Chapter 13

Techniques for Large-Scale Bacterial Genome Manipulation and Characterization of the Mutants with Respect to In Silico Metabolic Reconstructions

George C. diCenzo and Turlough M. Finan

Abstract

The rate at which all genes within a bacterial genome can be identified far exceeds the ability to characterize these genes. To assist in associating genes with cellular functions, a large-scale bacterial genome deletion approach can be employed to rapidly screen tens to thousands of genes for desired phenotypes. Here, we provide a detailed protocol for the generation of deletions of large segments of bacterial genomes that relies on the activity of a site-specific recombinase. In this procedure, two recombinase recognition target sequences are introduced into known positions of a bacterial genome through single cross-over plasmid integration. Subsequent expression of the site-specific recombinase mediates recombination between the two target sequences, resulting in the excision of the intervening region and its loss from the genome. We further illustrate how this deletion system can be readily adapted to function as a large-scale in vivo cloning procedure, in which the region excised from the genome is captured as a replicative plasmid. We next provide a procedure for the metabolic analysis of bacterial large-scale genome deletion mutants using the Biolog Phenotype MicroArray™ system. Finally, a pipeline is described, and a sample Matlab script is provided, for the integration of the obtained data with a draft metabolic reconstruction for the refinement of the reactions and gene-protein-reaction relationships in a metabolic reconstruction.

Key words Genome deletion, Site-specific recombinase, In vivo cloning, Phenotype microarray, Metabolic modeling, Functional genomics

1 Introduction

The explosion in the availability of complete bacterial genomes over the last decade has resulted in novel genes being identified at a rate that far exceeds our ability to characterize them. Approximately a third of the genes available through the Entrez Gene database are associated with the term hypothetical [1]. Highlighting this lack of understanding was the discovery that even a third of the essential *Mycoplasma mycoides* proteins are of unknown function [2]. The percentage of genes without a well-defined molecular function rises further when considering those with only general functional

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annotations, e.g., epimerase, without a known substrate. At the same time, high-throughput phenotypic analyses often lead to the identification of cellular capabilities while the underlying genetic determinants remain unclear [3, 4].

One approach to bridging the gap and associating functions to uncharacterized genes is to employ a large-scale genome deletion approach. Such a technique allows for the simultaneous analysis of tens to hundreds of genes, rapidly narrowing down the search for the genes underlying a particular phenotype. Our group has been using large-scale genome deletion and cloning approaches to study the genomics of *Sinorhizobium meliloti* since the late 1980s [5–7]. We and others have employed this approach in the study of both simple and complex biological traits including: identification of carbon transport and metabolic loci [4, 5, 8–10], ion transport systems [11], toxin-antitoxin modules [6], and essential genes [12], as well as in the study of regulatory networks [13], osmotic tolerance [14], soil colonization [15], magnetotaxis [16], cytochrome *c* respiration [10], and the rhizobium—legume symbiosis [10, 17]. The combination of large-scale genome reduction studies with in silico genome-scale metabolic reconstructions can be a particularly powerful approach. Metabolic characterization of the deletion mutants can facilitate the refinement of the gene-protein-reaction relationships in a metabolic reconstruction and assist in the annotation of novel gene functions [4].

In this chapter, we detail a procedure for the construction of bacterial strains with large-scale genome deletions in which the exact endpoints of each deletion are known. This procedure involves introduction of the recombinase recognition target sequences into the genome at a pre-selected position. Here, this is accomplished through single-plasmid cross-over recombination, although alternative methods of introducing the target sequences can be employed [18]. Subsequent expression of the corresponding site-specific recombinase results in recombination between the two target sequences and deletion of the intervening genomic region. A protocol for adapting this deletion method to function as an in vivo cloning tool is also described. Finally, metabolic characterization of these mutants with the Phenotype MicroArray™ system [19] and the application of the resulting data during the manual curation stage of an in silico genome-scale metabolic network reconstruction is described.

2 Materials

2.1 General Materials

1. Supplies and equipment required for general DNA manipulations, including PCR, restriction digest, DNA fragment purification, and cloning.
2. General laboratory supplies and equipment, such as centrifuge tubes, petri dishes, test tubes, and incubators.

3. Liquid and solid media permissive to growth of all bacteria used in this procedure. A common option is LB medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, and 15 g/L agar for solid medium).
4. Filter sterilized stock solutions of the appropriate selective agents.
5. Sterile 0.85% NaCl saline solution in water (if preferred, PBS can be used throughout the protocol in place of the 0.85% saline solution).
6. Autoclaved glass beads, or an L-shaped glass rod and 95% ethanol, or disposable sterile plastic rods for spreading of liquid culture on solid media.
7. An *E. coli* strain with a "helper plasmid" that expresses the RK2 *tra* genes (e.g., pRK600 [20]).
8. If performing in vivo cloning as described in Subheading 3.5 then an *E. coli* strain with a selectable marker (e.g., rifampicin resistant DH5α) is required.
9. NEBcutter (http://nc2.neb.com/NEBcutter2/) [21] for in silico determination of restriction enzyme digestion patterns.

2.2 A Site-Specific Recombinase System

The procedure for the construction of large, defined deletion mutants is based on a system involving a recombinase protein that detects and mediates recombination between two specific DNA sequences. The three main recombinase systems employed in bacterial genome editing are the Flp/*FRT*, Cre/*lox*, and phage ϕC31/*att* systems, and the key characteristics of these systems are described in Table 1. The choice of recombinase will influence the downstream applications (*see* Table 1), although all the systems can be employed for the construction of a basic deletion mutant. Our group employs the Flp/*FRT* system for construction of bacterial genome deletion mutants, and all our materials are freely available upon request.

2.3 A Vector Expressing the Site-Specific Recombinase

The minimum requirements for this vector are that:

1. The plasmid be replicative in the species of interest.
2. The vector is transferable via conjugation when the RK2 *tra* genes are expressed *in trans*.
3. The plasmid contains a selective marker.
4. The site-specific recombinase is expressed from a promoter active in the species of interest. In addition to these minimal requirements, two additional features are strongly recommended.
5. The vector expressing the recombinase is unstable in the organism of interest, such as pRK7813 [28], so that the vector can be readily lost from the population following the construction of the deletion (*see* **Note 1**); and
6. The site-specific recombinase is not constitutively expressed and is instead expressed from an inducible promoter (*see* **Note 2**).

