Consejo Superior de Investigaciones Científicas, Spain	EPP-SOP-CSIC02 Version 1.0

EPP - Standard Operating Procedure

(only for selected experiments intended to transfer results from one lab to the other)

Title: **ssDNA-based recombineering in *Pseudomonas putida***

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Instruction

ssDNA-based recombineering in *Pseudomonas putida*

1 Introduction / Purpose

Recombineering is the use of phage recombination proteins to improve and facilitate bacterial genome engineering. Depending on the nature of the DNA template, double-stranded or single-stranded, the system needs three proteins (Gam, Exo, Beta) or just one (Beta) to work properly. The use of this technique has been fundamental not only towards solving fundamental biological questions with reverse genetics but also for the generation of deep engineered *E. coli* chassis strains.

Unfortunately, the use of ssDNA recombineering is still limited to a narrow number of bacterial species. One of the reasons for that is the lack of proper recombinases to be efficiently used in different microorganisms and the lack of proper genetic tools to deliver and express these activity in a controlled way. Here, we describe a protocol to follow a simple workflow to identify, clone and quantify the function of the selected recombinases in the organism of choice by cloning and expressing them in standardized broad host range plasmids. As an example of the method we tested the use of the Ssr recombinase in *P. putida* EM42 by introducing a complete deletion of the target gene *pyrF*. The example shows how two parameters of the mutagenic oligo, i.e. length and phosphorothioate protection, affect the final outcome of the procedure.

2 Equipment and chemicals

2.1 Equipment

- 10-mL plastic tubes
- Erlenmeyer flasks
- Electroporator system with 2-mm gap width sterile electroporation cuvettes.
- Thermocycler and PCR tubes.
- 90 mm round sterile plastic Petri dishes.
- Sterile glass beads of 3-mm diameter (VWR International, PA, USA) to spread bacteria onto agar plates. Used glass beads can be recycled if they are washed with 70% (v/v) ethanol, dried and autoclaved.
- NanoVue™ or NanoDrop™ spectrophotometer to quantify DNA concentration.
- DNA horizontal electrophoresis system.

- Sterile 50 mL Falcon Tubes.

Kits:

- QIAprep Spin Miniprep™ kit (Qiagen Inc., Valencia, CA, USA)
- NucleoSpin™ Gel and PCR clean-up kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany)
- ExoSAP-IT™ PCR product cleanup kit (USB Molecular Biology, Affymetrix Ltd., Santa Clara, CA, USA).

2.2 Chemicals

- 10 mM of dATP, dCTP, dGTP, and dTTP in milliQ H_2O.
- 3 mL Tris-HCl 1M (pH 7.5)
- $MgCl_2$ 1M
- dithiothreitol (DTT) 1M
- polyethylene glycol (PEG) 8000
- NAD

Enzymes:

- Go*Taq*™ Flexi DNA polymerase (Promega Corp., Madison, WI, USA) with the 5x Green Go Taq Flexi buffer
- $MgCl_2$
- Q5 High-Fidelity DNA polymerase (NEB, Beverly, MA, USA).
- T5 exonuclease (Epicentre, Madison, Wi, USA)
- Phusion™ High-Fidelity DNA polymerase (NEB, Beverly, MA, USA)
- Taq ligase (NEB, Beverly, MA, USA).

2.3 Bacterial strains

P. putida KT2440 derivative EM42 as the host to perform the recombineering experiments

E. coli CC118 (°(*ara-leu*) *araD* °*lac*X174 *galE galK phoA20 thi-1 rpsE rpoB argE*-(Am) *recA1 as cloning host*

HB101 as the mating helper strain (F⁻ □⁻ hsdS20(r_B^- m_B^-) recA13 leuB6(Am) araC14 °(gpt-proA)62 lacY1 galK2(Oc) xyl-5 mtl-1 thiE1 rpsL20 glnX44(AS).

2.4 Other materials

2.4.1 O*ligonucleotides*

All oligonucleotides needed for PCR amplifications, sequencing and recombineering are specified below. Primers were purchased from Sigma-Aldrich, as desalted DNA, and resuspended in H_2O to obtain either 5 µM oligo solutions for PCR and sequencing or 100 µM stocks for recombineering. Primers are stored at –20 °C.

For the recombineering experiments, aiming to perform a single deletion of the *pyrF* gene, we tested different size oligonucleotides either 100, or 60, or 40 nt long. Therefore having 50, 30 or 20 bp, respectively of upstream and downstream genome homology flanking the *pyrF* gene.
Also, the influence of the presence of four phosphorothiate bonds at the 5´ end was studied.

