Stock Center). Before the actual P1 transduction of a selectable marker can be conducted, a fresh P1 stock lysate should be prepared from a WT *E. coli* strain to ensure a standard marker-free lysate. This lysate is required to generate donor lysates from strains containing desired mutations. Thereafter, the P1 stock lysate can be stored at 4°C for several months and can be used for the preparation of P1 donor lysates from strains in which a gene is replaced with an antibiotic marker.

Equipment

- Sterile disposable culture tubes (e.g., falcon tubes, microfuge tubes)
- Benchtop centrifuge
- Thermoshaker (e.g., Eppendorf Thermomixer)
- Standard PCR equipment
- Standard gel electrophoresis equipment, 1% agarose gels
- Syringe and 0.22-μm filter
- Laminar flow cabinet/Bunsen burner
- 37°C shaking incubator for bacteria cultivation
- 37°C plate incubator for bacteria cultivation

Buffers and reagents

- P1 salt solution: LB containing 10 m*M* $CaCl_2$ and 5 m*M* $MgSO_4$
- 1 *M* $CaCl_2$ solution
- 1 *M* Sodium citrate
- Nonselective and selective medium (e.g., LB liquid medium and LB agar plates) supplemented with antibiotics, depending on the introduced antibiotic cassette: 50 μg/mL kanamycin, 30 μg/mL chloramphenicol with and without 5 m*M* sodium citrate

Strains

- WT *E. coli* strain for the preparation of P1 stock lysate
- *E. coli* nonlysogenic donor strain (containing the genetic marker to be transduced)
- *E. coli* recipient strain (to be transduced with the genetic marker)
- P1 stock lysate (10^9–10^{10} pfu/mL)

Donor lysate preparation

1. Inoculate an overnight culture of your donor strain in 4 mL medium (e.g., LB) and culture at 37°C, shaking the culture tubes at 240 rpm in a shaking incubator.
2. Subculture your overnight strain 1:100 in 3 mL medium (e.g., LB) + 10 µL 1 *M* $CaCl_2$ (final concentration 5 m*M*) in a 50-mL flacon tube. Do not use reusable tubes to eliminate the chance of future contamination. The P1 phage requires $CaCl_2$ for activity.
3. Grow your subculture in a shaking incubator at 37°C, shaking at 240 rpm for 1 h (or until $OD_{600} \sim 0.3$, note: overgrown cultures will not lyse).
4. Add 50–100 µL of the P1 stock lysate to the *E. coli* subculture.
5. Continue incubation for 2–4 h until the culture is lysed (solution becomes clear with only cell debris visible). If no lysis occurs, stop the procedure and troubleshoot the P1 stock lysate. The first time a lysate is prepared, it is useful to run a parallel culture without the addition of P1 stock lysate, to estimate usual growth in comparison to lysis.
6. Transfer the lysed culture to 2-mL microfuge tubes and pellet the cell debris at 11,000 rcf for 1 min at room temperature in a benchtop centrifuge.
7. Remove the supernatant and filter it through a 0.22-µm filter into a new 2-mL tube.
8. Store the P1 donor lysate at 4°C (for up to 3 months).

Transduction

1. Inoculate an overnight culture of recipient strain in 4 mL medium (e.g., LB) and culture at 37°C, shaking the culture tubes at 240 rpm in a shaking incubator.
2. The next day, gently pellet 1 mL of the recipient culture at 4000 rcf for 5 min at room temperature in a benchtop centrifuge.
3. Discard the supernatant and resuspend the pellet in 0.5 mL P1 salt solution.

4. Add 100 μL of the recipient cell suspension to a sterile centrifuge tube.
5. Add 40–80 μL of P1 donor lysate containing the desired deletion to the suspension. If the transduction fails, try varying the amount of lysate.
6. Incubate for 30 min at 30°C to allow phage adsorption to the cells.
7. Add 1 mL medium (e.g., LB) + 100 μL of 1 *M* sodium citrate. Citrate chelates the calcium necessary for P1 phage activity and minimizes secondary infection.
8. Incubate the culture for 1 h at 37°C in a thermoshaker, shaking at 1000 rpm.
9. Centrifuge the culture at 11,000 rcf for 30 s at room temperature. Discard the supernatant and wash with 1 mL medium (e.g., LB). Repeat the washing process again.
10. Resuspend the pellet in 100 μL medium and plate the whole amount on selective plates containing 5 m*M* sodium citrate and the introduced antibiotic. Incubate overnight at 37°C in a plate incubator.
11. The next day, analyze colonies via PCR. Streak out the cells two times (or more) on plates (e.g., LB) supplemented with 5 m*M* sodium citrate and appropriate antibiotics to obtain a clean culture void of P1 phages or WT *E. coli*.

As the antibiotic cassette is flanked by FRT sites, the selection marker can be removed by expression of FLP (Senecoff et al., 1985). Assuming that the SIJ488 strain is used, please see the protocol above for the removal of the marker.

3. PATHWAY ENGINEERING: FINE-TUNE EXPRESSION OF ENZYMES

Establishing the activity of a synthetic pathway, or one of its modules, requires the appropriate expression of the genes encoding for its enzymes. Codon adaptation of enzymes from other organisms is highly recommended in order to avoid translation bottlenecks (Gustafsson, Govindarajan, & Minshull, 2004; Sørensen, Kurland, & Pedersen, 1989). For native enzymes that participate in the synthetic pathway, overexpression is only required when the endogenous activity fails to support pathway flux.

Gene expression can be established either on a plasmid or via integration into the host genome. The former provides a more flexible, easily tunable option (Sørensen & Mortensen, 2005), while the latter enables stable and robust expression and propagation (Bassalo et al., 2016; Srinivasan, Barnard, & Gerngross, 2003). In *E. coli*, gene expression from plasmids

usually provides a convenient way to test and optimize pathway activity. This is especially useful when the enzymatic components that need to be expressed are not fully predetermined: different orthologues may need to be tested or endogenous genes may require overexpression or modification. Genome integration, which is less flexible, should be used only once pathway enzymes and their expression parameters are determined.

The expression level of key enzymes can have a dramatic effect on pathway activity (Pitera, Paddon, Newman, & Keasling, 2007). This is especially true for enzymes that catalyze thermodynamically or kinetically limiting reactions, enzymes that generate reactive or toxic intermediates, and enzymes that function at a metabolic crossroads between different metabolic processes. We use three types of regulatory elements to span the expression space of pathway enzymes over several orders of magnitude: plasmid origins of replication, promoters, and ribosome-binding sites (RBSs). First, we integrate the required genes into synthetic operons using the method described by Zelcbuch et al. (2013). Each gene is cloned into a high copy cloning plasmid pNiv, where it is attached to one of six well-characterized RBS sequences that together span three orders of magnitude of expression (Fig. 5A). Using an augmented BioBrick cloning strategy (Shetty, Endy, & Knight, 2008), the RBS-modulated genes are combined into one plasmid to generate a synthetic operon (Fig. 5B). This method allows the cloning of any number of genes into a synthetic operon. Instead of cloning each gene to a single pNiv vector, the genes can be cloned into a library of pNiv vectors (i.e., a mixture of vectors), each carrying a different RBS. In this case, integrating the genes results in a library of operons, each characterized by a different pairing of genes and RBSs (Zelcbuch et al., 2013).

