CRISPR/Cas9-enhanced ssDNA recombineering for Pseudomonas putida

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Summary
Implementation of single-stranded DNA (ssDNA) recombineering in Pseudomonas putida has widened the range of genetic manipulations applicable to this biotechnologically relevant bacterium. Yet, the relatively low efficiency of the technology hampers identification of mutated clones lacking conspicuous phenotypes. Fortunately, the use of CRISPR/Cas9 as a device for counterselection of wild-type sequences helps to overcome this limitation. Merging ssDNA recombineering with CRISPR/Cas9 thus enables a suite of genomic edits with a straightforward approach: a CRISPR plasmid provides the spacer DNA sequence that directs the Cas9 nuclease ribonucleoprotein complex to cleave the genome at the wild-type sequences that have not undergone the change entered by the mutagenic ssDNA oligonucleotide(s). This protocol describes a complete workflow of the method optimized for P. putida, although it could in principle be applicable to many other pseudomonads. As an example, we show the deletion of the edd gene that encodes one key enzyme that operates the EDEMP cycle for glucose metabolism in P. putida EM42. By combining two incompatible CRISPR plasmids with different antibiotic selection markers, we show that the procedure can be cycled to implement consecutive deletions in the same strain, e.g. deletion of the pyrF gene following that of the edd mutant. This approach adds to the wealth of genetic technologies available for P. putida and strengthens its status as a chassis of choice for a suite of biotechnological applications.

Introduction
A large number of techniques have become available in recent years that ease the editing of the genomes of different types of bacteria. In the case of E. coli, the development of the lambda Red technology (Datsenko and Wanner, 2000) supposed a great leap forward, not only providing advances in the knowledge of its physiology but in the development of several genome-reduced strains (Kolisnychenko, 2002; Pósfai et al., 2006). A further development of this original approach involves the use of synthetic single-stranded DNA (ssDNA) as the agent for introducing the changes at stake in the replication fork stimulated by the action of the β recombinase of the Red system (Ellis et al., 2001). Alas, the method has an inherent low level of efficiency, which makes identification of mutated clones difficult in the absence of selection (Ellis et al., 2001; Aparicio et al., 2016). This can be alleviated by either multiplexing the process (such as in the case of the so-called multiplex automated genome engineering: MAGE; Wang et al., 2009) or by combining ssDNA recombineering with some type of counterselection. Since the Cas9 nuclease can be directed against a specific DNA by providing it with the desired sequence in the form of a CRISPR spacer, ssDNA recombineering can be combined with the nuclease for killing those clones that have not been modified (Jiang et al., 2013; Ronda et al., 2016). This approach is applicable not just to E. coli, but to other bacteria of biotechnological interest (Keasling, 2012; Calero and Nikel, 2019), including Pseudomonas putida (de Lorenzo, 2011; Martinez-García and de Lorenzo, 2019). A large number of molecular tools and strategies have

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been developed over the years to implement a suite of modifications in the genome of this bacterium (Martínez-García et al., 2014). This includes not only growingly efficient ways to enter changes through double-homologous recombination process (Martínez-García and de Lorenzo, 2011, 2012; Wirth et al., 2019), but also the sort of merged ssDNA recombineering-CRISPR/Cas9 just commented (Aparicio et al., 2016, 2017). An advantage of this technology is the possibility of selecting mutants with a reduced fitness that in the case of homologous recombination-based methods would be outnumbered by wild-type cells.

In this work, we report the formatting of the plasmid containing a CRISPR array following the Standard European Vector Architecture rules (Silva-Rocha et al., 2013; Martínez-Garcia et al., 2015). This new CRISPR plasmid was incorporated into the SEVA database (http://seva.cnbc.unicic.es/). On this basis, we describe a simple protocol to perform diverse types of genome modifications in P. putida. The procedure involves (i) ssDNA and spacer selection; (ii) cloning the spacer into the CRISPR plasmid; (iii) co-transform the desired host with the ssDNA and the CRISPR plasmid bearing the spacer; (iv) confirm the deletion; and (v) cure the plasmids from the deleted strain. Moreover, we show the possibility of combining the use of two mutually exclusive CRISPR plasmids with different antibiotic selection markers for cycling a multi-deletion process.

Protocol

The procedure described in this paper consists of combining together two simple techniques (i) ssDNA recombineering to introduce the desired DNA change in the genome and (ii) CRISPR/Cas9 to efficiently counterselect the non-modified bacterial clones to easily recover mutated clones with a non-conspicuous phenotype. Here, we are going to illustrate this protocol with a 1 kb deletion example but it could be applied as well for DNA insertions, single nucleotide changes or big chunk deletions.