Table 1
Site-specific recombinases employed in bacterial genome editing

Recombinase: Flp
Recognition site: *FRT* (5′-GAAGTTCCTATACTTTCTAGAGAATAGGAACTTC)
Notes: Following deletion formation, one *FRT* site remains in the genome that can be used in a second round of recombination to enlarge the deletion. This system cannot be used to delete two independent regions of the genome in the same strain; however, two existing deletions can be recombined into one strain. Additionally, once the two deletions are within the same strain, the region in between the two deletions can be removed by once again expressing the Flp recombinase
Examples: [6, 10, 22, 23]
Recombinase: Cre
Recognition site: *loxP* (5′-ATAACTTCGTATAGCATACATTATACGAAGTTAT) and variations [18, 24]
Notes: Following deletion formation, one *loxP* site remains in the genome that can be used in a second round of recombination to enlarge the deletion. Mutant lox sites have been reported that are recognized by the Cre recombinase but for which the *lox* site that is left over following recombination is a poor Cre substrate. Thus, using these mutant *lox* sites would allow for the construction of two or more independent deletions within the same strain
Examples: [16, 18, 24–26]
Recombinase: ϕC31
Recognitions site: *attB* (5′-GGGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGCGCGTA) and *attP* (5′-CCCCAACTGGGGTAACCTTTGAGTTCTCTCAGTTGGGG)
Notes: Following deletion formation, the *attB* and *attP* sites are no longer present and instead an *attL* or *attR* site remains in the genome, which are not recognized by the ϕC31 recombinase. This system therefore cannot be used in a second round of recombination to enlarge the deletion, but can be readily used for the construction of two or more independent deletions within the same bacterial strain
Examples: We are unaware of studies that use this system for bacterial genome deletions, but it has been used to integrate novel DNA into bacterial genomes [12, 17, 27]

2.4 Vectors for the Integration of the Recognition Targets into the Genome

Two vectors for the integration of the recombinase recognition target sequences into the genome must be designed. The minimum requirements for these vectors are that:

1. These vectors have a narrow host range origin of replication (e.g., ColE1, p15A) and function as a suicide vector in the species of interest.
2. They each contain a selective marker. The selective markers on the two plasmids must be different from each other and different from the selective marker on the recombinase expression vector.
3. They each contain a copy of the recombinase recognition target sequence (Table 1). If using the ϕC31 recombinase system, one vector will contain the *attB* sequence, and the other will contain the *attP* sequence.

4. A multiple cloning site for the introduction of a region homologous to the genomic region where the recognition target is to be integrated; and
5. The vectors must share limited homology to avoid recombination between the two vectors.

Careful consideration of the design of these vectors is necessary. The design of the vector influences the number of selective markers that remain once the deletion is made, and an improper design will result in an inversion, not a deletion, of the region of interest (Fig. 1). Additionally, if designed appropriately, the recombinase

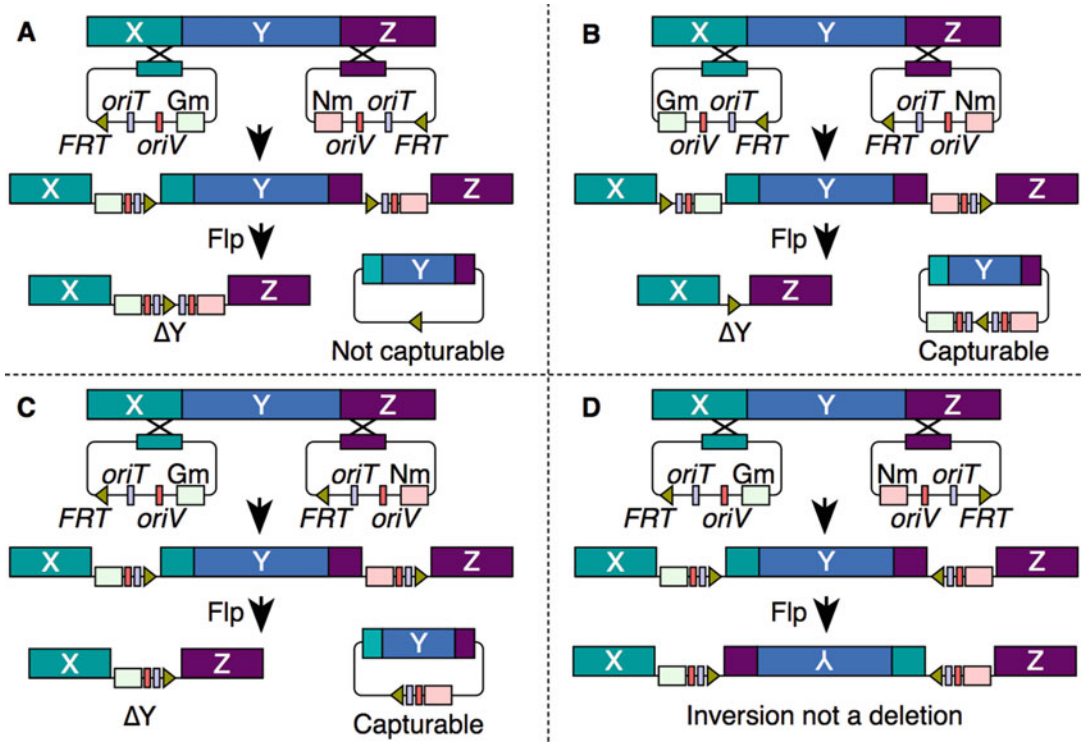


Fig. 1 Influence of vector design on the recombination end-product. A schematic illustrating how design of the vectors for the integration of the recombinase recognition target sequences into the genome will influence the structure of the resulting deletion. The Flp/*FRT* system is used as an example, but the schematic applies equally to all recombinase systems. Depending on how the integration vectors are organized, the final deletion may be marked by either (**a**) two selective markers, (**b**) zero selective markers, or (**c**) one selective marker. If the integration vectors are organized such that following recombination into the genome there are at least one *oriV*, *oriT*, and selective marker situated between the two FRT sites (**b**, **c**), the deleted region may be captured as a replicative vector in *E. coli*. Note that in all the cases, the region cloned into the vectors for recombination into the genome is not deleted following recombination between the *FRT* sites. In (**d**) an example is shown of how improper vector design will result in an inversion of the region of interest instead of a deletion. In this example, the *FRT* sites are introduced into the genome in the opposite direction of each, as opposed to direct orientation as desired, leading to the inversion

system can be used for both the construction of large-scale deletions and in vivo cloning of large genomic fragments (*see* **Note 3** and Fig. 1) [17, 29–31].