The synthetic operon (or the library of which) is subsequently transferred to a pZ expression vector. The pZ vector family comprises three genetic modules: origin of replication, antibiotic resistance marker, and promoter. These modules are integrated in a combinatorial manner to provide a variety of expression plasmids (Lutz & Bujard, 1997). We replaced the inducible promoters of the pZ vectors with constitutive ones based on *E. coli*'s endogenous *pgi* promoter (Froman, Tait, & Gottlieb, 1989), such that three origins of replication with different copy numbers are combined with three promoters of different strength. Fig. 5C shows that these combinations span almost three orders of magnitude of expression. Furthermore, each origin-promoter combination is available with three different antibiotic markers—kanamycin, streptomycin, and tetracyclin—allowing cotransformation with different expression vectors and enhancing the flexibility of the system.

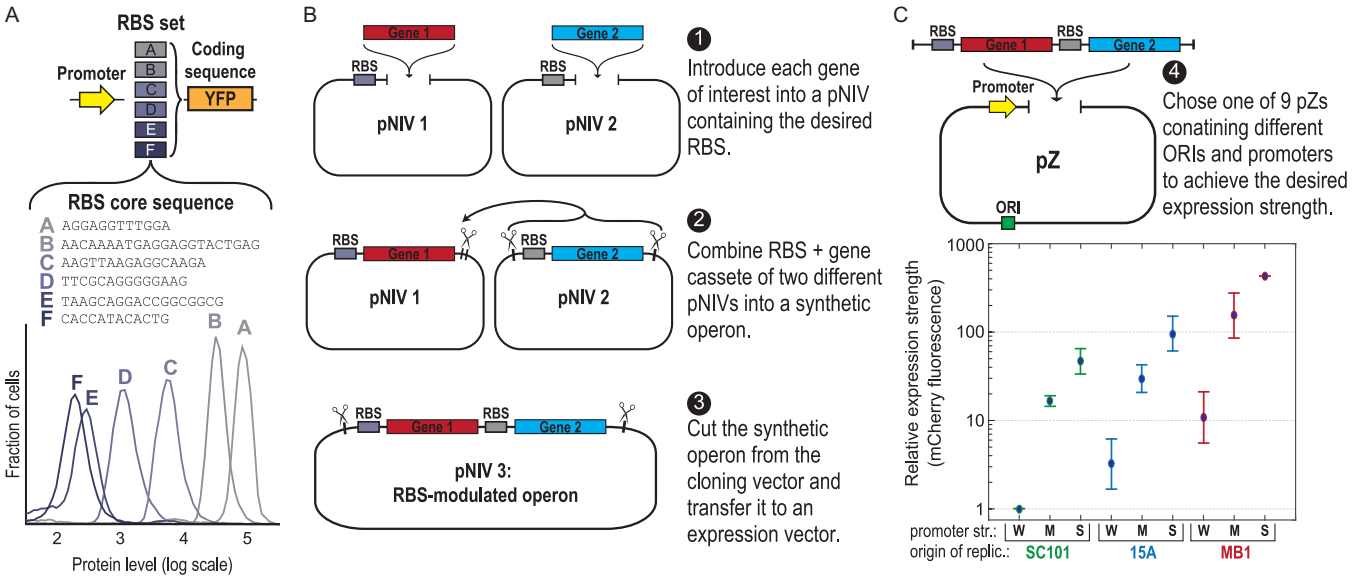

Fig. 5 Schematic representation of the expression strategy used to modify expression of pathway genes in a synthetic operon. (A) Six well-characterized RBS sequences span the expression over three orders of magnitude as shown by Zelcbuch et al. in a flow cytometry experiment. (B) Individual pathway genes are attached to an RBS of a desired strength by cloning into a corresponding pNIV vector. A BioBrick-based cloning approach allows combining of any number of RBS-gene cassettes into a synthetic operon. (C) The operon is transferred to a pZ vector that provides fine-tuning of expression level by combining origins of replication (ORI) of different copy numbers with promoters of different strength. mCherry fluorescence of all the different combinations of ORI and promoter was measured and shown to span almost three orders of magnitude. *Panel (A): Figure adapted from Zelcbuch, L., Antonovsky, N., Bar-Even, A., Levin-Karp, A., Barenholz, U., Dayagi, M., et al. (2013). Spanning high-dimensional expression space using ribosome-binding site combinatorics.* Nucleic Acids Research, 41. *https://doi.org/10.1093/nar/gkt151*.

By combining different RBS sequences with the pZ library, our system can span up to six orders of magnitude of expression. However, we usually find it unnecessary to explore the full expression space when testing a synthetic pathway. In most cases, a combination of a medium-copy-number origin (15A), with a medium or strong promoter, where all genes are regulated by a medium-strength RBS (RBS-C), suffices to establish satisfactory pathway activity. Only when this "default" combination fails or achieves suboptimal results, we explore regulatory combinations that span a larger expression space. For big operons, changing the order of the genes provides another way to modulate their expression, as the first genes tend to be expressed to a higher degree than those at the end of the operon (Zelcbuch et al., 2013).

3.1 Protocol for Integrating Genes in a Synthetic Operon

Native genes can be cloned directly from the genome. To enable cloning of a gene into a pNiv vector, the forward primer needs to include, upstream of the gene, the sequence ATGCATCATCACCATCACCAC which introduces an ATG + HIS-tag and an *Nsi*I restriction site (the primer-binding site needs to start downstream of the endogenous ATG). Equally, the reverse primer should include an *Xba*I restriction site downstream of the gene. If one of the following restriction sites is present in the gene sequence, it should be removed by the introduction of a synonymous mutation, e.g., by an overlap extension PCR: *Eco*RI, *Nhe*I, *Nsi*I, *Pst*I, *Sal*I, *Bcu*I, *Xba*I, *Xho*I, and *Vsp*I. It is recommended to codon optimize foreign genes for expression in *E. coli* (e.g., Jcat website (Grote et al., 2005)). The same restriction sites should be avoided in the codon-optimized sequence and the modifications described above should be included in the sequence.

We note that after each transformation, as described below, a colony PCR should be performed to confirm successful cloning events. To verify the construct, we usually extract the plasmid from two colonies that gave a positive PCR result and send them for sequencing. When assembling a synthetic operon, only the 5′ and 3′ ends of the operon are sequenced using vector-specific primers, as PCR-based mutations do not occur in the BioBrick-like assembly process.

Equipment

- DNA analysis software (e.g., Geneious®)
- Standard restriction enzyme digest cloning equipment
- Standard PCR equipment

- Standard gel electrophoresis equipment, 1% agarose gels
- Benchtop centrifuge
- Thermoshaker (e.g., Eppendorf Thermomixer)
- 37°C shaking incubator for bacteria cultivation
- 37°C plate incubator for bacteria cultivation

Buffers and reagents

- Standard PCR reagents
- High-fidelity polymerase (e.g., PrimeStar, TaKaRa; Phusion, Thermo Fisher Scientific)
- Restriction enzymes: *Nsi*I, *Xba*I, *Xho*I, *Bcu*I, *Sal*I, *Nhe*I, *Vsp*I, *Eco*RI, *Pst*I (e.g., Thermo Fisher Scientific)
- T4 ligase (e.g., Thermo Fisher Scientific),
- Blunt-end cloning kit (e.g., CloneJET PCR Cloning Kit, Thermo Fisher Scientific)
- PCR purification kit
- Gel purification kit
- Nonselective and selective medium LB liquid medium and LB agar plates supplemented with antibiotics, depending on the antibiotic cassette of the vector: 100 μg/mL ampicillin (amp), 50 μg/mL kanamycin, 100 μg/mL streptomycin, or 10 μg/mL tetracyclin

Strains

- Chemically competent *E. coli* DH5α

Cloning procedure

1. PCR amplify the gene of interest from the genome using primers that introduce an ATG + HIS-tag and an *Nsi*I restriction site upstream of the gene and a *Xba*I restriction site downstream of the gene.
2. Ligate the gene product into a linearized blunt vector (e.g., pJET) and transform the ligation product into chemically competent *E. coli* DH5α cells. Plate the transformants on selective LB plates (e.g., Amp for pJET).
3. After confirmation of the gene integrity (colony PCR and sequencing of the whole gene) cut out the gene from the vector using *Nsi*I + *Xba*I.