LD: 5'-
*A*C*A*G*GCATCGGTGGTTCGGCACAGGCCCTTGCTGGACAGCCGCAGGTTAA*GGGCAGGGTCT
CTTGGCAAGTCGAAAACGGCGCGCATTGTAAACGAAGTG-3'; ***(14)***.

LD-n: 5'-
*ACAGGCATCGGTGGTTCGGCACAGGCCCTTGCTGGACAGCCGCAGGTTAA*GGGCAGGGTCTCT
TGGCAAGTCGAAAACGGCGCGCATTGTAAACGAAGTG-3'.

LD-60: 5'-
*A*C*A*G*GCCCTTGCTGGACAGCCGCAGGTTAA*GGGCAGGGTCTCTTGGCAAGTCGAAAACGG-
3'.
LD-60n: 5'-
*ACAGGCCCTTGCTGGACAGCCGCAGGTTAA*GGGCAGGGTCTCTTGGCAAGTCGAAAACGG-3'.

LD-40: 5'-*G*C*T*G*GACAGCCGCAGGTTAA*GGGCAGGGTCTCTTGGCAAG-3'.

LD-40n: 5'-*GCTGGACAGCCGCAGGTTAA*GGGCAGGGTCTCTTGGCAAG-3'.

The asterisk within the DNA sequence denotes a phosphorothioate bond. Italics represent one half of the homology region.

2.4.2 Primers specific to SEVA vector backbone

Oligonucleotides hybridizing in specific sequences within the SEVA plasmid backbone are commonly used to either confirm the correct cloning of heterologous DNA into the plasmids by PCR or just to sequence the recombinant plasmids.

238F: 5'-GGTTTGATAGGGATAAGTCCAG-3'; ***(13)***.

PS2: 5'-GCGGCAACCGAGCGTTC-3'; (20).

2.4.3 *Primers to diagnose the pyrF deletion*

In order to confirm the complete deletion of the *pyrF* gene by either colony PCR or to sequence the genomic region of interest we used the following oligonucleotides:

PYRF-F: 5'-CGAGGGCTATGATGAGTATC-3'; ***(14)***.

PYRF-R: 5'-GTCAGGTGAAGAGCAAAGAG-3'; ***(14)***.

When employing the PYRF-F and PYRF-R primers for colony PCR the corresponding expected DNA amplicon lengths are 560 bp for the deleted strain or 1,262 bp in the case of wild type cells.

Plasmids

Plasmid	Description and relevant characteristics[a]	Reference
pSEVA258	Expression vector; oriV (RSF1010); *xylS*-Pm; MCS; Km^R	(20)
pSEVA258-*ssr*	pSEVA258 derivative; oriV (RSF1010); *xylS*-Pm □ *ssr*; Km^R	***(14)***
pRK600	Mating helper plasmid; *oriV*(ColE1), RK2(mob^+ tra^+); Cm^R	(39)

Antibiotic markers: Km, kanamycin; Cm, chloramphenicol.

3 Media and buffers

1. **LB medium**

   LB medium is used as the routine medium for growth of both *P. putida* and *E. coli*. 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl were dissolved and brought up to 1 L with deionized H_2O, and sterilized by autoclaving. LB was solidified by adding agar to the media at a final concentration of 1.5% (w/v) and autoclaved. After autoclaving, let it cool and distribute the culture medium into plastic Petri dishes (20 to 25 mL in a 90-mm plate). Then, let them solidify at RT.

2. **M9 minimal medium supplemented with citrate**

   In specific cases, when nutritional selection is required (mating experiments), we used the M9 minimal medium supplemented with citrate as the sole carbon source since it cannot be utilized by the *E. coli* donor cells commonly used for matings with *Pseudomonas* ***(18)***. The components of this mineral medium are prepared and autoclaved separately, and mixed just before use. The individual components can be stored at room temperature until their use. This medium is prepared as follows: a 10× stock of M9 salts is first made by dissolving 42.5 g of $Na_2HPO_4{\cdot}2H_2O$, 15 g of KH_2PO_4, 2.5 g of NaCl, and 5 g of NH_4Cl in deionized H_2O up to a final volume of 500 mL. A 1 M $MgSO_4$ solution is prepared by dissolving 12 g of anhydrous $MgSO_4$ in H_2O up to 100 mL. The carbon source stock is made as a 20% (w/v) sodium citrate solution. Components are mixed and diluted to have a final concentration of 1x M9 salt, 0.2% (w/v) of citrate and 2 mM $MgSO_4$ solution. For M9 minimal medium plates, prepare a 1.6% (w/v) agar suspension in H_2O, autoclave it separately from the other medium stock solutions, and then mix a suitable amount of this suspension with the rest of the components to obtain a final agar concentration of 1.4% (w/v).

