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Accelerated genome engineering of *Pseudomonas putida* by I-SceI-mediated recombination and CRISPR-Cas9 counterselection

Nicolas T. Wirth, Ekaterina Kozaeva and Pablo I. Nikel*

The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, 2800, Kongens Lyngby, Denmark.

Summary

*Pseudomonas* species have become reliable platforms for bioproduction due to their capability to tolerate harsh conditions imposed by large-scale bioprocesses and their remarkable resistance to diverse physicochemical stresses. The last few years have brought forth a variety of synthetic biology tools for the genetic manipulation of pseudomonads, but most of them are either applicable only to obtain certain types of mutations, lack efficiency, or are not easily accessible to be used in different *Pseudomonas* species (e.g. natural isolates). In this work, we describe a versatile, robust and user-friendly procedure that facilitates virtually any kind of genomic manipulation in *Pseudomonas* species in 3–5 days. The protocol presented here is based on DNA recombination forced by double-stranded DNA cuts (through the activity of the I-SceI homing meganuclease from yeast) followed by highly efficient counterselection of mutants (aided by a synthetic CRISPR-Cas9 device). The individual parts of the genome engineering toolbox, tailored for knocking genes in and out, have been standardized to enable portability and easy exchange of functional gene modules as needed. The applicability of the procedure is illustrated both by eliminating selected genomic regions in the platform strain *P. putida* KT2440 (including difficult-to-delete genes) and by integrating different reporter genes (comprising novel variants of fluorescent proteins) into a defined landing site in the target chromosome.

Introduction

Supported by the advances in synthetic biology and metabolic engineering made in the last few years, several microbes have emerged from a rather shadowy existence to become suitable biotechnological platforms (Calero and Nikel, 2019). Several members of the genus *Pseudomonas*, for instance, are being adopted by a steadily increasing number of laboratories worldwide as chassis for both fundamental and applied studies (Loeschcke and Thies, 2015; Benedetti et al., 2016; Dvořák et al., 2017; Nikel and de Lorenzo, 2018). *Pseudomonas* species, usually found in natural environments, distinguish themselves from other microorganisms by an unrivalled wealth of biochemical functions that are embedded in robust and flexible metabolisms (Poblete-Castro et al., 2017) – allowing bacteria to adapt to a diverse range of stressful conditions (Nikel et al., 2016). A rich set of synthetic biology tools has been developed to untap the metabolic potential of key representatives of the genus, e.g. *P. putida*, *P. taiwanensis* and *P. fluorescens* (Gomez et al., 2012; Domrös et al., 2017; Martínez-García and de Lorenzo, 2017; Cook et al., 2018; Wynands et al., 2018; Volke et al., 2019). A breakthrough in the standardization of a toolbox of reliable molecular tools for Gram-negative bacteria has been the creation of the Standard European Vector Architecture (SEVA) platform (Silva-Rocha et al., 2013; Martínez-García et al., 2015), including a variety...
of genetic parts (e.g. promoters, origins of replication and antibiotic-resistance determinants) that are functional in a number of *Pseudomonas* species. More recently, synthetic libraries of constitutive promoters have facilitated the predictable expression of a gene of interest with a desired strength (Elmore et al., 2017), particularly when the promoter of choice is to be combined with standardized translational couplers (Zobel et al., 2015).

Considerable efforts in the scientific *Pseudomonas* community have led to the development of tools for the construction of clean deletion mutants. Counterselection strategies adapted to *Pseudomonas* include a pyrF-based dual-selection system (Galvão and de Lorenzo, 2005), *sacB* as counterselectable marker (Gay et al., 1985) and the *upp* gene (uracil phosphoribosyltransferase) in an *upp*-deficient mutant (Graf and Altenbuchner, 2011). The λ *Red* system, widely used in *Escherichia coli* for recombineering (Murphy, 2016), has been combined with Cre/loxP site-specific recombination to enable genomic modifications with linear DNA fragments (Luo et al., 2016). Cook et al. (2018) introduced a recombineering method based on the λ *Red* system together with CRISPR-Cas9 counterselection. Such recombinease-based genome-editing approaches have recently been complemented by the design of a RecET system for *Pseudomonas* (Martínez-García and de Lorenzo, 2011), and Aparicio et al. (2018) further honed the CRISPR-Cas9 counterselection strategy, combining it with Ssr-based recombination for genome engineering. Among all these efforts, the standardized genome engineering method developed by Martínez-García and de Lorenzo (2011), based on the chromosomal integration of a suicide plasmid followed by the action of the homing nuclease I-SceI from *Saccharomyces cerevisiae*, excels for its efficiency and robustness to mediate any kind of genetic modification (e.g. large deletions, point mutations and allelic exchanges) – and is thus amongst the most intensively used by the scientific *Pseudomonas* community. The system relies on vector pEMG; this vector contains the conditional origin of replication R6K (Table 1), which requires the cognate Rep protein for replication. In bacterial strains that lack *pir*, vector pEMG behaves as a suicide plasmid. The vector contains a poly linker region (multiple cloning site) flanked by two I-SceI recognition sites cloned into the lacZα fragment, thus allowing for blue-white screening in *E. coli* cells carrying the lacZΔM15 mutation. A set of auxiliary SEVA plasmids harbours the gene encoding I-SceI under the control of the 3-methylbenzoate (3-MBz)-inducible XylS/Pm expression system from strain mt-2 (Martínez-García and de Lorenzo, 2011).

In this study, we present a smooth and quick workflow for genome editing of *P. putida* based on the action of I-SceI or CRISPR/Cas9 as a counterselection strategy. The integration of genes encoding fluorescent proteins (and antibiotic-resistance determinants) into the target locus circumvents experimental steps that would be needed to ensure the presence of the suicide vector. Furthermore, we propose the adoption of the USER assembly method (Smith et al., 1993; Bitinaite and Nichols, 2009; Nour-Eldin et al., 2010; Cavaleiro et al., 2015) to enable the quick and highly accurate assembling of homology regions (HRs) required for the genome engineering protocol. Helper plasmids for the constitutive expression of the gene encoding the homing I-SceI endonuclease from yeast were also designed to avoid the addition of chemical inducers during the process. The resulting streamlined protocol enables the introduction of virtually any genomic modification in *P. putida* within 5 days (for a single genetic manipulation including the construction of all necessary plasmids) down to 3 days (time required for each mutagenesis round once the relevant plasmids have been constructed), followed by plasmid curing. We demonstrate the potential of the technique by deleting both non-essential and difficult-to-knock-out metabolic genes in the platform strain KT2440. We furthermore show the integration of genes encoding different fluorescent proteins into a suitable landing pad in the *P. putida* chromosome and discuss their application as insertion reporters.