   Please note: Steps 2 and 3 are essential when preparing a library of operons. If only one defined operon is cloned, the PCR product from step 1 can be digested with *Nsi*I + *Xba*I and directly ligated into a pNiv vector (step 4).

4. Ligate the gene into an *Nsi*I/*Xba*I linearized pNiv vector such that an RBS is added upstream of the gene (it is also possible to use a mixture of pNiv vectors to obtain a library of RBS sequences regulating the gene, please see below).
5. Transform the ligation product into chemically competent *E. coli* DH5α cells and plate the transformants on selective LB plates (e.g., Amp for pNiv).
6. Confirm cloning of the gene into pNiv by colony PCR and sequencing.
7. If this is the only gene in the synthetic operon, jump to step 13.
8. If you are building an operon of two or more genes, clone all genes into pNiv vectors using the RBS sequences of your choice (or as a library of RBS sequences).
9. Incorporate the next gene into the operon as followed:
   i. Digest the acceptor vector—the vector that contains the initial part of the operon downstream of which the next gene shall be integrated—with *Nhe*I and *Xho*I.
   ii. Digest the donor vector—the vector that contains the gene to be incorporated into the operon—with *Bcu*I and *Sal*I to cut out the RBS:gene cassette.
   iii. Ligate the RBS:gene cassette from the donor vector into the acceptor vector.

   NheI and BcuI generate complementary sequences but once ligated form a scar sequence that is not recognized by any of the former restriction enzymes. The same is true for XhoI and SalI.
10. Transform the ligation product into chemically competent *E. coli* DH5α cells and plate the transformants on selective LB plates.
11. Confirm successful cloning by colony PCR and sequencing 5′ and 3′ ends of the operon.
12. Continue to add genes as described above until the synthetic operon is complete.
13. Digest the final pNiv vector—carrying all the required genes—with *Eco*RI/*Pst*I to obtain the entire operon cassette. Ligate the operon into an *Eco*RI/*Pst*I linearized pZ expression vector of choice (27 vectors available: 3 different ORIs, 3 promoters, and 3 antibiotic resistance markers).
14. Transform the ligation product into chemically competent *E. coli* DH5α cells and plate the transformants on selective LB plates (supplemented with antibiotics according to the pZ vector resistance).

15. After verification of the expression vector via colony PCR and sequencing, transform the appropriate selection strain with the pZ plasmid via electroporation (as described in the protocol for gene deletion in the SIJ488 strain).

Please note: For the preparation of a library of synthetic operons, each gene should be cloned into a mixture of pNivs. Therefore the *Nsi*I/*Xho*I-digested gene should be ligated into an equimolar mixture of *Nsi*I/*Xho*I linearized pNiv plasmids and transformed into competent cells. The next day, all resulting colonies are collected by adding 1–2 mL water and scraping gently into solution. Plasmid preparation should then proceed as if it were a dense overnight culture (a very high cloning efficiency that results in many colonies is vital to guarantee sufficient diversity of the library). The final pNiv library can be subsequently transferred into a pZ vector, thus constituting a library of expression vectors ready to be transformed into the appropriate strain.

3.2 Protocol for Genome Integration of Synthetic Operons

Chromosomal integration of a synthetic operon could eliminate plasmid-associated problems, such as genetic instability (plasmid loss) and the emergence of diverse population of plasmids—with slightly or significantly different sequences—within a single cell (Friehs, 2004; Silva, Queiroz, & Domingues, 2012). As gene copy number is substantially reduced when an operon is transferred from a plasmid to the genome, it is important to compensate by using stronger promoters or, as we found effective in multiple studies, replacing the default RBS-C with the stronger RBS-B.

Several genomic loci were previously found to be safe spots, i.e., facilitating both high-genome integration efficiency and high expression levels of genes without interfering with *E. coli*'s genetic elements (Bassalo et al., 2016). The synthetic operons are transferred into these spots using *E. coli's* endogenous homologous recombination mechanism. The availability of several safe spots is highly useful if multiple synthetic operons need to be overexpressed. However, if some of the genes within a synthetic operon also occur in the genome, the homologous recombination can fail to insert the operon into the right genomic locus. Deleting the genomic copy of the gene or shuffling the codon usage of the reintroduced copy —such that the genomic and plasmid sequences lose their homology—could relieve this problem.

We adapted the pDM4 vector (Milton, O'Toole, Hörstedt, & Wolf-Watz, 1996) to our BioBrick-based system, generating the genome integration vector pKI, and enabling subcloning of an operon from pNiv into pKI by restriction enzyme-based cloning. The pKI plasmid carries a chloramphenicol resistance gene (Cam^R), a levansucrase gene (*sacB*) which converts sucrose into the toxic compound levan (Gay, Le Coq, Steinmetz, Ferrari, & Hoch, 1983), and the conjugation gene *traJI* for the transfer of the plasmid. The plasmid replicates under the control of oriR6K, which does not replicate in WT *E. coli* but only in *E. coli* λpir strains (Kvitko et al., 2012). Thus, the acceptor strain can grow only under chloramphenicol selection if the plasmid has been successfully integrated into the genome by homologous recombination (Fig. 6A). For different integration loci, we constructed different pKI versions that contain two homology regions (HRs) of 600 bp length up- and downstream of the target safe spot. Between the HRs, the plasmid contains a strong promoter, a multiple cloning site for operon integration, and an FRT-flanked kanamycin cassette. After integration of the plasmid into the genome, a second homologous recombination between the integrated homology regions and their genomic homologues can occur. This removes from the genome either the complete plasmid or only the backbone genes (Fig. 6B). To select for the latter event, sucrose and kanamycin are added to the medium. Only cells in which the sacB gene was removed via the second homologous recombination (but that kept the kanamycin resistance gene) survive.

pKI plasmids can be transferred to an *E. coli* acceptor strain either by electroporation (see protocol for gene deletions) or by conjugation, which can be more successful when the recipient strain grows poorly, e.g., when several central metabolism genes are deleted. For conjugation, we use the *E. coli* ST18 strain which can only grow when 5-aminolevulinic acid (ALA) is added to the medium (Thoma & Schobert, 2009). Thus, after conjugation, culturing on a medium that does not contain ALA effectively eliminates the *E. coli* ST18 strain.

