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RESEARCH ARTICLE

Establishing a straightforward I-SceI-mediated recombination one-plasmid system for efficient genome editing in *P. putida* KT2440

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Abstract

Pseudomonas putida has become an increasingly important chassis for producing valuable bioproducts. This development is not least due to the ever-improving genetic toolbox, including gene and genome editing techniques. Here, we present a novel, one-plasmid design of a critical genetic tool, the pEMG/pSW system, guaranteeing one engineering cycle to be finalized in 3 days. The pEMG/pSW system proved in the last decade to be valuable for targeted genome engineering in *Pseudomonas*, as it enables the deletion of large regions of the genome, the integration of heterologous gene clusters or the targeted generation of point mutations. Here, to expedite genetic engineering, two alternative plasmids were constructed: (1) The *sacB* gene from *Bacillus subtilis* was integrated into the I-SceI expressing plasmid pSW-2 as a counterselection marker to accelerated plasmid curing; (2) double-strand break introducing gene *I-sceI* and *sacB* counterselection marker were integrated into the backbone of the original pEMG vector, named pEMG-RIS. The single plasmid of pEMG-RIS allows rapid genome editing despite the low transcriptional activity of a single copy of the I-SceI encoding gene. Here, the usability of the pEMG-RIS is shown in *P. putida* KT2440 by integrating an expression cassette including an *msfGFP* gene in 3 days. In addition, a large fragment of 12.1 kb was also integrated. In summary, we present an updated pEMG/pSW genome editing system that allows efficient and rapid genome editing in *P. putida*. All plasmids designed in this study will be available via the Addgene platform.

INTRODUCTION

Pseudomonas putida, a Gram-negative soil bacterium characterized by its versatile metabolism and remarkable stress resistance, has become an increasingly important chassis (Martínez-García et al., 2014; Martínez-García & de Lorenzo, 2024; Nikel & de Lorenzo, 2014, 2018; Weimer et al., 2020) for the production of value-added products such as aromatics (Schwanemann et al., 2020) and glycolipids (Tiso et al., 2020) from renewable carbon sources or waste streams (Ballerstedt et al., 2021). In recent years, several markerless genome editing methods have been developed and are extensively applied in the metabolic and genetic engineering of *Pseudomonas* (Aparicio et al., 2019; Galvão & de Lorenzo, 2005; Luo et al., 2016; Martínez-García & de Lorenzo, 2011; Sun et al., 2018). One prominent tool is homologous recombination-based editing tools, like the pEMG/pSW system established by Martinez Garcia et al. The system is well suited for successive scarless deletions, insertions or point mutations in *Pseudomonas* (Martínez-García & de Lorenzo, 2011; Wirth et al., 2020). The method is

based on two plasmids: a suicide vector with two I-SceI sites and a second vector providing the I-SceI endonuclease. Briefly, the utilization of this system for genome editing requires the integration of the pEMG vector into the genome by homologous recombination. An I-SceI encoding plasmid is transferred to the co-integrants and mediating double-strand breaks (DSB) in the I-SceI sites. The DSB are repaired by homologous recombination, and the merodiploid is resolved from the genome. The entire process typically takes approximately 6 days by using tri-parental mating plus some additional days for plasmid curing after confirmation of correct mutants (Volke et al., 2020, 2021; Wirth et al., 2020). It appears that there is potential for further optimization. Recently, cytidine deaminase-based toolsets that enable efficient multiplex editing in *P. putida* have been developed (Kozaeva et al., 2024; Volke et al., 2022; Yue et al., 2022). However, this approach necessitates several prerequisites and can potentially introduce unknown mutations beyond the spacer region (Volke et al., 2022).

The implementation of counterselection strategies is frequently contemplated and utilized in *Pseudomonas*, such as *pyrF/URA3* (Galvão & de Lorenzo, 2005), UPRTase (Graf & Altenbuchner, 2011) and Cre/*loxP* (Luo et al., 2016). Reyrat et al. (1998) presented an extensive overview of diverse counterselection markers, including *sacB*, *ccdB* and *pheS*. The *sacB* gene from *Bacillus subtilis* encodes the enzyme levansucrase and is frequently employed as a counterselection marker. When strains harbouring the *sacB* gene are plated, the presence of sucrose can cause death (Steinmetz et al., 1983).

In this study, we demonstrate the construction and applicability of a one-plasmid system for efficient genome editing in *P. putida* KT2440, based on the widely used pEMG/pSW system. The system comprises the pEMG vector backbone, an I-SceI endonuclease regulated by a strict inducible promoter and *sacB* gene as a counterselection marker. The new plasmid is integrated into the genome by homologous recombination, and I-SceI expression can be precisely induced without the need for the second plasmid delivery. Additionally, sucrose can facilitate the selection of merodiploid-resolved clones. The streamlined method enables genomic modification in *P. putida* within 3 days and obtains plasmid-free mutants. To our knowledge, this study presents the most rapid genome editing method in *P. putida* KT2440 reported thus far.