**Technical implementation**

The overall genome-editing procedure begins with the construction of donor DNA that is cloned into the suicide vector pGNW and derivatives thereof (Fig. 1). Such donor DNA, containing suitable HRs, serves as template for the endogenous homologous DNA recombination machinery. The procedure also includes the cloning of a target-specific synthetic guide RNA (sgRNA) into a dedicated CRISPR-Cas9 plasmid (when applicable), the integration of the donor DNA into the *Pseudomonas* chromosome, and the specific cleavage of chromosomal DNA by endonucleases (i.e. I-SceI or Cas9) for the resolution of co-integrates. The specific steps of the procedure are detailed in the sections below.

**Design and construction of plasmids**

**Construction of the suicide vector pGNW and derivatives** We first equipped plasmid pEMG with the gene encoding the monomeric superfolder GFP [msfGFP (Landgraf, 2012)] in the vector backbone while screening different combinations of promoters and translation initiation regions (TIR) that would give a high fluorescence output when the plasmid is integrated into the target chromosome. The fusion of the strong constitutive *P*14g...
promoter and a variation of the TIR sequence preceding msfGFP (Zobel et al., 2015) allowed for the direct visualization of Pseudomonas colonies harboring a single (chromosomal) copy of the novel vector pGNW2 (Table 1) by inspecting the plates on a blue-light transilluminator. A set of pGNW vectors was constructed by swapping the kanamycin resistance (KmR) determinant (i) required for bacterial conjugation, and a site-specific DNA insert that (i) is homologous to the target locus (i.e. HRs upstream and downstream to the region of interest) within the Pseudomonas genome and (ii) contains a desired modification. Such modification can be as short as a single base for a point mutation or arbitrarily long for the insertion of whole gene clusters.

<table>
<thead>
<tr>
<th>Name</th>
<th>Relevant features</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEMG</td>
<td>Suicide vector used for deletions in Gram-negative bacteria; oriT, traJ, lacZa, oriV(R6K); KmR</td>
<td>Martínez-Garcia and de Lorenzo (2011)</td>
</tr>
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<td>pRK600</td>
<td>Helper plasmid used for conjugation; oriV(ColE1), mob(RK2), tra(RK2); KmR</td>
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<td>pGNW4</td>
<td>Derivative of vector pGNW2; SmR</td>
<td>This work</td>
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<td>pGNW6</td>
<td>Derivative of vector pGNW2; GmR</td>
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<tr>
<td>pGNW2ΔnicX</td>
<td>Derivative of vector pGNW2 carrying HRs to delete nicX (PP_3945) in P. putida KT2440</td>
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<td>pS2313 ΔR</td>
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<tr>
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<td>pGNW2LP</td>
<td>Derivative of vector pGNW2 carrying HRs to insert P14g(BCD2)→mRFP1 into a landing pad in the chromosome of P. putida KT2440</td>
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<td>pSEVA128S</td>
<td>Helper plasmid; oriV(RK2), xylS, Pm→I-SceI; AmpR</td>
<td>Aparicio et al. (2015)</td>
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<td>Helper plasmid; oriV(RK2), xylS, Pm→I-SceI; KmR</td>
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<td>Aparicio et al. (2015)</td>
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<td>Aparicio et al. (2015)</td>
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<td>pSEVA1213S</td>
<td>Helper plasmid; oriV(RK2), P_EMS→I-SceI; AmpR</td>
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<td>Helper plasmid; oriV(RK2), P_EMS→I-SceI; GmR</td>
<td>This work</td>
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<td>pSEVA448</td>
<td>Expression vector; oriV(pRO1600/ColE1), xylS, Pm; SmR</td>
<td>Silva-Rocha et al. (2013)</td>
</tr>
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<td>pSEVA448.Cas9tr</td>
<td>Cloning vector; oriV(RK2), cas9, tracrRNA; SmR</td>
<td>Aparicio et al. (2018)</td>
</tr>
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<td>pS448 CsR</td>
<td>Derivative of vector pSEVA448 used for CRISPR-Cas9 counterselection; xylS (cured of Bscl restriction sites), Pm→cas9, P_EMS→sgRNA; SmR</td>
<td>This work</td>
</tr>
<tr>
<td>pS448CsRaceEF</td>
<td>Derivative of vector pS448 CsR carrying P_EMS→aceEF-targeting sgRNA; SmR</td>
<td>This work</td>
</tr>
</tbody>
</table>

a. Plasmids can be obtained from Addgene (www.addgene.org) with the following deposit numbers: pGNW2 (122086), pGNW4 (122088), pGNW6 (122093), pSEVA1213S (122095), pSEVA6213S (122094) and pS448 CsR (122096).

b. Antibiotic markers: Amp, ampicillin; Cm, chloramphenicol; Km, kanamycin; Sm, streptomycin; and Gm, gentamicin. HRs, homology regions.

### Table 1. Plasmids used in this work.

**Oligonucleotide design.** One primer pair is required to amplify each fragment that constitutes the pGNW derivative via PCR. Depending on the type of modification (see description below), this means that a minimum of three oligonucleotide pairs are required for assembly (Cavaleiro et al., 2001), Gibson assembly (Gibson et al., 2009) or USER assembly (Cavaleiro et al., 2015) that is recommended and further described here.

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cloning. A first pair of oligonucleotides (pGNW-USER_F and pGNW-USER_R, see Table S1) is used to reverse-amplify the pGNW backbone, and the resulting linear fragment can be used in a standardized way for every cloning procedure. Two further pairs of primers are required to amplify the homologous regions HR1 and HR2 from the Pseudomonas genome, flanking the mutagenesis region (MR) targeted. The sequences of the primers HR1_F and HR2_R (Fig. 2) are determined by the MR to be modified (or deleted), and by the type of modification (i.e. deletion, insertion or substitution).

i) Identify the ends of the MR sequence. The binding part of HR1_R starts upstream of the MR and extends into HR1, while the binding part of HR2_F starts downstream of the 3'-end of the MR and extends into HR2 (Fig. 2). Extend the sequence of both oligonucleotides so that they fulfil general primer design requirements satisfactorily (i.e. 18–25 nt length, 40–60% GC content and GC-clamp).