Equipment

- Standard restriction enzyme digest cloning equipment
- Standard PCR equipment
- Standard gel electrophoresis equipment, 1% agarose gels
- Benchtop centrifuge
- Thermoshaker (e.g., Eppendorf Thermomixer)

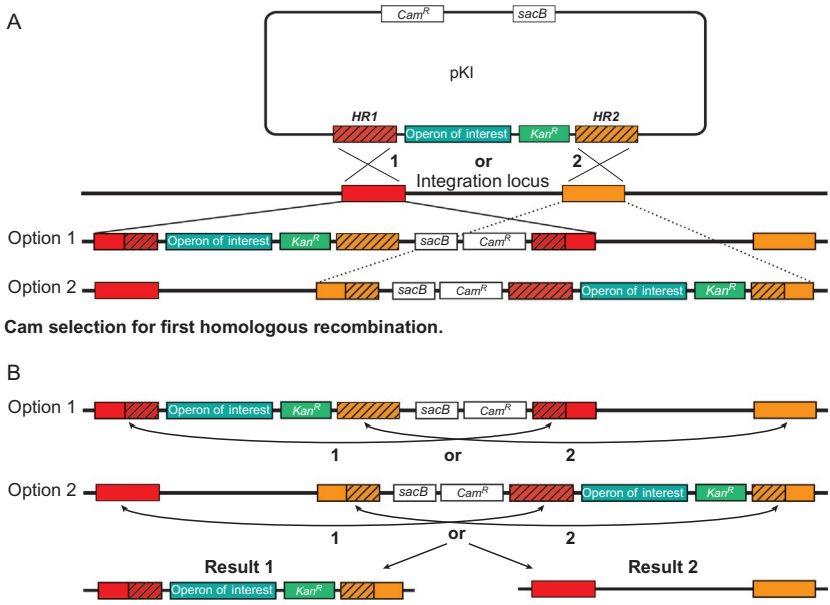

Fig. 6 Schematic representation of the pKI-mediated genome integration of a synthetic operon. (A) The operon of interest is cloned between two homology regions (HR1 and HR2) that are homologous to the desired integration locus, i.e., one of several characterized safe spots. A first homologous recombination occurs either between HR1 and its genomic homologue sequence (option 1) or between HR2 and its genomic homologue sequence (option 2), leading to the integration of the complete plasmid into the genome. As pKI carries a chloramphenicol (Cam) resistance gene, the first HRC can be selected for via cultivation on Cam. (B) Since the HR1 and HR2 and their genomic homologues remain within the genome, a second recombination within the integration region can occur. This can result either in the removal of the complete plasmid, leading to a WT genotype, or in the removal of unrequired backbone genes leading to the desired genotype. To select for the second homologous recombination sucrose is added to the medium. Only cells that have undergone second homologous recombination, such that the sacB gene—encoding for levansucrase an enzyme that converts sucrose into toxic levan—is removed, would survive. To select for the recombination events that resulted in the correct integration of the operon kanamycin (Kan) is added to the medium.

- 37°C shaking incubator for bacteria cultivation
- 37°C plate incubator for bacteria cultivation

Buffers and reagents

- Standard PCR reagents
- Restriction enzymes: *Bcu*I, *Nhe*I (e.g., Thermo Fisher Scientific)

- T4 ligase (e.g., Thermo Fisher Scientific)
- PCR purification kit
- Gel purification kit
- Nonselective and selective LB liquid medium and LB agar plates supplemented with antibiotics (depending on the introduced antibiotic cassette): 100 μg/mL ampicillin (amp), 50 μg/mL kanamycin, 30 μg/mL chloramphenicol
- Supplements for media and plates: ALA (final concentration 50 mg/mL), sucrose (final concentration 10%)

Strains

- Chemically competent *E. coli* DH5α λpir cells. This strain is used for cloning of the pKI vector as it does not require ALA for growth.
- Chemically competent *E. coli* ST18 for conjugation. An improved version of the S17-1λpir strain in which *hemA* is deleted. The strain grows only when ALA is added to the medium, which facilitates selection of positive acceptor strains after conjugation

Cloning the genome integration vector

1. Clone the operon of interest into a pNiv as described above. To achieve sufficient gene expression after genome integration, it is recommended to use RBS-B.
2. Cut out the operon from pNiv by digesting it with *Bcu*I/*Nhe*I.
3. Ligate the operon into a *Bcu*I/*Nhe*I linearized pKI genome integration vector containing homologous regions for the target locus and transform the ligation product into chemically competent *E. coli* DH5α λpir cells by a 42°C heat shock for 1 min. Recover the cells in LB medium for 1 h at 37°C, shaking at 1000 rpm in a thermoshaker.
4. Plate cells on selective LB plates supplemented with chloramphenicol and/or kanamycin (both resistance markers of pKI) and grow at 37°C in a plate incubator overnight.
5. Screen colonies by PCR for the correct insert.
6. Extract the plasmids from two positive colonies and validate the plasmid sequence.

Conjugation and genome integration (first homologous recombination)

1. Transform *E. coli* ST18 cells with the pKI genome integration vector by heat shock as described above (grow *E. coli* ST18 on LB plates supplemented with ALA and chloramphenicol and/or kanamycin).

2. Inoculate the ST18 donor strain and the recipient strain in 5 mL of appropriate medium (e.g. LB-ALA) in the morning and incubate at 37°C and shaking at 240 rpm in a shaking incubator until OD_{600} of 0.5.
3. Mix donor and recipient strain in a ~1:1 OD ratio by combining 500 µL of each culture in a microfuge tube. If the OD differs, harvest higher volume of the strain with the lower OD and resuspend in 500 µL. Mix donor and recipient strain by inverting the tube.
4. Centrifuged the mixture for 3 min at 4000 rcf in a benchtop centrifuge.
5. Discard the supernatant and resuspend the cell pellet in the remaining ~100 µL of the supernatant.
6. Pipette the entire cell suspension as one drop in the middle of an LB-ALA plate and incubate for 4–5 h upside up at room temperature under sterile conditions to allow for mating. Keep the lid of the plate open for the first 30 min to allow the spot to dry.
7. After 4–5 h scoop the cells from the mating spot using an inoculation loop and resuspend 100 µL LB medium. Vortex to stop the mating.
8. Plate 2 µL (20 µL of a 1:10 dilution), 20 µL and the rest of the cell suspension on plates optimized for the recipient strain (e.g., LB) containing just chloramphenicol and no ALA and incubate the plates at 37°C overnight in a plate incubator.
9. Pick single colonies and analyze plasmid integration by PCR.
10. Although *E. coli* ST18 cannot grow without the addition of ALA, plasmid integration strains should be transferred to a new plate at least two times by picking single colonies.

Plasmid removal by second homologous recombination

The second homologous recombination takes place between the integrated homologous sequences leading to either (i) removal of the plasmid backbone such that the synthetic operon is integrated into the genome, or (ii) removal of the entire plasmid resulting in a WT sequence that does not harbor the synthetic operon (Fig. 6B). We select for the former case by adding kanamycin to the medium:

1. Inoculate one or two transconjugants in 5 mL of appropriate medium (e.g., LB) in the morning and incubate at 37°C and 240 rpm in a shaking incubator for 4–5 h.
2. Plate 100 µL of a 10^{0}–10^{-4} dilution on plates containing 10% sucrose and kanamycin and grow for 24 h at 37°C.

Levansucrase, encoded by *sacB* which is encoded on the pKI backbone, converts sucrose into toxic levan; hence sucrose-tolerating cells are likely to have undergone the second homologous recombination.

3. Pick single colonies and streak them on two different LB plates in parallel, one supplemented with Cam and Kan, the other one with Kan only. Incubate overnight at 37°C in a plate incubator.
4. Test colonies growing on Kan but not on Cam and Kan by colony PCR.
5. If desired, the FRT/flanked kanamycin cassette can be removed by FLP as described in a previous section.

4. GROWTH EXPERIMENTS: IN VIVO SELECTION FOR PATHWAY/MODULE ACTIVITY

In most metabolic engineering projects, the synthetic pathway does not contribute to microbial growth; in fact, in most cases, it leads to reduced fitness. This complicates pathway testing and optimization and leads to genetic instability, that is, the activity of synthetic pathway is commonly lost after several generations as mutations suppressing it arise naturally and take over the microbial population (Koffas, Jung, & Stephanopoulos, 2003; Stephanopoulos & Vallino, 2009). In contrast, our metabolic engineering strategy is based on constructing gene-deletion strains in which the activity of a pathway, or a module of which, is coupled to microbial growth. This offers us a clear and simple readout for pathway activity and further ensures that the metabolic phenotype will not be lost during long-term cultivation.