2.5 Biolog Phenotype MicroArray™ Analysis

If an analysis of the metabolic capabilities of the deletion strains with the Biolog Phenotype MicroArray™ technology is desired, the following are required:

1. At least one of Biolog plates for testing metabolic capacity (http://www.biolog.com). The primary plates for this purpose are the PM1 (carbon metabolism), PM2A (carbon metabolism), PM3 (nitrogen metabolism), and PM4 (phosphorus and sulfur metabolism) plates; and
2. A standard 96-well plate reader, if an OmniLog plate reader is not used.

   The following list includes optional supplies and equipment, although their use is recommended where possible (*see* **Note 4**):
3. The Biolog redox dye.
4. Access to an OmniLog plate reader; and
5. Analysis software, such as "DuctApe" [32] which is written in python (https://combogenomics.github.io/DuctApe/, https://www.python.org) or "opm" and associated extensions [33, 34], which is an R package (https://r-forge.r-project.org/R/?group_id=1573, https://www.r-project.org). The authors of this chapter only have experience working with DuctApe, and consequently, this chapter only provides information on Phenotype MicroArray™ analysis with the DuctApe software.

2.6 Integration of the Biolog Data with a Metabolic Reconstruction

If the results of the Phenotype MicroArray™ experiments are to be used for refinement of a metabolic reconstruction, the following are required:

1. A draft metabolic reconstruction.
2. A text editor such as gedit (https://wiki.gnome.org/Apps/Gedit).
3. A modeling framework, such as the CobraToolbox [35] in Matlab (http://opencobra.github.io, http://www.mathworks.com/products/matlab/) or Cobrapy [36] in python (http://opencobra.github.io, https://www.python.org).
4. A list of all genes removed in each deletion, as well as a list of genes removed in each deletion that are also present in the draft metabolic reconstruction; and
5. A script to replicate the Phenotype MicroArray™ experiment in silico with the metabolic reconstruction (*see* **Note 5** for a sample script).

3 Methods

Subheadings 3.1–3.4 provide procedures for the construction and confirmation of deletions based on our experience in generating Flp/*FRT*-mediated deletions in the *S. meliloti* genome [6]. In Subheading 3.5 we detail how this procedure can be easily adapted for the in vivo cloning of large genome regions, with an upper size limit of at least 70 kb [17]. The final two Subheadings 3.6 and 3.7 provide protocols that have been used in the metabolic characterization of *S. meliloti* deletion mutants using Phenotype MicroArrays™, and the use of this data in refining a genome-scale metabolic reconstruction [4].

3.1 Flanking a Genome Region with Recombinase Recognition Target Sequences

1. Decide upon the nucleotide (nt) positions where the deletion will start and end (e.g., start at nt position 100,000 and end at nt position 200,000).
2. PCR amplify and purify fragments between ~500 and 1000 nt in length that directly border the region to be deleted (e.g., 99,000–99,999 and 200,001–201,000). Ensure that the primers have the necessary 5′ sequences for cloning into the vectors that contain the recognition targets (*see* **Note 6**).
3. Purify the two vectors that contain the recombinase recognition target sequences (e.g., *FRT* sites, *see* Subheading 2.4) and digest with an appropriate restriction enzyme(s) for the introduction of the PCR products produced in **step 2**.
4. Clone the PCR products from **step 2** into the vectors digested in **step 3** and transform into *E. coli*. One PCR product should be cloned into one of the vectors, and the second PCR product into the second vector (*see* **Note 6**).
5. Inoculate overnight cultures of the organism of interest in which the deletion is to be made, an *E. coli* strain carrying one of the vectors produced in **step 4**, and an *E. coli* strain carrying the conjugation helper plasmid (e.g., pRK600). All the strains can be inoculated in complex medium such as LB, and the appropriate selective agents should be included for growth of the two *E. coli* strains. Grow cultures at the appropriate temperature (37 °C for *E. coli*) overnight with shaking.
6. The next day, subculture the *E. coli* strains to a 1:50 dilution in a fresh complex medium with the selective agent and grow for a further 4–5 h at 37 °C with shaking (*see* **Note 7**).
7. Perform conjugations with the *E. coli* cultures of **step 6** and the overnight culture of the recipient as detailed in Subheading 3.4 (*see* **Note 8**).
8. Once transconjugant colonies have been obtained, purify a colony by dilution streaking on a fresh selective medium petri dish, and incubate.

9. Once the transconjugant colonies from the above purification (**step 8**) have grown, purify further by again dilution streaking one colony from the petri dish of **step 8** and incubating.
10. Repeat **steps 5–9**, starting with overnight cultures of the following strains: the strain purified in **step 9**, an *E. coli* strain carrying the second of the vectors produced in **step 4**, and an *E. coli* strain carrying the helper plasmid (e.g., pRK600). The final strain contains the genome region of interest flanked by the recombinase recognition target sequences.

3.2 Deletion of the Region Flanked with Recombinase Recognition Target Sequences

1. Inoculate overnight cultures of the strain produced in Subheading 3.1, the *E. coli* strain carrying the vector encoding the site-specific recombinase, and an *E. coli* strain carrying the helper plasmid (e.g., pRK600). All the strains can be inoculated in a complex medium such as LB, and the appropriate selective agents should be included for the growth of the two *E. coli* strains (*see* **Note 9**). Grow cultures overnight at the appropriate temperature with shaking.
2. The next day, subculture the *E. coli* strains to a 1:50 dilution in a fresh complex medium with the selective agent and grow for a further 4–5 h at 37 °C with shaking (*see* **Note 7**).
3. Perform conjugations with the *E. coli* cultures of **step 2** and the overnight culture of the recipient, as detailed in Subheading 3.4 but with the following change. If using a vector containing an inducible recombinase gene (recommended), and if you wish to immediately generate the desired deletion, plate the 10^0–10^{-4} dilutions (Subheading 3.4, **step 11**) on both selective medium with and without the inducer compound present. Alternatively, if you wish to simply transfer the recombinase vector to the recipient without inducing deletion formation, plate the 10^0–10^{-4} dilutions only on medium lacking the inducer (*see* **Notes 10** and **11**).
4. Once transconjugant colonies have been obtained, determine the frequency of conjugation separately for transconjugant cells recovered in the presence or in the absence of the inducing agent (*see* **Note 10**).
5. Purify at least four colonies from the selective medium containing the inducing compound by dilution streaking on medium selective for the recipient cells containing the recombinase vector (*see* **Note 12**). The inducer compound does not need to be included in these plates. Incubate the petri dishes at the appropriate temperature for the necessary length of time.
6. Confirm the structure of the deletion as described in Subheading 3.3.
7. If the recombinase plasmid is stably inherited, streak purify one of the confirmed deletion mutants from **step 5** by dilution

streaking a second time. In this case, this strain is the final deletion mutant. Otherwise, if the recombinase plasmid is an unstable plasmid, and you wish to lose the plasmid from the cell, proceed to **step 8**.