EXPERIMENTAL PROCEDURES

Bacterial strains, chemicals and DNA manipulations

The plasmids and bacterial strains used and generated in this study are listed in Tables 1 and 2. All oligonucleotides used in this study are listed in Table S1. *Escherichia coli* DH5α λ*pir* was used as a cloning host, and the *E. coli* HB101 strain, which harboured pRK2013, was used as the helper strain for tri-parental mating. Lysogeny broth (LB) liquid medium (tryptone 10 g L^{-1}, yeast extract 5 g L^{-1} and NaCl 5 g L^{-1}) and solid medium with 1.5% (w/v) agar were used for conventional cultivation of *E. coli* strains at 37°C and *P. putida* KT2440 at 30°C. Cetrimide agar was used to select *Pseudomonas* co-integrants after tri-parental mating. When required, 50 mg L^{-1} of kanamycin or 25 mg L^{-1} of gentamycin were added to the medium. All recombinant plasmids in this study were constructed with NEBuilder Hifi DNA Assembly Master Mix (New England Biolabs, Ipswich, Massachusetts, USA). All oligonucleotides were ordered, and all sequencing work was done at Eurofins Genomics (Ebersberg, Germany). DNA fragments for plasmid construction were amplified using Q5 DNA polymerase, and colony PCRs were performed with One*Taq* 2X Master Mix (New England BioLabs, Ipswich, Massachusetts, USA).

Plasmid constructions

For the construction of pEMG-RIS and pEMG-AIS plasmids, pEMG vector linearized by PCR amplification or digestion, *sacB* gene with the native promoter and terminator amplified from pLO3 (Lenz & Friedrich, 1998), P_{BAD} with *araC* gene amplified from pREDCas9 (Li et al., 2015) or P_{rhaB} with *rhaRS* genes amplified from pEcCas (Li et al., 2021), and the *I-sceI* gene amplified from pSW-2 (Martínez-García & de Lorenzo, 2011) were assembled by Gibson Assembly. Regarding the sequence in front of the *I-sceI* gene start codon, it was synthesized on the *I-sceI* gene amplification primer (I-SceI_F) containing the strong RBS (AGGAGG) and 8 bp (AATATACC) between RBS and start codon (Calero et al., 2016). To construct the pSW-2-*sacB* plasmid, the *sacB* gene amplified from pLO3 and pSW-2 vector linearized by PCR were assembled. For the construction of plasmid pEMG-RIS-0340-PYO, the linearized vector was amplified from pEMG-RIS-0340-*msfGFP* (this study) with primer HM1066 and HM1116, the fragment containing *nagR-phzA2B2CD1BFG* was obtained from pBNT.14.phz2 (Askitosari et al., 2019) by SspI digestion, and the fragment containing *nagR-phzMS* was amplified from pJNN.phzM+S (Schmitz et al., 2015) with primer HM1065 and HM1115 were assembled.

As the one-plasmid system is based on the pEMG vector, the plasmid construction can still follow the instructions for the pEMG vector to achieve the desired editing functions (Martínez-García & de Lorenzo, 2011; Wirth et al., 2020). First, pEMG-RIS or pEMG-AIS linearized by PCR amplification (pEMG_V2_F and pEMG_V2_R, Table S1) was used as the backbone. Other fragments required in subsequent plasmid assembly are contingent upon the specific

TABLE 1 Plasmids used in this study.

Plasmid	Description	Source
pEMG	Suicide vector used for deletions in Gram-negative bacteria; *oriT*, *traJ*, *lacZa*, *oriV(R6K)*, two I-SceI sites; Km^R	Martínez-García and de Lorenzo (2011)
pRK2013	Helper plasmid used for conjugation; *oriV*(*ColE1*), *mob*(*RK2*), *tra*(*RK2*); Km^R	Kessler et al. (1992)
pSW-2	Gm^R, oriRK2, *xylS*, $P_m \rightarrow$ *I-sceI* (*I-sceI* is transcriptional controlled by P_m)	Martínez-García and de Lorenzo (2011)
pSW-2-*sacB*	Derivative of vector pSW-2 carrying *sacB* gene; Gm^R	In this study
pEMG-RIS	Derivative of vector pEMG carrying P_{rhaB} controlled *I-sceI*, and native promoter-controlled *sacB* gene; Km^R	In this study
pEMG-AIS	Derivative of vector pEMG carrying P_{BAD} controlled *I-sceI*, and native promoter-controlled *sacB* gene; Km^R	In this study
pEMG-GRIS	Derivative of vector pEMG-RIS, Km^R is replaced by Gm^R	In this study
pEMG- *pta*	Derivative of vector pEMG carrying TSs for deletion of *pta* (PP_0774) in *Pseudomonas putida* KT2440; Km^R	In this study
pEMG-RIS-*pta*	Derivative of vector pEMG-RIS carrying TSs for deletion of *pta* (PP_0774) in *P. putida* KT2440; Km^R	In this study
pEMG-RIS-PP_3073	Derivative of vector pEMG-RIS carrying TSs for deletion of PP_3073 in *P. putida* KT2440; Km^R	In this study
pBG42	Km^R, Gm^R, *oriR6K*, Tn7L and Tn7R extremes, P_{14g}*(BCD2)–msfGFP* fusion	Zobel et al. (2015)
pEMG-RIS-0340-*msfGFP*	Derivative of vector pEMG-RIS, for insertion of P_{14g}*(BCD2)-msfGFP* into the site behind PP_0340; Km^R	In this study
pEMG-RIS-0340-PYO	Derivative of vector pEMG-RIS, for insertion of a 12.1 kb fragment into the site behind PP_0340; Km^R	In this study

TABLE 2 Bacterial strains used in this study.