As discussed above, several selection strains can be constructed for the testing of a metabolic route (pathway or module). Furthermore, many expression vectors—each combining different genes at different expression strengths—can be constructed. Altogether, testing pathway activity requires multiple parallel growth experiments which cannot be easily handled manually in flasks or cultivation tubes. For this reason, we perform growth experiments in 96-well plates, which are incubated within microplate readers at defined temperature, shaking, and gas composition. This method enables us to directly compare the growth characteristics of different strains, thereby identifying the most suitable genetic constructs to support a required activity.

There are two ways to monitor microbial growth in the microplate reader. The most common one is the change in absorbance at 600 nm.

A complementary approach is to measure the fluorescence of NADH (excitation at 340 nm, emission at 460 nm). We found that the absorbance and fluorescence curves usually correlate nicely. Deviations of the fluorescence curve from the absorbance curve can usually be interpreted as a major physiological shift and thus provide useful information on the metabolic state of the cell. The microplate growth experiments can be conducted both aerobically and anaerobically, where in the latter case the microplate reader is placed within an anaerobic chamber.

Growth experiments are performed in duplicates or triplicates. This enables us to decipher whether an observed growth is the result of a mutation: if all technical replicates are characterized by identical curves, it is reasonable to assume that the observed growth is not the result a mutation emerging in the population. On the other hand, if one replicate starts to grow and the others do not, it is clear that a mutant has taken over the population. In this latter case, we cannot be sure that the metabolic pathway being tested is indeed active or whether a metabolic bypass has emerged to enable growth under the selective conditions.

To exemplify pathway testing via growth experiments, we focus on the combined activity of the two modules of the PFL-threonine cycle, as shown in Fig. 7A: PFL-dependent condensation of acetate and formate to give pyruvate and threonine metabolism to produce glycine and serine. As all enzymes of these modules are endogenous to *E. coli*, it is unclear which enzyme might limit module activity and hence require overexpression. Clearly, we prefer to overexpress only genes that are strictly limiting rather than all pathway enzymes. As shown in Fig. 7B, we found that only a few enzymes require overexpression to support the combined activity of the modules in a minimal medium supplemented with acetate and formate: ThrA*, the first enzyme of threonine biosynthesis carrying a point mutation that makes it insensitive to product inhibition (Wang, Liu, & Qi, 2014), and Tdh and Kbl, which together catalyze threonine cleavage to acetyl-CoA and glycine (all genes were regulated by RBS-C and the expression vector had the medium copy number 15A origin and a strong promoter). Overexpression of Asd, ThrB, and ThrC—of the threonine biosynthesis pathway—leads to decreased growth which might be related to increased protein burden. Furthermore, the overexpression of PFL, its downstream enzymes leading to threonine biosynthesis, and the enzymes converting glycine to serine, are completely unnecessary as the endogenous expression levels suffice to support growth under the selective conditions.

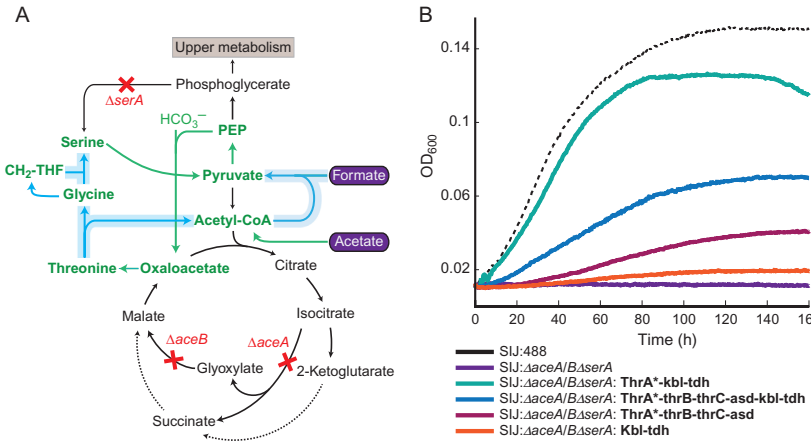

Fig. 7 Testing module activity, as exemplified by the PFL-threonine cycle. (A) The two metabolic modules selected for: PFL-dependent assimilation of acetate and formate together with glycine and serine biosynthesis from threonine metabolism. (B) A high-throughput growth experiment testing which endogenous genes must be overexpressed in order to achieve pathway activity. Strains were grown in a 96-well plate in minimal medium supplemented with 30 m*M* acetate, 30 m*M* formate, and 50 m*M* nitrate under anaerobic conditions. Growth was monitored in a Tecan Infinite® 200 PRO plate reader. As shown, overexpression of ThrA*—the first enzyme of threonine biosynthesis that is insensitive to product inhibition—as well as Tdh and Kbl—which cleaves threonine to glycine and acetyl-CoA—suffices to establish pathway activity and thus support growth. The endogenous activity of other enzymes seems to be sufficiently high as to support the desired flux.

4.1 Protocol for Growth Measurements With a Microplate Reader

Equipment

- Culture tubes for precultures
- Spectrophotometer (e.g., OD600 DiluPhotometer™, IMPLEN)
- Transparent 96-well plate (e.g., Nunclon™ Delta Surface, Cat. N° 167008, Thermo Scientific)
- Plate reader (e.g., Tecan Infinite® 200 PRO)
- Laminar flow cabinet
- Benchtop centrifuge
- Multichannel pipette
- Anaerobic chamber, if experiments are to be conducted anaerobically (e.g., COY Lab Products)
- Gas control unit (e.g., COY Lab Products)
- 37°C shaking incubator for bacteria cultivation

Buffers and reagents

5 × M9 stock solution

- Suspend 52.5 g M9 Medium Broth powder (Amresco) in deionized water
  - 33.9 g/L Na_2HPO_4
  - 15 g/L KH_2PO_4
  - 5 g/L NH_4Cl
  - 2.5 g/L NaCl
- Autoclave or filter sterilize

Trace elements stock solution ×100

- 5 g EDTA in 800 mL water, pH 7.5 (adjusted with NaOH)
- 498 mg $FeCl_3$ (anhydrous)
- 84 mg $ZnCl_2$
- 765 μL of 0.1 *M* $CuCl_2{\cdot}2H_2O$
- 210 μL of 0.2 *M* $CoCl_2{\cdot}6H_2O$
- 1.6 mL of 0.1 *M* H_3BO_3
- 8.1 μL of 1 *M* $MnCl_2{\cdot}4H_2O$
- Add water to a final volume of 1 L
- Autoclave or filter sterilize

M9 minimal medium (1 L)

- 200 mL of 5 × M9 stock solution (autoclaved)
- 10 mL of 100 × trace element mix (autoclaved)
- Add sterile ddH_2O to 1 L
- 2 mL of 1 *M* $MgSO_4$ (sterile filtered: 0.22 μm)
- 100 μL of 1 *M* $CaCl_2$ (sterile filtered: 0.22 μm)
- Mix well until the medium is clear

Strains

- *E. coli* WT strain (e.g., MG1655) for growth comparison
- *E. coli* selection strain—negative control
- *E. coli* selection strains transformed with different combinations of pathway enzymes

Day 1: Preculture

1. Inoculate strains in 5 mL relaxing medium: a nonselective minimal medium on which all strains should grow—that is, having all necessary carbon sources and supplements—even if the pathway/module is not active. Incubate the test tubes overnight in a shaking incubator at 37°C, shaking at 240 rpm.

Please note: Depending on the genetic background of the selection strain, precultures might take longer to grow to a dense culture.

2. Prepare all required selective media by supplementing M9 minimal medium with the appropriate concentration of the carbon source to be tested.

   *For anaerobic experiments: transfer media to the anaerobic chamber for overnight degassing. Vortex and open the lid to allow oxygen diffusion out of the media.