8. Inoculate a culture of a confirmed deletion mutant. Use a complex medium without selective agents, and incubate overnight at the appropriate temperature.
9. The next day, serial dilute the overnight culture in saline as described in **step 10** of Subheading 3.4, treating the overnight cultures as the 10^0 dilution.
10. Spread plate (*see* **Note 13**) the 10^{-4} to 10^{-7} dilutions on a complex medium that does not contain compounds selective for the recombinase vector, and incubate the petri dishes at the appropriate temperature until colonies form.
11. Replica plate (e.g., patching with sterile toothpicks) ~100 colonies onto complex medium selective for cells with the recombinase vector and onto complex medium lacking selective agents specific for the recombinase vector. Include a control strain that is expected to grow in the presence of the selective agent, and a control strain expected not to grow. Incubate the petri dishes until growth is observed.
12. Streak purify a strain that does not grow in the presence of the selective agent. Streak the colony onto medium both with and without the selective agent to confirm the genotype. Assuming the strain fails to grow in the presence of the selective agent, this strain is the final deletion mutant and lacks the recombinase vector.

3.3 Confirmation of Deletion Formation

It is possible that some or even all of the transconjugants recovered in Subheading 3.2 do not contain the desired deletion (*see* **Note 14**). It is therefore necessary to confirm that the transconjugants contain the correct deletion structure through testing of the selective marker resistance/sensitivity profile and by PCR, as well as optionally through whole genome sequencing such as with Illumina sequencing.

1. If deletion formation is associated with the removal of at least one selective marker (Fig. 1b, c), screen the transconjugants for the presence/absence of the selective marker(s). If the correct selective marker profile is observed, or if deletion formation is not associated with loss of a selective marker, proceed to **step 2** (*see* **Note 15**). Otherwise, *see* **Note 16**.
2. Use PCR to confirm the correct structure of the deletion by designing primers that amplify genome segments directly outside of both borders of the deletion and directly inside both borders of the deletion (*see* Fig. 2 for an example). If the PCR

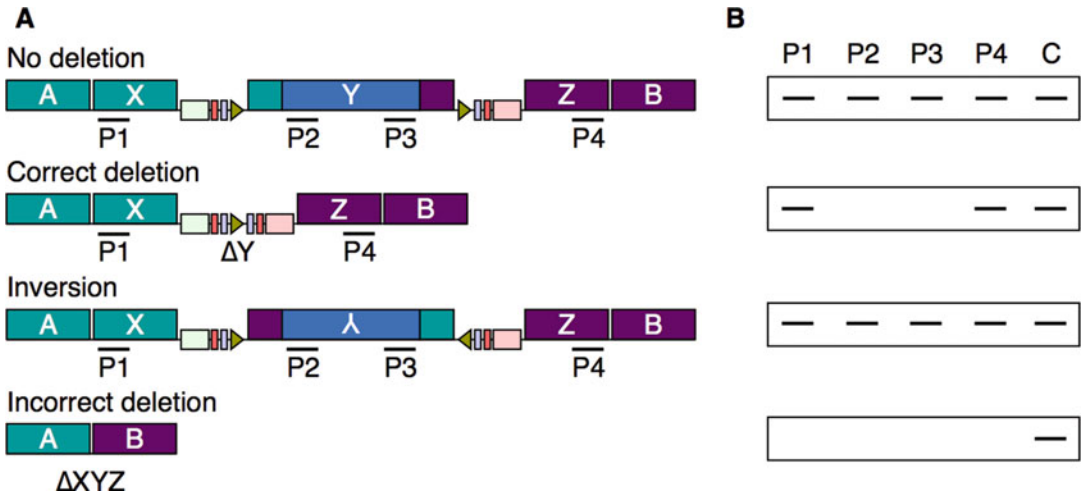


Fig. 2 Confirmation of deletion structure by PCR. (**a**) Schematic representations of sample genome structures following correct or incorrect deletion formation. The recommended positions of PCR products (P1, P2, P3, P4) for confirmation of the correct deletion structure are shown in all schematics. Ideally, the PCR products used for confirmation of the deletion structure do not overlap the fragments that were PCR amplified in **step 2** of Subheading 3.1. (**b**) A schematic representation of the expected banding pattern of the confirmation PCRs for each of the samples shown in (**a**). The PCR product "C" refers to a control PCR, which amplifies a genome segment situated far from the region to be deleted and is expected to always give a product

product profile is as expected, the correct deletion has been obtained. If the PCR product profile is not as expected, *see* **Note 16**.

3. Optionally, whole genome sequencing with the Illumina MiSeq or HiSeq platforms can be used to confirm the genome structure of the deletion mutants. Genomic DNA should be isolated and sequenced according to established procedures. An average genome coverage of at least 20× should be sufficient. The raw reads can be directly mapped to the reference genome of the organism using available software, such as Geneious (www.geneious.com), and the location of the deletion identified as a genome region with an absence of mapped reads. Compared to PCR, whole genome sequencing has the added benefit of allowing identification of whether a spontaneous second-site deletion, in addition to the desired deletion, is present in the recovered transconjugants.

3.4 Performing Conjugations and Isolating Transconjugants

1. Transfer 1 mL of each of the three cultures (recipient, donor, and helper) into separate 1.5 mL centrifuge tubes. Centrifuge the cells at 16,000 × *g* for 1 min, or as required for your organism of interest.
2. Remove the supernatant from all the three tubes, and resuspend the pellet in 0.9 mL of saline.