Bacterial strains, media and chemicals

The E. coli bacterial strains used in this work are CC118 (Manoil and Beckwith, 1985) as the cloning host and HB101 (pRK600) as the helper strain for tri-parental matings (Kessler et al., 1992). To perform the deletion experiments, we used the Pseudomonas putida KT2440 derivative named EM42 (Martínez-Garcia et al., 2014). The list of plasmids used in this paper is described in Table 1. LB medium is used as the routine medium for growth of both P. putida and E. coli. In specific cases, we used the M9 minimal medium (Sambrook et al., 1989) supplemented with 0.2% (w/v) of either glucose or citrate as the sole carbon source. The use of citrate as C-source is required for nutritional selection, such as in the case of matings to transfer plasmids from E. coli to P. putida. This C-source allows to counterselect the E. coli donor and mating helper strains from the mating mix (Martínez-García et al., 2017). Moreover, when required media were supplemented with 50 μg ml⁻¹ kanamycin (Km), 30 μg ml⁻¹ chloramphenicol (Cm), 10 μg ml⁻¹ or 15 μg ml⁻¹ gentamicin (Gm) for E. coli or P. putida, respectively, 100 μg ml⁻¹ streptomycin (Sm) for P. putida and 50 μg ml⁻¹ for E. coli, and 20 μg ml⁻¹ uracil. All oligonucleotides were purchased from Sigma-Aldrich, dissolved in H₂O to obtain either 5 μM oligo solutions for PCR and sequencing reactions or 100 μM stocks for ssDNA recombineering. The oligonucleotide stocks were stored at −20°C.

Construction of a bacterial strain harbouring plasmids with an inducible recombinase and Cas9

The first thing we need to start a deletion project in our selected host is to introduce two different plasmids that are required for the technique. One is the pSEVA658-ssr plasmid that expresses the Ssr recombinase in an inducible way (Aparicio et al., 2016); and the second one is the pSEVA421-Cas9tr that constitutively expresses the

Table 1. Plasmids used in this work

| Plasmid | Description and relevant characteristics | References |
|---------|------------------------------------------|------------|
| pSEVA658-ssr | xyS-Pm -> ssr, oriV RSF1010; GmR | Aparicio et al. (2017) |
| pSEVA421-Cas9tr | Cas9 and tracrRNA; oriV RK2; SmR²/SpR² | Aparicio et al. (2017) |
| pSEVA2316 | SEVA CRISPR array; oriV pBBR1; KmR | This work |
| pSEVA5316 | SEVA CRISPR array; oriV pBBR1; TelR | This work |
| pSEVA2316-edd1 | pSEVA2316 derivative bearing the edd1 spacer | This work |
| pSEVA231-C-edd1 | pSEVA231-CRISPR derivative bearing the edd1 spacer | This work |
| pSEVA231-C-edd3 | pSEVA231-CRISPR derivative bearing the edd3 spacer | This work |
| pSEVA5316-pyrF1 | pSEVA5316 derivative bearing the pyrF1 spacer | This work |
| pSEVA231-CRISPR | CRISPR array; oriV pBBR1; KmR | Aparicio et al. (2017) |
| pSEVA231 | MCS; oriV pBBR1; KmR | Silva-Rocha et al. (2013) |
| pSEVA531 | MCS; oriV pBBR1; TelR | Silva-Rocha et al. (2013) |
| pRK600 | Mating helper plasmid; oriV ColE1, RK2(mob* tra*); CmR | Kessler et al. (1992) |

Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; MCS, multiple cloning site; Sm: streptomycin; Sp: spectinomycin; Tel: tetracycline.
Cas9 nuclease and the tracrRNA (Aparicio et al., 2017). We recommend to do it serially, introducing one plasmid first and then the other. We tested both possible order combinations, first introducing the Ssr-containing plasmid and then the Cas9 vector; and the other way around, the Cas9 plasmid first and then the Ssr vector. Both permutations worked fine in different P. putida strains. To transform these plasmids, even though more time-consuming, we recommend the conjugation option as the choice method not only to increase the efficiency but also to maximize the correct integrity of these plasmids. For that reason, we describe below the mating protocol, and in the section dealing with the interference test, we will explain the electroporation procedure.

**Introduction of plasmids in the strain of choice**

The protocol described here is a simplified version of Martinez-Garcia et al. (2017) without measuring OD$\text{_{600}}$ of cultures and not using filters to lay the bacteria on.

i. From the –80°C frozen stocks grow aerobically overnight liquid cultures of:

   a. *E. coli* CC118 donor cells harbouring the plasmid to be transferred into *P. putida* (pSEVA658-ssr or pSEVA421-Cas9tr) in 2 ml LB with the appropriate antibiotic (Gm or Sm) at 37°C.

   b. *E. coli* HB101 helper strain (Boyer and Roulland-Dussoix, 1969), that encodes the transfer and mobilization functions in the plasmid pRK600, in 2 ml LB with Cm at 37°C.

   c. *P. putida* recipient strain in 2 ml LB at 30°C.

ii. Take 800 µl of the grown cultures, transfer them to a 1.5 ml Eppendorf tube and centrifuge at 9300 g for 2 min. Discard the supernatant and add 800 µl of 10 mM MgSO$_4$. Then, suspend the pellet gently.

iii. Centrifuge at 9300 g for 2 min. Discard the supernatant, add 800 µl of 10 mM MgSO$_4$ and suspend the pipetting up and down.

iv. Transfer 100 µl of each of the three bacterial strains to a new 1.5 ml Eppendorf tube. Centrifuge at 9300 g for 2 min. Discard the supernatant and add 20 µl of 10 mM MgSO$_4$.

v. Spot the 20 µl mating mixture onto a dried and pre-warmed LB agar plate. Let it dry for 5 min at room temperature and then incubate the LB agar plate at 30°C for 6 h in an upward position.

