CHAPTER

7

Natural transformation as a tool in *Acinetobacter baylyi*: Evolution by amplification of gene copy number

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Abbreviations

Ab^R	antibiotic resistance
ALE	adaptive laboratory evolution
Cit	citrate
cPCR	colony PCR
EASy	Evolution by Amplification and Synthetic biology
GDA	gene duplication and amplification
gDNA	genomic DNA
GOI	gene(s) of interest
Gua	guaiacol
Km	kanamycin
qPCR	quantitative PCR
SBF	synthetic bridging fragment
Sm	streptomycin
Sp	spectinomycin
TPA	terephthalic acid

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1 Introduction

Adaptive laboratory evolution (ALE) is a widespread and powerful tool to engineer microorganisms with enhanced tolerance, increased production capabilities, or new phenotypes (Dragosits & Mattanovich, 2013). By growing the cells under an appropriate selective pressure for multiple generations, the desired property can be attained due to the accumulation of beneficial mutations that lead to improved activities (i.e., optimization) or novel functions (i.e., innovation) (Barrick & Lenski, 2013). However, in the timescales typically employed in experimental evolution, the low frequency of spontaneous, beneficial mutations may impose constraints that are difficult to overcome. Specifically, in the early stages of selection, gene/protein expression may be insufficient to sustain growth. Low expression could be problematic if selection depends on the weak side activity of an enzyme, if toxic intermediates accumulate, and/or if non-native proteins are unstable. In nature, gene duplication and amplification (GDA) facilitate adaptation to new or changing environments by increasing expression via transient changes in gene dosage. This amplification is a reversible mechanism that allows the reduction of gene copy number once selective pressure is removed or other beneficial mutations are propagated throughout the population (Andersson & Hughes, 2009; Elliott, Cuff, & Neidle, 2013; Seaton et al., 2012).

Although gene duplications tend to occur significantly more frequently than point mutations (Anderson & Roth, 1981; Seaton et al., 2012), the probability remains low that a sufficient number of cells in the starting population of a culture will randomly have a duplication in the correct location to be selectively beneficial in ALE (Herrmann et al., 2022). However, if a tandem duplication encompassing the region of interest does exist, it provides long stretches of identical sequence that allow further amplification mediated by homologous recombination. Gene copy number then fluctuates stochastically, and if increased dosage is advantageous, cells will be selected that, on average, have the same level of increased expression (Reams & Neidle, 2003) (Fig. 1A). A paradigmatic example of the relevance of GDA in experimental evolution is the emergence of the aerobic citrate-utilizing (Cit^+) phenotype in *Escherichia coli* during a long-term evolution experiment (Blount, Barrick, Davidson, & Lenski, 2012). This event, which took place after >31,000 generations (15 years), was due to the GDA of *citT*, which encodes a citrate: succinate antiporter. Later works purposefully evolving *E. coli* for citrate utilization found that Cit^+ clones obtained after at least 30 days of selection also possessed multiple copies of *citT* (Van Hofwegen, Hovde, & Minnich, 2016).

To induce GDA in the laboratory at a precise position, the locus of interest may be artificially duplicated using DNA manipulation techniques (Stoudenmire et al., 2017) (Fig. 1B). Recently, we developed a method named Evolution by Amplification and Synthetic Biology (EASy) in *A. baylyi* ADP1 (Tumen-Velasquez et al., 2018). A schematic overview of this method is shown in Fig. 2. EASy takes advantage of the exceptional natural competence and efficient homologous recombination of *A. baylyi*. In a first step, the gene(s) of interest (GOI) and an antibiotic resistance (Ab^R) selection marker are integrated in the chromosome of *A. baylyi*. Next,

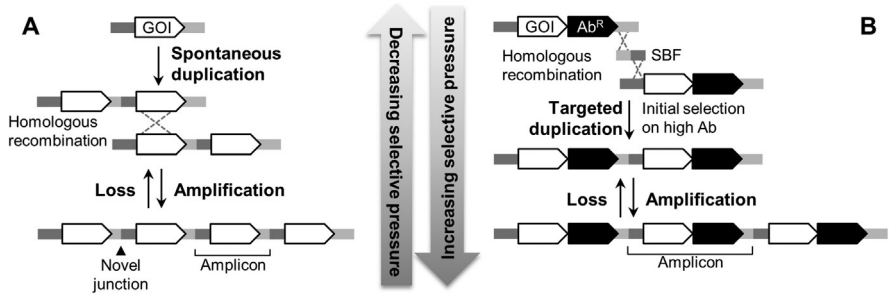

FIG. 1

Overview of natural and EASy-mediated gene duplication and amplification (GDA). (A) In natural GDA, the infrequent duplication of the gene of interest (GOI), by homologous or illegitimate recombination, in a few individuals in a population provides a stretch of sequence identity for subsequent homologous recombination. With increasing selective pressure, mutants with higher gene dosage are selected. (B) With EASy, the initial duplication of a targeted chromosomal segment is induced by transforming with a synthetic bridging fragment (SBF). High antibiotic concentrations allow the rapid selection of mutants with multiple copies of the antibiotic selection marker (Ab^R), which is present in the amplicon delimited by the SBF. Then, growth under selective conditions without antibiotic drives further GDA of the GOI.

a specific region of the chromosome, encompassing the GOI and the Ab^R marker, is amplified by transforming cells with a synthetic DNA bridging fragment (SBF). The SBF provides sufficient sequence identity for initial duplication by homologous recombination, and it precisely delimits the chromosomal segment that is duplicated, i.e., the amplicon. By including the Ab^R marker within the amplicon, mutants with multiple copies can be easily selected in the presence of high antibiotic concentrations. This step bypasses a potential bottleneck in the emergence of amplification mutants when direct selection for the desired phenotype is too demanding to the cells. Afterwards, the amplification mutants can be transferred to the selective conditions for ALE for the emergence of the desired phenotype. At this point, high antibiotic concentrations are removed from the medium. Thus, the only selective pressure driving GDA is the target condition for ALE (e.g., growth on a non-native substrate). Since chromosomal duplications are inherently unstable, beneficial mutations that result in a growth advantage will be selected during ALE and lead to a decrease in the average copy number of the amplicon in the evolving population. These beneficial mutations may be found in the GOI and/or in other regions of the chromosome.