3. Centrifuge the cell suspensions as in **step 1**. Discard the supernatant and resuspend the pellet in 1 mL of saline.
4. In a fresh 1.5 mL tube, mix 100 μL of both *E. coli* cell suspensions.
5. In a fresh 1.5 mL tube, mix 50 μL of the cell suspension from **step 4** with 25 μL of the recipient organism.
6. On a complex medium agar petri dish (e.g., LB agar), pipette into one spot all 75 μL of the mixture from **step 5**, separately pipette into one spot 50 μL of the mixture from **step 4**, and separately pipette into one spot 25 μL of the recipient organism (*see* **Note 17**). Each of the three solutions should be present as separate spots/pools and should not be spread on the plate. All three cell suspensions can be plated either on the same petri dish or each on a separate petri dish. Ensure no bubbles are created during pipetting of the cell suspensions as splashing will occur when the bubbles pop.
7. Incubate the petri dish(es) from **step 6** overnight at the optimal temperature for your organism of interest. Incubate the petri dish lid-up, and be sure that when transferring the petri dish to the incubator not to spill the liquid spots.
8. The next day, add 1 mL of saline to each of three 1.5 mL centrifuge tubes, and label each to correspond to each of the three mating mixtures from **step 6**. Prepare an additional six 1.5 mL centrifuge tubes, containing 0.9 mL saline and label each corresponding to the 10^{-1}–10^{-6} dilutions of the 75 μL mating spot from **step 6**.
9. Use a sterile wooden stick or sterile metal loop to individually collect each of the mating mixtures from **step 6** and transfer to the corresponding centrifuge tubes containing 1 mL of saline from **step 8**. Vortex, or use a pipette, to fully resuspend the cells in each of the centrifuge tubes. These tubes are the 10^{0} dilutions.
10. Serial dilute the sample containing the 75 μL mating spot. Transfer 100 μL of the 10^{0} dilution to the 10^{-1} centrifuge tube that contains 900 μL of saline, and mix the solution. Repeat for the 10^{-1} dilution to create the 10^{-2} dilution, and continue until 10^{-6}. It is not necessary to serial dilute the samples for the 50 μL or 25 μL mating spots.
11. Spread plate (*see* **Note 13**) the 10^{0}–10^{-4} dilutions for the 75 μL mating mixtures on medium selective for the recipient cells that received the appropriate vector. Also plate the 10^{0} dilution for the 50 μL and 25 μL mating mixtures on the same medium.
12. Spread plate (*see* **Note 13**) the 10^{-4} to 10^{-6} dilutions for the 75 μL mating mixtures on medium selective for all recipient cells regardless of whether or not they received any vector.

13. Incubate all the plates at the appropriate temperature for your organism of interest (*see* **Notes 18** and **19**). Once colonies have formed, count the number of colonies and determine the frequency of conjugation (CFU/mL of transconjugants in the 10^0 dilution/CFU/mL of total recipients in the 10^0 dilution).

3.5 Adaptation of the Deletion Method for In Vivo Cloning of Large Genome Fragments

The large-scale deletion approach described in the previous sections can be readily adapted for the cloning of large DNA fragments. Thus far, we have used this method to clone fragments as large as 70 kb [17], and expect it is possible to clone larger regions as well [7]. Note, however, that this procedure only works if the strain carrying the *FRT* sites is constructed appropriately (*see* **Note 3** and Fig. 1b, c).

1. Use the methods of Subheadings 3.1 and 3.2 to construct a strain that contains two *FRT* sites flanking the genome region to be cloned, and contains the recombinase expression vector. At this point, do not induce expression of the recombinase.
2. Inoculate overnight cultures of the strain produced in **step 1**, an *E. coli* strain carrying the helper plasmid (e.g., pRK600), and an *E. coli* recipient strain with a selectable marker (e.g., rifampicin-resistant DH5α). All the strains can be inoculated in a complex medium such as LB, and the appropriate selective agents should be included. For the strain from **step 1**, selective agents to ensure maintenance of the recombinase vector and maintenance of the genome region to be cloned (e.g., using the selective markers associated with the recognition target sites) must be included.
3. The next day, subculture the *E. coli* helper strain to a 1:50 dilution in a fresh complex medium with the selective agent and grow for a further 4–5 h at 37 °C with shaking (*see* **Note 7**).
4. Using the strains from **steps 2** and **3**, set up conjugations as described in Subheading 3.4, with the following changes.
   (a) In Subheading 3.4, **step 6**, include the inducer compound for induction of recombinase expression in the complex medium agar petri dish.
   (b) When spread plating (*see* **Note 13**) for transconjugants in Subheading 3.4, **step 11**, only plate the 10^{-0} through 10^{-2} dilutions as the conjugation frequency is expected to be low and on the order of 10^{-7}. Transconjugants are to be isolated on medium selective for the *E. coli* recipient that gained at least one of the selective markers associated with the recombinase recognition target sequences.
   (c) In Subheading 3.4, **step 13**, incubate the petri dishes at 37 °C for 1 or 2 days for colonies to form.
5. Once transconjugant colonies have been obtained, purify at least four colonies by dilution streaking on a fresh selective medium petri dish, and incubate.

6. Once the transconjugant colonies from the above purification (**step 5**) have grown, purify each further by again dilution streaking one colony from each of the petri dishes of **step 8** and incubating.
7. Purify the plasmids from each of the four strains of **step 6**. Do so using standard molecular biology techniques, such as with alkaline lysis [37] or commercial plasmid purification kits.
8. Digest the vector with appropriate restriction enzymes and separate the resulting fragments on an agarose gel according to standard procedures. Compare the digest pattern with the expected digest pattern of the plasmid as determined with available software, such as NEBcutter [21]. If the digestion pattern matches the predicted pattern, then the region of interest was successfully cloned and isolated on a plasmid. If the correct digestion pattern is not observed, *see* **Note 20**.