3. The antibiotics required for this protocol are prepared as concentrated stocks in H_2O, sterilized by filtration (0.45 µm) and stored at −20 ºC. The working concentration of the antibiotics are: kanamycin (Km), 50 µg/mL; chloramphenicol (Cm), 30 µg/mL.
4. To obtain an uracile stock of 20 mg/mL, dissolve 200 mg of uracile (Sigma-Aldrich, St. Louis, Mo, USA) in 10 mL of NaOH 0.5 N, filter sterilize, distribute into 1mL aliquots and store them at −20 ºC. Use it at a final concentration of 20 µg/mL.
5. 5-fluoroorotic acid (5FOA) was prepared by adding 5 mL of dimethylsulfoxide (DMSO) to 750 mg of 5FOA (Zymo Research, Irvine, CA, USA); to facilitate dissolution incubate at 37 ºC and vortex. Then, prepare aliquots containing 1mL and store them at −20 ºC. Use it at a final

concentration of 250 µg/mL.

6. In order to obtain electrocompetent cells of *P. putida*, prepare a 300 mM sucrose stock solution by dissolving 25.6 g of sucrose in H_2O up to a final volume of 250 mL, sterilize by filtration and aliquot it in 50 mL Falcon tubes. Keep them at room temperature.
7. To activate transcription under control of the *xylS-Pm* expression system, formulate a concentrated stock (0.5 M) of *m*-toluic acid (3-methyl benzoate; 3MB; # T36609; Sigma-Aldrich, St. Louis, Mo, USA) in H_2O adding NaOH pellets slowly until complete solubilization. Then, filter sterilze it.

4 Procedures

The following protocol describe a workflow to test the efficiency of different recombinases in a desired bacterial host (Fig. 1). The whole process starts upon identification of recombinase genes to be tested. The sequence from representatives of the different four single-strand annealing protein (SSAPs) superfamilies (Redß, ERF, Sak and Sak4; ***(21)***) can be obtained from the NCBI (Nacional Center for Biotechnology Information; https://www.ncbi.nlm.nih.gov) and perform a protein-protein BLAST (blastp; ***(22)***) against the non-redundant (nr) sequence database to identify possible homologues in the desired bacterial species. This informatic search can be restricted to a specific group (*Pseudomonas*, taxid: 286; *P. putida*, taxid: 136845; etc) or not depending on the particular interest.

For general biology techniques used see section 4.9 (at the end of the Procedures).

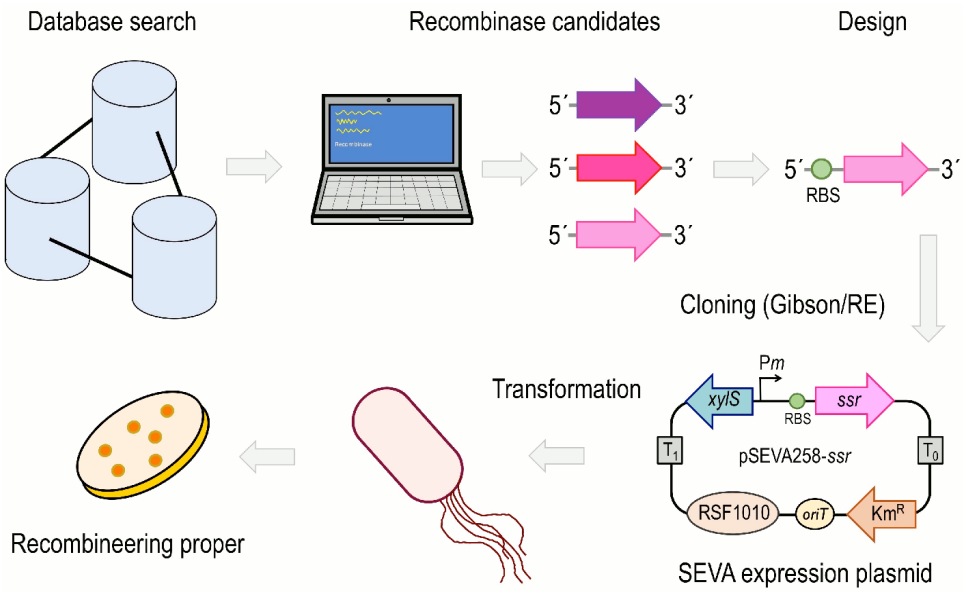

FIG. 1. Schematic representation of the experimental workflow described in this protocol. The process start by identifying possible candidates by bioinformatics analysis. Then, cloning the selected candidate/s in a standardized format into an inducible expression SEVA plasmid (in this particular example, the pSEVA258). Once obtaining the recombinant plasmids they have to be transferred to the desired bacterial species (*Pseudomonas*, or any Gram-negative bacteria in which the selected vector replicates). After that, one just has to perform the recombineering experiment as indicated in the protocol using an appropriate reporter gene (*pyrF*).

