1 **Running Title:** Natural transformation to engineer and analyze *A. baylyi*

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3 **Natural transformation as a tool in *Acinetobacter baylyi*: streamlined engineering and mutational**

4 **analysis**

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ABSTRACT

Natural transformation and homologous recombination in a soil bacterium, *Acinetobacter baylyi* ADP1, occur with exceptionally high efficiency. These genetic features can be harnessed to address a wide variety of fundamental and applied scientific topics. Recent advances in synthetic biology and laboratory evolution have led to renewed appreciation for the use of *A. baylyi* as a model organism. To complement several review articles that highlight new tool sets, this chapter focuses on simple protocols and examples of transformation assays that facilitate genetic analysis and engineering. Whole genome sequencing often reveals extensive genetic variation among closely related isolates that can confound the association of genotypic and phenotypic changes. In *A. baylyi,* such associations can be deciphered in unique ways by directly transforming cells with linear DNA fragments. The resulting allelic replacement, which occurs at high frequency, rapidly generates desired mutants via targeted chromosomal editing. Diverse screening and selection methods can be used to test hypotheses and streamline experimental strategies to reveal the significance of specific DNA sequences. Moreover, large procedural variations are well tolerated, and techniques can be readily adapted for new purposes. One goal of highlighting natural transformation methodology in *A. baylyi* is to expand the community of researchers using this versatile bacterial host.

Keywords

Acinetobacter baylyi ADP1; natural competence; allelic replacement; transformation; genome editing

38 **Abbreviations**

39 **Ap**	ampicillin
40 **CEMENT**	combinatorial evaluation of mutations examind by natural transformation
41 **Cm**	chloramphenicol
42 **EASy**	evolution by amplification and synthetic biology
43 **Gm**	gentamicin
44 **Km**	kanamycin
45 **LB**	lysogeny broth (also known as Luria-Bertani medium)
46 **MM**	minimal medium
47 **Pob**	*p*-hydroxybenzoate
48 **RAMSES**	rapid advantageous mutation screening and selection
49 **Sm**	streptomycin
50 **Sp**	spectinomycin

51 **1. INTRODUCTION**

52 *Acinetobacter baylyi* ADP1 has a remarkably efficient system of natural competence and
53 homologous recombination that is ideal for a wide variety of experiments. Several publications highlight
54 these features and describe new toolkits and optimized methods for this bacterium (Biggs et al., 2020; de
55 Berardinis, Durot, Weissenbach, & Salanoubat, 2009; Elliott & Neidle, 2011; Metzgar et al., 2004;
56 Santala & Santala, 2021; David M. Young, Parke, & Ornston, 2005). Our goal is to complement these
57 publications by illustrating the power of simple transformation techniques that tolerate large variations in
58 the amount and type of donor DNA, the state of the recipient cells, and the incubation conditions. The
59 technical simplicity of such transformation makes it possible to use and modify methods for diverse
60 applications. The provision of easy-to-follow protocols is intended to facilitate the increased adoption of
61 strain ADP1 as a model organism.

62 In the 1980s, we demonstrated the utility of allelic replacement to reveal mutations present on
63 relatively small DNA fragments in *A. baylyi* (previously designated *A. calcoaceticus*) (Neidle & Ornston,
64 1986). At that time, there were few molecular biology resources for bacteria other than *Escherichia coli*.
65 Chromosomal alteration by natural transformation enabled the modification of a defined genomic
66 segment to be displayed as a specific phenotype. Additionally, this approach obviated the need for
67 expression vectors, which were not yet available for *A. baylyi*. Decades later, the range of available
68 expression vectors and DNA constructs is nearly limitless. Nevertheless, the ability to analyze
69 chromosomal change via transformation-based methods in *A. baylyi* continues to push the boundaries of
70 current techniques.

71 A recently developed method, called rapid advantageous mutation screening and selection
72 (RAMSES), enhances adaptive laboratory evolution techniques by exploiting natural transformation (Luo,
73 McIntyre, et al., 2022). This method addresses two common issues that can impede the practical utility of
74 evolved strains selected for desired phenotypes. First, such strains typically carry multiple mutations.
75 Determining the significance of these mutations, alone or in combination, is usually laborious and time
76 consuming. Second, introducing these mutations into new strains with different genetic backgrounds can
77 be complicated, especially if multiple changes are needed. Both problems can be overcome using
78 experiments in which PCR-generated fragments carrying mutations from the adapted strain are added
79 directly to growing cultures. Under selective conditions, these specific mutations get incorporated into the
80 chromosome by allelic replacement. Cells with a competitive advantage emerge over time, and whole
81 genome sequence analysis of the competitive "winners" reveal beneficial mutations. This approach makes
82 reverse engineering easy and informative. Moreover, the construction of new strains for specific
83 applications can be readily accomplished with minimal effort (Luo, McIntyre, et al., 2022).

84 In addition to new applications, natural transformation remains a powerful tool for inexpensive
85 and rapid studies that can be done by students at all levels. A demonstration of genetic principles using *A.*
86 *baylyi* natural competence was developed as a college level laboratory exercise many years ago, using a
87 protocol that is still in use (Earnest & Rosenbaum, 1993). In a laboratory course at the University of

Georgia (U.S.A.), research using *A. baylyi* transformation permits students, with relatively little research experience, to conduct authentic projects that test novel hypotheses and yield publishable metabolic insights (Bedore et al., 2022; Stoudenmire et al., 2017).

A. baylyi is not unique in being naturally transformable, but it is unusual in being highly competent throughout its growth phase and early into stationary phase. This feature of nearly continuous competency, combined with an active homologous recombination machinery, makes the genetic manipulation of ADP1 unrivaled for ease and efficiency. For example, in a comparison of *A. baylyi* and *Bacillus subtilis* as hosts for a method based on recombinational capture of PCR products, *A. baylyi* was found to be approximately 100-fold more efficient than *B. subtilis* (Melnikov & Youngman, 1999). To date, there is no evidence of fundamental differences in recombination or mutational processes compared to other bacteria. For example, although we tout the plasticity of strain ADP1 and exploit gene amplification, the frequency of spontaneous duplication in *A. baylyi* is comparable to that in *E. coli* (Seaton et al., 2012).

One factor affecting allelic replacement, and its corresponding utility, could be a low level of DNA restriction and/or degradation following transformation. Small fragments and DNA from diverse sources are readily integrated into the genome following transformation. Recent attempts to improve recombinational frequencies by inactivating the single restriction modification system of strain ADP1 failed to have an effect, regardless of the source of DNA (Jiang et al., 2020). Thus, while future studies are needed to improve understanding of some aspects of natural transformation/recombination, the practical ease of genetic manipulation results in the ongoing expansion of metabolic engineering and synthetic biology applications for *A. baylyi* (Jiang et al., 2020; Luo, Efimova, Volke, Santala, & Santala, 2022; Suarez et al., 2020).

2. GENERAL CONSIDERATIONS

2.1. Convenience and optimization

Successful transformation of *A. baylyi* strains can be accomplished with large variations in methodology. In all cases, the ease of genome editing derives in large part from the ability to use linear donor DNA. Whether this donor DNA consists of PCR products, linearized plasmids, or genomic DNA, recombination with the recipient chromosome yields viable transformants only when allelic replacement occurs (**Fig. 1A**). In contrast, many systems depend on transformation with circular plasmids, wherein lowered rates of success (or other complications) may result because single recombination events integrate the entire plasmid in the genome. As illustrated in **Fig. 1**, a wide range of chromosomal changes can be made by allelic replacement, including large and small insertions, deletions, and/or the targeted replacement of specific genes or chromosomal regions. Markers can be used for selection and/or counter selection. In circumstances where selection for a specific replacement is not possible, it is feasible to screen for the desired transformants. Typically, it is sufficient to screen several hundred colonies from a nonselective plate following transformation. PCR or other methods can be used to identify the correct transformant(s) by checking individual or pooled isolates.

[INSERT FIGURE 1 HERE]

Since experiments can be done using different methods, the exact protocol chosen may depend on convenience. The care taken to optimize experimental procedures should be guided by the specific scientific goals. For example, in the case of simple allelic replacement with a selectable marker (**Fig. 1**), the highest possible efficiency of transformation and recombination should not be needed. For this purpose, cells may be used in almost any growth phase, and donor DNA may require very small stretches of sequence identity to promote homologous recombination with the chromosome. In contrast, if the isolation of the desired transformant requires screening, it may be worthwhile to obtain the highest allelic replacement efficiency that is possible. Throughout this article, and its companion ("Natural transformation as a tool in *Acinetobacter baylyi*: Evolution by amplification of gene copy number"), we indicate where variation in methodology may be tolerated. Moreover, genomic context can affect what

139 conditions are optimal for a specific purpose in ways that are not predictable (Seaton et al., 2012). Thus,
140 the use of various experimental protocols and/or trying multiple approaches in parallel can help ensure
141 success.

142 As in all transformation protocols, important issues include: 1) preparation of the recipient cells
143 and donor DNA, 2) introduction of DNA into cells and growth conditions following transformation, and
144 3) the isolation and confirmation of desired transformants. These issues are discussed below. In addition,
145 examples of transformation assays are provided to illustrate a range of applications that are uniquely
146 possible with this bacterial system.