3.6 Biolog Phenotype MicroArray™ Analysis

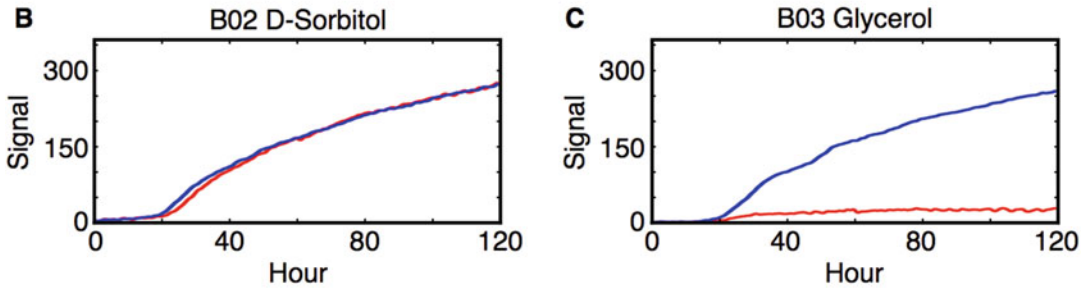
The deletions produced using the method described here can be characterized similarly to any single-gene deletion mutant. Characterization of their metabolic capabilities can provide invaluable information with respect to metabolic reconstructions. Many methods, including non-targeted metabolomics and Phenotype MicroArrays™, can be employed for this purpose [4, 38]. Here, we focus on the metabolic characterization of the large-scale deletion mutants using the Biolog Phenotype MicroArray™ technology [19] as the ease at which it can be performed with limited specialized equipment, analyzed, and integrated into the metabolic reconstruction makes it particularly useful. Many procedures for Phenotype MicroArray™ analysis can be found in the literature [3, 19, 39], and a protocol based on those procedures is described below.

1. Grow the wild type and all deletion strains of interest on any complex medium agar plates. Often it is possible to start the Phenotype MicroArray™ experiment from colonies grown on a solid medium, but in some cases it may be necessary to start from a liquid culture (*see* **Note 21**).
2. Use a cotton swab to resuspend the cells in 10 mL of a 0.85% NaCl solution to a final reading of 81% turbidity as determined with a Biolog turbidimeter, or to an OD_{600} ~ 0.1 in a 1 cm pathlength.
3. For each strain, combine 2 mL of each cell suspension from **step 2** with 22 mL of the desired minimal medium for the experiment. Ensure that the minimal medium used lacks the nutrient source to be tested (e.g., if using the carbon Biolog plates, the minimal medium must include all nutrients required for growth except for a carbon source).
4. Add 240 μL of Biolog redox dyeA into each 24 mL solution from **step 3** (*see* **Note 4**).

5. For each solution in **step 4**, add 100 μL to each well of the Biolog plates being employed.
6. Incubate all the plates in an OmniLog plate reader where available, otherwise a standard microtiter plate reader with temperature control may be employed (*see* **Note 4**). Incubate the plates at the appropriate growth temperature, and monitor reduction of the dye for 120 h, or however long is necessary for your specific organism and mutants.
7. Analyze the output from the OmniLog system using freely available software such as DuctApe [32] or opm [33, 34] (Fig. 3a, b; *see* **Note 22**).

A

			activity			
plate_id	well_id	chemical	Wild type	Mutant 1	Mutant 2	Mutant 3
PM01	A01	Negative Control	0	0	0	0
PM01	A02	L-Arabinose	9	3	9	9
PM01	A03	N-Acetyl-D-Glucosamine	9	9	9	9
PM01	A04	D-Saccharic acid	0	0	0	0



D

exchange reaction	Wild type	Mutant 1	Mutant 2	Mutant 3
EX_cpd00224_e0	0.55	0	0.55	0.42
EX_cpd00122_e0	0.80	0.80	0.80	0.80
EX_cpd00609_e0	0	0	0	0

Fig. 3 Sample output of the experimental and in silico Phenotype MicroArray™ experiments. (**a**–**c**) Sample output from the DuctApe analysis software. (**a**) A subset of an output table that provides a summary statistic summarizing how good each strain grew with each compound present in the tested Biolog plates. The higher the value, the better the growth. (**b**, **c**) Sample output curves illustrating the metabolic activity of wild type and one mutant strain in two different wells of the Biolog plate. (**d**) A subset of the table resulting from the script provided in **Note 5**. The objective function solution, which here is a specific growth rate, of each strain with each compound is shown. The first row was not printed by the script but added separately. The exchange reactions in column one can be switched with the corresponding compound name with simple scripts, or even in Excel, to allow direct comparison of the DuctApe output software. Sample data for this figure is taken from published work [4]

3.7 Incorporation of Phenotype MicroArray™ Data with a Draft Metabolic Reconstruction

The first step in using the experimental Phenotype MicroArray™ data to refine a draft metabolic reconstruction is to replicate the experiment in silico. Below, a procedure to do so and a sample Matlab script (*see* **Note 5**) are provided based on published work [4].

1. Create a list of all genes deleted in each of the deletion mutants examined.
2. Obtain a list of all genes present in the draft metabolic reconstruction, such as through the modelName.genes function in the cobraToolbox for Matlab.
3. Compare the list from **step 1** with the list from **step 2** in order to produce a comma separated list for each deletion mutant that indicates what genes are both removed in the mutant and also present in the draft metabolic reconstruction.
4. Use the data from **step 3** and a script such as that in **Note 5** to examine the in silico predicted phenotypes for each of the deletions studied with the Phenotype MicroArray™ analysis. In essence the script must perform the following: iteratively for the wild type and each mutant defined, the script must first delete all genes, and dependent reactions, that are removed in the deletion mutant and then iteratively provide a different nutrient source and test the ability of the model to produce biomass. The script provided in **Note 5** is a Matlab script making use of the cobraToolbox. Once the necessary user-defined variables are defined, this script can directly be used to replicate a Phenotype MicroArray™ experiment in silico (Fig. 3c).
5. Modify the draft metabolic reconstruction as necessary to reconcile the in silico predictions with the experimentally determined Phenotype MicroArray™ results. This can include modifying the gene-protein-reaction relationships present in the reconstruction, and/or addition or deletion of reactions.
6. Repeat **steps 4** and **5** as necessary until satisfied with the agreement between the in silico and experimental derived results. If during the curation new genes are added to the model, or genes are eliminated from the model, also repeat **steps 1–3**.

4 Notes

1. Usage of an unstable vector is not absolutely necessary, but is advantageous and can be required for certain applications. If it is desired to make a small deletion and enlarge it through successive rounds of deletion, the recombinase plasmid must be lost or eliminated from the cell following construction of the

initial deletion and prior to transfer of the next recombinase recognition target sequence containing plasmid. Similarly, if two unlinked deletions are to be recombined into the same strain, it is necessary to first remove the recombinase plasmid. Finally, the presence of the vector may impact the growth rate of the strain, it prevents re-usage of the associated selective marker, and other plasmids with the same origin cannot be co-maintained.