Strain	Description	Source
Escherichia coli		
DH5α λ*pir*	Cloning host, DH5a strain with λ*pir* lysogen	Platt et al. (2000)
HB101	Helper strain, carrying pRK2013 plasmid used for conjugation	Sambrook et al. (1989)
Pseudomonas putida		
KT2440	Wild-type strain, *P. putida* mt-2 derivative cured of the TOL plasmid pWW0	Bagdasarian et al. (1981)
KT2440 △*pta*	KT2440 with the deletion of *pta* (PP_0774) gene	In this study
KT2440 △PP_3073	KT2440 with the deletion of PP_3073 gene	In this study
KT2440 0340::GFP	KT2440 inserted with P_{14g}*(BCD2)-msfGFP* at the PP_0340 site	In this study
KT2440 0340::PYO	KT2440 inserted with a 12.1 kb fragment at the PP_0340 site	In this study

objective: (1) for gene deletions, the upstream and downstream targeting sequences TS1 and TS2 (~500 bp of each) of the deletion area amplified from *P. putida* KT2440 genome are required; (2) for gene insertions, additional DNA fragments are needed and should be positioned between TS1 and TS2; (3) for point mutations and short insertions, the mutation can be introduced within the primer overhangs. For more details on the primer design with pEMG vector, please refer to the provided descriptions by Wirth et al. (2020).

Tri-parental mating and cetrimide agar selection

For plasmid delivery, the conjugation (tri-parental mating) option is highly recommended as the method of choice (Aparicio et al., 2019). Briefly, this process includes overnight cultivation of the plasmid donor strain (*E. coli* DH5a λ*pir* harbouring the editing plasmid), helper strain (*E. coli* HB101 harbouring pRK2013), and the acceptor strain (*P. putida* KT2440 and its derivatives) in 4 mL LB medium with

corresponding antibiotics. Then, 200 μL of each culture was mixed in a 1.5 mL reaction tube and washed once with LB medium after centrifugation. Finally, it was resuspended with 100 μL LB medium, around 30 μL of the mixture was dropped on the pre-dried LB plate (Wirth et al., 2020), and incubated at 30°C for 6 ~ 8 h. For co-integrants selection, biomass on the plate agar surface was taken by a pipette tip and resuspended in 200 μL of LB medium. Next, 100 μL of that suspension was spread on a cetrimide agar plate supplemented with 50 mg L^{-1} of kanamycin and then incubated at 30°C for 14 ~ 16 h. Colony PCR was used to verify the co-integrants.

Induction of I-SceI endonuclease and selection of plasmid-free mutants

To introduce the I-SceI-mediated double-strand breaks, colonies of *P. putida* co-integrants with pEMG-RIS/pEMG-AIS vector from cetrimide agar plate mentioned above were picked into 4 mL LB medium supplemented with 10 mM rhamnose (pEMG-RIS) or 100 mM arabinose (pEMG-AIS) in test tubes. After 4 ~ 12 h, that culture was diluted and spread on the LB plates supplemented with 10% (w/v) sucrose. Then, they were incubated overnight, and several colonies were picked for PCR verification. Further verification was done by PCR amplification of the related sequences using Q5 DNA Polymerase after re-isolating the correct strain from the last step on the LB agar plate, and it was sent for sequencing after gel purification.

RESULTS AND DISCUSSION

Design a user-friendly genome editing tool for pseudomonads

The pEMG/pSW system, developed by Martínez-García et al. (2011), has been extensively employed in Gram-negative bacteria. It is a highly reliable and standardized tool that dramatically facilitates research involving pseudomonads (Martínez-García et al., 2014; Wynands et al., 2018). To further expedite the genome editing process, it is valuable to analyse the optimizable operations of the existing system and contemplate stepwise optimization. As shown in Figure 1, it is evident that the curing of the pSW-2 plasmid constitutes the most time-intensive aspect of this original system. Generally, a minimum of six successive passages (Wirth et al., 2020) in LB medium without selection pressure is required, which takes at least 2 ~ 3 days. Another seemingly laborious part is the delivery of the second plasmid pSW-2 using conjugation or electroporation.