We successfully used EASy to enable the catabolism of non-native aromatic carbon sources in *A. baylyi*, namely guaiacol and terephthalic acid (TPA) (Pardo et al., 2020; Tumen-Velasquez et al., 2018). Similarly, after the functional replacement of a native aromatic catabolic pathway with a non-native pathway, EASy was used to increase thermotolerance (Bedore, 2021). *A. baylyi* can utilize a broad range of aromatic compounds through the β-ketoadipate pathway, by which many different substrates are converted by peripheral routes into either catechol or protocatechuic acid (Fuchs, Boll, & Heider, 2011; Seaton & Neidle, 2018). The aromatic rings of these compounds are then cleaved by intradiol dioxygenases before metabolites

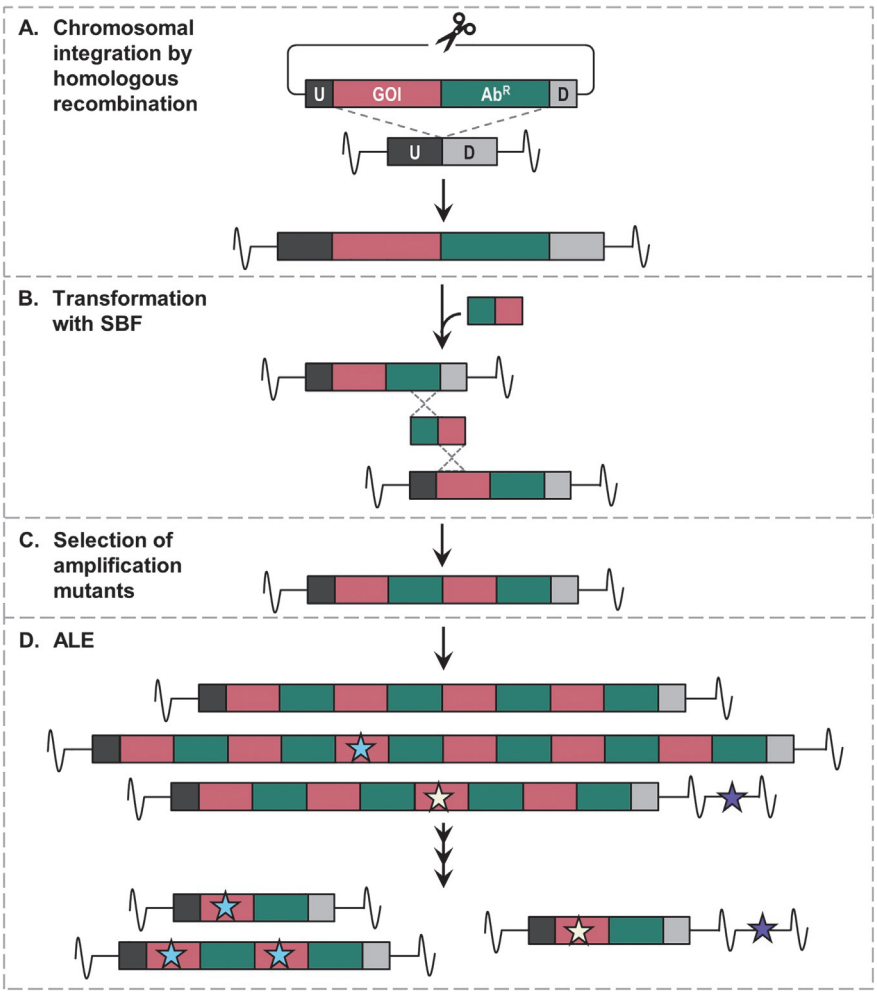

FIG. 2

Overview of the EASy method. (A) The gene of interest (GOI) and the antibiotic selection marker (Ab^R), flanked by sequences identical to the upstream (U) and downstream (D) regions of the targeted locus, are integrated in the *Acinetobacter baylyi* chromosome by homologous recombination. Alternatively, a native gene or chromosomal region can be targeted for amplification by simply introducing the Ab^R in an adjacent position. (B) Mutants are transformed with a synthetic bridging fragment (SBF), which delimits gene duplication and amplification to the desired chromosomal segment. (C) The amplification mutants that have incorporated the SBF are selected in the presence of high antibiotic concentrations. (D) The amplification mutants are subjected to adaptive laboratory evolution (ALE) under the desired selective conditions, in which antibiotic selection is removed. During ALE, the tandem array of amplicon copies may expand to sustain growth under the selective conditions. Beneficial mutations (shown as stars) may appear in the gene of interest or elsewhere in the chromosome. As the beneficial mutations accumulate, the copy number of the amplicon is reduced. Finally, alleles holding beneficial mutations are selected and fixed throughout the population.

are channelled into central metabolism. To enable *A. baylyi* to utilize guaiacol, we introduced into its chromosome the *gcoAB* genes from *Amycolatopsis* sp. ATCC 39116, which encode a guaiacol *O*-demethylase for the conversion of guaiacol to catechol (Fig. 3A). EASy-mediated GDA of *gcoAB* gave rise to mutants capable of growing on guaiacol as the sole carbon source (Gua^+ phenotype). After ~1000 generations of ALE, Gua^+ mutants with a single copy of *gcoAB* were obtained. These clones presented either point mutations in the heterologous *gcoAB* genes or translational fusions between *gcoAB* and *catA* (encoding the catechol dioxygenase). Other mutations in different loci were likewise selected (Tumen-Velasquez et al., 2018). In the second example, EASy was performed on strains harbouring the TPA catabolic genes *tph* from *Comamonas* sp. E6, which encode the necessary activities for the conversion of TPA to protocatechuic acid. To allow uptake of TPA into the cell, transport genes (*tpiAB*) were also integrated into the chromosome of *A. baylyi*. These genes, encoding the transmembrane proteins of the tripartite TPA transporter from *Comamonas* sp. E6, were maintained in single copy to avoid potential toxic effects resulting from overexpression (Fig. 3B). In these studies, mutants with a single copy of the *tph* genes were not obtained. Moreover, no mutations in the *tph* genes were identified in eight evolved strains. Nonetheless, EASy allowed the identification of native *A. baylyi* transporters that had evolved for improved uptake of TPA. We further demonstrated that the heterologous *Comamonas* transporter is not necessary for *A. baylyi* to grow on TPA. This outcome would not have been possible without prior amplification of the foreign catabolic genes, a step that was necessary for the emergence of the Tpa^+ phenotype (Pardo et al., 2020).

In this chapter, we provide some tips and best practices for performing EASy experiments with *A. baylyi*, particularly focused on the evolution of heterologous catabolic pathways. However, we note that this method is amenable to many variations, and we have successfully applied it in separate laboratories using slightly different approaches. We anticipate that EASy can be adapted for a range of applications.

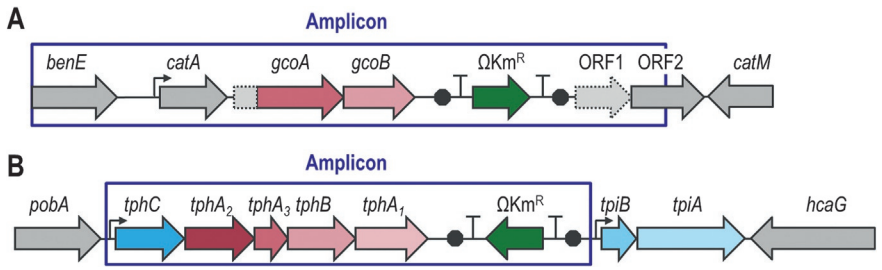

FIG. 3

Schematic representation of the chromosomal integrations in *Acinetobacter baylyi* for EASy of (A) guaiacol and (B) TPA utilization. The native *A. baylyi* genes are shown in grey. The foreign genes for guaiacol and TPA utilization are shown in pink (catabolic genes) and blue (transport genes). Promoters driving the expression of the heterologous genes are indicated with angled arrows. The ΩKm^R cassette used for antibiotic selection contains a kanamycin resistance gene (green) flanked by transcription and translation terminating signals, respectively shown as "T"s and black octagons. The open-reading frame disruption caused by insertion of *gcoAB* in panel (A) is indicated with dashed lines. The EASy amplicons are bound by purple boxes.