147

148 **2.2. Preparation of recipient cells and donor DNA**

149 Different culture conditions for *A. baylyi* may be used for transformation experiments. Systematic
150 studies to establish a standardized workflow determined that high transformation efficiency resulted from
151 growth at 30 °C, with liquid cultures aerated by shaking at 250 rpm (Biggs et al., 2020). In those studies,
152 cells used for natural transformation were grown in rich medium to make procedures as simple as
153 possible. Although it was shown that transformation efficiency decreased with growth at 37 °C, some
154 laboratories successfully and routinely grow *A. baylyi* at 37 °C rather than at 30 °C. The higher
155 temperature is convenient for the use of common equipment for culturing *A. baylyi* and *E. coli*. The
156 transformation efficiency at 37 °C is sufficient for nearly all applications. Moreover, minimal medium
157 rather than rich medium may be used to prepare competent *A. baylyi* cultures. Growth in minimal medium
158 with a single carbon source may reduce contamination (especially in undergraduate laboratory courses)
159 and, in some cases, a higher transformation efficiency is obtained compared to growth in rich medium.

160 To prepare cells for transformation, a starting culture of *A. baylyi* is typically grown overnight.
161 Such cultures are diluted, DNA is added, and additional growth is allowed for approximately 3-6 h, as
162 illustrated by the rightward arrow in **Fig. 2A** (Biggs et al., 2020). Using an alternative protocol, the
163 overnight culture may simply be supplemented with an additional aliquot of a carbon source to allow the

resumption of growth, as illustrated in **Fig. 2** by the downward arrow leading to panels **B** and **C**. In this case, cells require a short period of growth (approximately 30 minutes) prior to spreading competent cells on the surface of a plate with selective or non-selective growth medium. Although this approach may not yield maximal competency, the high concentration of recipient cells usually enables good results. To obtain higher transformation efficiencies, cell growth may be monitored to ensure that cultures are in late exponential phase prior to spreading competent cells on plates, and/or a cell concentration step may be used for a further increase in the number of recipient cells.

[INSERT FIGURE 2 HERE]

Genomic change in *A. baylyi* can be accomplished with diverse types of donor DNA. As described elsewhere, replicative plasmids may be introduced into recipient cells (Biggs et al., 2020). Here we focus on transformations with linear donor DNA to modify the chromosome via homologous recombination, a type of genome editing that is uniquely efficient in *A. baylyi*. This type of allelic exchange requires only that there is sequence identity between the donor and recipient DNA in regions upstream and downstream of the targeted change (**Fig. 1**). A simple way to prepare DNA from a donor *A. baylyi* strain involves heat-based lysis followed by filtration to remove whole cells. This method has the advantage of providing large stretches of DNA for homologous recombination. This method is typically used to combine mutations in different *A. baylyi* strains. In this case, the genomic location of all known mutations should be considered, as multiple allelic exchanges may take place, and several mutations can be co-transformed (Gerischer & Ornston, 2001). Localized or whole genome sequencing of the resulting transformant is recommended to confirm the genotype. Similarly, purified genomic DNA may be used to transform the recipient. If the results are likely to be complex, it may be preferable to use fragments of donor DNA to define the positions where homologous recombination will occur. When plasmid DNA is used, it should be linearized by restriction digestion with conditions that promote complete cutting (e.g., use of multiple enzymes, optimization of the time of restriction enzyme digestion, the amount of DNA,

190 and the amount of enzyme). Uncut plasmid DNA can result in the chromosomal integration of the entire
191 plasmid via a single recombination event. Thus, transformants should always be evaluated to ensure
192 allelic replacement rather than plasmid integration. PCR products from any type of single or multi-piece
193 assembly reaction may be used as donor DNA. Although PCR products need not be purified by gel
194 extraction or the use of a clean-up procedure, such purification steps can simplify experiments by
195 preventing the formation of undesired transformants and/or by improving transformation efficiencies.
196

197 **2.3 Introduction of DNA into cells, and growth conditions following transformation**

198 One transformation protocol involves the addition of DNA to diluted recipient cells followed by
199 continued growth. With this method, transformants can be plated directly on selective medium after
200 several hours of incubation (**Fig. 2A**). Alternatively, cells and DNA can be mixed in different ways on
201 plates (**Fig. 2B and 2C**). The lawn transformation method (**Fig. 2B**) enables rapid assays to study a range
202 of topics related to the significance of mutations. In ways that are not possible in other organisms, specific
203 genotypic and phenotypic effects can be readily correlated (David M. Young et al., 2005). These rapid,
204 and powerful genetic tests can streamline subsequent experimental strategies.

205 Several examples are used to demonstrate how lawn transformation assays can be used (1) to
206 screen libraries of mutated alleles, (2) to evaluate the effects of chromosomal context, and (3) to evaluate
207 multiple mutations (alone and in combination) that arise during adaptive laboratory evolution. In all these
208 examples, a culture sample is spread across the surface of a selective plate containing a medium
209 insufficient for this recipient to grow. In marked spots, cell-free linear DNA is dropped directly on the
210 plate surface. When these selections involve a change in the ability to use a carbon source (or other
211 selection that does not severely inhibit the recipient), rather than acquisition of antibiotic resistance, there
212 is no need for a recovery period involving growth on non-selective medium. Transformation and
213 homologous recombination take place within cells on the plate surface. When allelic replacement alters
214 the phenotype appropriately, growth of transformants is observed in regions corresponding to the
215 position(s) where DNA was dropped.

216 In the first example (**Fig. 3**), an auxotrophic mutant, which lacks a critical enzyme, is unable to
217 grow on minimal medium without amino acid supplementation. DNA encoding a different (non-
218 homologous) enzyme can compensate for the missing *A. baylyi* enzyme and confer growth. The
219 compensating DNA encodes a variant (positive control) of an *Escherichia coli* enzyme whose native
220 sequence does not confer growth (negative control). Alleles encoding different variants of this *E. coli*
221 enzyme can be tested in separate spots. The results not only indicate which variants permit prototrophic
222 growth, but the time needed for colonies/confluent growth to appear in each spot provides preliminary
223 information that correlates with the growth rate of the mutant. This type of rapid analysis enables the
224 identification of mutants with desired phenotypes. Such data can hone subsequent mutagenic strategies
225 and focus further analyses on the most promising candidates.
226
227 **[INSERT FIGURE 3 HERE]**
228
229 In the second example (**Fig. 4**), a similar assay was used to understand a mutation that arose
230 during experimental evolution. In this case, a foreign pathway was introduced into a mutant that was
231 unable to grow on *p*-hydroxybenzoate (Pob) as the carbon source. Isolates that grew on this substrate
232 (Pob^+ derivatives), were obtained with Evolution by Amplification and Synthetic biology (EASy)
233 (Tumen-Velasquez et al., 2018), a method that is described more fully in our companion chapter
234 ("Natural transformation as a tool in *Acinetobacter baylyi*: Evolution by amplification of gene copy
235 number"). One critical mutation for this acquired phenotype inactivates a native ADP1 enzyme (here
236 termed EnzB) that has functional overlap to an enzyme encoded by the foreign pathway (here termed
237 EnzA). Since these enzymes have significant sequence differences, the preference for EnzA could relate
238 to enzyme activity. However, using the transformation assay (**Fig. 4**), it was shown that either enzyme
239 enables Pob^+ growth if the corresponding gene is in the chromosome within the region of the foreign
240 DNA. In contrast, neither enzyme was sufficient for growth if its gene was in the position normally
241 occupied by *enzB*. These results suggest that some type of regulation related to proximity of the

242 associated genes and/or proteins affects metabolic function. This example illustrates the use of
243 chromosomal alteration by natural transformation and allelic replacement to investigate chromosomal
244 context, an issue that is typically difficult to study in other organisms.
245
246 **[INSERT FIGURE 4 HERE]**
247
248 A third example of a transformation assay (**Fig. 5**) illustrates a novel approach to assess the
249 significance of mutational combinations. This plate-based assay is essentially a variation of the RAMSES
250 method described above (Luo, McIntyre, et al., 2022). Strains derived from laboratory evolution
251 experiments are typically found to carry multiple mutations. However, to determine which mutations are
252 required for the selected phenotype can be difficult. In the example shown, a Pob^- parent strain (unable to
253 use Pob as the carbon source), evolved to yield a Pob^+ derivative. Some of the approximately twelve
254 mutations in various locations of the chromosome that were revealed by whole genome sequencing are
255 depicted by asterisks (**Fig. 5A**). In this case, the parent strain was spread on selective growth medium
256 with Pob as the carbon source. Individual linear fragments were obtained that each carry one mutation
257 from the Pob^+ isolate as well as surrounding regions of sequence identity to allow allelic replacement of
258 the corresponding region of the parent strain. After testing various mutations, alone and in combination,
259 the assay revealed that a combination of three specific mutations allowed growth on the selective Pob
260 plate. We have not yet determined the limit of how many individual mutations can be efficiently
261 transformed in this fashion. Nevertheless, this approach, together with RAMSES, can streamline
262 mutational analyses as well as the construction of strains with multiple defined mutations.
263
264 **2.4 Isolation and confirmation of desired transformants**
265 Transformants generated by any of the methods shown in **Fig. 2** should be confirmed after streak
266 purification. Such confirmation can be accomplished using PCR, screening for drug resistance, localized
267 DNA sequencing and/or whole genome sequencing. When linearized plasmids are used as the donor