4.1 Cloning the recombinase into a pSEVA expression plasmid system

Once the user has chosen a recombinase/s it has to be placed first into an appropriate expression plasmid that allows its production upon addiction of the inductor. For these type of experiments, we routinely used the pSEVA258 (20, 23) as the plasmid backbone. This vector has the following characteristics: Km^R, medium-high copy (*oriV* RSF1010) and a XylS-*Pm* expression cassette. This inducible system is composed of a transcriptional factor, XylS, that upon addiction of *m*-toluate (or other benzoate derivatives) became active and drives the expression of the heterologous DNA placed under the control of the *Pm* promoter: for a review see ***24)***. Some of the advantages of the XylS-*Pm* system are: (i) tightly regulation; (ii) a high induction ratio; (iii) independent of the cellular physiology ***(25)***; (iv) and that the system has proven active in many bacterial species (26).

Then, the second element of the system is the desired recombinase/s to be tested. The particular gene can be PCR amplified from genomic DNA or colony PCR of the bacteria with the recombinase

gene. However, in the most common scenario of not having a suitable PCR template, one can use the sequence information of that particular recombinase retrieved from the databases to directly obtain a synthetic DNA from any of the Gene synthesis companies. It is important to take into account that the pSEVA258 lacks an RBS, relying on the user to select the most appropriate (20). Then, for comparison purposes, it is important that all recombinase/s to be tested contain the same 5´ untranslated region (5´-UTR) (*see* **section 3.1.2** for more detail). The recombinase can be cloned by enzymatic restriction adding an EcoRI site to the 5´-end and a BamHI sequence to the 3´-end of the PCR primer pair (*see* **Note 1**). Also, the other option is to clone the recombinase gene into the destination plasmid (pSEVA**X**58; where **X** means any of the Ab^R cassettes available at the SEVA collection) by Gibson assembly.

In this protocol, we are going to describe the cloning process of the *ssr* recombinase gene using the Gibson assembly option with a synthetic DNA. Briefly, we need first to obtain the two DNA molecules, the linearized plasmid and the recombinase gene, to join. Then, prepare an assembly homemade kit to perform the isothermal assembly with the DNA fragments and transform the mixture into an appropriate *E. coli* strain. Thus, once selected a positive clone we need to transfer the recombinant plasmid to the *P. putida* EM42 strain. To finally perform the recombineering experiment to validate the function of the selected recombinase.

4.2 Preparation of the plasmid DNA for the assembly

1. Purify the pSEVA258 plasmid using a commercial column kit from a 20-mL saturated *E. coli* culture.
2. Digest the plasmid with the appropriate restriction enzymes (EcoRI and BamHI in the example).
   Restriction mix:
   - 8 µL Buffer 10x
   - 8 µL BSA 10x
   - 61 µL of plasmid DNA
   - 1.5 µL EcoRI
   -1.5 µL BamHI
3. Purify the linearized plasmid DNA (~7.2 kb) after proper separation in a 1% (w/v) agarose gel electrophoresis with a commercial column kit.

4. Quantify the DNA concentration using a NanoDrop or by inspection in an agarose gel.

4.3 Outsourcing the recombinase for DNA synthesis

In order to use Gibson assembly 40-bp from pSEVA258 before EcoRI and after BamHI were included in the 5´and 3´ synthetic DNA, respectively. Underlined is marked the EcoRI and BamHI sites, in bold the added RBS, and in italics the entire 5´ UTR sequence added to the recombinase gene.

1. Add the following 56-bp DNA sequence to 5´-end of the recombinase gene: 5´-TGGAGTCATGACCATGCCTAGGCCGCGGCCGCGCGAATTC*AGA**AGGAGA**ATATACC*-3´.
2. Incorporate the following 40-bp DNA sequence to the 3´-end of the recombinase: 5´-GGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGCGG-3´.
3. Request the DNA sequence for gene synthesis to your favourite company.
4. Once received quantify its concentration (**Note 2**).

4.4 Arrangement of the isothermal assembly reaction

1. Mix both DNA fragments (~100 ng vector plus equimolecular ratio of the synthetic DNA) in a final volume of 5 µL (**Note 3**).
2. Add the 5 µL of the mixed DNA pieces to 15 µL of the freshly thawed isothermal assembly mixture (*see* **Section 2.3**). Blend gently while keeping the mixture in ice.
3. Immediately, place the reaction tube in a thermocycler and incubate at 50 °C for 1 hour.
4. Take 10 µL of the reaction and transform *E. coli* chemical competent cells (CC118) (**Note 4**).
5. Plate cells onto LB plus Km and incubate at 37 °C overnight.
6. Pick colonies and check the presence of the correct integration of the *ssr* recombinase gene into the plasmid by whole-cell colony PCR using primers 238F/PS2 (expected DNA band size: ~1kb).
7. Select a positive clone and send to sequence with 238F and PS2 primers.
8. Once confirmed a correct recombinant clone prepare a frozen stock to conserve it.