Accelerating the plasmid curing process of pSW-2

To accelerate the process of plasmid curing, the *sacB* gene, which is commonly employed as a counterselection marker, has been considered and incorporated into the pSW-2 plasmid. The resulting plasmid is named pSW-2-*sacB* (Figure 2A). Thus, the utilization of sucrose during the plasmid curing procedure enables the isolation of plasmid-free mutants. To validate the optimized plasmid in *P. putida* KT2440, we conducted an experiment involving deleting the *pta* gene (PP_0774, 2.1 kb, encoding the phosphate acetyltransferase), which the deletion was studied in the past (Nikel & de Lorenzo, 2013). For the deletion of the *pta* gene, it worked as well as the original pSW-2 plasmid, and they both demonstrated 100% merodiploid re-solvation efficiency. To evaluate the plasmid curing efficiency, a dilution series of a liquid culture of a correct colony was spread on LB agar plates supplemented with different concentrations of sucrose (0%, 0.5%, 1%, 2% w/v). As shown in Figure S1A, thousands of colonies grew on the plate lacking sucrose, whereas only tens of colonies grew on the plates with sucrose. Subsequently, we tested these colonies for the loss of pSW-2-sacB using LB-Gm plates, as shown in Figure S1B. As expected, colonies from the plates supplemented with sucrose exhibited 100% curing efficiency on plasmid loss. In comparison, only 3.7 ± 1.3% was obtained without sucrose selection (Figure S1C). These results indicated that the pSW-2-*sacB* plasmid can facilitate the isolation of plasmid-free clones on LB-sucrose plates and works as well as the pSW-2. This prevents the need for multiple passaging, resulting in a time-saving of approximately 3 days.

Constructing a one-plasmid system

A one-plasmid system was then considered to streamline the process further. However, the system requires incorporating the *I-sceI* gene and the two I-SceI sites in a single vector, which may result in 'self-cleavage'. Therefore, it is crucial to control the I-SceI expression strictly to avoid this issue. Calero et al. (2016) characterized several inducible promoter systems in *P. putida* and demonstrated that the XylS/P_m promoter exhibits a high leakage. The promoters P_{rhaB} and P_{BAD} show a very low basal expression level and are, therefore, suitable for the inducible expression of heterologous genes (Calero et al., 2016). For this reason, we replaced the XylS/P_m promoter, used for I-SceI expression on plasmid pSW-2, with AraC/P_{BAD} or RhaRS/P_{rhaB}. The inducible promoters were fused with *I-sceI* and integrated into the pEMG vector alongside the native promoter-controlled *sacB* gene, yielding pEMG-RIS