(ii) For deletions of chromosomal sequences, use the last ~ 100 bp of HR1 and the first ~ 100 bp of HR2 as input sequences for the AMUSER tool (Genee et al., 2015), available online at http://www.cbs.dtu.dk/services/AMUSER/, to identify suitable USER overhangs for the two primers. Add the required bases to the 5'-end of both oligonucleotides. For gene deletions to have minimum polar effects and to avoid the potential creation of toxic, truncated polypeptides, we recommend leaving the START and STOP codons of the target gene intact and deleting the interjacent sequence. Upon deletion, this approach will leave e.g. 5'-ATG TGA-3' in lieu of the coding sequence, where ATG and TGA are the START and the STOP codons, respectively.

Fig. 1. Overview diagram of the genome engineering procedure in Pseudomonas putida. On the delivery date of the designed oligonucleotides, homologous regions are amplified from the Pseudomonas genome and assembled into a suitable pGNW vector. The resulting plasmid is then delivered into E. coli pir cells via chemical transformation. Furthermore (if applicable), a synthetic spacer, completing a specific synthetic guide RNA (sgRNA), is separately prepared and cloned into the CRISPR-Cas9-vector pS448-CsR. On day 2, E. coli transformants are screened for the correct pGNW insert size (and spacer insertion in vector pS448-CsR, if applicable) via colony PCR, the resulting amplicons are sent out for sequencing, and the corresponding clones are used to inoculate liquid cultures. Day 3 includes the verification of sequence integrity of the insert in the pGNW vector, the purification of individual plasmids, and their delivery into Pseudomonas either via electroporation or tri-parental mating. The resulting co-integrants are enriched in liquid LB cultures during the course of day 4 and transformed with either an I-SceI—bearing plasmid or plasmid pS448-CsR carrying an appropriate sgRNA. Finally, Pseudomonas colonies without fluorescence are tested on day 5 for their genotype via colony PCR.
(iii) For larger insertions (exceeding 29 bp), additional DNA fragments should be amplified separately and assembled with HR1 and HR2 to form the insert to be cloned in the pGNW vector of choice. The AMUSER tool can be used to design primers with suitable overhangs for their assembly.

ii Design HR1_F at a distance of ~500 bp upstream of HR1_R with a similar melting temperature. Design HR2_R at ~500 bp downstream of HR2_F (Fig. 2). Add the motif 5'-AGA TCC U-3' as the primer overhang to HR1_F, and 5'-AGG TCG ACU-3' as overhang to HR2_R. These two overhangs match the ones that have been used in the primers to reverse-amplify vector pGNW.

iii Design one pair of 'g-check' primers to test for the genotype of individual colonies after the genome engineering procedure. For deletions and insertions larger than 30 bp, the primers bind upstream and downstream of the MR, respectively, and amplify a product that can be distinguished from the wild-type sequence with agarose gel electrophoresis according to its size (a size difference of >10% is recommended). For substitutions of identical size, one primer should be able to bind only within the MR. The wild-type sequence should thus give no amplification. For substitutions of only a few base pairs, the mutation-containing region must be amplified and the genotype confirmed by restriction analysis (if applicable) or sequencing.

**Amplification and assembly of DNA fragments**

i Amplify vector pGNW using 5 ng of plasmid as template and primers pGNW-USER_F and pGNW-USER_R (Table S1) using Phusion™ U Hot Start DNA polymerase. Use standard reaction conditions as recommended by the manufacturer, with an elongation time of 3 min as well as an annealing temperature ‘touchdown’ from 65 to 59°C (Don et al., 1991), with a decrement of −1°C per cycle and a final annealing temperature of 58°C for 30 further cycles. We recommend to perform gel purification on the amplified pGNW fragment and to use the purified product as template for further PCRs. In this way, digestion with Dpn I to remove circular plasmids (i.e. template) can be omitted. We further recommend generating a large amount of linearized...
pGNW vector in several parallel PCRs for repeated use in USER assembly.

ii Perform separate amplification reactions on purified genomic *Pseudomonas* DNA with the adequate primer pairs (HR1_F/HR1_R and HR2_F/HR2_R) to generate the HR1 and HR2 fragments. Perform, if needed, additional PCRs to generate the DNA fragments required for insertions. We generally recommend the use of a touchdown PCR protocol (Don *et al.*, 1991) to reduce the formation of unspecific by-products.

iii Analyse a 3- to 5-µl aliquot of each PCR by agarose gel electrophoresis to verify the correct amplification of the products. The concentrations of the fragments can be roughly estimated from the intensities of their bands. If agarose gel electrophoresis reveals the presence of non-specific by-products, the desired bands have to be purified from a gel prior to cloning. If the product appears clean, the PCR reaction can be used directly in the assembly reaction.

iv In a PCR tube, combine equimolar amounts of insert fragments in a volume of 7 µl with 3 µl of the linearized pGNW vector. Add 1 µl of 1 U µl⁻¹ USER enzyme (New England Biolabs, Ipswich, MA, USA). Set up a thermocycler and run a reaction programme as follows: *deoxyuracil* excision, 30 min at 37°C; annealing 1, decrement from 28°C to 20°C, −2°C per step, 3 min step duration; and annealing 2, ≥ 10 min at 10°C. If a plasmid was used as template for the amplification of one of the fragments that contains the same antibiotic resistance as the employed pGNW vector, add 0.5 µl of FastDigest DpnI (Thermo Fisher Scientific, Waltham, MA, USA) to the reaction mix prior to incubation at 37°C.

v Transform 50 µl of chemically-competent *E. coli* DH5α λpir cells with 5 µl of the assembly reaction from the previous step. Plate the cells on LB medium agar supplemented with the respective antibiotic for the pGNW vector used. If a circular plasmid was used as template in the PCR for pGNW linearization, and if the reaction was directly employed for the assembly reaction (rather than using a gel-purified plasmid), spread the transformed *E. coli* DH5α λpir cells on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal) at 40 µg ml⁻¹.