vi. Using a bended yellow tip scrape the mating spot and suspend it in 1 ml of 10 mM MgSO$_4$.

vii. Plate different dilutions (normally, 10$^{-3}$, 10$^{-2}$ and 10$^{-1}$) onto M9 minimal medium with 0.2% (w/v) citrate supplemented with the appropriate antibiotic (15 µg ml$^{-1}$ Gm for pSEVA658-ssr or 100 µg ml$^{-1}$ Sm for pSEVA421-Cas9tr). Incubate overnight at 30°C.

viii. Select a few colonies and re-streak them into M9 + 0.2% (w/v) citrate+ antibiotic and check the presence of the correspondent plasmid by miniprep and restriction.

ix. Prepare a frozen stock of the correct strain in LB 20% (v/v) glycerol and store at –80°C.

x. Repeat the whole process to introduce the second plasmid.

**Cloning the spacer into the CRISPR plasmid**

This section explains how to design and anneal the appropriate spacers for their cloning into the empty CRISPR plasmids.

**Construction of a SEVA CRISPR plasmid**

Even though the pSEVA231-CRISPR plasmid was proven to be fully functional as stated by Aparicio et al. (2017), it does not match the SEVA standardization rules (Silva-Rocha et al., 2013; Martinez-Garcia et al., 2015). This is because of the presence of a PshAI restriction site in the natural sequence of the leader region of the CRISPR array. Together, both PshAI and Swal are two key enzymes for the SEVA standard since they are required to swap the antibiotic resistance marker in those plasmids. For that reason, we decided to apply the SEVA format (Silva-Rocha et al., 2013; Martinez-Garcia et al., 2015) to the CRISPR module as a new cargo for the collection and test its functionality. To do that, we needed to eliminate PshAI restriction site present in the promoter region of the CRISPR element. So, we changed the natural sequence present in the pSEVA231-CRISPR plasmid 5'-GACTGAAGTC-3’ for the newly designed 5'-CATCAGAGTC-3’. On this basis, we outsourced the complete synthesis of the 395 bp CRISPR DNA module to GeneCust. Since the resulting synthesized DNA was cloned in the pUC57 vector, the module was excised with the flanking enzymes EcoRI and BamHI and cloned into those sites in the cargo region of pSEVA231 and pSEVA531 plasmids. This new module was assigned with the SEVA cargo code #16 (Fig. 1A) and the resulting plasmids named pSEVA2316 and pSEVA5316.

**CRISPR plasmid extraction**

This protocol is used here to prepare the CRISPR plasmid (pSEVA2316, pSEVA5316 or pSEVA231-CRISPR; Table 1) that is going to be the receptor of the spacers but it also works to extract the plasmids containing the spacers for the electroporation required at the final steps of the deletion procedure. For plasmid extraction, we normally use the QIAprep Spin Miniprep™ Kit (Qiagen Inc., Valencia, CA, USA) and follow the manufacturer’s instructions.
i. From the frozen stock, inoculate with the E. coli strain that harbours the pSEVA2316 (or any other of the CRISPR plasmid) in a 100 ml flask containing 20 ml of LB plus the appropriate antibiotic and grow it aerobically at 37°C overnight.

ii. Transfer the culture to a 50 ml Falcon tube and centrifuge the whole culture at 3220 g at room temperature.

iii. Discard the supernatant and proceed with the plasmid extraction adding the volume recommended for 4 reactions of buffer 1 (in the particular case of Qiagen, we add 1000 µl of buffer 1).

iv. Vortex to re-suspend the pellet and distribute the total 1 ml into four Eppendorfs containing 250 µl each.

v. Proceed as indicated in the instructions of the plasmid extraction kit provider.

vi. Elute the plasmid DNA by adding 100 µl of H2O to each tube. Then, concentrate the DNA in a Speed-Vac for 30 min and finally mix the liquid of the four Eppendorfs into one tube.

vii. Quantify the plasmid DNA concentration with a NanoVue Plus (GE Healthcare, Chicago, IL, USA).

Restriction of the CRISPR plasmid with BsaI and backbone purification

The empty CRISPR plasmid has to be digested with the restriction enzyme BsaI or BsaI-HF (NEB, Ipswich, MA, USA) to clone the designed spacers (Figs 1B and 2A).

Prepare the following restriction mix:

i. Add 10 µl of the 10× CutSmart buffer.

ii. Add 10 µl of the 10× bovine serum albumin (BSA).

iii. Complete with 78 µl of plasmid DNA

iv. 2 µl of BsaI

v. Incubate for 2 h at 37°C.

vi. Add 17 µl of 6× Gel loading Dye (NEB, Ipswich, MA, USA).

Once the plasmid is digested, proceed to purify the plasmid backbone DNA from an agarose gel. This ensures the elimination of the DNA buffer sequence from the restriction mixture.

i. Prepare a 1% (w/v) agarose gel and load the restriction sample.

ii. Purify the 3.3 kb linear fragment of the pSEVA2316 plasmid (or 3.7 kb for pSEVA5316) using an appropriate DNA extraction kit. We normally use the NucleoSpin™ Gel and PCR clean-up kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany).

iii. Quantify the purified plasmid DNA concentration spectrophotometrically. Also, visualize the DNA by inspection on a 1% (w/v) agarose gel. Purified plasmid DNA can be kept at −20°C until further use.