2 General considerations

2.1 Design of the amplicon and synthetic bridging fragment

To design an EASy experiment, the first step is to determine the precise chromosomal region to be amplified, i.e., the amplicon. In most experiments, this design involves the chromosomal insertion of target DNA that will be amplified, although in some experiments, we have amplified a region of the wild-type chromosome (Stoudenmire et al., 2017). As depicted in Fig. 1A, the endpoint of the amplicon is created by a novel junction that joins two chromosomal regions that are not normally adjacent. This novel junction is engineered by the choice of DNA segments connected on the SBF (Fig. 1B). When used as donor DNA, this SBF promotes homologous recombination with chromosomal DNA to duplicate the desired region. Also essential to the experimental design is inclusion of an Ab^R marker in the chromosome within the intended amplicon. This marker provides a means to select and maintain increased gene dosage after transforming with the SBF.

The simplest way to construct the parent strain is to insert the GOI (or larger region of interest) into the chromosome together with the Ab^R marker (Fig. 2). We have used Ω fragments encoding either resistance to kanamycin (Km^R) or streptomycin/spectinomycin (Sm^R/Sp^R) (Eraso & Kaplan, 1994; Prentki & Krisch, 1984). In these fragments, the Ab^R marker is flanked by transcription and translation termination signals (Fig. 3). This configuration ensures that the insertion of the Ab^R marker does not interfere with the expression of upstream and downstream genes, independent of the orientation. However, any DNA construction that isolates the expression of an Ab^R marker from the rest of the amplicon is expected to work.

An important consideration when designing the amplicon is the potential toxicity caused by overexpression of certain genes. For example, when engineering TPA transport and catabolism in *A. baylyi*, we found that high expression levels of genes encoding transmembrane proteins were lethal to the cells, even when in single copy in the chromosome (Pardo et al., 2020). Therefore, we decided to insert them as a separate transcription unit that was not included in the amplicon (Fig. 3B). The architecture of the amplicon can also be tailored to different gene expression strategies. For EASy of guaiacol utilization, the *gcoAB* genes were inserted as transcriptional fusions directly downstream of *catA*, so that they were under control of the *cat* promoter (Fig. 3A). On the other hand, the *tph* genes were placed under control of the *tac* promoter (de Boer, Comstock, & Vasser, 1983), a strong constitutive promoter in *A. baylyi*, so that they were independently transcribed during EASy TPA consumption (Fig. 3B). Synthetic promoter sequences, of varying strength, for use in strain ADP1 have recently been characterized (Biggs et al., 2020).

Once the desired amplicon is planned, the SBF is designed accordingly to delimit GDA to the chromosomal segment of interest. For this purpose, the last ~1000 bp of the 3′-end of the amplicon are fused to the first ~1000 bp of the 5′-end, in tandem. This length provides a sufficiently long stretch of sequence identity to enable homologous recombination in *A. baylyi*. Again, this step is amenable to variations. The SBF can be designed to limit the amplicon to the foreign DNA sequence (Fig. 4, amplicon 1), or to include the native upstream and downstream sequences of the insertion locus (Fig. 4, amplicon 2). We have successfully used both approaches.

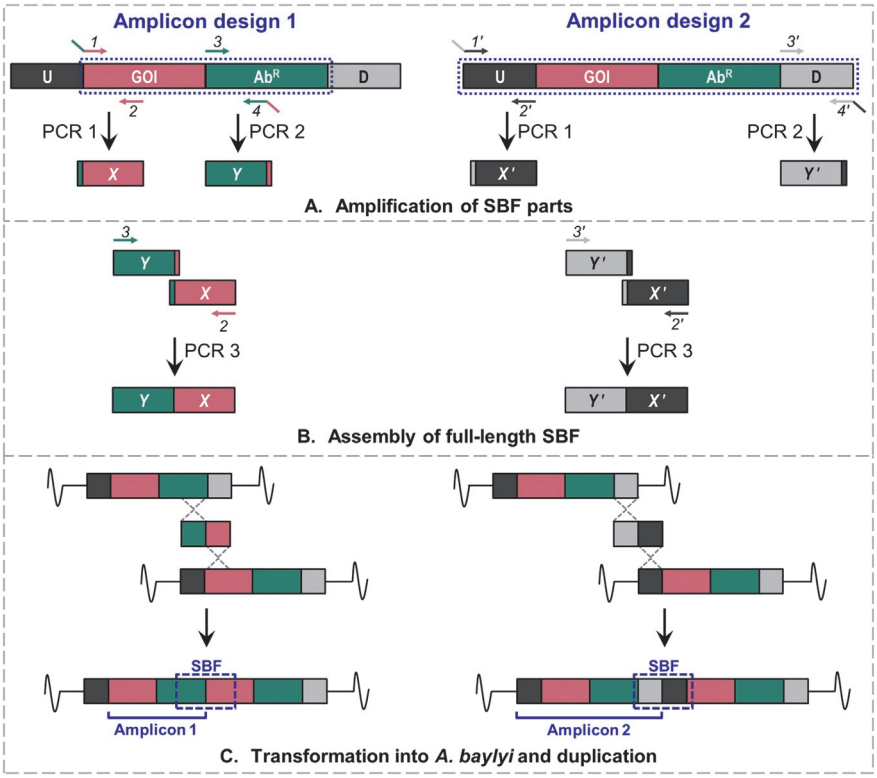

FIG. 4

Workflow for the construction of the synthetic bridging fragment (SBF) for EASy. (A) In a first step, the target chromosomal segment that will be amplified is determined (i.e., amplicon, bound by a dotted line). In the figure, amplicon design 1 (left side) is limited to the gene of interest (GOI) and the antibiotic selection marker (Ab^R), whereas amplicon design 2 (right side) includes the upstream (U) and downstream (D) chromosomal regions of the targeted insertion locus. Once the amplicon is designed, the first and last ~1000 bp of the target amplicon are amplified by PCR. The forward primer to amplify the 5′-end of the amplicon (primer *1*) is designed to have a ~20 bp overhang identical to the 3′-end of the target amplicon. Likewise, the reverse primer to amplify the 3′-end of the target amplicon has a ~20 bp overhang that is the reverse complement of the 5′-end of the amplicon (primer *4*). (B) The full-length SBF is assembled in 15 cycles of a primer-less PCR by sequence overlap extension, using the PCR products form the first round of PCR as self-priming templates (fragments *X* and *Y* in panel A). Then, primers *2* and *3* are added to amplify the full-length SBF in another 15 cycles of PCR. (C) The SBF is transformed into the recipient strain to precisely delimit GDA to the target chromosomal region, with the integrated SBF forming part of the novel junction (bound by dashed lines). Depending on the design of the SBF, the resulting amplicon will be different (indicated with brackets).