Verification of clones.

i Use primers Seq-pGNW_F and Seq-pGNW_R (Table S1) in a colony PCR (20 µl reaction volume for each PCR) to amplify the HR insert in pGNW of eight individual *E. coli* transformants that show green fluorescence under blue-light exposure (and are white under white light if Xgal was added to the plate).

ii Analyse a 3- to 5-µl aliquot of each PCR by agarose gel electrophoresis to verify the correct insert size. Purify the remaining volume of the PCRs with the correct insert size and send the samples for sequencing to verify sequence integrity.

iii Inoculate 3–5 ml LB cultures (add the corresponding antibiotic) with six individual clones that were tested for a correct insert size. Incubate the culture at 37°C for 12–18 h in a shaking incubator at 180–250 rpm (depending on the type of incubator). In order to continue with the genome engineering procedure in *Pseudomonas* on the subsequent day (section ‘Genome editing in *Pseudomonas putida*’), launch a pre-culture of strain KT2440 as well, and incubate with shaking (180–250 rpm) at 30°C.

iv Purify plasmid DNA from the *E. coli* cultures that tested positive by colony PCR and send the purified plasmid DNA for sequencing in addition to (or instead of) step 2.

**Cloning of sgRNAs into vector pS448-CsR for counterselection of mutants.** To adopt CRISPR-Cas9 as a counterselection tool, vector pS448 CsR was constructed by USER assembly from vector pSEVA448 (Table 1) via reverse amplification with primers pS448_F and pS448_R (Table S1) and assembled with (i) cas9, amplified from plasmid pSEVA421.Cas9tr with primers Cas9_F and Cas9_R and (ii) a *P. aeruginosa*-sgRNA module (synthesized by Integrated DNA Technologies, Leuven, Belgium; and amplified with primers sgRNA_F and sgRNA_R). This module contains a single guide RNA construct fused to a trans-activating CRISPR RNA (tracrRNA)-part and two *BsaI* recognition sites for the easy insertion of a spacer. The two *BsaI* recognition sites are placed in inverse orientation immediately upstream of the tracrRNA part to enable the creation of incompatible, single-stranded overhangs during linearization by *BsaI*. This step allows for the insertion of a double-stranded DNA (dsDNA) fragment with suitable overhangs. If CRISPR-Cas9 is to be used as counterselection tool, vector pS448-CsR must be equipped with an adequate spacer (Bhayya *et al.*, 2011), which targets the original sequence that becomes modified, as disclosed below.

**Spacer design.** For the use of CRISPR-Cas9 as counterselection tool, a spacer is inserted into vector pS448 CsR to target the original sequence that becomes modified after genome engineering. The spacer represents the CRISPR RNA (crRNA)-part of the sgRNA and is defined by the 20-nt sequence upstream of a spacer adjacent motif (PAM, 5’-NGG-3’) on either of both DNA strands within a target DNA region. Strand specificity is not relevant when CRISPR-Cas9 is employed for counterselection, and the most important
parameter is the sgRNA specificity for its target. To choose a suitable spacer sequence for counterselection, we recommend the use of the online tool CRISPY-web (Blin et al., 2016) as indicated below.

i. Upload a GenBank file with the complete genomic sequence of your Pseudomonas species into CRISPY-web, available at https://crispy.secondarymetabolites.org/.

ii. Specify either the gene that is to be deleted/substituted or the range of the MR.

iii. Pick a suitable 20-nt spacer candidate sequence from the top entries in the list provided by the CRISPY-web application.

iv. Design and order two oligonucleotides that are complementary to each other. The first oligonucleotide represents the 20 nt of the spacer sequence obtained in the previous step. The second oligonucleotide is the reverse complement of the first oligonucleotide. Add a 5′-AAC-3′ overhang to the 5′-end of the second oligonucleotide and a single C residue to its 3′-end. Add a 5′-GCGCG-3′ overhang to the 5′-end of the first oligonucleotide. For example, if aceF (PP_0338) is to be targeted, the sequences would be oligonucleotide 1 (aceEF_F), 5′-GCGCG CTC ATT CGC GTA CCT GAC ATG-3′; and oligonucleotide 2 (aceEF_R), 5′-AAAC ATG TCA GGT ACG CGA ATG AG C-3′ (Table S1 in the Supporting Information; additions are indicated in boldface). For ligation, the oligonucleotides need to be phosphorylated at the 5′-OH terminus. The oligonucleotides can either be purchased with terminal phosphorylation or phosphorylated in situ using T4 polynucleotide kinase (PNK, see below).

DNA preparation and construction of derivatives of vector pS448 CsR.

i. Digest vector pS448-CsR with Bsal or Eco31I (Thermo Fisher Scientific) according to the manufacturer's recommendations. Use agarose gel electrophoresis and gel purification to isolate the linearized plasmid (9.9 kb) from the non-restricted fraction.

ii. Dilute the annealed and phosphorylated oligonucleotides 1:200 with water, i.e. to a final concentration of dsDNA of 50 nM.

iii. Let the cells recover for 2 h at 30°C and plate them onto the LB medium agar supplemented with streptomycin (100 µg ml⁻¹). Incubate the plates for overnight at 30°C.

iv. Dilute the annealed and phosphorylated oligonucleotides 1:200 with water, i.e. to a final concentration of dsDNA of 50 nM.

v. Ligate the dsDNA encoding the sgRNA-spacer into the linearized pS448-CsR vector in a 10-µl reaction containing 3 µl of diluted insert from the previous step, 10–30 ng of Bsal-digested pS448-CsR vector, 1 µl of T4 ligase buffer, 1 µl of T4 DNA ligase (New England BioLabs) and water, if needed, to reach the final volume.

vi. Purify plasmid DNA from three individual E. coli transformants and verify the sequence integrity by sequencing with primer SEVA-T0_F (Table S1).