Spacer selection for crRNA

In order to use the CRISPR/Cas9 system as a counterselection method, we first need to clone a proper spacer...
into the BsaI sites of the plasmid that contains the CRISPR array (pSEVA2316, pSEVA5316 or pSEVA231-CRISPR; Table 1). The cloned spacer is transcribed and processed into a proper crRNA that together with the tracrRNA guides the Cas9 nuclease to the target position in the chromosome (Gasiunas et al., 2012; Jinek et al., 2012) (Fig. 2B). Once the Cas9:tracrRNA:crRNA complex finds its genomic target and it is adjacent to a PAM, the nuclease introduces double-strand breaks (DSB) that are lethal if not repaired. For that reason, the spacer sequence has to be contained within the region that is intended to be deleted or substituted in order to allow mutant clones to escape the scan of the Cas9 nuclease. In the case of a gene/operon deletion, the spacer can be located anywhere within the eliminated DNA. When intending to perform single base substitutions, it is crucial that the distance of the modified nucleotide to the PAM is no more than 3-nt away (Aparicio et al., 2017). This requirement limits the possibility of single base substitutions to the proximity of PAM sequences in that area. Also, it is important that the mismatch between the genome and mutagenic oligo is loosely recognized by the endogenous MMR system, otherwise it will be automatically repaired (Aparicio et al., 2017). The selection of spacer sequences could be done manually or by the use of a specific online software tool such as CRISPOR (http://crispor.org) (Haessler et al., 2016) and CRISPy-web (https://crispy.secondarymetabolites.org/#/input; Blin et al., 2016). However, in this section we describe the manual procedure applied to the deletion of the edd gene of P. putida EM42. We strongly recommend to select at least two or three spacers and test them at the same time to ensure that at least one of them works properly. Even consuming more resources, this approach could save time. Then, use the spacer that shows the best efficiency in the interference test (see below).

i. Start by scanning your target gene or region of interest to identify different protospacer-adjacent motifs (PAM: 5'-NGG-3') in any of the DNA strands.

ii. Then, select the 30 nucleotides immediately adjacent to the PAM in the 5' direction, such as 5'-N<sub>nt</sub>-NGG-3' (where N could be any of the four nucleotides) and order the 30-nucleotide sense oligo (S), importantly without including the PAM in its sequence, and the

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Annealing of spacer oligonucleotides

i. Add the required volume of H₂O to the lyophilized spacer oligonucleotides to obtain a concentration of 100 μM. Vortex both tubes for 30 s and incubate them at RT for 5 min to dissolve them.

ii. Prepare the annealing mix by adding 45.5 μl of H₂O, 2.5 μl of 1.0 M NaCl, 1 μl of oligo S (100 μM) and 1 μl of the oligo AS (100 μM) into a PCR tube.

iii. Place the PCR tube with the mix in a thermocycler and dilute it with 90 μl of H₂O to obtain a 0.2 μM concentration. The annealed oligonucleotides stocks can be stored at −20°C for future use.

Cloning of spacers into the CRISPR plasmid

In the text below, pSEVA2316 (Km²) is used as example but the same procedure applies to any other CRISPR plasmid of choice. If using a different CRISPR plasmid, change accordingly the antibiotic used for selection during the protocols of cloning, interference and recombineering-CRISPR/Cas9. To start this process, thaw the BsaI-restricted pSEVA2316 plasmid and the diluted annealed oligonucleotides and prepare the following ligation mixture:

i. Add 10 μl of the 2× quick ligation buffer

ii. Include 6 μl of H₂O

iii. Add 50 to 100 ng of the linearized pSEVA2316.

iv. Add 1 μl of the diluted annealed oligonucleotides.

v. 1 μl of quick ligase

vi. Incubate 5 min at room temperature

After the ligation process, we can directly proceed with the transformation step. To do that, we must have already prepared chemically or electrocompetent E. coli cells.

vii. Take 10 μl of the ligation mixture and add to competent cells of your favourite laboratory strain of E. coli (we normally use chemically competent cells of E. coli CC118).

viii. Incubate 15 min in ice.

ix. 1 min and 30 s at 42°C.

x. Place for 5 min in ice.

xi. Add 900 μl of LB to the 100 μl of competent cells and incubate for 1 h at 37°C aerobically.

xii. Centrifuge at 7200 g for 2 min; discard 900 μl of the supernatant and use the 100 μl leftover to suspend the pellet. Then, plate everything onto an LB agar plate plus 50 μg ml⁻¹ of Km. Incubate overnight at 37°C.

xiii. Re-streak a few clones to a fresh LB agar plate supplemented with Km to isolate individual clones.

xiv. Extract plasmids from re-streaked clones and send to sequence with either oligonucleotide PS1 or PS2 (Table 2) to verify the presence of the spacer.

xv. Select a correct clone and re-streak in a fresh LB+Km agar plate and incubate it at 37°C overnight. Then, prepare a frozen stock by adding 2.5 ml of LB 20% (w/v) glycerol to the agar plate and with the help of a bended yellow tip scrape all cells, transfer the supernatant to cryovial and store it at −80°C.