2. The use of an inducible promoter has two applications. First, it can assist in troubleshooting when the desired deletion cannot be recovered. If transconjugants carrying the recombinase plasmid are recovered less frequently when plated on media with the inducer compound than without the inducer compound, this indicates that the deletion is lethal. Otherwise, if transconjugants are recovered at the same frequency in both the cases, but none of the transconjugants carry the expected deletion, there is another issue (e.g., recognition targets are incorrectly integrated, the recombinase is not expressed). Second, it allows for recovery of strains carrying both recognition targets and the uninduced recombinase, which can subsequently be induced during specific environmental conditions and/or to study temporal effects [25]. For example, recombinase expression can be induced in a liquid culture to examine the effect on cell viability [6], or deletion formation can be induced under varying conditions to examine the effect of different factors on the phenotype of the deletion.
3. When designed appropriately, it is possible to not only delete a genome region but also capture the deleted region as a plasmid. This can be done by ensuring that an *oriT*, a narrow host range *oriV*, and a selective marker are present between the two integrated recognition targets (Fig. 1b, c). Induction of expression of the recombinase will result in the excision of the region intervening the two recognition targets, which will be present as a non-replicative vector that is lost from the cell. However, if the transfer genes are provided *in trans*, this non-replicative vector can be conjugated to a permissive host such as *Escherichia coli*, where it can be recovered as a replicative plasmid as described elsewhere [17].
4. The OmniLog plate reader can be replaced with a standard 96-well plate reader, with redox dye reduction measured at 590 nm. If desired, the ability of a deletion mutant to grow with the different compounds can be directly measured, such as with OD_{600}, instead of using a redox dye as an indicator of active metabolism. The use of published analysis software is not necessary and can be replaced with manual analysis, for example in Excel or with custom scripts. However, it is recommended to make use of the OmniLog plate reader, the redox dye, and

existing analysis software as this allows for a simple experimental and computational workflow that will provide meaningful data for use in metabolic reconstructions without an excessive effort.

5. A sample Matlab script for replicating a Phenotype MicroArray™ experiment in silico with a metabolic reconstruction is provided below. It is based on previously used code [4].

```
%% Load the model
model = readCbModel('modelName.xml'); % import the model
%% Define the strains to be studied
wildType = {}; % an empty string for the wild type
deletion1 = {'gene1','gene2','gene3'}; % list all genes removed in deletion 1 and that are present in the reconstruction
deletion2 = {'gene4','gene5'}; % list all genes removed in deletion 2 and that are present in the reconstruction. Repeat for all deletions
strains = {wildType,deletion1,deletion2}; % list all the strains defined above
%% Define the base medium composition
exchangeList = {'exchange1';'exchange2'}; % list all the exchange reactions in the model
model = changeRxnBounds(model, exchangeList, 0, 'l'); % set the lower bound of all exchange reactions to 0
baseMedium = {'nitrogen','sulfur','phosphorus',`etc'}; % list all exchange reactions that define the base medium. Should contain all essential nutrients except for the nutrient class being tested (e.g. except for a carbon source)
model = changeRxnBounds(model, baseMedium, -100, 'l'); % set the lower bound of the exchange reactions of the base medium to be non-growth rate limiting
%% Define the compounds to be studied
testCompounds = {'carbon1','carbon2','carbon3'}; % list the exchange reactions for all compounds to be tested
%% Define the output variables
testCompounds2 = {'blank';'blank';'carbon1';'carbon2';'carbon3'}; % The same as the `testCompounds' string, except it begins with two fields containing `blank' and compounds are separated by `;' not `,'
outputBiolog = {}; % the variable that will contain the final data
outputBiolog = testCompounds2; % add the list of tested compounds to the output variable
temp = {}; % a temporary variable
%% Perform the analysis
for strains = {wildType,deletion1,deletion2} % start of a for loop to iteratively test each of the strains defined earlier
```

```
    strain = strains; % set the 'strain' variable equal to the
current strain being tested
    genes = strain{1}; % set the 'genes' variable equal to all
the genes present in the current strain
    temp = {'blank';'blank'}; % add two blank cells to the temp
variable and clear existing data
      [modelMutant,hasEffect,constrRxnNames,deletedGenes] =
deleteModelGenes(model,genes); % delete all genes in the
'genes' variable from the model.  Save the model as modelMu-
tant, and all reactions that should no longer exist are saved
in the constrRxnNames variable
   modelRxnsRemoved = removeRxns(modelMutant,constrRxnNames);
% delete all reactions from the model that should no longer
exist
      for testCompounds = {'carbon1','carbon2','carbon3'} %
start a second for loop to iteratively add one test compounds
and test biomass production
       compoud = testCompounds; % set the 'compound' variable
equal to the current compound
        model2 = changeRxnBounds(modelRxnsRemoved, compound,
-5, 'l'); % add the tested compound to the growth medium at a
growth rate limiting concentration
        solution = optimizeCbModel(model2, 'max'); % optimize
the model
           temp(end+1) = {solution.f}; % add the objective
function flux solution to the temp variable
     end % finish the for loop for testing of the various
compounds
    outputBiolog = horzcat(outputBiolog,temp); % add the data
in the temporary file to the output file
end % finish the for loop for testing the various strains
clearvars -except output; % clear all intermediate variables
leaving just the output.  The first column of the output file
will contain the different exchange reactions, and each
following column will contain the output for each of the
strains in the order that they were listed in the 'strains'
string (a value of 0 means the strain failed to grow with the
corresponding compound listed in the first column)
```

6. Any method can be used for cloning of the PCR products into the vectors containing the recombinase recognition targets. Possible methods include classical ligation [37], Gibson cloning [40], SLiCE [41], or SLIC [42], among others. We work primarily with SLIC (*s*equence- and *l*igation-*i*ndependent *c*loning) for cloning of PCR products and we recommend the use of a ligation-independent cloning method as this precludes the need to digest the PCR product and therefore eliminates issues with identifying compatible restriction enzymes that can be

used to digest both the vector and PCR product without cleaving the PCR product or vector in half.