Test efficiency of the sgRNA for counterselection. This step is optional and is meant to provide an estimation of the efficiency of the sgRNA in targeting the locus targeted during the procedure (thus providing an estimate of the success rate of the whole counterselection procedure).

i. Inoculate 10 ml of LB medium with P. putida KT2440 and grow the cells overnight at 30°C with agitation.

ii. Wash the cells four times with 1 ml of 300 mM sucrose (filter-sterilized) and finally resuspend in 400 µl of 300 mM sucrose.

iii. Individually electroporate 100 ng of the empty pS448-CsR vector and 100 ng of plasmid pS448-CsR carrying the sgRNA of choice into 100-µl cell suspension aliquots with a voltage of 2.5 kV, 25 µF capacitance and 200 Ω resistance (e.g. in a Gene Pulser Xcell™ Electroporation System; Bio-Rad Laboratories, Hercules, CA, USA).

iv. The plate with cells harbouring vector pS448-CsR with a functional sgRNA should have considerably less colonies (if any at all). We typically see a number of CRISPR-Cas9 escapers in the low double-digit range (i.e. 10–20 colonies) while Pseudomonas transformed with an empty pS448-CsR vector will form several hundred colonies within 16 h.

Genome editing in Pseudomonas putida

In order to introduce mutations into the Pseudomonas genome, the suicide plasmid pGNW (constructed in the previous section) is integrated into the chromosome at the target location employing the native homologous
recombination mechanism (Fig. 3). After selection of positive co-integration events, the meganuclease I-SceI or the CRISPR-Cas9 system, respectively, is delivered into the cells on a replicative plasmid. The expression of the DNA modifying enzymes – and thus restriction of pGNW within the chromosome – enforces a second homologous recombination that can yield either the mutant genotype or a revertant genotype (i.e. wild-type sequence).

Integration of pGNW derivatives into the chromosome. The suicide vector pGNW carrying the corresponding HRs to the MR has to be delivered into the cells to enable its chromosomal integration via homologous recombination. This step can be achieved either by electroporation or by tri-parental mating. The latter procedure is recommended if multiple manipulations are conducted within the same strain. Thereby, the plasmid bearing I-SceI (e.g. pSEVAX28S, where X indicates any antibiotic resistance; see Table 1) can be maintained within the cells by addition of the respective antibiotic during all cultivation steps. A leaky expression of the gene encoding I-SceI, however, interferes with the integration of pGNW – significantly decreasing the transformation efficiency. This issue can be countered by a much more efficient plasmid delivery achieved by tri-parental mating.

Plasmid delivery by electroporation
i Grow an overnight culture of P. putida KT2440 in 5–10 ml of LB medium. The Pseudomonas culture can be started at the same time that the overnight cultures of E. coli DH5α λpir with pGNW (Section ‘Verification of clones’, step 3).

ii For the electroporation of cells, we adopted the procedure by Choi et al. (2006). In brief, the cell suspension is distributed into four microcentrifuge tubes and pellet the cells in a microcentrifuge at 11 000 g for 1 min. Wash the cells twice with 1 ml of 300 mM sucrose for each pellet and finally resuspend and combine them in a total of 100 μl of 300 mM sucrose in a single tube. Add 500 ng of pGNW vector to the suspension and transfer the mixture into an electroporation

![Fig. 3. Workflow for targeted genomic manipulations in Pseudomonas. Pseudomonas cells are transformed with pGNW via electroporation or tri-parental mating. One to five individual green fluorescent colonies are combined and enriched in liquid LB medium cultures and transformed either with plasmid pSEVAX2YS or with a derivative of vector pS448-CsR carrying an appropriate sgRNA. Note that the antibiotic resistance could be different depending on the vector chosen for the deletion. Expression of the gene encoding the I-SceI meganuclease mediates the excision of the pGNW sequence from the chromosome, leading to non-fluorescent colonies under blue-light exposure. These clones are tested via colony PCR and sequencing for a revertant (i.e. wild-type) or mutant genotype. Asterisks indicate the relevant genotypes as well as the molecular events happening in the Pseudomonas chromosome (A) during the corresponding steps of the genome engineering protocol (B).](image-url)
cuvette with 2 mm gap width. Apply an electric field pulse (2.5 kV voltage, 25 μF capacitance, and 200 Ω resistance) and quickly add 1 ml of LB medium. All steps of the electroporation protocol can be performed at room temperature.

iii Combine 100 μl of cell suspension into a sterile test or centrifuge tube (5–50 ml) and recover the cells with shaking at 30°C for 2 h.

iv Harvest the cells by centrifugation (11 000 g; 1 min) at room temperature, and plate the whole suspension onto LB medium agar supplemented with the respective antibiotic for the pGNW vector used (either 50 μg ml⁻¹ Km, 10 μg ml⁻¹ Gm or 100 μg ml⁻¹ Sm). Incubate the cells overnight at 30°C.

v Inspect the plate on a blue-light transilluminator. Transformants should show green fluorescence (usually all colonies).

Plasmid delivery by tri-parental mating

i Grow overnight cultures of Pseudomonas, E. coli DH5α λpirpGNW and E. coli HB101/pRK600 in at least 1 ml of LB medium each. Incubate at 30°C for Pseudomonas or 37°C for E. coli respectively.

ii Dry an LB medium agar plate without added antibiotics in a clean bench for 1 h.

iii Combine 100 μl of each pre-culture into a reaction tube and pellet the cells in a microcentrifuge at 10 000 g for 1 min at room temperature. Wash the cells with 1 ml of fresh LB medium and finally resuspend them in 20–30 μl of LB medium. Pipette the whole suspension onto the pre-dried LB medium agar plate. In order for bacterial conjugation to work, the cells have to be brought into close proximity within a non-planktonic state. A dried agar plate will absorb a small volume of media, allowing the bacterial cells to form a biofilm-like structure on the agar surface.

iv Incubate the cells at 30°C for 3–5 h.

v Use an inoculation loop to take the biomass up from the agar surface and resuspend it in 1 ml of LB medium. Spread 100 μl of this suspension directly onto a cetrimide agar plate supplemented with the antibiotic to select for the pGNW vector used, as well as 50 μg ml⁻¹ ampicillin, and the antibiotic for the I-SceI plasmid (if already delivered at this step, see below). Concentrate the remaining cells to about 100 μl by centrifugation, and resuspend and spread them onto a second plate. The chosen concentration of Amp was found to not affect the growth of P. putida KT2440 that is naturally resistant to β-lactam antibiotics while completely suppressing the growth of most E. coli strains, which may show some residual growth even in the presence of cetrimide.

vi Incubate the plates at 30°C for 16–24 h until clear colonies have formed that show green fluorescence under blue-light exposure.