2.2 Chromosomal integration by natural transformation

Having designed the DNA cassette that will be integrated into the chromosome of *A. baylyi*, it needs to be flanked by upstream and downstream sequences identical to the chromosomal locus targeted for insertion (Figs. 2 and 6). These regions of identity can be as small as 300 bp (de Berardinis et al., 2008), although longer stretches of identical sequences increase the efficiency of homologous recombination and facilitate the integration of larger DNA fragments. The construction of the DNA cassette flanked by the homology regions can be performed using any DNA assembly technique (e.g., restriction cloning, sequence overlap extension PCR, Gibson assembly, Golden Gate assembly, etc.). Since linear DNA fragments are used for the integration into the chromosome, the assembly products can be directly transformed into *A. baylyi* or sub-cloned into a plasmid for sequence verification and maintenance. If the latter option is used, a simple digestion with a restriction enzyme to linearize the plasmid backbone needs to be performed before transformation. In all cases, the genotype of the resulting transformant should be confirmed.

As noted above, *A. baylyi* has a remarkable competency for natural transformation, which allows using many different variations of the same two-step protocol: (1) incubation of *A. baylyi* with transforming DNA; and (2) selection or screening to identify desired transformants (Biggs et al., 2020; Metzgar et al., 2004; Santala, Karp, & Santala, 2016; Suárez et al., 2020; Suárez, Renda, Dasgupta, & Barrick, 2017). The incubation step can be done in liquid or solid medium, which can be either minimal or rich. The same protocol can be used for replicative plasmids or linear DNA fragments. For chromosomal integrations, unpurified linear DNA fragments can be added directly from assembly or digestion reactions, and even genomic DNA from lysed cells can be used. Bedore et al. (2023) describe in detail the protocols that we routinely use in the laboratory, although these may be entirely customized. Any one of the transformation protocols given can be used throughout the different steps of EASy.

2.3 Selection of amplification mutants

As noted in the introductory section, the SBF provides sufficient sequence identity for initial duplication of the target chromosomal segment. Further amplification of the duplicated region can occur and be maintained if selectively advantageous. Therefore, following transformation with the SBF, amplification mutants are selected in the presence of high antibiotic concentration. This selection is used to maintain a tandem array of multiple copies of the amplicon. Under the conditions described in the Procedures section, we use a concentration of 1 g/L Km or Sm/Sp (using a combination of both Sm and Sp) which inhibits growth of mutants carrying a single copy of the Ab^R gene. Nevertheless, we have found that this concentration may need to be optimized when using resistance genes from different sources or growing on different media (unpublished data). In particular, the ΩKm^R fragment we typically use (positions 10,499–12,705 in GenBank MN266288.1) harbours the *nptII* gene from Tn*5*,

whereas the ΩSm/Sp^R fragment (GenBank M60473.1) harbours the *aadA* gene from plasmid R100 (Fellay, Frey, & Krisch, 1987; Prentki & Krisch, 1984). Researchers implementing EASy in their laboratory should first optimize the selection conditions that only permit growth of the amplification mutants. Spontaneous drug-resistant mutants are not observed. Typically, 5–10 copies of the drug marker are selected by these antibiotic concentrations.

Once colonies are obtained that are resistant to high antibiotic concentrations, GDA can be checked by colony PCR (cPCR) or quantitative real-time PCR (qPCR). In the first approach, a pair of primers that specifically amplify the novel junction created by the SBF can be used (e.g., primers used for PCR 3 in Fig. 4). Although this method is not able to quantify copy number, it is a simple and fast way to screen possible amplification mutants. To quantify gene copy number, qPCR is used instead. Primers targeting any region of the amplicon can be employed. However, primers that specifically bind the Ab^R marker have the advantage that they can be re-used in different EASy experiments to give reproducible estimations of the gene copy number. In previous work, we have shown that the estimated copy number is virtually the same using primers binding the Ab^R marker or the GOI (Pardo et al., 2020). Clones confirmed to have multiple copies of the amplicon can then be transferred to the selective medium for ALE. The antibiotic is omitted at this point so that gene dosage of the amplicon can vary and be selected based on the desired phenotype.

In a variation of this approach, two different chromosomal regions of the same parent strain can be amplified. This approach was recently demonstrated using the ΩSm/Sp^R marker to select amplification in one chromosomal region and the ΩKm^R marker in a different region (unpublished data). In this case, the two distinct regions were manipulated sequentially in the construction of a synthetic metabolic pathway. The goal is to build complex pathways in a modular fashion. This approach is designed to alleviate problems caused by imbalances between the synthesis and degradation of metabolic intermediates that might be toxic. After moving the doubly-amplified populations to selective growth medium, without antibiotics, the new pathway may evolve to reduce the accumulation of toxic metabolites via variation in the gene dosage of different parts of the pathway.

2.4 Interpreting gene copy number estimations and obtaining single-copy mutants

GDA is a highly dynamic process, where gene copy number rapidly fluctuates, and copy number decreases in the absence of selection. Therefore, genomic DNA extracted from cells growing under the selective conditions should be used for an accurate estimation of the gene copy number by qPCR. It is important to note that this number represents the average for all the cells in a population. On occasions, the gene copy number for an evolving population will stabilize at 2–4 copies, but a significant proportion of individual cells will carry a single copy of the amplicon. In Section 4.4, we provide two approaches to isolate single-copy mutants. The first

approach is based on the screening of isolated colonies from the evolving population for the absence of the SBF, indicative of a single copy of the amplicon (Section 4.4.1). The second approach consists of replacing the tandem repeats of the amplicon with a single copy of the GOI (Section 4.4.2). An overview of this allelic replacement method is shown in Fig. 5. In a first step, the tandem amplicon array in an evolved isolate is replaced with a selectable/counter-selectable marker, such as the *sacB*-Km^R cassette (Jones & Williams, 2003). The counter-selectable marker allows the isolation of mutants in a second transformation step, in which a single copy of the non-evolved amplicon is re-introduced. It should be noted that if the original GDA was selected with one Ab^R marker (such as Km^R) a different marker (such as Sm/Sp^R) should be used in combination with the *sacB* counter-selectable marker.