4.5 Transforming P. putida cells with the recombinase expressing plasmid

The next step in the protocol is to introduce the recombinase expressing plasmid into the desired *P. putida* strain. The delivery process could be performed by either conjugation or by electroporation. Even though more time consuming, we recommend the conjugation option that shows higher frequencies (**Note 5**). To perform a mating experiment, one just needs to pile up three different strains to catalyze the mating process; (i) the *E. coli* donor cell harboring the pSEVA258-*ssr* plasmid, (ii) the recipient cell (*P. putida* KT2440), (iii) and a helper strain bearing the conjugative plasmid (pRK600) that provide the DNA transfer function in *trans*. For this protocol, we describe a simple and easy mating procedure. For a more detailed mating protocol see ***(18)***.

1. Grow the three bacterial strains in LB with proper antibiotics overnight.
2. Collect 1 mL of the overnight grown cells, centrifuge at 7,200 x*g* for 2 minutes at room temperature, discard supernatant, and suspend with 1 mL of 10 mM $MgSO_4$. Repeat this process at least two times.
3. Take 100 µL of each bacterial type (ratio 1:1:1) and centrifuge at 7,200 x*g* for 2 minutes at room temperature, discard supernatant, and suspend cells in 20 µL of 10 mM $MgSO_4$. Then, spot the mixture onto an LB agar plate and incubate at 30 °C for 8 h.
4. Using a sterile curved yellow tip collect the mating mixture and suspend in 1 mL of 10 mM $MgSO_4$.
5. Plate different dilutions (10^{-1}, 10^{-2}, 10^{-3}; **Note 6**) and it is necessary to try other ones until obtaining isolated colonies until a correct clone is isolated) onto M9 supplemented with citrate plus Km at 50 µg/mL.
6. Re-streak colonies onto M9-cit plus Km at 50 µg/mL.
7. Grow several colonies and check the presence and correct integrity of the plasmid by miniprep and restriction. In the case of the pSEVA258-*ssr*, digestion with EcoRI renders a 7.6 kb and 0.36 kb DNA bands (**Note 7**).
8. Once confirmed that a *P. putida* clone bears the correct plasmid prepare a frozen stock in 20% (v/v) glycerol in LB to preserve it.

4.6 Selecting a target gene for the recombineering experiment

Phage recombinases used in recombineering protect the synthetic ssDNA and mediates its allelic exchange with the homologous genomic counterpart, providing an efficient way to generate mutants in bacteria. Then, the next step in the process is to choose a good target gene candidate to screen the proper function of the selected recombinase to introduce desired mutations. The most important requisite of the target reporter is that should produce an easy and detectable phenotype when the gene is altered.

An example of possible candidates, the *pyrF* gene that encodes the enzyme orotidine 5´-phosphate decarboxylase involved in *de novo* synthesis of the pyrimidine precursor uridine monophosphate *(27)*. This is an essential enzyme making *pyrF* cells auxotrophs for uracile. However, at the same time wild type cells convert the uracile analog, 5-fluorootic acid (5FOA), into a toxic compound (28, 29). In the case of disrupting the gene mutated cells can be selected on M9-cit+Ura+5FOA. A caveat of this system is the spontaneous appearance of $5FOA^R$ mutants that are not related to the *pyrF* gene at low frequency (~10^{-7}; *(29)*). Nevertheless, this problem can be solved by double streaking on M9-cit+Ura+5FOA (*pyrF* and spontaneous $5FOA^R$ are able to grow) and M9-cit (spontaneous $5FOA^R$ are able to grow but not *bona fide pyrF* mutants).

Other option is to select genes that upon specific point mutation confer resistance to antibiotics. One of these is the *rpsL* gene that encodes the 30S ribosomal protein S12. Within this protein, the single amino acid change K43T confer resistance to streptomycin (13, 30). Other possible target gene candidates include the *gyrA* gene encoding the DNA gyrase subunit A, where the D87N alternative confers resistance to nalidixic acid to bacterial cells (31, 32); and *rpoB* whose product is the beta subunit of the RNA polymerase, where the mutated variant Q518L confers resistance to rifampicin *(33)*.

4.7 Design of the recombineering oligonucleotides

Once the target gene has been selected the next step is to design the proper mutagenic oligonucleotide to perform the recombineering experiment. In terms of length, 90 or 100-bp usually work fine, including the desired change in the middle of the sequence (i.e. single base substitutions).