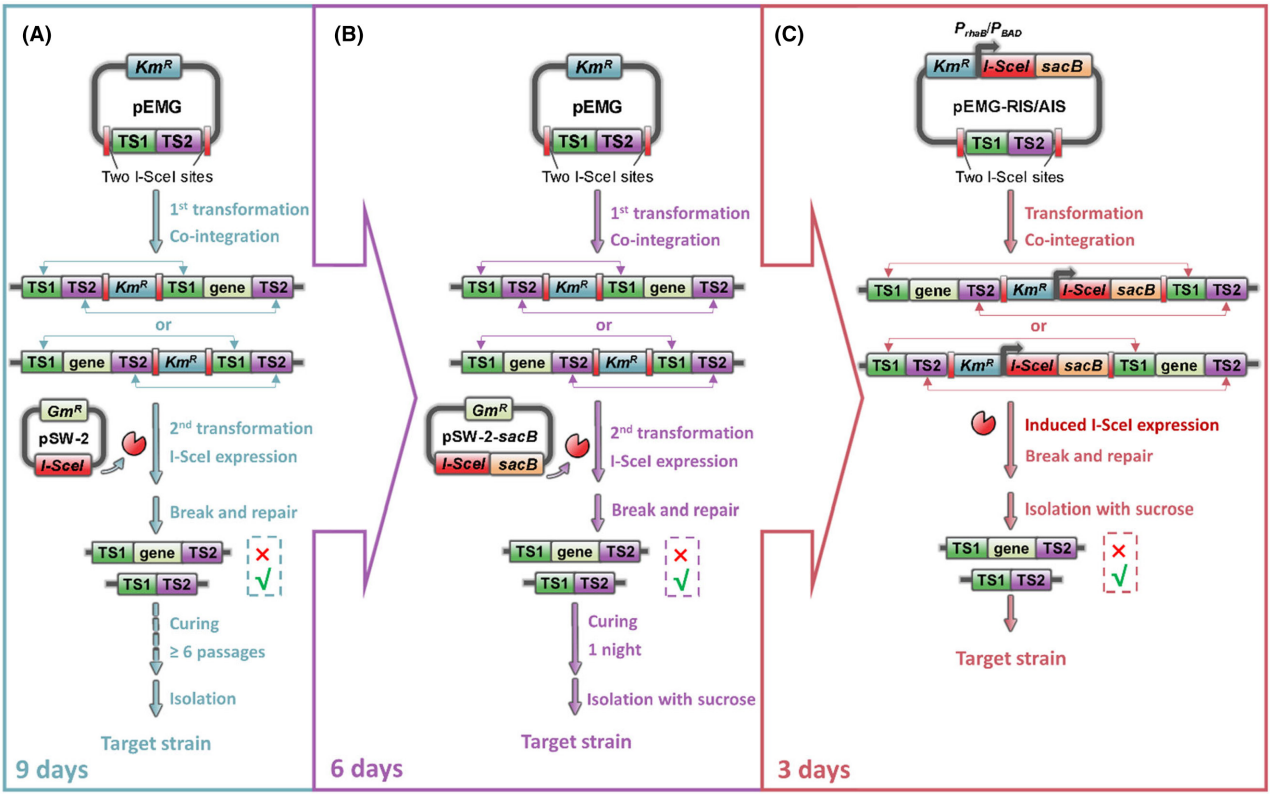

FIGURE 1 Overview of the workflow with the original pEMG/pSW system and the implementation of two alternative strategies. (A) The original pEMG plasmid bearing a Km^R marker, an origin of replication *oriR6K*, an *oriT*, and two I-SceI sites on the backbone. For gene deletions, the upstream and downstream targeting sequences (TS1 and TS2) are assembled into this plasmid between the two I-SceI sites. Firstly, this suicide vector was co-integrated into *Pseudomonas putida* KT2440 genome via tri-parental mating. The integration is either done by TS1 or TS2. Secondly, the I-SceI endonuclease was introduced by the delivery of the pSW-2. Then, the resulting DSB caused by I-SceI was repaired by homologous recombination. Successful re-solvation of merodiploid is tested by kanamycin sensitivity. Next, a plasmid curing procedure was conducted after the genotype verification through colony PCR. (B) Based on the pEMG/pSW system, a counterselection marker *sacB* was introduced onto pSW-2, resulting in pSW-2-*sacB*. Then, the utilization of sucrose facilitated the isolation of plasmid-free strains, avoiding the curing of pSW-2 and reducing the overall time required for the procedure from 9 to 6 days. (C) In a one-plasmid system, the *I-scel* gene controlled by P_{rhaB} or P_{BAD} and *sacB* gene were assembled into the backbone of the pEMG vector, named pEMG-RIS and pEMG-AIS, respectively. Then, after co-integration, I-SceI was expressed with the induction of rhamnose or arabinose in LB liquid cultures and plated on LB agar plates supplemented with 10% (w/v) sucrose. The streamlined procedure was accomplished within 3 days.

or pEMG-AIS (Figure 2B). This allowed us to control I-SceI expression strictly by the addition of rhamnose (pEMG-RIS) or arabinose (pEMG-AIS). Moreover, unique restriction sites remain in the multiple cloning site (MCS) between the I-SceI sites, enabling plasmid linearization by restriction endonuclease digestion (Figure 2B). Consequently, a one-plasmid system has been established, which theoretically enables one round of genome editing process within 3 days (Figures 1C and 4A).

To verify the applicability and efficiency of the newly constructed plasmids, the gene *pta* in *P. putida* KT2440 was deleted. The obtained plasmids pEMG-RIS-*pta* and pEMG-AIS-*pta* were co-integrated into the genome, and afterward, I-SceI expression induced by either rhamnose or arabinose for 10 h to introduce DSB in the co-integrants. Both plasmids performed satisfactorily, achieving a positive ratio (successful modification) of 21/28 (75%) and 12/28 (42.9%), respectively. Notably, the plasmid containing P_{rhaB} performed better than the P_{BAD} inducible variant, as confirmed through repeated experiments. Calero et al. showed that the P_{rhaB} promoter in *P. putida* KT2440 is stronger and requires lower concentration of inducer (Calero et al., 2016), which may contribute to better performance. Consequently, the pEMG-RIS vector was selected for subsequent demonstrations.

However, upon co-integrated into the genome, the functional efficiency of the SacB and I-SceI can get lower expression. In contrast to the consistently high expression on plasmids, expression on the genome is not only down-regulated due to single copy but also influenced by the genomic context (Englaender et al., 2017; Espah Borujeni et al., 2014; Köbbing et al., 2024). We have observed some co-integrants on the LB plate supplemented with 1% (w/v) sucrose, which revealed that some cells did not demonstrate targeted cleavage mediated by I-SceI after induction when