Once mutants have been isolated in which the evolved amplicons have been replaced with a single copy of the original version of this DNA, further analyses are possible (Fig. 5B). If these mutants grow in the selective medium, then the mutations accrued throughout the chromosome are sufficient to sustain growth with a single copy of the non-evolved GOI. If such mutants do not grow in the selective medium, they can be transformed with individual copies of the evolved GOI alleles, amplified from the multi-copy population by PCR. Beneficial mutations introduced by allelic replacement can be selected and identified (Fig. 5C). We note that recombination between different alleles of the GOI may occur during PCR or transformation. Therefore, mutations that were present in separate copies of the gene may be combined in the same allele. Likewise, two mutations that are sufficiently distant in the same copy of the GOI may be segregated. By plating cells transformed with the evolved alleles on solid ALE selective medium, faster-growing clones that acquire a single copy of an allele with beneficial mutations can be identified and isolated for sequencing. However, given enough time, spontaneous amplification mutants may also arise. The novel junction may occur anywhere in the chromosome—even several kbp apart from the GOI. In this case, screening for the absence of the SBF does not assure that mutants with the desired phenotype will present a single copy of the GOI. Similarly, a PCR using primers that bind the upstream and downstream regions flanking the insertion locus of the amplicon may still give a band resembling that of the single-copy parent strain before GDA. Therefore, the presence of the GOI in single copy needs to be confirmed by qPCR or whole-genome sequencing. Any method of whole-genome sequencing should be sufficient for such purposes.

The final goal of EASy is to isolate beneficial mutations that improve cell fitness under the selective conditions. Ideally, the epistatic effect of the mutations accrued during ALE allows growth of evolved mutants with a single copy of the amplicon. These mutations are identified with whole-genome sequencing of isolated evolved clones and mapping to the reference genome sequence with appropriate software (Deatherage & Barrick, 2014). The latest annotated genome sequence for *A. baylyi* APD1 is deposited in GenBank under accession number NC_005966. It is possible, however, that evolved mutants with a single copy of the amplicon cannot be obtained, despite following the protocols mentioned above. For example, it may not be possible to remove and reintroduce amplicon copies as illustrated in Fig. 5. One issue that may arise with ALE experiments is a reduction in the transformability

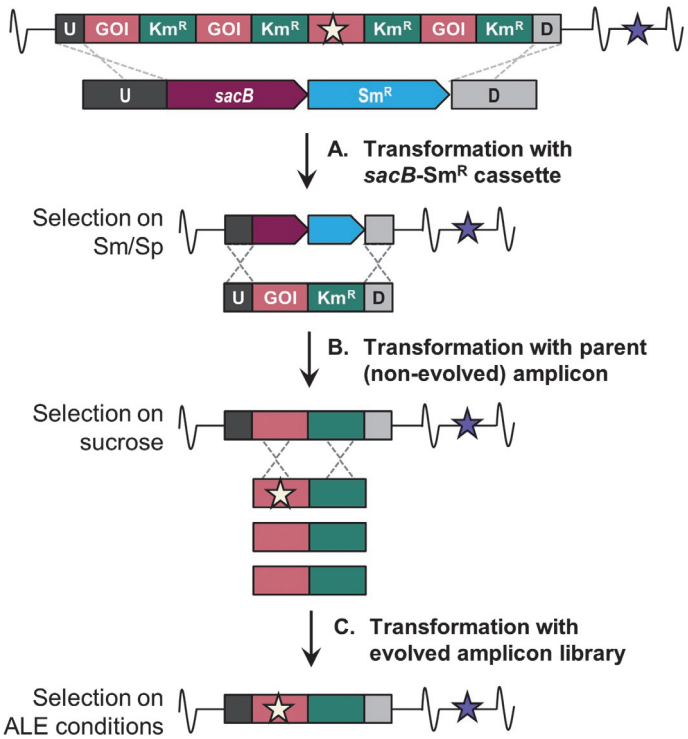

FIG. 5

Overview of the allelic replacement strategy to obtain single-copy mutants from EASy experiments. (A) An evolved isolate from an EASy experiment carries four copies of the amplicon, which encompasses the gene of interest (GOI) and a kanamycin selection marker (Km^R). One of the copies of the gene of interest presents a beneficial mutation (white star). A second beneficial mutation has been selected elsewhere in the chromosome (purple star). The multi-copy amplicon array is replaced by a DNA cassette carrying a streptomycin/spectinomycin selection marker (Sm^R) and a *sacB* counter-selection marker, flanked by sequences identical to upstream (U) and downstream (D) chromosomal regions for homologous recombination. Transformants are selected on Sm/Sp plates. (B) Mutants are then transformed with the parent, non-evolved amplicon. Transformants are selected on medium with sucrose. (C) Mutants obtained in step B are then transformed with single copies of the amplicon derived from the initial EASy isolate. Transformants that incorporate mutated alleles that provide a growth benefit are selected on the same medium used for ALE.

of ADP1. This problem can be avoided using a transposon-free strain, ADP1-ISx (Suárez et al., 2017). The choice of parent strain may depend on the goal of the experiment. In some cases, we prefer to use ADP1, in which the insertion sequences are intact, because transposition generates beneficial genomic mutations during ALE. Regardless of whether a single copy of the GOI is obtained, valuable information can be gained from whole-genome sequencing data, as we discuss in the Section 5.

3 Material and equipment

3.1 Strains and culture media

- *A. baylyi* ADP1 (from culture collections ATCC 33305 or DSM 24193). A transposon-free derivative of ADP1 is also available, ADP1-ISx (Suárez et al., 2017).
- Chemically-competent *E. coli* cells to be used as hosts for plasmid construction and maintenance (any host strain). Typical strains used for this purpose include *E. coli* strains DH5α and XL1-Blue (Agilent Technologies).
- Metals 44 solution (per litre, 2.5 g EDTA; 10.95 g $ZnSO_4 \cdot 7H_2O$; 5 g $FeSO_4 \cdot 7H_2O$; 1.54 g $MnSO_4 \cdot 7H_2O$; 392 mg $CuSO_4 \cdot 5H_2O$; 250 mg $Co(NO_3)_2 \cdot 6H_2O$; and 177 mg $Na_2B_4O_7 \cdot 10H_2O$)
- Concentrated base solution (per litre, 20 g nitriloacetic acid dissolved in 600 mL H_2O with 14.6 g KOH; 28.9 g $MgSO_4$; 6.67 g $CaCl_2 \cdot 2H_2O$; 18.5 mg $Mo_7O_{24} \cdot 4H_2O$; 198 mg $FeSO_4 \cdot 7H_2O$; and 100 mL of Metals 44 solution)
- Minimal medium for *Acinetobacter* (per litre, 25 mL 0.5 M KH_2PO_4; 25 mL 0.5 M Na_2HPO_4; 10 mL 10% $(NH_4)_2SO_4$; 1 mL concentrated base) supplemented with an appropriate carbon source (e.g., 20 mM pyruvate, succinate, or acetate). Although organic acids tend to be preferred to sugars, glucose (25–50 mM) can be used. A longer lag phase will be observed with glucose. In addition, casein amino acids are good carbon sources and can be added in concentration 0.2% (with or without other carbon sources). For plates, 1.5% agar is used.
- YTS agar plates for sucrose counterselection (autoclave 3 g yeast extract, 6 g tryptone, 11 g agar in 300 mL water; then add 300 mL filter-sterilized 50% sucrose)