Interference test: checking the efficiency of spacers

Not all spacer sequences have the same efficiency guiding the Cas9 nuclease and it is not well understood what
The aim of this optional experiment was to test the efficiency of the selected spacers to guide the Cas9 to target the chromosome and kill the cell. This procedure will allow us to select the most efficient spacer in a fast, easy way. Having one spacer that works efficiently ensures the successful use of CRISPR/Cas9 as a counterselection technique in a ssDNA recombineering experiment. Briefly, P. putida EM42 previously transformed with the plasmids pSEVA658-ssr and pSEVA421-Cas9tr, as described before, is electroporated in parallel with the CRISPR plasmids containing the spacer and also the control plasmid (pSEVA231-CRISPR or pSEVA2316). Cells transformed with the control plasmid should produce a Cas9 complex without an effective target within the P. putida genome, rendering viable transformant clones (Fig. 3A). When using a different organism, it is important to ensure that the buffer sequence of the control plasmid has no match in that bacterium as previously checked for P. putida KT2440 (Aparicio et al., 2017). Nevertheless, in the case of cells transformed with the plasmids harbouring the designed spacers, the Cas9 complex will be able to identify the target adjacent to a PAM motif, introducing double-strand breaks (DSB) in the genome that lead to bacterial death (Fig. 3A). To perform the interference experiment, proceed as follows:

i. Start by purifying and quantifying the control plasmid (pSEVA2316 or pSEVA231-CRISPR) and the CRISPR vectors harbouring the appropriate spacers (pSEVA231-C-edd1, pSEVA231-C-edd3 and pSEVA2316-edd1; Table 1). To do that, follow the procedure described in the CRISPR plasmid extraction section.

ii. Inoculate a P. putida strain that harbours the pSEVA658-ssr and pSEVA421-Cas9tr in a 100 ml flask containing 20 ml of LB supplemented with Gm and Sm. Grow that culture aerobically overnight at 30°C.

iii. Collect the culture in a 50 ml Falcon tube and proceed to prepare electrocompetent cells.

iv. Centrifuge at 3200 g for 10 min at room temperature (RT) and discard supernatant.

v. Add 10 ml of 300 mM sucrose and gently mix to suspend the cellular pellet.

vi. Centrifuge at 9300 g for 2 min at RT. Discard supernatant.

vii. Add 1 ml of 300 mM sucrose and gently mix and transfer the supernatant to 2 ml Eppendorf tube.

viii. Centrifuge at 9300 g for 2 min at RT. Discard supernatant gently (bacterial pellet could be loosely attached to the tube), add 800 μl of 300 mM sucrose and re-suspend cells.

ix. Repeat step viii at least three times.

### Table 2. Oligonucleotides used in this work

| Name       | Sequence 5’ → 3’                                      | Usage                     | References                                |
|------------|--------------------------------------------------------|---------------------------|-------------------------------------------|
| PS1        | AGGGCGGCGGATTTGTCC                                     | To sequence the cargo region of pSEVA plasmids | Silva-Rocha et al. (1989)                  |
| PS2        | GCGGCAACCGAGCGTTC                                      | To sequence the cargo region of pSEVA plasmids | Silva-Rocha et al. (1989)                  |
| PS3        | GAACGCTCGGTTGCCG                                       | To sequence the gadget and selection marker of pSEVA plasmids | Silva-Rocha et al. (1989)                  |
| edd-40     | GCCCTGGAAGCGCACCACCGCGACAAAGTCACGCT                   | Mutagenic oligo to delete a 1 kb fragment of the edd gene | This work                                 |
| cr-edd-1-S | AAAACGCACAACATGTTCGACGCGGCACTCATGCTG                   | Oligonucleotide to obtain the edd-1 spacer     | This work                                 |
| cr-edd-1-AS| AAAACTTCGGGCATTGTCGAAACCCTGTGTGACAAG                  | Oligonucleotide to obtain the edd-1 spacer     | This work                                 |
| cr-edd-3-S | AAACCCGCAGCCTGGGCGATCGCCGGGATGTGCAG                   | Oligonucleotide to obtain the edd-3 spacer     | This work                                 |
| cr-edd-3-AS| AAAACTGCACATCCCGGCGATCGCCCAGGCTGCGG                  | Oligonucleotide to obtain the edd-3 spacer     | This work                                 |
| edd-check-F| TAAACCGCCCTTACAATTAG                                     | Diagnostic oligo for deletion of the edd gene  | This work                                 |
| edd-check-R| ACCAACGCAACCTTGTAG                                     | Diagnostic oligo for deletion of the edd gene  | This work                                 |
| pyrF-B-np  | AACAGGCATCGGTGGTTCGGCACAGGCCCTTGCTG                   | Mutagenic oligo to delete the complete pyrF gene | This work                                 |
| cr-pyrF-1-S | AAACTTCGGGCATTGTCGAAACCCTGTGTGACAAG                  | Oligonucleotide to obtain the pyrF spacer      | Aparicio et al. (2017)                    |
| cr-pyrF-1-AS| AAAACTTGTCACACAGGGTTTCGACAATGCCCGAA                  | Oligonucleotide to obtain the pyrF spacer      | Aparicio et al. (2016)                    |
| pyrF-F      | CGAGGGCTATGATGAGTATC                                     | Diagnostic oligo for deletion of the pyrF gene | Aparicio et al. (2016)                    |
| pyrF-R      | GTCAGGTGAAGAGCAAAGAG                                     | Diagnostic oligo for deletion of the pyrF gene | Aparicio et al. (2016)                    |