268 DNA, it is important to ensure that chromosomal changes result from allelic replacement rather than
269 plasmid integration. Another issue to consider is whether the isolation method could lead to the selection
270 of unanticipated mutations. For example, in the experiments shown in **Figs. 3-5**, transformants are
271 selected directly for growth on a given substrate, which could result in the isolation of spontaneous
272 mutations that are not introduced by the donor DNA. While such mutations may be rare, it is important to
273 consider all possibilities. For example, in **Fig. 3**, the introduction of a mutated allele could lead to slow
274 growth that increases the chance of selecting spontaneous mutations that increase the growth rate. To test
275 the phenotypes of transformants that have never been exposed to the selective carbon source, an
276 alternative approach would be to select allelic replacement using expression of the adjacent antibiotic
277 resistance marker. When using drug resistance, the recipients should be allowed to grow on non-selective
278 medium to allow time for recovery and expression before selection in the presence of the antibiotic.
279
280
281 **3. MATERIAL AND EQUIPMENT**
282 **3.1. Strains and culture media**
283 • *Acinetobacter baylyi* ADP1 (from culture collections ATCC 33305 or DSM 24193); genome
284 sequence in GenBank under accession number NC_005966. A transposon-free derivative of
285 ADP1 is also available, ADP1-ISx (Suárez, Renda, Dasgupta, & Barrick, 2017).
286 • Chemically competent *Escherichia coli* cells, to be used as hosts for plasmid construction and
287 maintenance (any host strain). Typical strains used for this purpose include *E. coli* strains DH5α
288 and XLI Blue (Agilent Technologies).
289 • Minimal medium (MM) for *A. baylyi* (per liter, 25 mL 0.5 M KH_2PO_4; 25 mL 0.5 M Na_2HPO_4;
290 10 mL 10% $(NH_4)_2SO_4$; 1 mL concentrated base) supplemented with an appropriate carbon
291 source (e.g. 20 mM pyruvate or 10 mM succinate). Common carbon sources such as glucose and
292 acetate (25-50 mM) also work well, although a longer lag phase is observed with glucose. In

addition, casein amino acids serve as an excellent carbon source (especially if strains overexpress certain proteins) and can be added in concentration 0.2% (with or without other carbon sources). For solid media, 1.5% agar is used.

  - Concentrated base solution (per liter, 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).
  - Metals 44 solution (per liter, 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 $Na_2B_4O_7{\cdot}10H_2O$).

- Minimal medium, different formulations: alternative recipes may also be used for *A. baylyi*. Examples of alternative defined media are described elsewhere (Biggs et al., 2020; Hartmans, Smits, van der Werf, Volkering, & de Bont, 1989).
- Rich medium, such as Lysogeny Broth (LB, also known as Luria Bertani medium), (per liter, 10 g Bacto-tryptone, 5 g yeast extract and 10 g NaCl); Note: an alternative composition with only 1 g per liter NaCl may be used to improve *A. baylyi* growth.
- Supplements added as needed. Antibiotics are used at the following final concentrations: kanamycin (Km), 25 $\mu g\ ml^{-1}$; spectinomycin (Sp) and streptomycin (Sm), 12–15 $\mu g\ ml^{-1}$ (each); ampicillin (Ap), 150 $\mu g\ ml^{-1}$, chloramphenicol (Cm), 25-50 $\mu g\ ml^{-1}$, and gentamicin (Gm), 15 $\mu g\ ml^{-1}$. Note: ADP1 has some natural resistance to Ap. If testing *A. baylyi* transformants, check the resistance level of the recipient in comparison to the transformant, and, if needed, the drug concentration can be increased to discriminate between natural and acquired resistance.
- 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. Buffers and reagents for DNA preparation and manipulation

- Gel electrophoresis methods may be used with any standard agarose and buffers.
- Lysis buffer can be used to generate genomic donor DNA (0.05% sodium dodecyl sulfate [SDS] in 0.15 M NaCl-0.015 M citrate, trisodium salt).
- Molecular biology reagents include standard materials for PCR, restriction digestion, cloning, and DNA isolation. Such materials depend on the experiment and may include: PCR primers, cloning vectors, plasmids carrying the ΩKm^R or $\Omega Sm/Sp^R$ fragment, e.g. pHP45Ω-Km (Fellay et al., 1987), pUI1637, and pUI1638 (Eraso & Kaplan, 1994), DNA polymerase and PCR components (such as buffer and dNTPs), restriction enzymes, and T4 DNA ligase.
- Purification methods/kits: donor DNAs need not be purified. However, in some cases, experimental strategies or efficiencies can be improved by using a commercial clean-up kit or by excising and purifying a specific DNA band from an agarose gel. Any purification method or commercial kit may be used.
- Salt Sodium Citrate (SSC) buffer is used for the heat-based cell lysis method: 0.15 M NaCl and 0.015 M citrate, trisodium salt.
- Syringe filter units can be used to ensure that whole cells are removed from donor DNA (disposable, polyethersulfone (PES) membrane; pore size 0.2 μm, diameter 13 mm).

3.3. Equipment

- Bacterial growth will require standard equipment, such as incubators and a method for the aeration of liquid cultures (such as an orbital shaker or a roller drum for test tubes).
- Gel electrophoresis equipment will be used for DNA analysis, requiring an electrophoresis chamber and a power supply. To visualize DNA in the gels, a UV transilluminator is needed.
- Quantification of DNA can be done using any equipment for any method (such as a spectrophotometer, fluorometer, or specialized quantification machine). For most purposes, DNA quantification is not required.
- A thermocycler will be needed for PCR.

- For RAMSES, a microplate reader is needed (e.g., Tecan Spark multimode microplate reader, Tecan, Switzerland)

4. EXPERIMENTAL PROCEDURES

4.1 Design and preparation of donor DNA

Using allelic replacement to modify the chromosome of an *A. baylyi* recipient strain requires the donor DNA to carry sequences identical to the chromosome in regions upstream and downstream of the targeted modification (**Fig. 1**). All methods of obtaining cell-free genomic DNA from *A. baylyi* donor strains will produce linear fragments that have long sequence stretches matching the recipient chromosome. These long stretches of adjacent DNA promote homologous recombination and increase the efficiency of allelic replacement. Using smaller fragments lowers this efficiency but can be advantageous by defining the chromosomal change and enabling the design/synthesis of mutations.

The design of PCR products or plasmids should take into consideration the DNA needed for recombination. Typically, approximately 500 bp to 2 kbp of sequence identity between donor and recipient DNA on either side of the target modification enables high-efficiency allelic replacement (Biggs et al., 2020). However, much smaller regions of sequence identity can be sufficient. For example, in one study, the introduction of a point mutation was evaluated using different donor DNA fragments (Gerischer & Ornston, 1995). All fragments had 385 bp of identity with the chromosome in the region downstream of the mutation. In the region upstream of the mutation, 122 bp was sufficient for generating frequent transformants. Surprisingly, transformants were also obtained, albeit at low frequency, when the donor DNA in the region upstream of the mutation had only 4 bp of identity with the recipient chromosome. Suitable constructs may be made by any molecular biology method (restriction cloning, PCR, any DNA assembly method, etc.) or by commercial synthesis. One convenient method for plasmid construction takes advantage of (*in vivo*) assembly in *E. coli* (Kostylev, Otwell, Richardson, & Suzuki, 2015).

369 *4.1.1 Preparation of cell-free genomic DNA by a heat-based lysis method*

370 Any method of purifying genomic DNA may be used. A simple method for generating donor
371 DNA involves minor modifications of past protocols (Juni, 1972; Neidle & Ornston, 1986). In general,
372 these lysates are highly effective as donor DNA for combining mutations from different strains.

373

374 1. Inoculate a 5 ml overnight liquid culture of a donor strain in minimal medium with a carbon
375 source such as pyruvate (20 mM).
376 2. Harvest cells by centrifugation and suspend in 0.5 ml sterile prewarmed lysis buffer.
377 3. Incubate at 65 °C for 1 to 3 h.
378 4. Dilute 10-fold in SSC buffer and remove any remaining cells from 1 ml of the diluted lysate by
379 filter sterilization (using a disposable unit).
380 5. The filtered lysate and different dilutions (10-fold and 100-fold) of it may be used. In some cases,
381 using more dilute samples of the lysate can be helpful, perhaps by diluting SDS in the lysis buffer
382 or inhibitors in the lysate. DNA concentration need not be determined.

383

384 *4.1.2 PCR-products or linearized plasmids as donor DNA*

385 Any method of PCR may be used to generate donor DNA. A preferred method for adding
386 flanking DNA and/or cassettes with selectable or counter-selectable markers is (*in vivo*) assembly of
387 DNA fragments in *E. coli* (Kostylev et al., 2015). Another good method is splicing by overlap-extension
388 PCR (Horton, Cai, Ho, & Pease, 1990). If high fidelity PCR is desired, the following polymerases are
389 good options: PrimeSTAR (Takara Biosciences), Phusion (NEB), and Q5 (NEB). However, low fidelity
390 or mutagenic PCR conditions may be used deliberately to mutate the donor DNA (Kok, Young, &
391 Ornston, 1999; Young, Kok, & Ornston, 2002). Any method of restriction digestion may be used to
392 linearize a circular plasmid. The following points should be considered.