3.2 Reagents for DNA manipulation

- Custom PCR primers
- Cloning vector, such as pUC18 or pUC19 (Yanisch-Perron, Vieira, & Messing, 1985)
- Plasmid carrying the ΩKm^R or $\Omega Sm/Sp^R$ fragment, e.g., pHP45Ω-Km (Fellay et al., 1987), pUI1637, pUI1638 (Eraso & Kaplan, 1994)
- High-fidelity DNA polymerase and additional components (e.g., NEB Phusion)
- Taq polymerase and reagents for colony PCR (e.g., Bioline MyTaq HS Red Mix, NEB LongAmp Taq DNA polymerase)
- NEBuilder HiFi DNA assembly master mix (NEB)
- Restriction enzymes
- DNA ligase (e.g., NEB Quick ligation kit, T4 DNA ligase)
- TaqMan probes
- TaqMan Gene Expression Master Mix (Thermo Scientific)
- Agarose
- TAE (Tris Acetate EDTA) buffer, final working solution for electrophoresis running buffer (1×): 40 mM Tris, 20 mM acetic acid, and 0.4 mM EDTA
- Plasmid purification kit (e.g., Zymo Research ZR Plasmid miniprep kit)

- PCR purification kit (e.g., Zymo Research DNA Clean & Concentrator kit)
- Genomic DNA (gDNA) extraction kit (e.g., Zymo Research Quick-DNA Miniprep Plus kit)

3.3 Equipment

- Orbital shaker
- Incubator
- DNA electrophoresis chamber
- UV or blue-light transilluminator
- NanoDrop or comparable DNA quantification instrument
- Thermocycler
- Real-time PCR cycler
- Spectrophotometer or device for following cell growth

4 Experimental procedures

4.1 Construction of the amplicon and chromosomal integration

As indicated in Section 2, different DNA manipulation techniques can be used to assemble the cassette that will be integrated into the *A. baylyi* chromosome. Here, we describe a procedure using NEBuilder HiFi DNA assembly for plasmid cloning as described in Pardo et al., 2020 (Fig. 6):

1. Design PCR primers for the amplification of the gene of interest (including 5′ and 3′ untranslated regions) and the upstream and downstream homology regions (~1 kbp each) of the targeted insertion locus. These oligos should have 15–20 nt overhangs complementary to each other and the cloning vector for subsequent assembly. Additionally, the reverse primer amplifying the gene of interest and the forward primer amplifying the downstream homology region should include a restriction site for the insertion of the ΩKm^R fragment in Step 7.
2. Amplify the upstream and downstream homology regions using *A. baylyi* genomic DNA (gDNA) or whole cells as template with a high-fidelity DNA polymerase (e.g., Phusion) following the manufacturer's indications. If whole cells are used, include an initial denaturation step at 95 °C for 5 min in the PCR program. Follow the same protocol to amplify the gene of interest from gDNA of a heterologous host, plasmid, or synthetic DNA.
3. Linearize the cloning vector (e.g., pUC19) by PCR or digestion with restriction enzymes.
4. Purify the PCR and/or digestion products and measure DNA concentration with NanoDrop (or comparable instrument for quantification).
5. Prepare NEBuilder HiFi DNA assembly reaction including the upstream and downstream homology regions, the gene of interest, and the linearized cloning vector following the manufacturer's indications.

6. Transform an aliquot of the assembly reactions into chemically-competent *E. coli* and select on LB agar with the appropriate antibiotic.
7. Purify plasmids by miniprep and verify correct assembly by sequencing or other methods.
8. Digest the plasmid carrying the ΩKm^R fragment (e.g., pUI1637) with a restriction enzyme whose target sequence is present on the inverted flanking polylinkers. Purify the ΩKm^R fragment in an agarose gel (2.4 kbp). Digest the plasmid carrying the gene of interest (recipient) with the same enzyme.
9. Prepare a T4 ligase reaction with the recipient plasmid and the ΩKm^R fragment.
10. Transform an aliquot of the ligation reactions into chemically-competent *E. coli* cells and select on LB agar +50 mg/L Km.
11. Purify plasmids by miniprep. Optionally, the orientation of the ΩKm^R fragment can be checked by PCR or diagnostic restriction digestion.
12. Digest the resulting plasmid with a restriction enzyme cutting in the vector backbone for transformation into *A. baylyi*. No purification of the DNA or heat inactivation of the enzyme is necessary.
13. Transform DNA into *A. baylyi* using any of the protocols provided in Bedore et al. (2023).

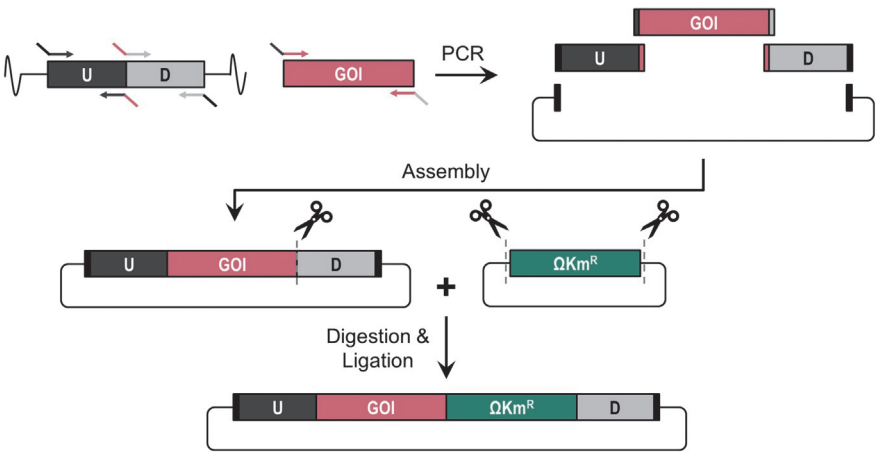

FIG. 6

Workflow for the construction of the amplicon in a replicative plasmid for subsequent integration in *Acinetobacter baylyi*. The gene of interest (GOI) and the flanking upstream (U) and downstream (D) chromosomal regions for homologous recombination are amplified by PCR, using primers with appropriate 15–20 nt overhangs for NEBuilder HiFi DNA assembly into a cloning vector. The resulting plasmid (recipient) and the donor plasmid carrying the ΩKm^R fragment are digested with the same restriction enzyme. Then, the ΩKm^R fragment is ligated into the recipient plasmid between the GOI and the downstream homology region.

4.2 Construction of the SBF and amplification of gene copy number

Once the desired DNA cassette has been integrated into the *A. baylyi* chromosome, the resulting mutant (i.e., parent strain) is transformed with the SBF to facilitate gene duplication. Here, we describe the construction of the SBF using sequence overlap extension PCR (Fig. 4), although other DNA assembly techniques can be used (or can be commercially synthesized). For future experiments, it is convenient to construct a plasmid carrying the SBF that can be stored and used as needed.