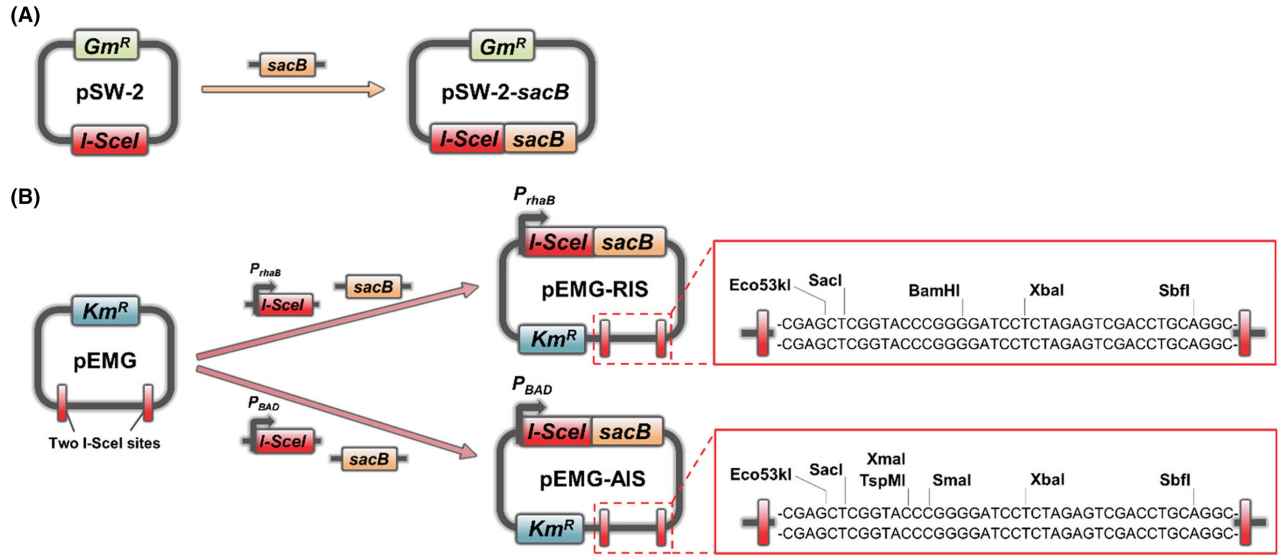

FIGURE 2 Design of the construction of pSW-2-*sacB* (A) and the construction of pEMG-RIS and pEMG-AIS (B). The DNA sequences flanked by I-SceI sites depict the multiple cloning sites in both plasmids. These sites enable plasmid linearization of pEMG-RIS or pEMG-AIS by restriction endonuclease digestion.

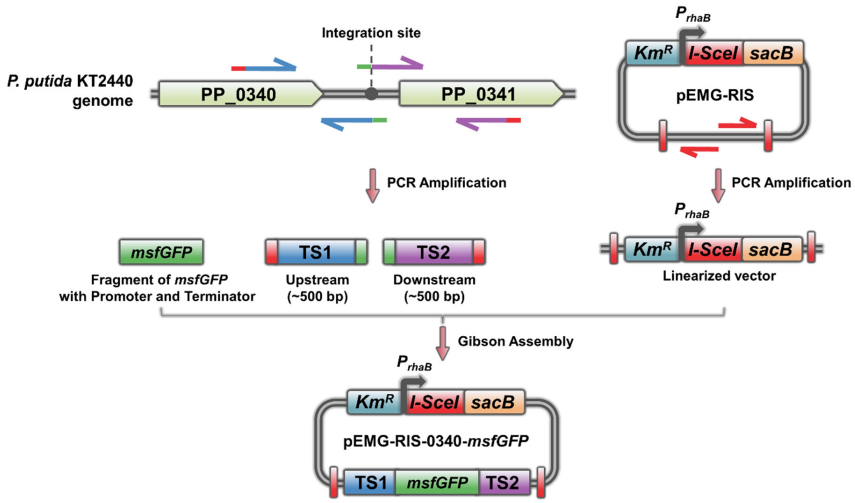

FIGURE 3 Workflow for constructing the pEMG-RIS-0340-*msfGFP* plasmid for gene insertion in *Pseudomonas putida* KT2440.The upstream and downstream targeting sequences (TS1 and TS2) amplified by two pairs of primers from *P. putida* KT2440 genome, linearized pEMG-RIS vector and the *msfGFP* gene with P_{14g} promoter and T_0 terminator were assembled via Gibson Assembly. Then, the assembled pEMG-RIS-0340-*msfGFP* plasmid is introduced into *Escherichia coli* DH5a λ*pir*. The annotation of PP_0340 is '*glnE* glutamate-ammonia-ligase adenylyltransferase', and PP_0341 is '*waaF* ADP-heptose: LPS heptosyltransferase II', and the exact position of that PP_0340 site on the genome is '414,043::414,044' (referring to a reference genome, NC_002947.3).

utilizing the one-plasmid system. Moreover, the selection condition of 1% (w/v) sucrose employed for pSW-2-*sacB* was inadequate here, which led to a re-evaluation of counterselection on the LB plate supplemented with different concentrations of sucrose (see Figure S2). As expected, the growth of uninduced strains decreased substantially with increasing sucrose concentrations, and a 10% (w/v) sucrose concentration sufficiently fulfils the counterselection requirements of the genome editing system in *P. putida* KT2440.

Targeted integration of *msfGFP* in the genome of *P. putida* KT2440

To visualize the efficiency and reliability of our one-plasmid system, we integrated a gene expression cassette containing *msfGFP* in the genome *P. putida* KT2440. For targeted integration, we chose the genomic locus next to PP_0340 (Between PP_0340 and PP_0341, Köbbing et al., 2024). To facilitate the insertion, a pEMG-RIS vector containing the upstream

and downstream homologous regions was constructed. The expression cassette consists of an *msfGFP* reporter gene driven by a constitutive σ^{70}-dependent promoter P_{14g} (Zobel et al., 2015), a *BCD2* element (Mutalik et al., 2013) and T_0 terminator (Figure 3).

The resulting *E. coli* DH5a λ*pir* strain containing plasmid pEMG-RIS-0340-*msfGFP* was then used as donor strain as described in Figure 4A. After 4 hours induction, 50 μL of that culture was spread on LB plates with or without 10% (w/v) sucrose selection and incubated overnight, respectively. Then, photographs were taken on a blue tray (Figure 4B), and colonies were counted and verified by colony PCR. Through PCR, we confirmed that only the merodiploid-resolved strains could grow in the presence of 10% (w/v) sucrose. At the same time, colonies derived from regular LB plate resulted in a mixture of co-integrant, GFP inserted strain or wild-type (parent type) (Figure 4B). The evaluation of integration efficiency of the expression cassette alone into the genome was then performed through direct observation of clones with or without green fluorescence on a blue tray (Figure 4B). On plates supplemented with 10% (w/v) sucrose, 50.6 ± 0.2% (140/277) of the colonies contained the desired *msfGFP* genomic integration (green spots). And on plates without sucrose, only 4.9 ± 0.1% (137/2816) of the colonies were the wild-type strains (dark spots), which means most of the colonies were merodiploid-unresolved (Figure 4C). These results showed that the addition of 10% (w/v) sucrose to LB plates effectively functioned as a counterselection, resulting in a notable decrease in the number of PCR samples needed to obtain the target strain. As expected, these results provided a comprehensive and visual validation of our method, achieving the anticipated theoretical efficiency of 50%.