393 1. If plasmid DNA is used as the template for a PCR product, a small amount of circular
394 template in the donor DNA may get integrated by a single recombination event in the

395 transformant. Thus, using digestion conditions that promote complete cutting of the plasmid
396 should be used, and transformants should be carefully characterized. It should be noted that
397 most cloning vectors used in *E. coli* to generate donor alleles (such as pUC19 and other
398 vectors with ColE1-based origins of replication) are not stably maintained in *A. baylyi* as
399 independent replicons. However, they may persist in the cell for an undetermined amount of
400 time (Gralton, Campbell, & Neidle, 1997).
401 2. Similarly, if genomic DNA is used as the template for PCR, a small amount of genomic
402 template in the PCR product may be able to modify the chromosome of the recipient. While
403 such issues occur infrequently, it is important to be aware of all possibilities and to note that
404 very little DNA is needed for allelic replacement to occur.
405
406 **4.2 Liquid transformation method (Fig. 2A)**
407 The following transformation method works well with as little as 25 ng of DNA added to the culture
408 (Biggs et al., 2020). However, while it is not critical to quantify the amount of DNA that is added to cells,
409 a range of 25 ng to 2 μg DNA in a volume of 1 to 50 μl is typical.
410 1. Grow the recipient *A. baylyi* strain overnight in 5 mL liquid MM with a non-selective carbon
411 source (e.g., 20 mM pyruvate), with aeration (such as shaking at 250 rpm) at 30 °C (or 37 °C).
412 Alternatively, rich medium (LB) may be used.
413 2. Combine 70 μL of the recipient culture with 1 mL fresh growth medium and linear donor DNA in
414 a culture tube. Prepare a similar culture without adding DNA to serve as a negative control.
415 3. Incubate culture and DNA for 3- 6 h, with aeration (such as shaking at 250 rpm) at 30 °C (or 37
416 °C).
417 4. Spread 100 μL transformed cell culture onto a selective medium agar plate. Do the same for the
418 control culture. Note: to obtain isolated transformants on the selective medium, the culture may

419 need to be diluted or concentrated. Use the same dilutions or concentrations for the control
420 culture.
421 5. Incubate plate(s) at 30 °C (or 37 °C). Depending on the selection, colonies typically appear
422 within 1-3 days.
423
424 **4.3 Lawn transformation (Fig. 2B)**
425 Transformations can be done directly on selective growth medium when conditions are not lethal
426 to the recipient. As shown in **Figs. 3-5**, selection for growth on a new carbon source enables rapid assays
427 for a range of metabolic investigations as well as for strain engineering. Such direct selection is not
428 appropriate for antibiotic selection. For antibiotic selection, spread recipients to non-selective plates and
429 allow growth/recovery before moving transformed cells to selective medium (after overnight incubation).
430 1. Grow the recipient *A. baylyi* strain overnight in 5 mL liquid MM with a non-selective carbon
431 source (e.g., 20 mM pyruvate), with aeration (shaking at 250 rpm) at 30 °C (or 37 °C).
432 2. Add additional carbon source (100 µL 1 M pyruvate) and incubate with shaking for an additional
433 30 min to initiate cell growth.
434 3. Spread 100-200 µL culture onto solid selective medium (35 x10 mm plate). Note: depending on
435 the selection, it may be helpful to wash the cells and/or concentrate them by centrifugation and
436 suspension in MM without a carbon source. Carryover of some growth substrate when spreading
437 the recipient cells can sometimes be helpful by allowing cells to grow and increase the
438 transformation efficiency. In other cases, such carryover can cause background growth that
439 interferes with the experiment. While a negative control without added DNA can help to identify
440 the impact of carryover substrate, for lawn transformation assays the difference between growth in
441 spots where DNA was dropped compared to the background growth (negative control) on the plate
442 helps assess the effect of recipient growth and transformation by DNA. The background region
443 and plating of cells without DNA can also provide information about reversion rates and/or

444 spontaneous mutation rates of the parent culture. When possible, it is best to add donor DNA that
445 should confer growth to serve as a positive control to assess the competency of the parent culture.
446 Washing cells can be helpful in removing excess carbon source and can also be used to
447 concentrate cells to increase the number of recipient cells. However, a disadvantage of washing the
448 cells is that during the centrifugation and suspension steps the competency of the culture may be
449 reduced. If one method proves insufficient, minor changes to the plating method can be beneficial.
450 4. Allow culture to dry on the plate such that the surface is not visibly wet. This step should not
451 require more than approximately 1-2 min.
452 5. Mark on the plastic side of dish where DNA will be dropped and label appropriately. A small
453 circle or region drawn on the plate is sufficient to demarcate the location of the DNA (and can be
454 seen in **Figs. 3-5**).
455 6. Drop 1-5 μL linear DNA (in isotonic solution) on top of the dried culture on the plate. Such drops
456 typically contain 25 – 500 ng of DNA. For a negative control, use an isotonic solution without
457 DNA. DNA that should not confer growth should also be used as a negative control.
458 7. Allow the DNA drop to air dry, until the surface is no longer visibly wet (several min).
459 8. Incubate at 30 °C (or 37 °C). Growth is typically observed in 1-3 days, depending on the
460 experiment and the selection.
461
462 **4.4 Spot transformation (Fig. 2C)**
463 *A. baylyi* may also be transformed in small spots on the surface of a non-selective agar plate.
464 Recipient cells and donor DNA can be mixed in drops and incubated together. One advantage of this
465 method is that different amounts of cells and DNA can easily be combined in multiple spots on the same
466 plate. Such variation may facilitate obtaining the desired transformants. Although altering the cell/DNA
467 ratio is typically not needed, the constraints of different experiments and selective conditions can be
468 unpredictable. The initial steps are the same as for the lawn transformation method.

469 1. Grow the recipient *A. baylyi* strain overnight in 5 mL liquid MM with a non-selective carbon
470 source (e.g., 20 mM pyruvate), with aeration (shaking at 250 rpm) at 30 °C (or 37 °C).
471 2. Add additional carbon source (100 µL 1 M pyruvate) and incubate with shaking for an additional
472 30 min to initiate cell growth.
473 3. To increase the concentration of recipient cells, the culture may be concentrated by
474 centrifugation. The cell pellet can be suspended in a small amount of growth medium
475 (approximately 100 -500 µL MM, with or without carbon source).
476 4. Mark on the plastic side of dish where DNA will be dropped and label the spot(s) appropriately.
477 5. Drop samples of the recipient culture in spot(s) on the plate. Drops of different amounts may be
478 used. However, procedures are simpler if spots do not mix/run along the plate surface. Volumes
479 of 1-10 µL may be used regardless of the cell concentration.
480 6. Drop samples of the donor DNA (in isotonic solution) to mix with cells. A volume of 1-5 µL is
481 appropriate (regardless of the DNA concentration). Typically, 25-500 ng of DNA will be added.
482 If the volume is too large, the spot will spread. For a negative control, mix the same volume of
483 liquid without DNA with cells.
484 7. Allow cell-DNA mixtures to air dry, until the spots will not spread when the plate is moved.
485 8. Incubate at 30 °C (or 37 °C) overnight before transferring cells to selective (or non-selective)
486 medium by streak purification or suspending cells in a small amount of medium and spreading on
487 an agar plate.
488
489 **4.5 Isolation, screening, and genotypic/phenotypic confirmation of transformants**
490 Regardless of the transformation method, the resulting isolates should be streak purified. Where
491 possible, growth on selective medium will yield individual colonies that can be tested further. However, if
492 selection is not possible, transformation efficiencies are usually high enough to obtain desired
493 transformants by screening 100 – 300 colonies. Depending on the screening method, candidate colonies

494 can be pooled to reduce the effort. For example, screening for a genotype by PCR can be done in the
495 absence of a phenotypic screen. After individual transformants are identified, they are characterized
496 further by one or more of the following steps.