In the particular case of complete gene deletions, the oligo contains 45/50 nucleotides of homology flanking the region to be deleted *(14)*. Since a clear bias for the lagging strand was observed in *E. coli* it is recommended to design the oligos to that strand *(5)*. The lagging strand replicates discontinuously by the Okazaki fragments, for that reason, it is important to know the genomic coordinates of the *oriC* and *dif* regions in the interested organism *(34)* to define the two replichores and the leading/lagging strand in each one *(14)*. In the case of not knowing those features it can be solved by designing oligonucleotides for the two strands and test them both. For single base substitutions, it is important to take into account the effect of mismatch repair system (MMR) of the bacterial host. In *E. coli* and probably in other Gram-negatives some mismatches between the oligo and targeted region are recognized and repaired better (G-G > G-T > A-A > C-T, A-C) than others (A-G, T-T, C-C) *(35)*. Another important aspect to take into account is the folding energy of the oligo, one should try to minimize it as much as possible (preferentially ΔG > -12.5 kcal/mol) (6, 7) (**Note 8**). Normally, between 2 to 5 phosphorothioate linkages at the 5´end of the ssDNA are included to evade nuclease degradation *(6, 7, 36)*.

4.8 Testing the efficiency of the selected recombinase with a target gene

With this information in mind, we aimed to test (i) the influence of the size of the ssDNA oligo template in the efficiency of the recombineering (Fig. 2) and and (ii) the protection exerted by the phosporothiote bonds in the 5´-terminus of the oligo (Fig. 3). For that, we selected the *pyrF* gene as our proxy to parametrize the efficiency of recombineering attending these two variables.

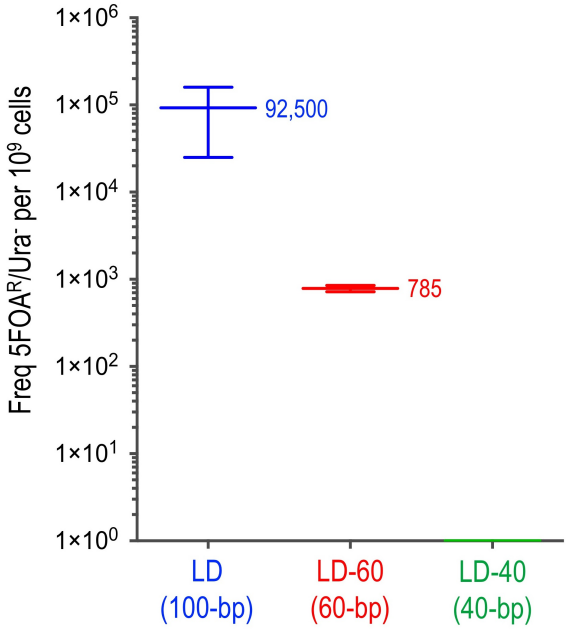

FIG. 2. Influence of the ssDNA size in recombineering efficiency. Ssr induced *P. putida* EM42 cells containing the pSEVA258-*ssr* plasmid were transformed with the different size mutagenic ssDNAs (either 100-bp, or 60-bp or 40-bp). The chart shows the frequency of $5FOA^R/Ura^-$ clones normalized by a total of 10^9 cells and represents the data from at least duplicate experiments. The three mutagenic oligos (LD, LD-60 and LD-40) contained phosphorothiate bonds at their 5´ terminus; their complete DNA sequence is shown in section 2.4.1. The plot shows a general decrease in the recombineering efficiency as the homology region of the oligo is reduced. The efficiency dropped about two orders of magnitude when the total size of the oligo is 60 bases and no *pyrF*$^-$ recombinant clones were observed when the size is reduced to 40 bases. This fact is consistent with was already seen for *E. coli* ***(5)*** and with the observation that recombinase proteins binds poorly to short ssDNAs ***(40)***.