Insertion of a large fragment using the one-plasmid system

Strain engineering often requires the integration of larger fragments into the genome, which can be

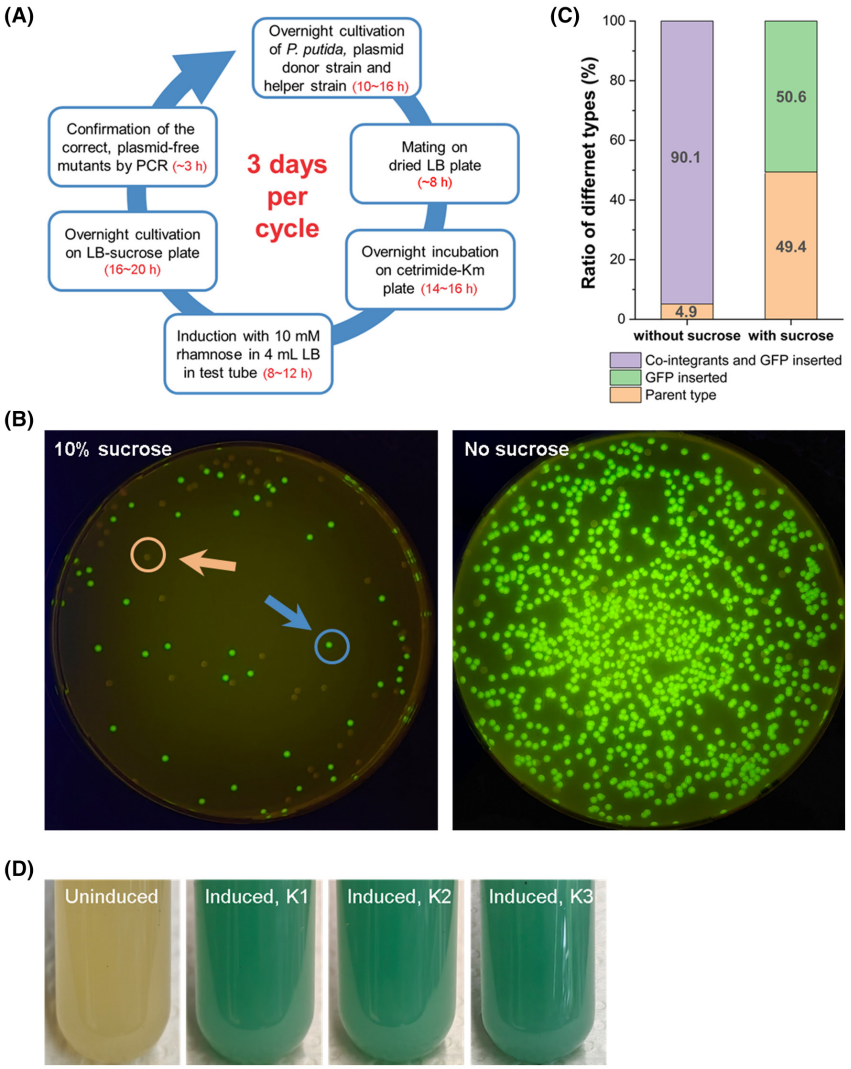

FIGURE 4 A 3-day workflow (A) for integrating the *msfGFP* gene into *Pseudomonas putida* KT2440 using the one-plasmid system. The plasmid donor strain is *Escherichia coli* DH5a λ*pir* harbouring pEMG-RIS-0340-*msfGFP*, and the helper strain is *E. coli* HB101 harbouring pRK2013. (B) A blue light screen was used to easily detect the presence of *msfGFP* in the grown colonies. Cultures were plated on agar plates with (left plate) or without (right plate) sucrose. Colonies showing no fluorescence represent the wild type, while colonies with green fluorescence either have the desired integration or the entire pEMG-RIS is still present in the genome. (C) The composition of colonies from the triplicate experiments of B is displayed (D). The intense colour formation of the pyocyanin (PYO) production is shown after induction with 0.1 mM salicylate for 36 h.

challenging for genome editing methods. To evaluate the efficiency of the one-plasmid system in addressing this challenge, we conducted an experiment involving the insertion of a 12.1 kb fragment into the genome next to PP_0340 (Köbbing et al., 2024). This inserted long fragment contains the pyocyanin-producing gene that can show intense colour formation after induction (Figure 4D) (Askitosari et al., 2019; Schmitz et al., 2015), which could confirm the successful insertion reliably. Surprisingly, despite the considerable size of the resulting integration plasmid (21.2 kb), it showed a commendable performance with a positive ratio of 11/96 (11.5%). Consequently, we can confidently assert that this one-plasmid system can fully meet any conventional gene insertion requirements.