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ssDNA recombineering with CRISPR/Cas9 counterselection

Upon selection of a target gene, one has to start by designing a proper mutagenic oligonucleotide to perform the desired deletion experiment. The total length of the ssDNA should be around 80 or 90 bp, containing around 40 nucleotides of upstream and another 40 bp of downstream homology in the regions flanking the area to be deleted (Fig 2A). In the case of single substitutions, the mutation should be included in the middle part of the recombinogenic oligonucleotide and is important to take into account the effect of the mismatch repair system (MMR) of the bacterial host (Babic et al., 1996; Aparicio et al. 2017). Then, it is recommended to design the mutagenic oligo against the lagging strand (Ellis et al., 2001). This requires to know, a priori, the genomic coordinates of the oriC and dif regions in the organism of choice to be able to define the two replicohores, positioning the leading/lagging strand in each one (Carnoy and Roten, 2009). In the case of not knowing those features, just design oligonucleotides for the two strands and test them both. The last aspect to consider is to minimize as much as possible the folding energy of the mutagenic oligo (preferentially $\Delta G > -12.5$ kcal mol$^{-1}$ for E. coli and $>-16$ kcal mol$^{-1}$ for P. putida). For more details, see Aparicio et al. (2016).
Perform colony PCR to check the target deletion using

i. Start by re-streaking a number of colonies on a fresh

PCR to check the deletion.

ii. In order to confirm the complete deletion of the target
gene in the transformant colonies, design oligonu-

merase (following vendor

directions) into each PCR

tube and proceed with the reaction.

iii. Prepare a 1/1000 dilution, vortex and transfer 3 µl of the
grown culture (even though no

visible growth is observed) to a new tube with 3 µl of

fresh LB.

iv. Incubate overnight at 30°C with shaking.

v. Finally, use the grown liquid culture to streak a LB

agar plate to obtain separate colonies.

vi. Screen several colonies by streaking them onto LB

agar plates with and without the proper antibiotics

(Sm, Gm and Km).

vii. Prepare a positive clone and send to sequence the

complete deletion of the target

gene.

viii. If required, proceed to cure the three different

plasmids from the strain that harbours three different plasmids and the last

step of this protocol is to eliminate those plasmids from the new engineered strain. A diagram of a general plasmid curation process is represented in Fig. 3B.

The steps that we have to follow in the laboratory are the following:

i. inoculate from the frozen stock a 10 ml test tube

containing 3 µl of LB.

ii. Incubate overnight at 30°C with shaking.

iii. Prepare a 1/1000 dilution, vortex and transfer 3 µl to

tube with 3 µl of LB.

iv. Incubate for 6 h at 30°C aerobically.

v. Transfer 3 µl of the grown culture (even though no

visible growth is observed) to a new tube with 3 µl of

fresh LB.

vi. Incubate overnight at 30°C with shaking.

vii. Repeat the process from step iii to vi at least 10

times.

viii. Finally, use the grown liquid culture to streak a LB

agar plate to obtain separate colonies.

ix. Screen several colonies by streaking them onto LB

agar plates with and without the proper antibiotics

(Sm, Gm and Km).

x. Select a colony that is sensitive to all antibiotics used

(Sm, Gm and Km) and check again by PCR that is

the desired mutated strain (Fig. 3B). Then, prepare a

glycerol stock and maintain it at −80°C. If colonies are still resistant to any of the antibiotics, repeat the process from step iii to ix.

Application examples

With this information in mind, we aimed to test (i) the

functionality of the standardized CRISPR plasmid by

deleting a target gene (ii) and the possibility of cycling

the deletion process by eliminating the curation step of

the CRISPR plasmid, speeding the process of making

serial deletions into the same strain, increasing the effi-
ciency of the process.

Deletion of the edd gene of P. putida using ssDNA recombinase and CRISPR/Cas9

The glucose catabolism in P. putida occurs almost entirely through the Entner–Doudoroff (ED) pathway and

EDEMP cycle (Chavarria et al., 2013; Nikel et al., 2015). The first enzyme of this pathway is a phosphogluconate

dehydratase (EC 4.2.1.12) that catalyses the transformation of 6-phospho-D-gluconate (6PG) to 2-keto-3-deoxy-

6-phospho-D-gluconate (KDPG). This enzyme is encoded by the edd (PP_1010) gene. A disruption of this
gene prevents the growth of those clones in glycolytic carbon sources but not in gluconeogenic ones or rich media (Nikel et al., 2015). For that reason, we selected the *edd* (PP_1010) gene as our deletion target because mutants would show a detectable phenotype (impaired growth on glucose). Moreover, the deleted strain could be interesting *per se* for certain laboratory applications, such as counterselection to discriminate donor and recipient cells in mating-based experiments between two *Pseudomonas putida* strains.