497 1. Several colonies from the same isolate should be screened on different growth media (as
498 appropriate) by patching each colony to multiple plates with a sterile toothpick (Bedore et al.,
499 2022). Growth, antibiotic resistance and/or screening for counter-selectable markers, such as *sacB*
500 (Jones & Williams, 2003) or *tdk* (Metzgar et al., 2004) help to confirm or detect phenotypic
501 differences between the transformants and parent (recipient) strain. Drug resistance (typically
502 ampicillin resistance encoded on the vector backbone) can identify transformants that result from
503 plasmid integration rather than allelic replacement.
504 2. PCR with multiple sets of primers may be used to confirm expected genotypes. These tests can be
505 done rapidly using colony PCR or with purified DNA templates. When possible, the primers used
506 should bind the chromosome outside of the surrounding identical sequences included in the donor
507 DNA to promote homologous recombination (e.g., when using linearized plasmids or PCR
508 products as donor DNA). In this way, allelic replacement in the targeted locus can be verified.
509 3. DNA sequencing of localized regions, and/or of the whole genome will identify genetic changes.
510

511 **4.6 Method for rapid advantageous mutation screening and selection (RAMSES)**

512 The agar plate assays described above, and shown in **Figs. 3-5**, reveal the significance of
513 mutations when transformants form colonies on selective media. However, such assays are unable to
514 distinguish more subtle phenotypes that reflect growth differences under diverse selective conditions. The
515 RAMSES method, introduced earlier, provides a powerful means to monitor the growth of transformants
516 in a microtiter plate reader. As illustrated in **Fig. 6**, this method readily demonstrates which of the many
517 genomic mutations, typically observed in a strain derived by laboratory evolution, are most beneficial for
518 the desired phenotype (Luo, McIntyre, et al., 2022). RAMSES allows seamless introduction of the
519 individual mutated alleles (alone and in different combinations) into a recipient strain that does not grow

520 under the selective conditions. Mutated PCR fragments, added directly to the cells, generate transformants
521 that become enriched in the population when they have acquired selectively advantageous mutations. This
522 method enables both the use of incremental concentrations of the selective compound, such as high
523 concentrations of an aromatic compound, and the simultaneous comparison of multiple variations of
524 donor DNA combinations. For RAMSES, either liquid transformation or spot transformation can be used.
525 Here we describe the method based on spot transformation.

526 1. Analyze the whole genome sequencing data of any isolate(s) with an improved or novel
527 phenotype to identify and choose the mutations to be studied**.**
528 2. Design PCR primers that cover the mutated areas in the genome. The amplified DNA
529 fragments should each contain at least 500 bp of sequence identity with the recipient
530 chromosome on each side of the mutated region to ensure successful allelic replacement.
531 3. Use genomic DNA of the mutant isolates as template for PCR to amplify the targeted region.
532 To avoid unintended co-transformation of different mutated alleles, each PCR product should
533 be purified using a gel extraction kit. We recommend not placing different PCR products in
534 adjacent wells of the agarose gel to avoid cross-contamination.
535 4. Streak the recipient strain on LB agar and incubate at 30°C overnight. For RAMSES, we
536 recommend using a transposon-free recipient, such as *A. baylyi* ADP1-ISx (Suárez et al.,
537 2017) or a strain derived from it. Such recipients have increased transformation efficiency
538 and genomic stability to help avoid the emergence of undesired spontaneous mutations.
539 However, ADP1 and ADP1-derived strains can also be used as recipients. Add purified DNA
540 (0.5-2 μL) onto single colonies and mix well by pipetting up and down. Incubate overnight at
541 30°C.
542 5. Scrape the colonies treated with DNA and suspend in 1-2 ml MM supplemented with a low
543 concentration of the selective substrate (for example, 5 mM of aromatic compounds). Prepare
544 a control culture using a colony without DNA treatment. Another good control is to use a
545 colony and add DNA for transformation that would not be expected to confer a growth

546 benefit (such as a fragment identical in sequence between the mutant isolate and the recipient
547 strain). Incubate the suspensions at 30°C with aeration (shaking at 300 rpm) for up to 10 h.

548 6. Use the suspensions to inoculate 200 µl of MM supplemented with elevated concentrations of
549 the selective substrate (for ferulate, use e.g., 20 mM, 40 mM, 60 mM, and 80 mM) in a 96-
550 well plate. Prepare triplicate cultures for each concentration and mutated allele / combination.
551 Incubate the plate in a microplate reader (e.g., Tecan Spark multimode microplate reader,
552 Tecan, Switzerland) at 30°C and monitor optical density for 24-48 h.

553 7. If the cells treated with the mutated allele(s) show improved growth over the controls at the
554 elevated aromatic concentrations, 5 µL of the cells can be taken from the well to further
555 inoculate 5 mL of MM containing the same (or higher) concentration of the corresponding
556 aromatic substrate for further mutant enrichment and storage.

557 8. Streak cells from the liquid culture on LB agar. Verify the mutated alleles in clones using
558 PCR analysis and/or sequencing.

559

560 **4.7 Modified gap-repair method**

561 A recombinational capture method, termed gap repair (Gregg-Jolly & Ornston, 1990), allows
562 very large genomic segments to be cloned on a plasmid, as illustrated in **Fig. 1C**. Compared to PCR-
563 based cloning, this method avoids complications related to polymerase mediated replication fidelity and
564 problems with generating large-sized PCR products. In mutants isolated by adaptive laboratory evolution
565 and EASy, the gap-repair method can help identify whether mutations within a target region are sufficient
566 to confer a selected phenotype (Bedore, 2021). As the first step in such analyses, the *A. baylyi* region of
567 interest is captured on a plasmid that replicates in *E. coli*. In this step, a plasmid-borne drug marker is
568 used (such as ampicillin resistance in **Fig. 1C**). Next, the purified plasmid can be linearized by restriction
569 enzyme digestion in a region that does not carry *A. baylyi* DNA (i.e., in the backbone of the plasmid). If
570 the linearized plasmid DNA also carries a drug-resistance marker within the region of interest (shown as
571 Km^R in this example), it is easy to move the captured region into different genetic backgrounds by

572 transformation and drug selection. For example, a linearized version of the "gap-repaired" plasmid could
573 be used as donor DNA to transform a strain that did not undergo laboratory evolution. After selection for
574 allelic replacement, a transformant would have the genomic configuration shown in **Fig. 1D**, wherein, the
575 only mutations are those introduced in the region of interest from the mutant. Using a comparable series
576 of steps, the chromosomal region of a strain prior to evolution could be introduced into an evolved mutant
577 such that the only mutations are outside the region of interest (**Fig. 1E**). In this fashion, the significance of
578 mutations localized to specific chromosomal regions can be investigated.

579 The original gap-repair method was developed to capture chromosomal DNA on a vector that
580 replicates and is stably maintained in *A. baylyi*, pRK415 (Keen, Tamaki, Kobayashi, & Trollinger, 1988).
581 More recently, better results have been obtained using a smaller plasmid that is not stably maintained in
582 *A. baylyi.* With a linearized "capture plasmid" derived from a pUC18 or pUC19 vector (Yanisch-Perron,
583 Vieira, & Messing, 1985), homologous recombination in *A. baylyi* generates sufficient "gap-repaired"
584 plasmid to be isolated for use in the subsequent transformation of *E. coli*. Thus, a plasmid resulting from
585 homologous recombination in *A. baylyi* can be characterized and propagated in *E. coli*. The following
586 protocol using pUC18 can be modified as needed to employ different vectors that replicate in *E. coli*.
587
588 *4.7.1 Design of a "capture-plasmid" for gap repair*

589 As in the design of any plasmid intended to promote homologous recombination with
590 chromosomal DNA, the choice of *A. baylyi* DNA to clone is important (grey boxes depicted on the
591 plasmid, **Fig. 1C**). The appropriate *A. baylyi* sequences can be added to a cloning vector (e.g., pUC18)
592 using any method such as splicing by overlap PCR, restriction cloning, *in vitro* assembly, *in vivo*
593 assembly, or commercial synthesis (Horton et al., 1990; Kostylev et al., 2015). Several factors to consider
594 in the design of the capture plasmid are indicated below.

595 1. The plasmid-borne *A. baylyi* sequences, which correspond to those in the chromosome upstream
596 and downstream of the target, should be sufficiently large. Compared to other protocols so far
597 described, longer sequences (1-2 kbp on each side of the target region) may be needed to

accommodate the large chromosomal segment separating these sequences in the recipient strain. Long stretches of sequence identity facilitate plasmid-chromosomal alignment for the necessary homologous recombination events.

2. A sequence that can be cleaved by a restriction enzyme needs to be engineered between the two regions of *A. baylyi* DNA on the plasmid. This sequence must be a unique site such that cleavage with the appropriate enzyme will yield a linear fragment, as depicted in **Fig. 1C**. Both ends of the linear fragment will be generated by digestion at this restriction site (RS in **Fig. 1C**).
3. Restriction site analysis of the entire region should be considered. If the gap-repaired plasmid is later to be used as linear donor DNA to introduce the captured region into a different recipient chromosome, there must be a site or sites in the backbone for subsequent restriction digestion (indicated by a black triangle in **Fig. 1C**). These site(s) must not be present within the *A. baylyi* DNA. Depending on the size and sequence of the region of interest, it may be difficult to identify appropriate sites. If no appropriate sites are identified, it is possible to introduce such a site at the junction between the *A. baylyi* DNA and the plasmid DNA to allow cleavage at this boundary. It is important to consider this possibility at the outset such that the capture plasmid will be useful for all intended purposes.

<u>*4.7.2 Transformation of an* A. baylyi *recipient with a linearized capture plasmid*</u>

1. Digest the capture plasmid with an appropriate restriction enzyme to linearize it (RS in **Fig. 1C**). Note: if both the capture and gap-repaired plasmids confer the identical drug resistance (e.g., Ap^R), it is important to ensure complete digestion of the capture plasmid to prevent uncut plasmid from being selected at the end of the experiment. If the gap-repaired plasmid will confer additional drug resistance (e.g., Ap^R and Km^R), this pattern can be used to distinguish uncut capture plasmid from the gap-repaired plasmid generated by recombination.