Introduction of endonucleases

i Combine 1–5 individual colonies of Pseudomonas with a co-integrated pGNW vector into 5–10 ml of LB medium in a test or centrifuge tube (5–50 ml) and incubate with shaking at 30°C for at least 5 h (see next step). Using several individual colonies increases the chances of having cells with both the two possible genetic configurations after pGNW co-integration (Fig. 5A), and reduces the impact of unspecific insertion of the suicide plasmid at untargeted chromosomal sites. Furthermore, the more biomass is used to inoculate the LB medium culture, the quicker the cell density required for the next step is reached so that the protocol can be continued on the same day. If the cells still contain an inducible I-SceI—plasmid (i.e. pSEVAX28S) from prior mutagenesis procedures, continue directly with Step 3 below.

ii After the cells have reached an optical density at 600 nm (OD₆₀₀ nm) of at least 0.3, use 20–100 ng of an I-SceI plasmid (i.e. pSEVAX2YS, where the expression of I-SceI could be either constitutive or inducible; see Table 1) or 100–200 ng of plasmid pS448 CsR to transform Pseudomonas co-integrants by electroporation as described in the previous section (Section ‘Plasmid delivery by electroporation’). Transfer the cell suspension into a sterile test or centrifuge tube and recover the cells shaking at 30°C for 1 h. If a plasmid containing the XylS/Pm expression system (i.e. pSEVAX28S or pS448CsR) is used, add 3-mBz to a final concentration of 3 mM to the LB medium used for cell recovery.

iii Plate 70 μl of cell suspension on LB medium agar supplemented with the respective antibiotic for pSEVAX2YS (i.e. 500 μg ml⁻¹ Amp, 50 μg ml⁻¹ Km, 10 μg ml⁻¹ Gm or 100 μg ml⁻¹ Sm), or Sm for pS448-CsR selection. Incubate the cells overnight at 30°C. If a plasmid containing the XylS/Pm expression system (i.e. pSEVAX28S or pS448-CsR) is used, add 3-mBz to a final concentration of 3 mM to the LB medium plates to ensure proper induction of the system.

iv Inspect the plate on a blue-light table. Pseudomonas clones that have lost vector pGNW through a second homologous recombination event will show no green fluorescence.

v Test ≥8 individual non-fluorescent colonies via colony PCR using the g-check primers adequate for their genotype. If CRISPR-Cas9 counterselection was employed, all non-fluorescent clones are expected to harbour the mutation. The fraction of cells that have
maintained fluorescence after counterselection is expected to be significantly higher when using CRISPR-Cas9 as compared to I-SceI, due to a higher probability of escaping restriction by mutating the sgRNA recognition sequence.

Plasmid curing

The last step in the procedure, once the mutation has been confirmed, is to cure the mutant cells from the plasmid(s) used during genome editing (Fig. 4).

i Use a single colony of mutant Pseudomonas to inoculate 3–10 ml of LB medium in a test or centrifuge tube.

ii Culture the cells shaking at 30°C. Every 4–12 h, transfer 1 μl of the culture into 3–10 ml of fresh LB medium. Continue the culturing process for 2–3 days, and pass the cells into fresh medium even if you cannot see the formation of biomass in the tubes. This procedure ensures that the cells are kept in a state of maximum division rate. If your mutant cells have severe growth deficiencies due to the deletion of important genes or the introduction of DNA that could impair growth, the curing of a plasmid can require continuous cultivation for an extended period.

iii Dilution-streak the culture on an LB medium agar plate without added antibiotics.

iv Test individual colonies for the loss of the plasmid by re-streaking on LB medium agar with the respective antibiotic.

Application examples

Deletion of nicX in Pseudomonas putida KT2440 mediated by I-SceI activity

Pseudomonas putida KT2440 is able to grow on nicotinic acid as a sole carbon source (Belda et al., 2016).

The degradation pathway, encoded in the nic gene cluster, involves the hydroxylation of nicotinate to 6-hydroxynicotinate, its further reduction to 2,5-dihydroxy-

Fig. 4. Plasmid curing after successful genome engineering of Pseudomonas. The biomass from a single mutant Pseudomonas colony is used to inoculate 5–10 ml of LB medium and the suspension is incubated at 30°C with shaking. Two to three times per day, a small volume of this culture is transferred into fresh LB medium. After 2–3 days (i.e. at least six passages), the cells are isolated on LB medium agar plates and tested for the loss of plasmids based on their antibiotic-resistance profile.

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for their genotype via colony PCR using primers nicX-g-check_F/nicX-g-check_R (Table S1). The different colorations of mutant and revertant colonies allowed for their direct identification. With all three plasmid combinations, the fraction of mutant colonies was always close to 50% (53%, 46% and 48% respectively).

**Deletion of aceEF in Pseudomonas putida KT2440 using CRISPR-Cas9 counterselection**

As an obligate aerobic bacterium, *P. putida* strongly relies on an active tricarboxylic acid cycle for the generation of reducing equivalents that are used, *inter alia*, to generate ATP through oxidative phosphorylation (Nikel et al., 2014). The pyruvate dehydrogenase complex (PDHc), which catalyses the entry reaction to the tricarboxylic acid cycle, plays a central role in the central carbon metabolism of *Pseudomonas* (Sudarsan et al., 2014; Nikel et al., 2015). The PDHc is a multimeric system composed of three different proteins, AceE, AceF and Lpd (Reed et al., 1975; Angelides et al., 1979). In *P. putida* KT2440, the genes encoding AceE (PP_0339) and AceF (PP_0338) form an operon (Belda et al., 2016). Considering its central role in sugar catabolism, genes encoding PDHc are considered a difficult knock-out to achieve if relying on an untargeted selection strategy, and the resulting mutant would become bradytroph for C2 units as the reaction converting pyruvate into acetyl-coenzyme A is blocked. Because of this reason, CRISPR-Cas9 was adopted for counterselection of the correct mutants in this study. The homology regions to delete *aceE* and *aceF* were amplified from chromosomal DNA of *P. putida* KT2440 using the primer pairs aceEF_HR1_F/aceEF_HR1_R and aceEF_HR2_F/aceEF_HR2_R (Table S1) and cloned into vector pGNW2. *Pseudomonas* co-integrants were then transformed with 200 ng of plasmid pS448/CsR_aceF (Table 1) and selected on LB medium agar supplemented with Sm, 3-mBz and 5 mM nicotinic acid to enhance the growth of bradytrophic mutant cells. After 2 days, 10 out of 39 colonies of normal size and a majority (29 out of 39) of very small colonies could be identified on the plate (Fig. S2). Inspection under blue-light exposure revealed that all larger colonies had kept green fluorescence and thus had escaped CRISPR-Cas9-mediated restriction. All smaller colonies were tested by...