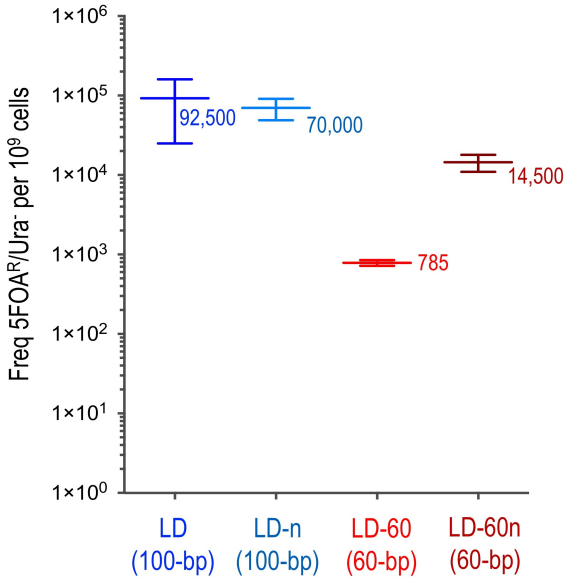

FIG. 3. Effect exhorted by the phosphorothioate protection of the 5´terminus ssDNA in the recombineering efficiency. As above, the expression of Ssr was induced in *P. putida* EM42 cells bearing the pSEVA258-*ssr* plasmid by adding 1mM of 3MB for 30 min and then cells were transformed with different mutagenic oligos containing or not phosphorothiate bonds at one end. Interestingly, we did not observe a difference in recombination by including the phosphorothioate protection for the bigger size oligos (100 bases). Surprisingly, in the reduced size oligo (60 bp) we did observe a negative effect exhorted by the 5´-end DNA protection. This could be caused by the fact the phosphorothioate bonds may reduce the actual size of ssDNA to which the recombinase efficiently binds but this phenomenon should be further investigated. The chart plots the frequency of $5FOA^R/Ura^-$ clones normalized by a total of 10^9 cells and shows the data from at least duplicate experiments. The DNA sequence of the oligos is shown above.

4.9 Induction of the Ssr recombinase

1. Inoculate a 10-mL tube containing 3 mL of LB plus Km directly from the frozen stock of *P. putida* EM42 (pSEVA258-*ssr*) and incubate at 30 °C aerobically overnight.
2. Dilute the overnight grown culture to an OD_{600} of 0.1 in a 250-mL flask containing 60 mL of LB with Km and incubate at 30 °C aerobically until OD_{600} reach ~0.5.
3. Induce the expression of the Ssr recombinase by adding 1 mM 3-methylbenzoate (3MB).
4. Incubate the induced culture for 30 minutes at 30 °C aerobically.

4.10 Preparation of P. putida electrocompetent cells

1. Divide the whole induced culture, 60 mL, in two sterile Falcons by adding 30 mL to each.
2. Centrifuge at 3,220 x *g* for 10 minutes at room temperature (RT). Discard supernantant.
3. Add 10 mL 300 mM sucrose and gently mix to suspend the cellular pellet.
4. Centrifuge at 3,220 x *g* for 5 minutes at RT. Discard supernantant.
5. Add 5 mL 300 mM sucrose and gently mix to suspend the cellular pellet.
6. Centrifuge at 3,220 x *g* for 5 minutes at RT. Discard supernantant.
7. Add 1 mL 300 mM sucrose and gently mix to suspend the cellular pellet and transfer it to 1.5 mL Eppendorf tubes.
8. Centrifuge at 10,600 x *g* for 5 minutes at RT. Discard supernantant.
9. Add 300 µL 300 mM sucrose to each, gently mix to suspend the pellet and mix both cultures into one tube, making a final volume of 600 µL (finally, cells are washed and 100 x concentrated).
10. Distribute 100 µL aliquots into 1.5 mL Eppendorf tubes.

4.11 ssDNA electroporation, recovery and plating

1. Prepare a 100 µM stock solution of the ssDNA mutagenic oligo.
2. Add 1 µL of the ssDNA oligo (~1 µM) to the fresh prepared electrocompetent cells.
3. Gently transfer the mix to a 2-mm gap width electroporation cuvette (**Note 9**).
4. Electroporate at 2.5 kV and quickly add 1 mL of LB Ura.
5. Measure the OD_{600} and dilute the electroporated culture to obtain a final OD_{600} of ~0.1 in a 150 mL Erlenmeyer flask containing 20 mL of LB+Ura.

6. Let cells to recover for 6 hours at 30 °C aerobically (**Note 10**).
7. First, plate dilutions (10^{-3}, 10^{-4}) onto M9 with 0.2% (w/v) citrate plus Ura to obtain an estimate number of viable cells. Also plate dilutions (10^{-1}, direct) on M9 with 0.2% (w/v) citrate plus Ura+ 5FOA to get the number of mutated clones (**Note 11**).
8. Incubate plates at 30 °C for 24 h.
9. Enumerate CFUs on different plates.
10. To confirm that the selected colonies are true *pyrF* deletions a number of colonies were double streaked on M9 with 0.2% (w/v) citrate and M9 with 0.2% (w/v) citrate plus Ura+ 5FOA. Clones that did not grow on the first plate (M9cit) but did on the second (M9cit+Uta+5FOA) were counted as true *pyrF* mutants. Calculate the *pyrF*/5FOA^R ratio and use that value to correct the total number obtained on M9cit+Ura+5FOA plates. Then, that figure is divided by the total viable cells (M9cit+Ura) and normalized to 10^9 cells.
11. In order to verify the proper deletion (1.2 kb vs. 0.5 kb), the genomic DNA surrounding the *pyrF* gene was PCR amplified using primers PYRF-F / PYRF-R from a number of positive clones. The PCR DNA fragment was purified and sequenced with primer PYRF-F (**Note 12**).