7. The use of fresh cultures increases conjugation efficiency, and thus this subculture step is to achieve a higher plasmid transfer rate. However, this step can normally be skipped if necessary due to time constraints.
8. Assuming a plasmid transfer frequency of ~10^{-2} to 10^{-3} per recipient cell, and assuming the plasmid recombines into the recipient genome at a frequency ~10^{-3} to 10^{-4} per recipient cell that received plasmid, the overall conjugation frequency is expected to be in the range of ~10^{-5} and 10^{-7}.
9. Selective agents can be included for growth of the strain produced in Subheading 3.1, although in most cases this is not necessary as the integrated vectors should not be easily lost.
10. Comparing the amount of transconjugants recovered on medium with the inducing agent versus without the inducing agent provides insight into the viability of the deletion. Assuming a plasmid transfer frequency of ~10^{-2} to 10^{-3} per recipient cell, the frequency of conjugation is expected to be ~10^{-2} to 10^{-3} per recipient regardless of whether the inducer compound is included in the selective medium. A large decrease (>tenfold different) in the recovery of transconjugants in the presence of the inducing compound compared to without the inducing agent indicates that the majority of cells in which the deletion was made failed to grow and form colonies. In such cases, it may not be possible to recover colonies containing the deletion.
11. Transferring of the recombinase vector to the recipient without the inducer compound present allows for the isolation of transconjugants carrying the uninduced recombinase without forming the deletion. Such a strain can be purified, and then grown with the inducer compound present at a later time to induce the deletion, which can be useful for studies as discussed in **Note 2**.
12. If attempting to isolate transconjugants carrying the recombinase vector but without forming the deletion, it is best to continuously select for a selective marker that is lost once the deletion is formed. Even without actively inducing the expression of the recombinase, we have found that background expression of the recombinase can be sufficient to facilitate the formation of the deletion in a subset of the population. Thus, it is necessary to continually select for strains not containing the deletion to ensure the genotype of the cell population is as desired.
13. To spread plate the cell suspensions, transfer by pipette 100 μL of the desired suspension to the petri dish. Spread the culture

either by placing sterile glass beads on the petri dish, shaking, and then discarding the glass beads, or by using a sterile "hockey stick" glass rod to spread the culture (sterilize the glass rod in between uses by dipping it in 95% ethanol and then burning the ethanol by very briefly bringing the glass rod in contact with the flame of the Bunsen burner—leaving the glass rod in the flame for an extended period will cause the rod to break).

14. There are several reasons for why a recovered transconjugant may not contain any deletion. The primary reason is that it is possible for either of the vectors integrated into the genome through single cross-over plasmid integration to recombine back out of the genome at a frequency ~10^{-3} to 10^{-4}, leaving only one recombinase recognition target sequence in the genome. Additionally, at a frequency not greater than 10^{-7} there may be a spontaneous mutation within the recombinase gene or either recognition sequence that would inactivate the system. Another possibility is that the deletion strategy was incorrectly designed, and the recognition targets are not in the same orientation, and so an inversion is produced instead of a deletion (Fig. 1d).
15. A correct selective marker profile confirms that a deletion has been made. However, our own experience tells us that this should not be considered sufficient evidence that the correct deletion has been made. It is possible the plasmids were targeted to the incorrect genome location, or a spontaneous genome deletion event removed the selective marker(s) while making an incorrect deletion. Therefore, PCR confirmation is strongly recommended even if the correct selective marker profile is detected.
16. If the structure of the deletion is not confirmed, then more transconjugant colonies can be screened through replica patching ~100 of the transconjugant colonies obtained in Subheading 3.2, **step 4**. If the correct deletion is still not detected, then conjugation of the recombinase expression vector can be repeated. If the correct deletion is still not obtained, and especially if there is a large difference in the number of transconjugants obtained on media with and without the inducing agent, deletion of the region under the tested conditions may be lethal (*see* **Note 10**). However, if there is no difference in the number of transconjugants obtained on media with and without the inducing agent, then it may be that the deletion construct is incorrectly designed (Fig. 1d) and should be re-examined.
17. The 50 and 25 μL mating spots serve as negative controls. When eventually plated on medium selective for recipient

cells that had gained the vector containing the recombinase recognition target, no colonies should form on these plates. If colonies do appear, there was a source of contamination at some point of the experiment, and the experiment should be repeated.

18. It may be necessary to incubate the petri dishes a day or two longer than normal for colonies to grow due to the large number of cells plated on the petri dish.
19. This note is applicable only to conjugations involving a vector that functions as a suicide vector in the recipient cell. In these cases, it is common for there to be greater background growth on the petri dishes on which the 75 μL spot was streaked compared to the petri dishes on which the 50 μL and 25 μL spots were streaked. This is due to slight growth of recipient cells that gain the vector containing the recombinase recognition target but in which the vector did not recombine into the recipient's genome.
20. It is necessary to confirm that the recovered plasmid is correct as there are two main reasons for why the incorrect plasmid might be obtained. It is possible for the vectors containing the recombinase recognition target sequence to recombine back out of the genome at a frequency ~10^{-3} to 10^{-4}, and for just one or both of these vectors to be transferred to the *E. coli* recipient. Additionally, if the recognition target sequences were introduced into the incorrect genome location, the wrong genome region would be cloned.
21. In some cases, a deletion mutant might experience an extra long lag phase compared to the wild type upon inoculation into the liquid minimal medium. This can negatively impact the experiment and analysis. In such cases, it is recommended to not start the Phenotype MicroArrayTM experiment from solid agar, but to instead use liquid cultures as the inoculum. To do so, grow all appropriate mutants and wild type in a minimal medium containing all nutrients required to grow until the cultures have reached a sufficiently high density. Pellet and wash the cells from each culture twice with 0.85% saline, and resuspend the washed pellets in 1 mL of 0.85% saline. Use this cell resuspension to inoculate 10 mL of a 0.85% NaCl solution to 81% turbidity as determined with a Biolog turbidimeter, or to an OD600 ~0.1 in a 1 cm pathlength, and proceed to **step 3** of Subheading 3.6. In some cases, significant background growth in the negative control may be observed with this method, indicating that the cells may need to be starved of the nutrient type (e.g., nitrogen) being tested. If this occurs, after washing and resuspending the cultures, dilute to an OD600 ~0.1 in medium lacking the nutrient class to be tested

(e.g., no nitrogen source) and incubate overnight, or longer as necessary. Then wash the cells with saline as before, and proceed to preparing the 10 mL saline cell resuspension.

22. The DuctApe analysis software allows automated prediction of genes whose deletion in a mutant may be responsible for the observed metabolic phenotype. In order to make use of this function, it is necessary to produce a fasta proteome file (.faa) for the wild type and for each mutant. The wild-type proteome file should contain the amino acid sequences for all proteins encoded by the wild-type genome. The mutant proteome files should contain the amino acid sequences for all proteins that are encoded by the wild type but not by the mutant (i.e., the proteins deleted in the mutant).

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