The location of gene in the genome can influence its modification

We have observed that the genomic context can influence the effectiveness of the SacB and I-SceI in the one-plasmid system. We evaluated the merodiploid-resolving efficiency of the deletion of the PP_3073 gene, compared with the deletion of the *pta* gene and gene insertion at the PP_0340 site. Our findings indicate that longer induction times led to increasingly higher resolving efficiency. The resolving efficiency at the PP_3073 site was significantly lower compared to the PP_0340 site and *pta* gene (Figure 5).

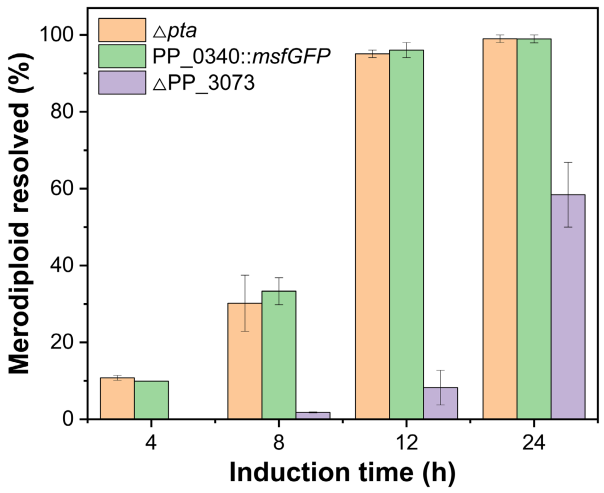

FIGURE 5 Time dependency of resolving the merodiploid from the genome of *Pseudomonas putida* KT2440. Different times of I-SceI induction during gene insertion at PP_0340 or gene deletion of *pta* or PP_3073 were tested. The experiment was conducted by cultivating the co-integrants in LB medium supplemented with 10 mM rhamnose. These samples were then diluted and spread on LB plates. After overnight incubation, single colonies were picked and tested using LB-Km plates for sensitivity. The colonies that could not grow on LB-Km plates correspond to cells that resolved the merodiploid. The plot shows the result of triplicate experiments and at least 100 colonies per sample.

Furthermore, the strain with the co-integration at the PP_3073 site could still grow on LB plates supplemented with 10% (w/v) sucrose. However, the size of the colonies was much smaller than those that resolved the merodiploid (Figure S3). These results indicated that the genome locus sometimes could significantly affect genome editing with the one-plasmid system, and an induction time of 12 hours or even longer might be more effective.

For comparison, we also tested the merodiploid-resolving efficiency by spreading diluted uninduced culture samples on LB plates or LB plates with 10% (w/v) sucrose. After incubation, colonies from LB plates were picked and tested with LB-Km plates for sensitivity. As expected, all the 900 colonies we tested grew on LB-Km plates, showing that all merodiploids were genetically stable under the tested conditions. Besides, only a few colonies grew on the LB-sucrose plates, and over half were unresolved. That indicates the low efficiency and high false positive rates while only relying on the sucrose counterselection for genomic modification.

CONCLUSION

In this study, we demonstrate a one-plasmid genome editing tool, accelerating the engineering cycle from 9 to 3 days. Based on the pEMG backbone, a tightly controllable *I-sceI* gene and a constitutive *sacB* gene were assembled on a single plasmid. Consequently, the requirement of a second plasmid transformation and a laborious plasmid curing process can be circumvented. Notably, the new pEMG-RIS single plasmid system is also reliable for large fragment insertion.

Some techniques are based on the SacB counterselection alone, such as the pK18-*mob-sacB* vector (Schäfer et al., 1994), which is used in many laboratories (Chung et al., 2011; Johnson & Beckham, 2015), or CRISPR-based modification systems (Aparicio et al., 2019; Cook et al., 2021; Sun et al., 2018; Zhou et al., 2020). The SacB-based methods are based on a passive second cross-over event followed by selecting target strains and killing unresolved merodiploid strains with SacB. In our hands and other laboratories, the resolution of the merodiploid frequently causes false positives (Pang et al., 2024). The CRISPR-based methods currently require two or even three plasmids and at least two different sgRNA-containing plasmids. In comparison, our one-plasmid system is simple, fast, and has a relatively high editing efficiency (50%, theoretically).

To further accelerate genome editing, we also attempted to simultaneously execute two gene deletions by employing pEMG-RIS plasmids containing distinct antibiotic markers. Regrettably, this attempt proved unsuccessful, potentially due to the long identical sequences on the plasmid backbone, which may impede the co-integration.

To effectively address the growing demand for genome editing in *P. putida*, we present here our novel one-plasmid system, pEMG-RIS. We hope this 3-day method can expedite the exploration of *P. putida* and expand its use in biological and environmental applications. All plasmids designed in this study will be available via Addgene.

AUTHOR CONTRIBUTIONS

Hao Meng: Conceptualization; data curation; formal analysis; investigation; methodology; software; validation; visualization; writing – original draft; writing – review and editing. **Sebastian Köbbing:** Conceptualization; data curation; formal analysis; investigation; methodology; project administration; resources; software; supervision; validation; visualization; writing – review and editing. **Lars M. Blank:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; software; supervision; validation; visualization; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

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SUPPORTING INFORMATION

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

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