622 2. It is helpful to use any type of DNA clean up kit and to elute the digested plasmid in a small
623 volume of isotonic solution (the same range of DNA amounts and concentrations as described
624 above for typical allelic replacement experiments will work). Save DNA until needed.
625 3. Inoculate a 5-ml culture of the *A. baylyi* recipient strain in non-selective medium (MM and 20
626 mM pyruvate or alternative carbon source) and culture with aeration (shaking at 250 rpm) at 30
627 °C (or 37 °C) overnight.
628 4. Subculture 120 µl of overnight culture into 3 ml of fresh medium and incubate cells with aeration
629 (shaking at 250 rpm) at 30 °C (or 37 °C) for 2-3 h.
630 5. Use the spot transformation method (**Fig. 2C**) and drop different amounts of recipient cells and
631 donor DNA in multiple spots on an LB plate. For example, use some samples of recipient cells
632 that have not been concentrated and others that have been concentrated by centrifugation.
633 6. Incubate the LB plate spotted with different mixtures of cells and DNA at 30 °C (or 37 °C) for 6-
634 8 h.
635 7. Take as many cells as possible from the spots and transfer by heavy patching to plate(s) of LB
636 with appropriate antibiotics associated with the drug-resistance markers on the expected gap-
637 repaired plasmid. However, keep in mind that the ColE1 origin of replication in pUC18 does not
638 lead to stable plasmid maintenance.
639 8. Incubate plate overnight at 30 °C (or 37 °C).
640
641 *4.7.3 Isolation of gap-repaired plasmid and use in* E. coli *transformation*
642 1. Scrape as many *A. baylyi* recipient cells as possible from the incubated plate (LB and antibiotics)
643 and suspend in 20 ml LB plus antibiotics. This slightly larger volume of cells than typically used
644 for plasmid minipreps helps to recover sufficient plasmid DNA to transform *E. coli*.
645 2. Grow culture with aeration (shaking at 250 rpm) at 30 °C (or 37 °C) for 12 -24 h.

3. Pellet all cells in the 20-ml culture by centrifugation, and isolate plasmid DNA using any miniprep protocol. Expect relatively low plasmid concentrations (based on the origin of replication) and suspend in a small volume.
4. Use the isolated plasmid to transform *E. coli* (any host strain) using chemically competent (or electrocompetent) cells. Follow typical methods and appropriate antibiotics to select plasmid-carrying *E. coli*.
5. Isolate and characterize plasmids from drug-resistant *E. coli* transformants.
6. Once the gap-repaired plasmid is isolated and checked by DNA sequencing, it may be digested and used as donor DNA in allelic replacement experiments following methods described earlier.

5. SUMMARY AND CONCLUDING REMARKS

We describe a variety of methods and applications for natural transformation and allelic replacement in *A. baylyi*. Although the ease and efficiency of such methods for genome editing are unrivaled, this naturally competent bacterium remains relatively obscure. It is often confused with similarly named bacteria or with a problematic pathogenic species that is not highly competent for natural transformation, *Acinetobacter baumannii*. However, the potential benefits for *A. baylyi* to become better known and more commonly chosen as a model organism are highlighted by vast amounts of DNA sequence data that continue to accumulate in databases. Synthetic biology and adaptive laboratory evolution, coupled with affordable and quick whole genome sequencing, are identifying mutations at a rate that exceeds our ability to understand genetic variation. *A. baylyi* offers simple methods to analyze mutations and to engineer strains with multiple and targeted mutations. These rapid techniques, which help link genotypic and phenotypic changes, can be used to extract critical biological information from DNA sequence data.

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678

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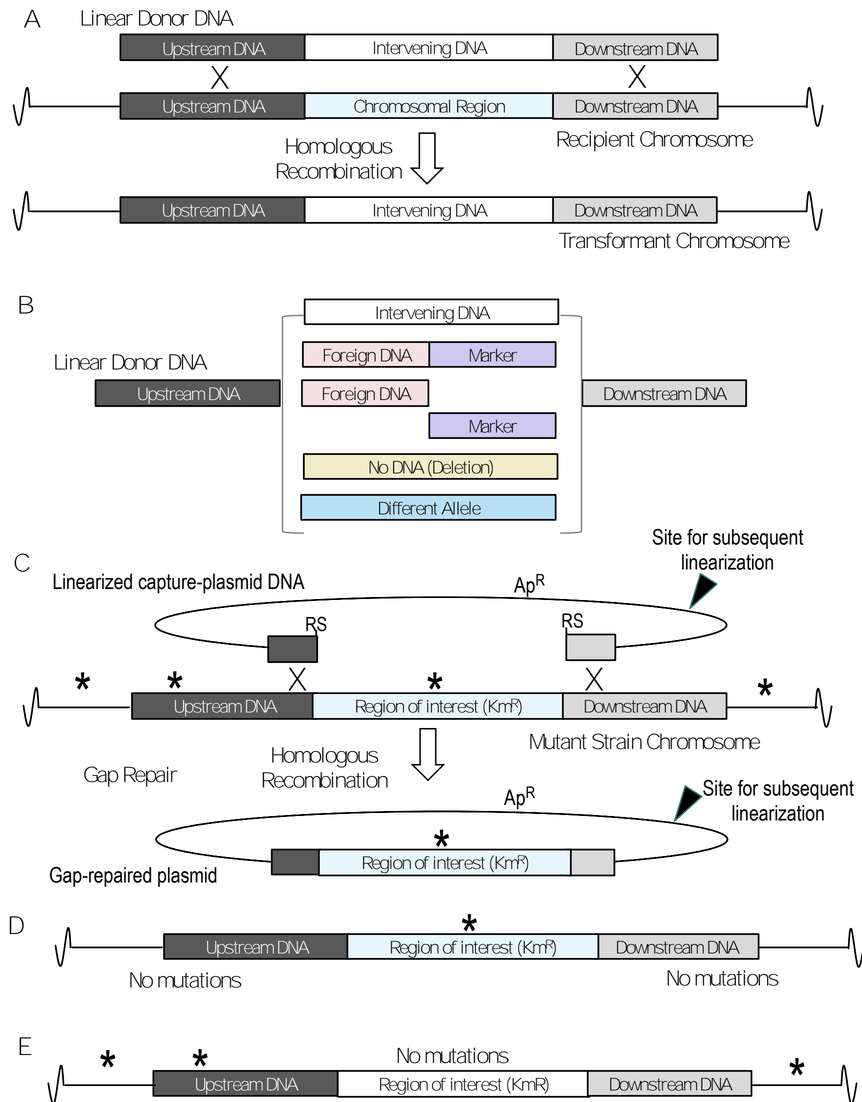
774

775 FIGURE LEGENDS



776

777 **Figure 1.** Allelic replacement techniques in *A. baylyi*. (**A**) Homologous recombination events (X) will
778 replace a chromosomal region with intervening DNA of the donor. A targeted insertion will result if there
779 is no DNA between the regions of homology in the recipient. (**B**) The type of intervening donor DNA can
780 vary and may include a selectable and/or counter-selectable marker. (**C**) A recombinational gap-repair
781 method can clone a chromosomal region of interest from a strain with multiple mutations (*). A "capture
782 plasmid" is used as donor DNA after being cleaved at a unique engineered restriction site (RS).

783 Homologous recombination (X) generates a "gap-repaired" plasmid that can be propagated in *E. coli*.

784 Subsequent linearization of this resulting plasmid facilitates transfer of the captured DNA into the

785 chromosome of a new recipient strain. To use the plasmid as donor DNA, a restriction site is employed

786 that is outside the *A. baylyi* DNA region (black triangle). (**D**) Allelic replacement of the analogous region

787 of a recipient with no other genomic mutations (than those of an original strain being investigated) can be

788 selected with a drug-resistance marker (Km^R) in the region of interest. The resulting transformant can be

789 used to assess the significance of mutation(s) in the region of interest. (**E**) Comparable allelic-replacement

790 steps to those described in (D) can be used to introduce unmutated DNA into the chromosome of the

791 mutant that has multiple chromosomal mutations. Allelic replacement of the region of interest allows

792 analysis of the effects of genomic mutations that reside outside the target region.