4.12 General Molecular Biology Techniques

1. Plasmid DNA was purified from bacteria using the QIAprep Spin Miniprep™ kit (Qiagen Inc., Valencia, CA, USA) by following the manufacturer's instructions.
2. Whole-cell colony PCR is routinely used for DNA amplifications. Colonies are picked directly from the agar plate with a sterile toothpick and placed into the PCR reaction tube containing the proper amount of H_2O. For screening PCRs, we commonly used the Go*Taq*™ Flexi DNA polymerase (Promega Corp., Madison, WI, USA) with the 5x Green Go Taq Flexi buffer and adding 1.5 mM $MgCl_2$ and 0.2 mM dNTPs.
3. DNA required for cloning purposes or Gibson assembly was obtained through PCR amplification using Q5 High-Fidelity DNA polymerase (NEB, Beverly, MA, USA).
4. Prepare a stock solution of deoxynucleotide triphosphates (dNTPs) containing 10 mM of dATP, dCTP, dGTP, and dTTP in milliQ H_2O. Store the solution at −20 ºC.
5. To purify PCR products, we used the NucleoSpin™ Gel and PCR clean-up kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) or the ExoSAP-IT™ PCR product cleanup kit (USB Molecular Biology, Affymetrix Ltd., Santa Clara, CA, USA).

6. For Isothermal assembly, make a homemade mix by preparing a 5x reaction by combining 3 mL Tris-HCl 1M (pH 7.5), 300 µL $MgCl_2$ 1M, 60 µL of dNTPs 100 mM, 300 µL dithiothreitol (DTT) 1M, 1.5 gr of polyethylene glycol (PEG) 8000, 20 mg of NAD and H_2O to 6 mL. Then, add 1.5 µL of T5 exonuclease (Epicentre, Madison, Wi, USA), 20 µL of Phusion™ High-Fidelity DNA polymerase (NEB, Beverly, MA, USA), and 160 µL of Taq ligase (NEB, Beverly, MA, USA). Prepare 15 µL aliquots and store them at −20 ºC until use (19).

5 remarks/troubleshooting:

1. Before selecting the restriction enzymes for cloning the recombinase gene it has to be checked first that it does not contain any of those selected restriction sites within its DNA sequence.
2. If the DNA concentration of the synthesized product is not enough it can be PCR amplified to increase the yield.
3. When the DNA concentration of any of the fragments it is not enough they can be concentrated in a centrifugal evaporator (SpeedVac) to obtain the desired amount in a final 5 µL.
4. If electroporation is going to be the method of choice for transformation the Gibsozined sample has to be dialyzed first to eliminate salts.
5. When introducing plasmid where the expression of the heterologous DNA may be detrimental for the bacterial host we have observed that conjugation tends to maintain the plasmid integrity better than electroporation.
6. Keep the mating mixture at 4 °C until the end of the experiment. To make sure the dilutions plated render isolated colonies. If not the case, two things could have happened: (i) a lawn of cells is observed on the plates, then use a higher dilution; and (ii) no colonies are observed on plates, thus use a lesser dilution.
7. it is recommended to use a restriction enzyme that cuts within the recombinase gene to confirm the plasmid restriction pattern.
8. To calculate the folding of the oligo (ΔG) we recommend to use the web-based tool mfold-UNAFold (http://unafold.rna.albany.edu; *(37)*). In *E. coli* the threshold was set up at -12.5 kcal/mol but in other organisms with different GC% content and optimal growth temperature a different figure might be advised (i.e. for *P. putida* we routinely use folding energies from -13 to -16 kcal/mol *(14)*).
9. In the case that air bubbles are observed within the electroporation cuvette try to eliminate them.

It is also a good idea to clean the electroporation cuvette surface with a paper towel before the pulse.

10. For the particular case of the *pyrF* gene, where mutated cells are overgrown by wild type counterparts even in the presence of uracile, the recovery time was restricted to 6 hours. However, other gene reporter systems may require different growth timing conditions.
11. It is recommended to test different dilution factors the first time the experiment is performed and choose the more appropriate for the next rounds.
12. Take into account that for big size oligonucleotides may contain errors in their DNA sequence (~1 error per 100 bp) due to the intrinsic synthesis process (12, 38).

6 Biosafety

7 References

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8 Acknowledgements

This project has received funding from the European Union's Horizon 2020 *research and innovation programme under grant agreement No 635536.*