Day 2: Setup of the growth experiment

1. Harvest ~1 mL of densely grown precultures into 1.5 mL microfuge tubes and centrifuge for 3 min at 4000 rcf in a benchtop centrifuge.
2. Carefully discard the supernatant, using a pipette.
3. Add 1 mL of fresh M9 minimal medium, resuspend the pellet, and centrifuge for 3 min at 4000 rcf.
4. Repeat washing of cells (steps 2 and 3) two more times.
5. Resuspend the pellet in 1 mL M9 minimal medium and determine the OD_{600} of the cell suspension (e.g., by measuring the OD_{600} of a 1:10 dilution in a spectrophotometer).
6. Determine the volume of cell suspension that needs to be transferred to 1 mL of selective medium to receive the desired starting OD_{600}. (A starting OD_{600} of 0.005 is recommended.)
7. Transfer the determined volume of selection strains to microfuge tubes containing 1 mL of selective medium.

   *For anaerobic experiments: transfer the resuspended cells to the anaerobic chamber. Open the lids for degassing before transferring the appropriate volume of cells to the selective media.
8. Mix cells and selective medium by inverting the tubes.
9. Transfer 150 μL of the cells in selective medium to each well of the 96-well plate (using a multichannel pipette if possible). Technical duplicates or triplicates are highly recommended. Starting OD_{600} in each well is 0.005.
10. Cover the 150 μL of cell suspension in the well with 50 μL mineral oil to avoid evaporation during the experiment. Mineral oil enables free diffusion of gas.
11. Transfer the 96-well plate to the plate reader. Take off the lid and start the experiment by starting the program (parameters given below) on the computer.

Set up the following (or similar) program to run long-term growth measurements (app. 9 days) on a Tecan Infinite® 200 plate reader (Table 1).

Table 1 Parameters for Setting up a Long-term Growth Measurements on a Tecan Infinite® 200 Plate Reader

Incubation Parameters for an Aerobic Growth Experiment		Incubation Parameters for an Anaerobic Growth Experiment
1000 kinetic cycle of the following steps		1000 kinetic cycle of the following steps
1. Absorbance measurement:		*1. Absorbance measurement:*
Wavelength: 600 nm N° of flashes: 3 Settle time: 50 ms		Wavelength: 600 nm N° of flashes: 3 Settle time: 50 ms
2. Shaking/incubation (12 min)		*2. Shaking/Incubation (12 min)*
Linear shaking for 60 s Amplitude: 3 mm Frequency: 452.1 rpm	3 times	Wait: 10 min 30 s
Orbital shaking for 60 s Amplitude: 3 mm Frequency: 218.3 rpm		
Linear shaking for 60 s Amplitude: 2 mm Frequency: 579.8 rpm		Orbital shaking for 60 s Amplitude: 3 mm Frequency: 218.3 rpm
Orbital shaking for 60 s Amplitude: 2 mm Frequency: 280.8 rpm		Wait: 30 s
3. Return to step 1		*3. Return to step 1*

5. PATHWAY CONFIRMATION: ^{13}C LABELING OF PROTEINOGENIC AMINO ACIDS

Growth experiments provide a clear readout for the activity of a pathway or a module. However, even if all proper control and repetition experiments have been carefully conducted, it could still be the case that the observed growth does not correspond to the desired activity but rather stems from the emergence of an unrelated metabolic bypass. We use steady-state ^{13}C-labeling experiments to follow the metabolic conversion of a substrate and thus unequivocally confirm or disprove pathway activity (Szyperski, 1995). In these experiments, one of the carbon sources in the selective medium is replaced with an analog in which one or more carbon atoms are substituted

with the stable isotope ^{13}C. The cells are cultivated in this medium until reaching late exponential stage, where they are harvested and boiled in concentrated acid. This procedure extracts the proteinogenic amino acids, whose labeling can then be measured using liquid chromatography–mass spectrometry (LC–MS) (Opiteck, Lewis, Jorgenson, & Anderegg, 1997). The labeling patterns of these amino acids confirm or disprove pathway activity.

A comprehensive metabolomics analysis of small molecules could potentially give a more accurate picture of cellular metabolism (Bennett, Kimball, & Gao, 2009). However, in almost all cases, this level of resolution is not required, and the more accessible proteinogenic amino acids, whose extraction is simple and straightforward, provide a clear view of the key metabolic fluxes. Hence, the rather complex quenching and extraction procedures associated with quantifying the levels and labeling of small molecules can usually be avoided.

Several LC–MS/MS procedures can further fragment the amino acids and test the mass associated with each fragment. This can reveal which of the carbons in the amino acid is labeled, and thus allows a more precise view of the metabolic flow of carbon. In some cases, the identity of the labeled carbon could be inferred even if further breakdown of the amino acids is not possible. For example, if alanine was found to be labeled once, pyruvate could be labeled on each of its three carbons. However, if valine is also labeled once, we can deduce that pyruvate is labeled on its carboxylic carbon. Conversely, if valine is labeled twice, the labeled carbon of pyruvate cannot be the carboxyl. We can deduce this as the biosynthesis of valine involves the condensation of two pyruvate molecules, in which one carboxylic group is lost; therefore, valine contains only one carboxylic carbon from pyruvate but two carbons that originate both from the second and third positions of pyruvate.

To demonstrate the use of ^{13}C-labeling experiments, we focus on formate assimilation into the tetrahydrofolate system—an essential part in the PFL-threonine cycle (Fig. 2). As shown in Fig. 8, the C1 moiety of 10-formyltetrahydrofolate contributes one carbon to histidine (carbon colored in yellow), and the C1 moiety of 5-methyltetrahydrofolate provides a carbon for methionine (carbon colored in pink). Hence, the assimilation of labeled ^{13}C-formate via the tetrahydrofolate system is expected to result in singly labeled histidine and singly labeled methionine. Indeed, and as we elaborate in a previous study (Yishai et al., 2017), the expression of a formate-tetrahydrofolate ligase within a $\Delta glyA$ $\Delta gcvTHP$ strain—which cannot produce the essential C1-activated THF molecules from serine

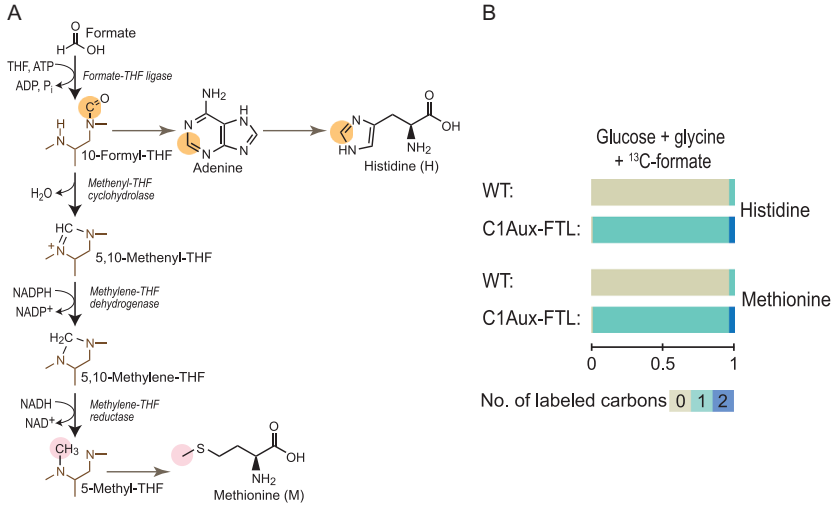

Fig. 8 A demonstration for the use of ^{13}C labeling to confirm the activity of a metabolic module. (A) Assimilation of labeled formate into the tetrahydrofolate (THF) system can be detected by monitoring the labeling of histidine and methionine, where one of the carbons of the former amino acid is derived from 10-formyltetrahydrofolate (marked in *yellow*) and one of the carbons of the latter amino acid originates from 5-methyltetrahydrofolate (marked in *pink*). (B) The results of the labeling experiment: histidine and methionine are singly labeled in a Δ*glyA* Δ*gcvTHP* strain in which formate serves as the only source of C1-activated tetrahydrofolate; these amino acids are unlabeled in a WT strain in which C1-activated tetrahydrofolate molecules are derived from serine and glycine.

and glycine—resulted in fully labeled histidine and methionine once ^{13}C-formate was added to the medium (Fig. 8B). As expected, these amino acids were unlabeled when ^{13}C-formate was added in the cultivation of a WT strain.