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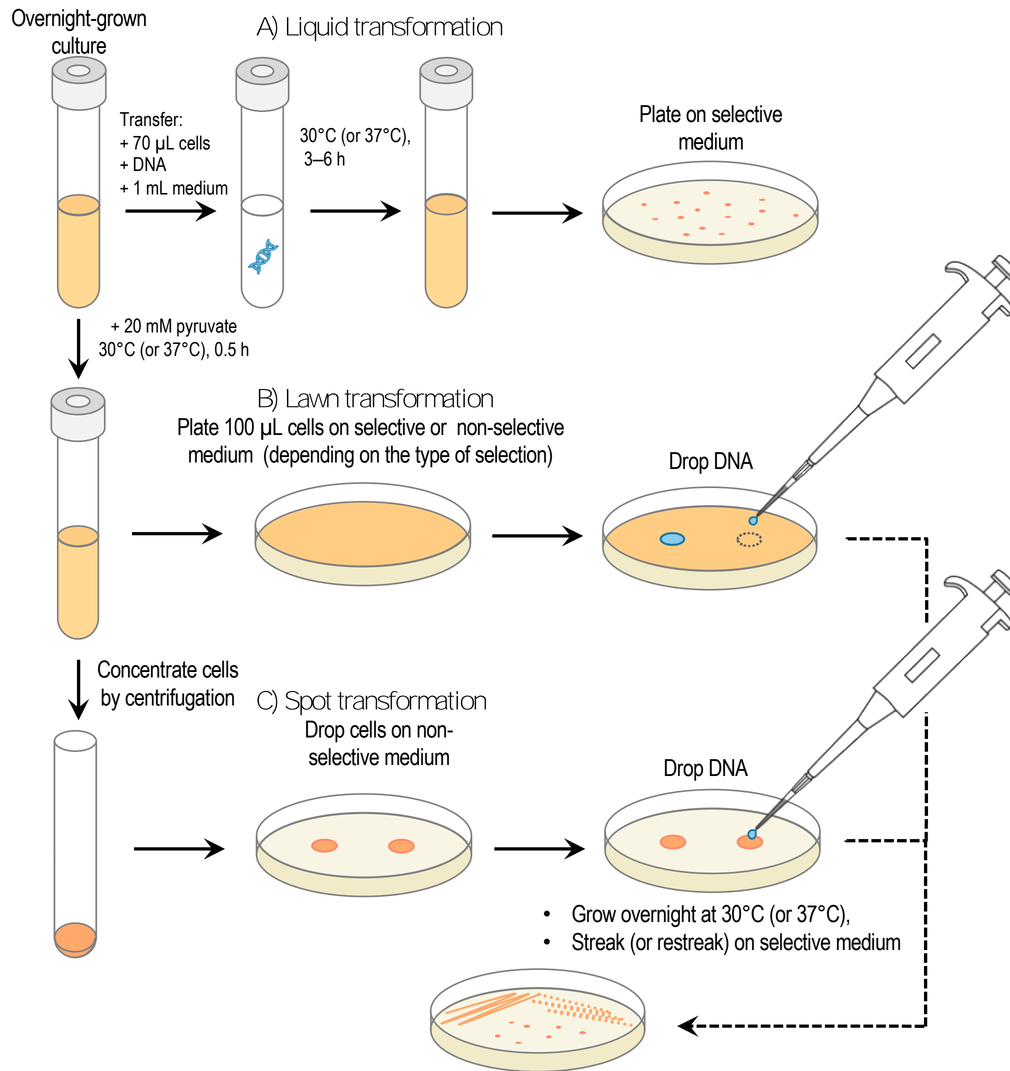

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795 **Figure 2.** Different methods for transforming *A. baylyi.* (**A**) Liquid transformation is highly efficient. (**B**).
796 Lawn transformation can be done on selective medium if recipient cells can survive (see **Figs. 3-5**). For
797 selections based on antibiotic resistance, a recovery period on non-selective medium is needed. (**C**) Spot
798 transformations on a non-selective medium allows different concentrations and proportions of DNA and
799 cells to be mixed prior to selection or screening. Variations and combinations of these methods are well
800 tolerated. Modifications may be tailored to specific needs, convenience, or available resources. If no
801 selection is available, screening of individual or pooled transformants will typically identify allelic
802 replacement (with linear donor DNA) at a frequency of approximately 10^{-2} to 10^{-3}.

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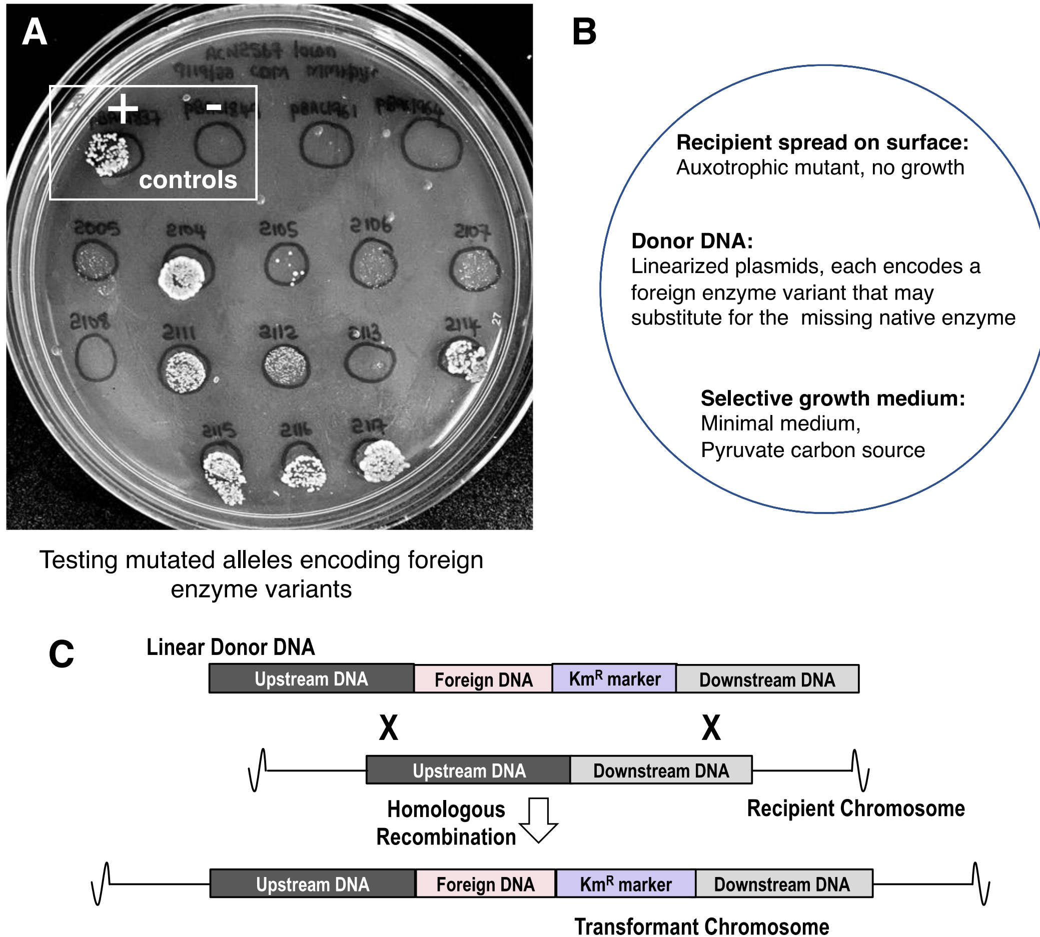

804

805 **Figure 3.** Lawn transformation assay on selective medium. (**A**) A recipient strain, which is unable to

806 grow on the agar plate medium, was spread across the surface. Cell-free linear DNA dropped in marked

807 spots can transform the recipient and replace the corresponding chromosomal region via homologous

808 recombination. Growth of transformants indicates that allelic replacement enables expression of a foreign

809 enzyme to compensate for a missing enzyme in the auxotrophic recipient. (**B**) Details corresponding to

810 the experimental results shown in panel (A). (**C**) Schematic illustration of the allelic replacement. In the

811 experiment shown, selection depends on prototrophy without antibiotics. An alternative way to assess

812 transformants involves antibiotic resistance, using a marker adjacent to the foreign enzyme gene and a

813 method that includes a recovery period on non-selective medium (**Fig. 2**). If Km^R-transformants are

814 selected, their growth can be evaluated without of any prior selection for prototrophy.

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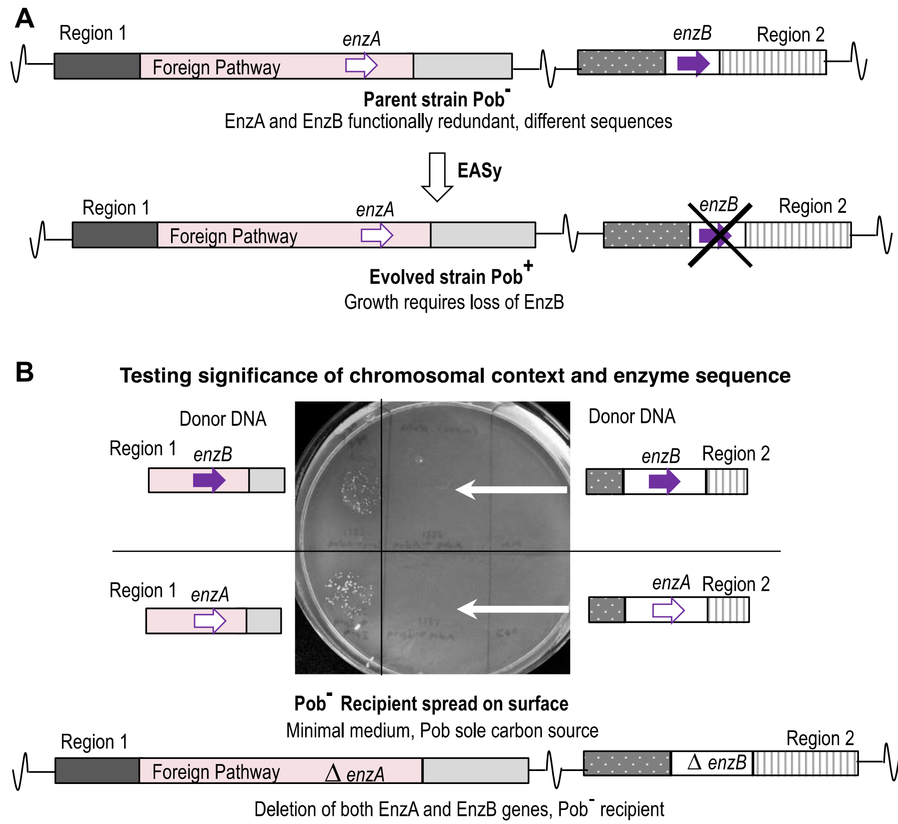