**Interference test to identify a functional spacer**

As recommended in the interference test, it is always a good idea to perform a quick-and-dirty experiment to test the functionality of various spacers to choose the most efficient one for the deletion part. For that, we chose two potential spacers that would direct the Cas9 nuclease complex against different regions of the *edd* gene. Thus, we selected two regions of 30 nucleotides that are adjacent to a PAM sequence (Fig. 2 shows the specific example of spacer edd-1). Then, we cloned the spacers by annealing the oligos cr-edd-1-S with cr-edd-1-AS and cr-edd-3-S with cr-edd-3-AS (Table 2) to yield plasmids pSEVA231-C-edd1 and pSEVA231-C-edd3 (Table 1). The idea of this simple experiment is that cells containing the Cas9 and the recombinase vector would be transformed with either a CRISPR control plasmid that would not be able to assay a target within the genome and transformed cell thus would be viable (Fig. 3A). On the other hand, cells would be also transformed with a CRISPR plasmid that contain a spacer that would direct the Cas9 complex to a specific genomic target located adjacent to a proper PAM, introducing in that way a DSB that would result in cell death (Fig. 3A).

To select the most efficient spacer, we performed an experiment with just one replica. However, in order to properly compare the different plasmids to test, it is important to use the same batch of electrocompetent cells. *P. putida* EM42 (pSEVA658-ssr and pSEVA421-Cas9tr) was transformed with the following plasmids: pSEVA231-CRISPR (control), pSEVA231-C-edd1 and pSEVA231-C-edd3. After 2 h of recovery at 30°C in LB+Sm+Gm, we plated dilutions of transformed cells on LB+Sm+Gm to estimate the number of total viable cells and on LB+Sm+Gm+Km to enumerate the number of transformants. The efficiency of the interference test was calculated, in each case, dividing recombinant clones by viable cells and normalizing that number to 10⁶ cells. To represent the data charted in Fig. 4A, we plotted the ratio of the transformation efficiencies of the control by the CRISPR plasmids. Bigger values represent better interference efficiencies. This is an important parameter since it is going to determine the usability of the spacer. We have observed that values close to 100 allow to perform an efficient counterselection when combined with ssDNA recombineering to delete a gene (Aparicio et al., 2017). In this case, plasmid pSEVA231-C-edd1 showed a higher interference than pSEVA231-C-edd3 (Fig. 4A). So, we selected spacer edd1 for future experiments and discarded the spacer edd3.

**Assaying the functionality of the new pSEVA2316 plasmid**

Our next objective was to evaluate the functionality of the new constructed CRISPR module. Therefore, we cloned the most efficient spacer, edd1, in plasmid pSEVA2316 to render pSEVA2316-edd1 (Table 1). Then, we proceed to compare the efficiency of the SEVA rule violating, pSEVA231-C-edd1, and the SEVA plasmid, pSEVA2316-edd1 (Fig. 4B). In this case, we performed three biological replicates and represented the average and standard deviation. As shown in Fig. 4B, both plasmids display similar interference values (*p* value of 0.79; unpaired t test with a significance threshold set at 0.05), demonstrating that the SEVA version of the CRISPR plasmid shows the same functionality. On these terms, we select the SEVA version for the following experiments.

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Target edd gene deletion

Once selected an efficient spacer and tested the functionality of the pSEVA2316-edd1, the next step was to proceed with the ssDNA recombineering to delete a 1 kb DNA fragment of the edd gene and the counterselection exohorted by the CRISPR/Cas9 system as a proof of concept. A scheme of the whole process is depicted in Fig. 5A. Briefly, cells loaded with the Ssr recombinase are co-transformed with 1 μl of the ~100 μM mutagenic oligonucleotide edd-40 (Table 2) and 100 ng of the CRISPR plasmid that has the appropriate spacer (pSEVA2316-edd1; Table 1) to kill unmodified cells. Transformed cells were recovered for 2 h at 30°C in LB+Sm+Gm and plated on selective LB media containing Sm+Gm+Km to recover clones with the three plasmids (Fig. 5A). Two different morphologies, big and small colonies, were clearly visible in the plate. Then, individual clones of each morphology were subjected to colony PCR, using oligos edd-check-F and edd-check-R (Table 2), to verify whether they have the desired mutation or not. It turned out that all small colonies were deleted strains while the big ones corresponded to wild-type cells. In order to calculate the editing efficiency, we considered all colonies, big and small, that appeared on the selective plate. We repeated the experiment twice and plotted the average with the standard deviation. The observed deletion efficiency of the edd gene was 73% while 27% of the colonies remained wild type (Fig. 5B).

Moreover, we selected a total of six mutated clones and the genomic region of edd was sequenced to validate the accuracy of the deletion. All clones showed the expected sequence in the deleted region. Therefore, this approach establishes a powerful and reliable genome engineering tool, allowing to obtain a deleted strain within a few days.