5.1 Protocol for ^{13}C-Labeling Analysis of Proteinogenic Amino Acids

Equipment

- Benchtop centrifuge
- Thermoshaker (e.g., Eppendorf Thermomixer)
- Cap locks for sealing microfuge tubes
- Air stream for drying the samples
- Standard equipment for bacteria culture
- 37°C shaking incubator for bacteria cultivation

- LC–MS device: Liquid chromatography system (e.g., Waters Acquity UPLC System)/Mass spectrometer (e.g., Exactive mass spectrometer, Thermo Scientific)
- LC–MS data analysis software (e.g., Xcalibur, Thermo Scientific)

Buffers and reagents
- M9 minimal medium
- ^{13}C-Labeled carbon source
- 6 *M* hydrochloric acid (HCl)
- Amino acid standards (Sigma-Aldrich)

Strains
- Transformed selection strain that showed clear pathway activity in growth experiments
- WT *E. coli* strain (used for the creation of the selection strain) as control

Growth on labeled carbon sources

Inoculate strains into 2 mL selective medium where one of the carbon sources is replaced with an analogue in which one or more carbon atoms are substituted with ^{13}C. Incubate the test tubes in a shaking incubator at 37°C and 240 rpm.

Amino acid extraction
1. At the late exponential stage ($OD_{600} \sim 0.8$), harvest the equivalent of 1 mL of culture at $OD_{600} = 1$ by spinning down the cells in a benchtop centrifuge for 1 min at 18,500 rcf.
2. Discard supernatant and resuspend the pellet in 1 mL of water.
3. Spin down cells in a benchtop centrifuge for 1 min at 18,500 rcf and discard the supernatant.
4. Add 1 mL of 6 *M* HCl to the cell pellet, seal the tubes with a cap lock, and boil at 95°C in a thermoshaker overnight to hydrolyze the cells.
5. Open the lid of the tubes and dry the samples at 95°C under a constant stream of air (4–8 h).
6. When the samples have completely dried, dissolve the pellet in 1 mL water.
7. Centrifuge the solution in a benchtop centrifuge at 18,500 rcf for 5 min and transfer supernatant to a new tube.
8. Submit the samples for analysis by LC–MS.

Mass analysis by LC–MS
1. Separate hydrolyzed amino acids with an LC system (e.g., Waters Acquity UPLC system), using a reversed phase column (e.g., HSS T3

C_{18}, 100 mm × 2.1 mm, 1.8 μm; Waters) at a flow rate of 0.4 mL/min and elute off the column using a hydrophobicity gradient.

2. Use the buffers (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile with the following gradient for the mobile phase:
   - 0–3 min—100% of A;
   - 3–9 min—100% of A to 100% of B;
   - 9–13 min—100% of B;
   - 13–14 min—100% of B to 100% of A;
   - 14–20 min—100% of A;
3. Use a mass spectrometer coupled to the LC device for the acquisition of mass spectra of the proteinogenic amino acids (e.g., Exactive mass spectrometer, Thermo Scientific).
4. Use the following parameters for the acquisition of amino acid spectra:
   a. Use argon as the collision gas with a flow rate of 0.22 mL/min
   b. Cone voltage 25 V
   c. Capillary set to 3 kV
   d. Source temperature 150°C
   e. Desolvation temperature 500°C
   f. Desolvation gas flow 700 L/min
   g. Source offset 50
   h. Cone gas flow 250 L/min
   i. Collision energy 14 eV
5. Positive ionization mode is sufficient for identification of most of the desired amino acids.
6. The mass/scan range should be 50.0–300.0 m/z to detect all amino acids from glycine (the lightest amino acid) to tryptophan (the heaviest amino acid).
7. Record mass spectra for 20 min.
8. Analyze MS data using an appropriate analysis software (e.g., Xcalibur, Thermo Scientific).

When analyzing MS data for the first time, the accurate retention times and ionization parameters of the amino acids need to be determined and optimized by including and analyzing amino acid standards.

6. CONCLUDING REMARKS

In this chapter, we presented our systematic approach for the rewiring of microbial central metabolism. The key aspects of our approach involve the division of a synthetic pathway into small modules whose activity

can be selected for in dedicated deletion strains, construction of expression vectors that can span the expression space of the pathway enzymes, high-throughput growth experiments to validate pathway activity, and ^{13}C labeling of proteinogenic amino acids to confirm the desired metabolic flux. While the protocols provided here relate to the metabolic engineering of *E. coli*, they can be easily adapted for the rewiring of central metabolism of other bacteria and yeast hosts.

One aspect we have not covered relates to question: what can we do when selection for pathway activity fails after testing multiple expression strategies? In this case, the best approach is to apply adaptive laboratory evolution (Dragosits & Mattanovich, 2013), in which the strain, expressing the necessary genes (preferably after genome integration), is continuously cultivated under selective conditions that promote the emergence of mutations that facilitate pathway activity. For example, to establish the activity of the full PFL-threonine cycle, long-term cultivation at saturating amounts of formate but limiting amounts of acetate is expected to slowly evolve the strain toward reduced utilization of the latter feedstock, until growth on formate as sole carbon source is reached. The combination of a direct selection approach as described in this chapter with adaptive laboratory evolution has the potential to dramatically reshape microbial metabolism into novel and biotechnologically productive architectures.

ACKNOWLEDGMENTS

This work was funded by the Max Planck Society. The authors thank Charlie Cotton, Hai He, and Hezi Tenenboim for discussions and critique reading of the manuscript.

REFERENCES

Anthony, C. (2011). How half a century of research was required to understand bacterial growth on C1 and C2 compounds; the story of the serine cycle and the ethylmalonyl-CoA pathway. *Science Progress*, *94*, 109–137.

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., et al. (2006). Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: The Keio collection. *Molecular Systems Biology*. *2*, https://doi.org/10.1038/msb4100050.

Bar-Even, A. (2016). Formate assimilation: The metabolic architecture of natural and synthetic pathways. *Biochemistry*, *55*, 3851–3863.

Bassalo, M. C., Garst, A. D., Halweg-Edwards, A. L., Grau, W. C., Domaille, D. W., Mutalik, V. K., et al. (2016). Rapid and efficient one-step metabolic pathway integration in *E. coli*. *ACS Synthetic Biology*, *5*, 561–568.

Bennett, B., Kimball, E., & Gao, M. (2009). Absolute metabolite concentrations and implied enzyme active site occupancy in *Escherichia coli*. *Nature Chemical Biology*, *5*, 593–599.

Datsenko, K. A., & Wanner, B. L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proceedings of the National Academy of Sciences of the United States of America*, *97*, 6640–6645.

Dragosits, M., & Mattanovich, D. (2013). Adaptive laboratory evolution—Principles and applications for biotechnology. *Microbial Cell Factories*, *12*, 64. https://doi.org/10.1186/1475-2859-12-64.