colony PCR with primers aceEF_check_F/aceEF_check_R (Table S1) and were found to have aceEF deleted. In a separate experiment, co-integrant cells were transformed with both pSEVA628S and pS448/CsR_aceF (500 ng of each plasmid). Selection on LB medium agar plates containing Sm, 3-mBz, and acetate (as a direct source of acetyl-coenzyme A) led to the formation of only three colonies, all of which had aceEF deleted.

Integration of fluorescent protein-encoding genes into a landing pad in *Pseudomonas putida* KT2440

To evaluate the suitability of the system for the insertion of DNA at a defined position, a landing pad was chosen within the intergenic region between PP_0013 (gyrB) and PP_5421, close to the chromosomal origin of replication of *P. putida* KT2440 (Nelson et al., 2002; Reynolds and Gill, 2015; Belda et al., 2016). First, plasmid pGNW2 LPR was assembled from four fragments in a USER assembly reaction. To this end, vector pGNW2 was amplified with pGNW-USER_F and pGNW-USER_R. The two HRs framing the landing pad were amplified from genomic DNA with the primer pairs LP_HR1_F/LP_HR1_R and LP_HR2_F/LP_HR2_R (Table S1). A fourth fragment containing the gene encoding the monomeric red fluorescent protein mRFP1 (Campbell et al., 2002) and the rrnB-T1 terminator element was amplified with RFP4LP_F and RFP4LP_R (Table S1) from vector pS2313 R. The primers RFP4LP_F and LP_HR1_R furthermore contained extended overhangs to introduce the constitutive $P_{EM7}$ promoter upstream of the mRFP1 coding sequence.

![Integration of fluorescent proteins](image)

**Fig. 6.** Integration of different fluorescent proteins into a landing pad in the chromosome of *Pseudomonas putida* KT2440.

A. Genes encoding the fluorescent proteins mRFP1, mOrange2, mTurquoise2, msfGFP, and mBFP2 [placed under transcriptional control of a P$_{gal}$BCD2 regulatory element], were integrated into a chosen landing site in the chromosome of *P. putida* KT2440 using the insertional vectors pGNW2, pGNW4, or pGNW6. After selection of co-integrants, the second homologous recombination was mediated by the inducible expression of the gene encoding the I-SceI meganuclease from pSEVAX28S vectors.

B. Images of the plates were taken after incubation for 16 h at 30°C followed by 7 days at 4°C to allow for proper fluorescent protein maturation. mBFP2-integrated colonies showed no visible fluorescence under blue-light exposure (not shown). The blue arrows indicate colonies of mutated strains displaying different integrated fluorescent proteins; white arrows identify colonies of cells displaying a revertant genotype (i.e. wild-type) without fluorescence.
Table 2. Bacterial strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> DH5α</td>
<td>Cloning host; F−, endA1, glnX44(As) thiE1, recA1, relA1, spoT1, gyrA96(NalR) rfbC1, deoR, rupG-680(lacZAM15) Δ(argF-lac)U169, hsdR17 (F− λpir)</td>
<td>Hanahan and Meselson (1983)</td>
</tr>
<tr>
<td>DH5α λpir</td>
<td>Cloning host; same as DH5x but λpir lysogen</td>
<td>Platt (2000)</td>
</tr>
<tr>
<td>HB101</td>
<td>Helper strain used for tri-parental mating; F− thi-1, hsdS20(λ− mB2) supE44, recA13, ara-14, leuB6, proA2, lacY1, galk2, psiL20(SmR) xyl-5, mtl-1</td>
<td>Boyer and Roulland-Dussoix (1969)</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em> KT2440</td>
<td>Wild-type strain, derived from <em>P. putida</em> mt-2 (Worsey and Williams, 1975)</td>
<td>Bagdasarian et al. (1981)</td>
</tr>
<tr>
<td>KT2440 ΔaceEF</td>
<td>Same as KT2440, but with an in-frame deletion of the aceEF genes (PP_0038-PP_0339)</td>
<td>This work</td>
</tr>
<tr>
<td>KT2440 ΔnicX</td>
<td>Same as KT2440, but with an in-frame deletion of the nicX gene (PP_0395)</td>
<td>This work</td>
</tr>
<tr>
<td>KT-LPR</td>
<td>Same as KT2440, but carrying a P14g-BCD2→mRFP1 element integrated between PP_0013 (gyrB) and PP_5421</td>
<td>This work</td>
</tr>
<tr>
<td>KT-LPG</td>
<td>Same as KT2440, but carrying a P14g-BCD2→msfGFP element integrated between PP_0013 (gyrB) and PP_5421</td>
<td>This work</td>
</tr>
<tr>
<td>KT-LPB</td>
<td>Same as KT2440, but carrying a P14g-BCD2→msfF2 element integrated between PP_0013 (gyrB) and PP_5421</td>
<td>This work</td>
</tr>
<tr>
<td>KT-LPO</td>
<td>Same as KT2440, but carrying a P14g-BCD2→mOrange2 element integrated between PP_0013 (gyrB) and PP_5421</td>
<td>This work</td>
</tr>
<tr>
<td>KT-LPT</td>
<td>Same as KT2440, but carrying a P14g-BCD2→mTurquoise2 element integrated between PP_0013 (gyrB) and PP_5421</td>
<td>This work</td>
</tr>
</tbody>
</table>

a. Antibiotic markers: Nal, nalidixic acid; and Sm, streptomycin.

Since this promoter was found to give too low expression (data not shown), its sequence was exchanged with the strong P14g promoter (Zobel et al., 2015) and the translational coupler BCD2 (Mutalik et al., 2013) by amplifying pGNW2-LP::P_Emp-mRFP with oligonucleotides P14 g-BCD2_F/P14 g-BCD2_R and a BCD2-fragment from a gBlock containing this regulatory element (pur-chased from Integrated DNA Technologies) with BCD2-P14g_F/BCD2-P14g_R. The resulting plasmid was termed pGNW2-LP2 (Table 1).