1. Amplify the first ~1000 bp of the target amplicon using a forward primer with a 15–20 bp overhang identical to the 3′-end of the amplicon, and a standard reverse primer. Use a high-fidelity DNA polymerase and the plasmid or mutant strain carrying the DNA cassette constructed in Section 4.1 as template.
2. Similarly, amplify the last ~1000 bp of the target amplicon using a standard forward primer and a reverse primer with 15–20 bp overhang that is the reverse complement of the 5′-end.
3. Purify the PCR and/or digestion products and measure DNA concentration.
4. Prepare a primer-less PCR mix using ~30 ng of each of the of the purified DNA fragments and amplify for 15 cycles. The 30–40 bp stretch of sequence identity generated by the overlapping primers should have a melting temperature of 55–65 °C. Then, add the standard forward and reverse primers used in steps 1 and 2, respectively (i.e., primers *3* and *4* in Fig. 4). Amplify the full-length SBF for another 15 cycles.
5. Check correct assembly of the full-length SBF in an agarose gel (expected size of ~2 kbp). If needed, purify the correct band by excision from the agarose gel.
6. Transform the SBF fragment into the parent *A. baylyi* strain containing a single copy of the region targeted for amplification. For selection of amplification mutants, plate cells on minimal medium containing 1 g/L Km.
7. We recommend streak purification of colonies at least three times on 1 g/L Km plates to rule out false positive clones. Often, colonies grow poorly on the first transfer.
8. Confirm integration of the SBF in the high-antibiotic resistant colonies by PCR, using the same primers used for amplification of the SBF in step 4. Amplification mutants should be maintained under selective pressure, such as in the presence of high antibiotic concentration, to prevent undesired decrease of the amplicon copy.

4.3 Adaptive laboratory evolution and monitoring of gene copy number over time

Selection of the amplification mutants in high antibiotic concentrations ensures obtaining colonies in 1–2 days, which can be quickly checked for increased gene dosage by colony PCR or qPCR. The confirmed amplification clones can then be transferred to the desired solid selective medium without antibiotic, e.g., minimal medium

with a non-native carbon source. In this way, the only selective pressure is the target condition for adaptive laboratory evolution (ALE). In our experience, it can take up to 2 weeks for colonies to appear on the selective agar plates, although growth will be faster when re-streaked on a second selective plate. These clones can then be used to initiate an ALE experiment, which we describe below following a serial passage method. Generally, as in any ALE experiment, it is recommended to start with a moderate selective pressure and gradually increase it over time, e.g., substrate concentration, temperature, dilution, transfer rate. Optionally, the changes of gene copy number over time can be monitored by qPCR. Here we describe a method using fluorescent probes, e.g., 6FAM-MGBNFQ labelled TaqMan™ probes (Thermo Scientific). Fluorescent DNA-binding dyes such as SYBR Green can also be used, but we find reduced background noise when using probes. The qPCR protocol described below specifies the DNA concentrations that we normally use and that give suitable amplification curves to estimate gene copy number. We note that we have been able to detect copy numbers above 100 in an EASy experiment.

4.3.1 Adaptive laboratory evolution by serial transfer

1. Inoculate sterile test tubes containing at least 2 mL liquid selective medium with the amplification clone(s). Use a heavy inoculum for this first transfer to liquid medium. Incubate under the appropriate conditions.
2. Once stationary phase is reached, transfer an aliquot of the culture to fresh medium, diluting cells at least 100-fold. Repeat this operation as many times as needed until the desired phenotype is reached or no further improvements are observed.
3. While performing the serial transfers, periodically remove an aliquot of the culture for preservation of the cells as glycerol stocks and extraction of genomic DNA for the quantitation of the average gene copy number of the evolving population (e.g., once a week).
4. Extract genomic DNA with a molecular biology kit (e.g., Quick-DNA Miniprep Plus kit from Zymo Research) following the manufacturer's instructions and measure DNA concentration with NanoDrop. Purified DNA can be stored at −20 °C or −80 °C for long-term storage.

4.3.2 Gene copy number analysis by quantitative PCR

1. Design primer pairs and probes targeting the amplicon and a reference gene that is present in single copy in the chromosome using an appropriate software. In Table 1, we provide the sequences used for qPCR of the Tn*5* *nptII* gene present in the ΩKm^R cassette with *rpoA* as reference gene.
2. Prepare separate master mixes (without DNA) for quantitation of the *nptII* (Km^R) and *rpoA* genes with the TaqMan™ Gene Expression Master Mix. The final reaction mixes should contain each primer and probe at concentration of 0.2 µM.
3. Dispense the master mixes into separate wells in a real-time PCR 96-well plate, with at least 3 technical replicates per sample and target. Add DNA to a final

Table 1 Primer and probe sequences for qPCR.

Target	Forward primer	Reverse primer	Probe
nptII[a]	gcgttggctaccc gtgata	ggaagcggtcagcccatt	tgaagagcttggcggc
rpoA[b]	gctcgacgccttc tatttcaa	tttacgtcgcattctatt gtcttctt	tcaaccacagcagcgc caggc

[a]*Sequence within the ΩKm^R cassette, used as a measure of amplicon gene dosage.*
[b]*Sequence within a housekeeping gene, used as a measure of a single-copy chromosomal gene.*

concentration of 0.05 ng/μL to each well such that the final reaction volume is 20 μL (1 ng DNA per well). Genomic DNA from the parent strain containing a single copy of the amplicon should be included as control.

4. Using the genomic DNA from the parent strain as template, prepare a calibration curve with increasing amounts of DNA. We typically use a 5-point curve from 0.02 to 12.5 ng gDNA per well, prepared as 5-fold serial dilutions.
5. Cover the plate with an optically-clear adhesive film appropriate for real-time PCR and run a program with the following cycles: 50 °C for 2 min; 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min.
6. Using appropriate software, quantify the amount of *nptII* and *rpoA* for each sample. The *nptII*:*rpoA* ratio is considered to be equivalent to the relative gene copy number of the amplicon. The ratio for the parent strain should be equal to one.

4.4 Obtaining single-copy mutants from EASy

The ultimate goal of EASy is to obtain mutants with a single copy of the amplicon thanks to the accumulation of beneficial mutations. These mutations may appear in the GOI and/or in other chromosomal loci that provide a growth benefit to the cell when combined. On occasions, a single copy of the amplicon will not be reached following ALE. Nevertheless, single-copy mutants can sometimes be obtained by screening or by allelic replacement.

4.4.1 Isolation and screening by colony PCR

The gene copy number that results from qPCR analysis is an average of the cells that make up the population. Therefore, in a population with a copy number of 4 or less some cells will present a single copy of the amplicon. These can be isolated using the following procedure:

1. Dilute and plate cells from the evolving population on the same selective medium used for ALE with agar. To obtain well isolated colonies, it is generally sufficient to plate 100 μL of a 10^{-6} dilution from a saturated culture ($OD_{600} \sim 2$).