Erb, T. J., Jones, P. R., & Bar-Even, A. (2017). Synthetic metabolism: Metabolic engineering meets enzyme design. *Current Opinion in Chemical Biology*, *37*, 56–62.

Friehs, K. (2004). Plasmid copy number and plasmid stability. *Advances in Biochemical Engineering/Biotechnology*, *86*, 47–82.

Froman, B., Tait, R., & Gottlieb, L. (1989). Isolation and characterization of the phosphoglucose isomerase gene from *Escherichia coli*. *Molecular and General Genetics MGG*, *217*, 126–131.

Gay, P., Le Coq, D., Steinmetz, M., Ferrari, E., & Hoch, J. A. (1983). Cloning structural gene sacB, which codes for exoenzyme levansucrase of Bacillus subtilis: Expression of the gene in *Escherichia coli*. *Journal of Bacteriology*, *153*, 1424–1431.

Grote, A., Hiller, K., Scheer, M., Münch, R., Nörtemann, B., Hempel, D. C., et al. (2005). JCat: A novel tool to adapt codon usage of a target gene to its potential expression host. *Nucleic Acids Research*, *33*, W526–W531. https://doi.org/10.1093/nar/gki376.

Gustafsson, C., Govindarajan, S., & Minshull, J. (2004). Codon bias and heterologous protein expression. *Trends in Biotechnology*, *22*, 346–353.

Ikeda, H., & Tomizawa, J. I. (1965). Transducing fragments in generalized transduction by phage P1: III. Studies with small phage particles. *Journal of Molecular Biology*, *14*, 120–129.

Jensen, S. I., Lennen, R. M., Herrgård, M. J., & Nielsen, A. T. (2015). Seven gene deletions in seven days: Fast generation of *Escherichia coli* strains tolerant to acetate and osmotic stress. *Scientific Reports*, *5*, 17874. https://doi.org/10.1038/srep17874.

Keasling, J. D. (2010). Manufacturing molecules through metabolic engineering. *Science*, *330*, 1355–1358.

Koffas, M. A. G., Jung, G. Y., & Stephanopoulos, G. (2003). Engineering metabolism and product formation in Corynebacterium glutamicum by coordinated gene overexpression. *Metabolic Engineering*, *5*, 32–41.

Kvitko, B. H., Bruckbauer, S., Prucha, J., McMillan, I., Breland, E. J., Lehman, S., et al. (2012). A simple method for construction of pir+ Enterobacterial hosts for maintenance of R6K replicon plasmids. *BMC Research Notes*, *5*, 157. https://doi.org/10.1186/1756-0500-5-157.

Lennox, E. S. (1955). Transduction of linked genetic characters of the host by bacteriophage P1. *Virology*, *1*, 190–206.

Lutz, R., & Bujard, H. (1997). Independent and tight regulation of transcriptional units in *Escherichia coli* via the LacR/O, the TetR/O and AraC/I1-I2 regulatory elements. *Nucleic Acids Research*, *25*, 1203–1210.

Milton, D. L., O'Toole, R., Hörstedt, P., & Wolf-Watz, H. (1996). Flagellin A is essential for the virulence of Vibrio anguillarum. *Journal of Bacteriology*, *178*, 1310–1319.

Murphy, K. C. (1998). Use of bacteriophage λ recombination functions to promote gene replacement in *Escherichia coli*. *Journal of Bacteriology*, *180*, 2063–2071.

Opiteck, G. J., Lewis, K. C., Jorgenson, J. W., & Anderegg, R. J. (1997). Comprehensive on-line LC/LC/MS of proteins. *Analytical Chemistry*, *69*, 1518–1524.

Pitera, D. J., Paddon, C. J., Newman, J. D., & Keasling, J. D. (2007). Balancing a heterologous mevalonate pathway for improved isoprenoid production in *Escherichia coli*. *Metabolic Engineering*, *9*, 193–207.

Senecoff, J. F., Bruckner, R. C., & Cox, M. M. (1985). The FLP recombinase of the yeast 2-micron plasmid: Characterization of its recombination site. *Proceedings of the National Academy of Sciences of the United States of America*, *82*, 7270–7274.

Shetty, R. P., Endy, D., & Knight, T. F. (2008). Engineering BioBrick vectors from BioBrick parts. *Journal of Biological Engineering*, *2*, 5. https://doi.org/10.1186/1754-1611-2-5.

Silva, F., Queiroz, J. A., & Domingues, F. C. (2012). Evaluating metabolic stress and plasmid stability in plasmid DNA production by *Escherichia coli*. *Biotechnology Advances*, *30*, 691–708.

Sørensen, M. A., Kurland, C. G., & Pedersen, S. (1989). Codon usage determines translation rate in *Escherichia coli*. *Journal of Molecular Biology*, *207*, 365–377.

Sørensen, H. P., & Mortensen, K. K. (2005). Advanced genetic strategies for recombinant protein expression in *Escherichia coli*. *Journal of Biotechnology*, *115*, 113–128.

Srinivasan, S., Barnard, G. C., & Gerngross, T. U. (2003). Production of recombinant proteins using multiple-copy gene integration in high-cell-density fermentations of Ralstonia eutropha. *Biotechnology and Bioengineering*, *84*, 114–120.

Stephanopoulos, G., & Vallino, J. J. (2009). Network rigidity and metabolite engineering in metabolic overproduction. *Science*, *252*, 1675–1681.

Szyperski, T. (1995). Biosynthetically directed fractional ^{13}C-labeling of proteinogenic amino acids. An efficient analytical tool to investigate intermediary metabolism. *European Journal of Biochemistry*, *232*, 433–448.

Thoma, S., & Schobert, M. (2009). An improved Escherichia coli donor strain for diparental mating. *FEMS Microbiology Letters*, *294*, 127–132.

Thomason, L. C., Costantino, N., & Court, D. L. (2007). *E. coli* genome manipulation by P1 transduction. *Current Protocols in Molecular Biology*. Chapter 1, 1.17.1–1.17.8.

Wang, Q., Liu, X., & Qi, Q. (2014). Biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) from glucose with elevated 3-hydroxyvalerate fraction via combined citramalate and threonine pathway in Escherichia coli. *Applied Microbiology and Biotechnology*, *98*, 3923–3931.

Yishai, O., Goldbach, L., Tenenboim, H., Lindner, S. N., & Bar-Even, A. (2017). Engineered assimilation of exogenous and endogenous formate in *Escherichia coli*. *ACS Synthetic Biology*, *6*, 1722–1731.

Zelcbuch, L., Antonovsky, N., Bar-Even, A., Levin-Karp, A., Barenholz, U., Dayagi, M., et al. (2013). Spanning high-dimensional expression space using ribosome-binding site combinatorics. *Nucleic Acids Research*, *41*, e98. https://doi.org/10.1093/nar/gkt151.

Zelcbuch, L., Lindner, S. N., Zegman, Y., Vainberg Slutskin, I., Antonovsky, N., Gleizer, S., et al. (2016). Pyruvate formate-lyase enables efficient growth of Escherichia coli on acetate and formate. *Biochemistry*, *55*(17), 2423–2426.

Zelcbuch, L., Razo-Mejia, M., Herz, E., Yahav, S., Antonovsky, N., Kroytoro, H., et al. (2015). An in vivo metabolic approach for deciphering the product specificity of glycerate kinase proves that both E. coli's glycerate kinases generate 2-phosphoglycerate. *PLoS ONE*, *10*(3), e0122957.

Zhang, Y., Buchholz, F., Muyrers, J. P. P., & Francis Stewart, A. (1998). A new logic for DNA engineering using recombination in *Escherichia coli*. *Nature Genetics*, *20*, 123–128.