To exchange mRFP1 in the landing pad with different fluorescent proteins and to transfer the landing pad to vectors pGNW4 or pGNW6, two- or four-fragment USER assembly reactions were performed with interchangeable modules. These modules were amplified and assembled as indicated in Table S1. The msfGFP gene was amplified from vector pGNW4. The genes encoding the other fluorescent proteins were amplified from the plasmids pS2313-B, pS2313-O and pS2313-T that had been constructed as follows: The sequences of the genes mBFP2, mOrange2 and mTurquoise2 were extracted from their original publications (Table 1), cured from protein tags and restriction sites to make them SEVA-compatible and ordered as custom genes (Integrated DNA Technologies). The synthetic DNA fragments were then cloned into the expression vector pSEVA2313 via USER assembly using the primer pairs pS2313_F/pS2313_R (for pS2313), pS_BFP2_F/pS_BFP2_R (for mBFP2) and pS_Ora_Tq_F/pS_Ora_Tq_R (for mOrange2 and mTurquoise2; Table S1), yielding plasmids pS2313-B, pS2313-O and pS2313-T respectively.

The resulting plasmids pGNW2-LPR, pGNW2-LPG, pGNW2-LPB, pGNW4-LPO and pGNW6-LPT (Table 1) were individually integrated into the chromosome of *P. putida* KT2440 as indicated above. The backbones were removed from the co-integrants by the delivery and induction of plasmid pSEVA128S (for pGNW2-LPR), pSEVA628S (for pGNW2-LPG and pGNW2-LPB), pSEVA228S (for pGNW4-LPO) and pSEVA428S (for pGNW6-LPT) respectively (Fig. 6A). On the following day, the plates with the resulting strains (*P. putida* KT-LPR, KT-LPG, KT-LPB and KT-LPT; Table 2) were placed at 4°C to let the fluorescent proteins mature, since none of them showed any visible coloration under blue-light exposure. After 7 days, photographs of the plates were taken on a blue-light transilluminator (Fig. 6B). *Pseudomonas* colonies with integrated mBFP2 (strain KT-LPB) showed not visible fluorescence even after further prolonged incubation (data not shown). To determine the kinetics of the fluorescent proteins in *P. putida* KT2440, at least three biological replicates of the mutant strains (verified by colony PCR with primers Seq-LP_F and Seq-LP_R, Table S1) and the wild-type strain were grown at 30°C in 96-well plates (Greiner CELLSTAR™; Sigma-Aldrich, St. Louis, MO, USA; poly-styrene, round bottom) with 200 µl of de Bont minimal medium (Hartmans et al., 1989) per well, supplemented with 30 mM citrate, and covered with a sealing
membrane (Diversified Biotech Breathe-Easy™; VWR, Radnor, PA, USA). The kinetics of bacterial growth and fluorescence were acquired by measuring the OD₆₃₀ as well as the excitation/emission values of mRFP1 at 582 nm/609 nm, mBFP2 at 385 nm/450 nm, mOrange2 at 541 nm/567 nm and mTurquoise2 at 451 nm/477 nm (Fig. S1). Although under the transcriptional control of the same regulatory elements, the five fluorescent proteins differed in both the intensity of their signal as well as their expression pattern. The fluorescence intensity of only msfGFP increased steadily for the whole cultivation time of 50 h; the signal of the remaining four reporter proteins reached stagnation after 15 h (mOrange2), 8 h (mRFP1 and mBFP2) and 25 h (mTurquoise2). Since all cultured strains continue to grow until the end of the experiment, the biomass-specific fluorescence decreased continuously (Fig. S1).

Discussion

The present genome engineering protocol reduces the hands-on work of the original procedure by Martínez-García and de Lorenzo (2011). We further streamlined the protocol with (i) the adoption of the USER assembly method that enables a standardized workflow (Cavaleiro et al., 2015), (ii) a reporter function (i.e. fluorescence) for the donor plasmid that allows for its direct visualization within host cells (Calero et al., 2016), (iii) additional antibiotic resistances to broaden the host spectrum and (iv) an efficient CRISPR-Cas9 counterselection, particularly relevant for the construction of difficult knock-outs. The protocol described herein yields reliable results for targeted mutagenesis in Pseudomonas species within a standard workweek. We have illustrated the flexibility of the system by combining different molecular elements of the toolbox to delete genomic regions in P. putida KT2440 and demonstrated the functionality of four new fluorescent proteins (mRFP1, mOrange2, mTurquoise2 and, to some extent, mBFP2) in this host via their targeted integration into a landing site in the chromosome. While we optimized the critical steps of the protocol that yield the desired mutation, the subsequent curing of replicating plasmids still adds up time required to generate the final, plasmid-free strain. One possible solution could be to implement conditional origins of replication in the current plasmid system, e.g. temperature-sensitive derivatives of oriV(RK2) (Valla et al., 1991) that has been shown to function in P. putida (Choi et al., 2018). This will be of particularly value for the curing of plasmid DNA from cells that are severely impaired in growth and are thus less prone to lose plasmids during proliferation. A reliable plasmid curing after each cycle of mutagenesis could also solve issues encountered with the non-induced, basal expression of the XylS/Pm expression system that can be observed particularly with increased copy numbers of the xylS gene (Gawin et al., 2017) or would allow to rely exclusively on the constitutive system. Furthermore, if combined with the first pre-culturing step of Pseudomonas in this protocol, plasmid curing would not affect its time requirements. While the synthetic biology toolbox for Pseudomonas is subjected to continuous improvement, the protocol discussed herein represents the fastest extant procedure for genome editing of P. putida and related species.

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Conflict of interest

None declared.

References


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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** Oligonucleotides used in this work.

**Table S2.** Assembly of pGNW plasmids with genes encoding fluorescent proteins within a chromosomal landing pad.

**Fig. S1.** Kinetics of the accumulation of selected fluorescent proteins measured in *P. putida* KT2440.

**Fig. S2.** Deletion of aceEF in *P. putida* KT2440 using a synthetic CRISPR-Cas9 device for counterselection.