2. Screen isolates for the absence of the SBF by colony PCR (cPCR), using the same pair of primers as in Section 4.2, step 7. Only colonies that still possess multiple copies of the amplicon should give a product of the expected size. However, since the qPCR-determined copy number represents the average in a population, colonies need to be screened to identify those with a single copy of the region of interest. If the average copy number is 2 or higher, we recommend screening at least 100 colonies.
3. Confirm the phenotype of the SBF-negative isolates by streak purification (at least three times) on selective medium to ensure a pure clonal population. We also recommend genotypic and phenotypic confirmation of the presence of the Ab^R gene that was initially present in the parent strain prior to amplification and evolution (Section 4.1).
4. Extract genomic DNA from the selected isolates and repeat the PCR to check for the presence of the SBF. Simultaneously, perform additional PCRs using primers that can distinguish alternative conformations. For example, with a primer pair, each of which binds outside of the amplicon, only clones that harbour a single copy of the amplicon should give an appropriately sized product. However, experimental design may need to be tailored to the specific situation.
5. Clones that are confirmed to have a single copy of the amplicon can be further analysed by Sanger sequencing of the GOI or whole-genome sequencing to identify beneficial mutations.

4.4.2 Allelic replacement in evolved populations

This method consists of a series of transformations to replace the tandem amplicon array from an isolated clone of the evolved population with a single copy of the amplicon. This method facilitates analysis of the fitness contribution of mutations in the GOI (or in other regions of the chromosome). However, the success of this method may be limited by the known loss of natural competency of *A. baylyi* during ALE (Renda, Dasgupta, Leon, & Barrick, 2015).

1. Transform an isolated clone from the evolved population with a DNA cassette carrying a Sm/Sp^R selectable marker and a *sacB* counter-selectable marker. This DNA cassette should be flanked by at least 1000 bp of sequence identical to the upstream and downstream regions of where the tandem array of the amplicon is inserted. The same DNA assembly strategy as the one used for the initial integration of the amplicon can be used (Section 4.1).
2. Select mutants on agar plates containing 25 mg/L Sm/Sp. Lower transformation efficiency may occur due to the loss of natural competency during ALE.
3. Confirm Sm/Sp^R colonies for correct allelic replacement by cPCR. Also confirm sensitivity to Km and the inability to grow on the ALE selective medium.
4. Transform mutants from step 3 with a single copy of the non-evolved amplicon, as in Section 4.1. Select for sucrose-resistant transformants on YTS. Since sucrose selection can have high background growth, re-streak colonies on fresh YTS plates at least three times before confirming by cPCR.

5. Amplify individual copies of the amplicon from the evolved isolate by PCR, using primers that bind the ends of the amplicon. Alternatively, amplify only the GOI.
6. Transform cells containing a single copy of the non-evolved allele with the PCR products obtained in step 5. These PCR products can be integrated by direct recombination with the allele present in the chromosome. Include a no DNA control.
7. Select transformants on plates containing the selective medium used for ALE. Colonies growing faster than the control are expected to have acquired evolved alleles with beneficial mutations.
8. Confirm that mutants growing on the selective medium present a single copy of the gene of interest by qPCR.

5 Summary and concluding remarks

In this chapter, we provide general guidelines to engineer new phenotypes in *A. baylyi* via controlled GDA followed by ALE. Although we focus on expansion of the catabolic capabilities of *A. baylyi*, the EASy method has potential for use in any application in which the desired phenotype can be linked to improved growth. For example, EASy might facilitate the isolation of mutations that increase detoxifying activities or that modify the substrate preference of promiscuous enzymes. The principle of EASy is to compensate for initial low gene expression or enzymatic activities with increased gene dosage. Indeed, the single-copy parent strains used in our EASy experiments were unable to grow under selective conditions, making it impossible to use direct selection in ALE without increased gene dosage. Furthermore, we did not observe spontaneous GDA in the parent strains. With EASy, one can target the amplification of the GOI by transforming with a SBF. Since direct selection for the desired phenotype may be too demanding to the cells, GDA is first induced in the presence of high antibiotic concentrations. Growth under these conditions is achieved by introducing an Ab^R marker in the target chromosomal segment, i.e., the amplicon. With this strategy, multi-copy mutants can be obtained within 1–2 days to be transferred to ALE conditions.

The EASy method is highly tuneable and can be adapted to various standard DNA manipulation techniques. This flexibility is enabled by the natural competence and genetic malleability of *A. baylyi*. These same host properties can be exploited to screen beneficial mutations acquired from ALE by reverse engineering (Bedore et al., 2023; Luo et al., 2022). However, EASy presents some limitations. Importantly, there is no "counter-selective" pressure to drive the decrease of gene copy number, other than the inherent instability of the tandem amplicon array. Therefore, it is possible that a single-copy of the amplicon is not fixed in the evolving population after several generations of ALE. In some cases, when the average copy number is 2–4, it is possible to obtain single-copy mutants, as

we have described in Section 4.4.2. In others, average gene copy numbers above 10 make this task more difficult, as we observed for EASy of TPA utilization.

While selection for high-level gene dosage using antibiotic selection is convenient, there are situations where it may be preferable to use direct selection after transformation with the SBF. Our initial EASy studies to achieve growth on guaiacol and TPA did not allow direct selection. However, recent studies using selection for growth without antibiotic markers have been more successful (unpublished results). By monitoring growth in liquid cultures, the positive effect of gene amplification on growth has been observed without first using antibiotic selection. While this approach is still under development, one benefit could be that gene dosage will more closely match that needed for the target selection. Thus, if selection initially demands low-level amplification, the problem of incomplete allele segregation may be reduced.

Regardless of problems that result if a haploid state is not obtained at the end of EASy experiments, valuable information can still be obtained from the whole-genome sequence data of isolates that maintain multiple amplicons. Beneficial mutations that are selected elsewhere in the chromosome can be identified and reverse engineered into the single-copy parent strain to evaluate their phenotypic effect. For example, during EASy of TPA utilization, we identified mutations that improved TPA uptake in *A. baylyi*. When these mutations were engineered into the single-copy parent strain, we observed spontaneous GDA of the *tph* catabolic genes that led to a Tpa^+ phenotype, in contrast to the unmodified parent strain. This result proved that uptake of TPA was the first limiting step to obtain TPA-utilizing mutants. The EASy-mediated or spontaneous GDA can be identified with whole-genome sequencing by an increased coverage of certain regions of the chromosome, usually delimited by short sequence stretches with a high density of polymorphisms—indicative of novel junctions. For the analysis of whole-genome sequencing data, we refer the reader to the open-source *breseq* computational pipeline, which we have found is optimal for the detection of novel junctions formed during GDA (Deatherage & Barrick, 2014).

In conclusion, EASy is a powerful tool to engineer improved cell catalysts through laboratory evolution. Additionally, EASy can be applied in more fundamental research addressing gene regulation or the dynamics of GDA in a microbial population. In all, the simplicity and versatility of the EASy method in combination with the natural competence of *A. baylyi* make this tool accessible to most microbiology laboratories.

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