816

817 **Figure 4.** Lawn transformation assay to investigate mutations in a Pob+ evolved strain derived by the

818 EASy method from a Pob- parent strain. (**A**) Schematic representation of chromosomal configurations in

819 the parent and evolved strains. The acquired phenotype of the evolved mutant required both expression of

820 a foreign pathway, including a gene (*enzA*), integrated in the chromosome (region 1), and additional

821 mutations such as the inactivation of a native *A. baylyi* gene (*enzB*) in a different chromosomal locus

822 (region 2). (**B**) A new Pob- strain was generated by deleting both *enzA* and *enzB,* which encode enzymes

823 that catalyze the same reaction despite some significant differences in their sequences. Linear DNA

824 fragments were generated in which each gene (*enzA* or *enzB)* resides within sequences corresponding to

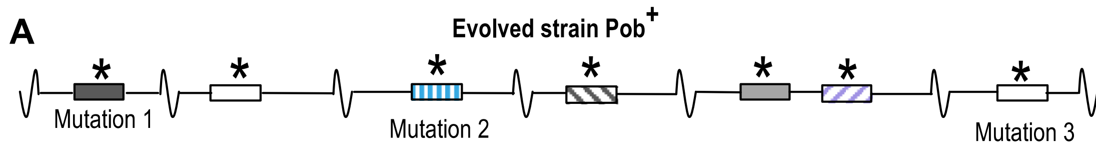
825 region 1 or region 2. The Pob- deletion strain was used as the recipient in a lawn transformation assay

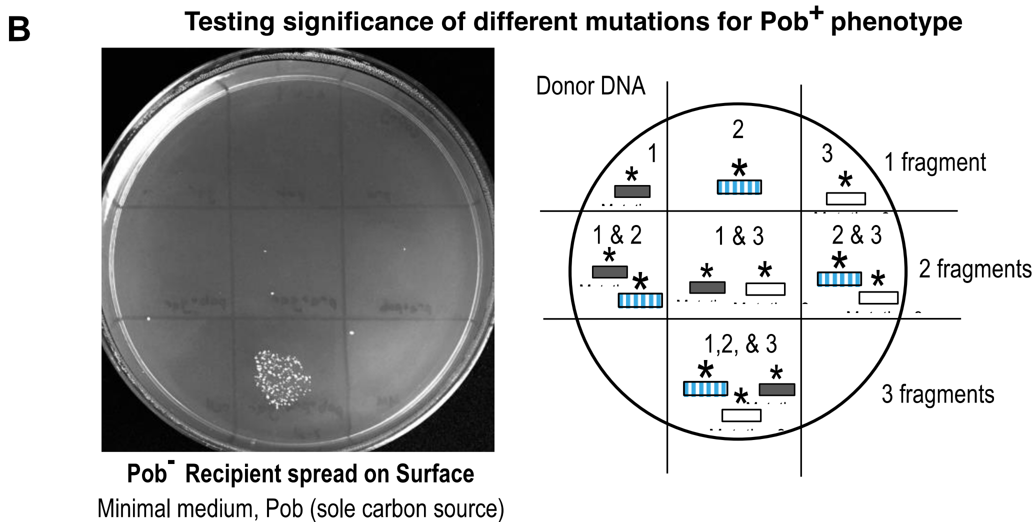
826 with cell-free donor DNA for each of the four configurations (*enzA* in region 1, *enzA* in region 2, *enzB* in

827 region 1, and *enzB* in region 2). Transformants grew on Pob when either gene could be inserted in region

828 1, whereas neither conferred growth in region 2. These results suggest that genomic context, rather than

829 the enzyme sequence is most important for a Pob+ phenotype.





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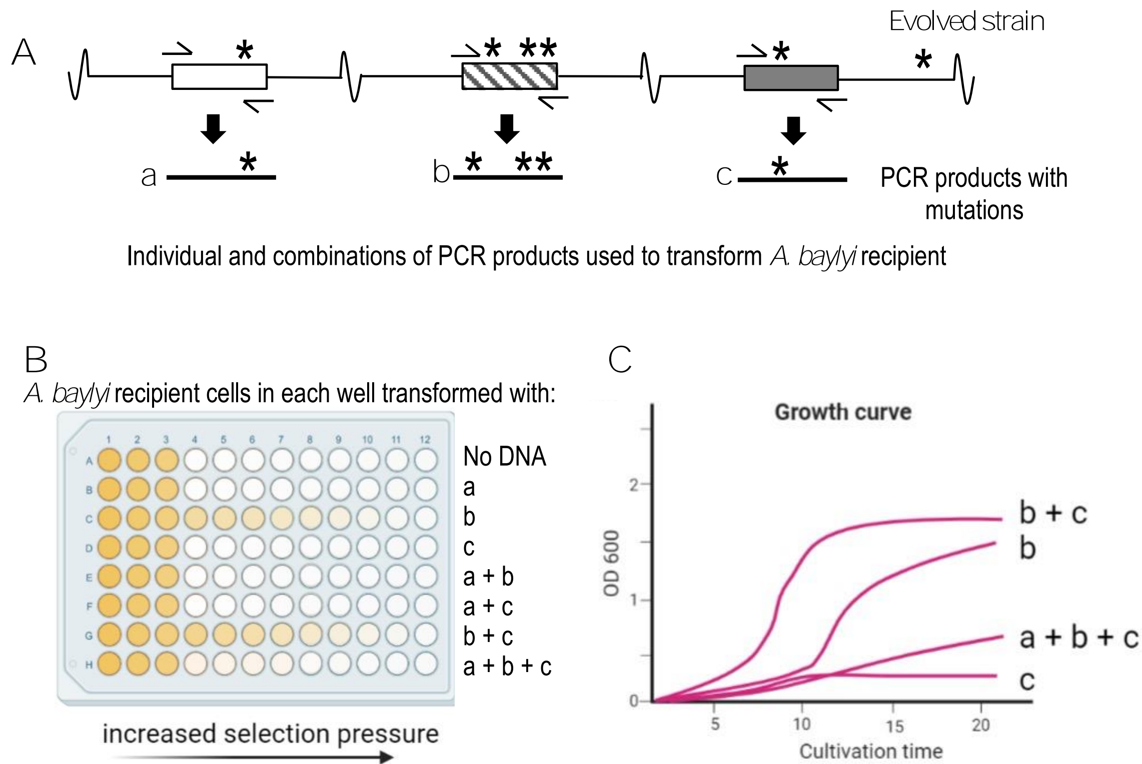
831 **Figure 5**. Combinatorial evaluation of mutations examined by natural transformation (CEMENT). (**A**)
832 Schematic representation of the chromosomal configuration of a Pob^{+} mutant that evolved from a Pob^{-}
833 parent strain. Whole genome sequencing revealed multiple mutations (*) throughout the chromosome.
834 Linear DNA fragments were generated that each corresponded to a genomic region (rectangle). (**B**) Cell-
835 free linear fragments, each carrying a known mutation from the evolved strain, were used as donor DNA
836 in a lawn transformation assay. The recipient strain, spread on the surface of a selective plate with Pob as
837 the carbon source, was the Pob^{-} parent from which the evolved mutant was derived. Multiple fragments
838 were tested, and a combination of three specific fragments (mutations) proved sufficient to transform the
839 recipient to grow on the Pob plate. Growth was observed only when all three fragments were dropped in
840 the same spot (bottom center). The diagram at the right indicates the regions of the plate where the DNA
841 fragments were dropped. Each was tested individually and as a pair with one other fragment as well as in
842 the three-fragment combination. The results highlight the importance of this combination of mutations for
843 Pob^{+} growth.



844

845 **Figure 6.** Use of the RAMSES method to identify beneficial mutations in evolved strains. (**A**) Schematic

846 representation of the chromosomal configuration of an evolved strain with multiple mutations (*). Using

847 genomic DNA of the evolved strain as template, PCR with specific primers generates linear DNA

848 fragments that are purified and used as donor DNA to transform an appropriate reference strain (e.g., *A.*

849 *baylyi* ADP1-ISx). These PCR products, which carry known mutations, are tested for the ability to affect

850 the phenotype of the recipient. This example illustrates the use of different PCR products (a, b, and c) in

851 individual and combined transformations. The transformed cells are first incubated under selective

852 conditions. (**B**) Cells are then transferred to 96-well plate with various concentrations of the selective

853 compound. Cell growth is monitored by optical density (OD_{600}). A negative control should be included

854 that uses comparable conditions with a DNA fragment that should not confer benefit (random DNA or a

855 fragment that has the same sequence in the donor and the recipient). Comparably treated cells to which no

856 DNA is added could serve as an additional negative control. (**C**) The growth curves indicate which

857 mutations (and which combinations) confer selective growth advantage.