Cycling the deletion process

In certain cases, it is necessary to introduce more than one deletion in the same strain. We wanted to test the
possibility of cycling the deletion process without curing the CRISPR plasmid. Meaning by that, to directly electroproporate the strain with a deletion in the Gene Of Interest-1 (GOI-1) with both mutagenic oligo#2 and CRISPR plasmid-spacer #2. Since both CRISPR plasmids share the same replicon (pBBR1), it is important to use two plasmids with different selection markers (Km\(^R\) and Tet\(^R\) in this example). Once confirmed the second deletion event (strain GOI-2), a third round could potentially be introduced by re-using again the Km\(^R\) CRISPR plasmid-bearing spacer #3. Then, a fourth round could be done using the Tet\(^R\) CRISPR plasmid-bearing spacer #4. An illustration of the process is depicted in Fig. 6A. So as to test whether this cycling process is doable, we planned to perform two deletions within the same strain. The selected targets were the previously deleted \(edd\) and the \(pyrF\) gene (PP_1815), whose disruption generates a strain auxothoph for uracil (Aparicio et al., 2016). The spacer pyrF1 was designed and tested in a previous work into the pSEVA231-CRISPR plasmid (Aparicio et al., 2017). To perform this cycled experiment, we cloned the pyrF1 spacer into a Tet\(^R\) CRISPR plasmid (pSEVA5316) to obtain pSEVA5316-pyrF1 plasmid (Table 1).

Once obtained a \(P.\ putida\) EM42 with an \(edd\) deletion (as described above), we transformed that strain with the pyrF-B-np oligo (Table 2) and the pSEVA5316-pyrF1 plasmid (Table 1) and plated dilutions on LB+S+m+Gm+Tet+Ura. The experiment was done twice and all colonies tested \((n=20)\) corresponded to pyrF deleted strains, accounting a 100% efficiency (Fig. 6B). Of those, we selected 6 mutated clones and confirmed the deletion by sequencing the appropriate genomic region. All clones had the expected sequence throughout the deletion boundaries. Then, we tested whether the second CRISPR plasmid (Tet\(^F\)) displaced the first one (Km\(^R\)) or both were able to coexist in bacterial cells. To do that, we selected a number of clones with a double deletion that were Sm\(^G\)Gm\(^R\)Tet\(^R\) and re-streaked those on similar plates supplemented with Km. Of a total of 35 clones, \~83%\ were Km\(^R\), denoting that were able to maintain both CRISPR plasmids, while \~17%\ were Km\(^S\), indicating that they lost the first CRISPR plasmid and only kept the second one. The plasmid displacement occurred at a doable frequency, allowing to establish a serial deletion protocol that might speed up multiple deletions.

Finally, after having the desired \(\Delta edd\ \Delta pyrF\) strain, we proceeded to cure the three working plasmids (Fig. 3B). To start this process, we selected a double mutant clone that was Km\(^S\) and inoculated an LB tube without antibiotics, performing five consecutive passages of curing. After those, we checked 20 individual colonies and confirmed that they lost the pSEVA421-Cas9tr (Sm\(^R\)) and pSEVA5316-pyrF1 (Tet\(^F\)) but all of them still had the high/medium copy plasmid pSEVA658-ssr (Gm\(^R\)). Then, we started the process again and continued it for an extra 10 more cycles. After that, we analysed a total of 150 colonies and 98% did lose the pSEVA658-ssr
plasmid. Doing this protocol, we constructed a *P. putida* strain (EM42ΔeddΔpyrP) that is unable to grow on glucose as C-source and also shows auxotrophy for uracil. To phenotypically confirm that, we streaked the wild type, the Δedd mutant and double mutant (ΔeddΔpyrP) on four different media (Fig. 7). On M9 + cit+uracil, as expected, the three strains were able to grow. Then, on M9 + citrate only the wild type and Δedd mutants grew. Similarly, when using glucose as C-source, neither of the *edd* mutants were able to grow.

**Outlook**

In this article, we examined the combined use of ssDNA recombineering with CRISPR/Cas9 as the counterselection technique to re-write bacterial genomes. The present protocol allows to do genome engineering in *P. putida* not only in a shorter time than homologous recombination-based approaches, but it also demands less laboratory work than other procedures (Martinez-Garcia and de Lorenzo, 2011, 2012). One simple cloning step for the construction of the CRISPR plasmid containing the spacer is required. Here, we extensively described all the steps needed to do a genome modification using *P. putida* as the example organism. Fig. 8 summarizes all the steps of the process in a simple workflow. However, this protocol could be adjusted easily to work in any *Pseudomonas* species. To facilitate that, we also SEVARized the CRISPR array, eliminating the PshAI restriction site present within the leader region, to render the new cargo assigned with the code #16. We included that cargo into the pSEVA231 and pSEVA531 plasmids to yield the pSEVA2316 and pSEVA5316 vectors that would be included in the SEVA database. Since these plasmids are modular now, the antibiotic selection marker of these plasmids could be easily swapped by the use of Swal and PshAI restriction enzymes with any other cassette of the collection at user’s will. The use of these two CRISPR plasmids together with the pSEVA421-Cas9-tr and pSEVA658-ssr allows to cycle the deletion process without the need to cure the previously used CRISPR plasmid. This cycled process is especially indicated for cases where one needs to do several genome modifications within the same strain. This protocol could be further optimized, for instance by reducing the number of working plasmids, but at the end of the day the general idea and the steps of this procedure would be similar.

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**Conflict of interest**

None declared.

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