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A CRISPR/Cas9-based single-stranded DNA recombineering system for genome editing of *Rhodococcus opacus* PD630

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ABSTRACT

Genome engineering of *Rhodococcus opacus* PD630, an important microorganism used for the bioconversion of lignin, is currently dependent on inefficient homologous recombination. Although a CRISPR interference procedure for gene repression has previously been developed for *R. opacus* PD630, a CRISPR/Cas9 system for gene knockout has yet to be reported for the strain. In this study, we found that the cytotoxicity of Cas9 and the deficiency in pathways for repairing DNA double-strand breaks (DSBs) were the major causes of the failure of conventional CRISPR/Cas9 technologies in *R. opacus*, even when augmented with the recombinases Che9c60 and Che9c61. We successfully developed an efficient single-stranded DNA (ssDNA) recombineering system coupled with CRISPR/Cas9 counter-selection, which facilitated rapid and scarless editing of the *R. opacus* genome. A two-plasmid system, comprising Cas9 driven by a weak *Rhodococcus* promoter Pniami, designed to prevent cytotoxicity, and a single-guide RNA (sgRNA) under the control of a strong constitutive promoter, was proven to be appropriate with respect to cleavage function. A novel recombinase, *Rr*RecT derived from a *Rhodococcus ruber* prophage, was identified for the first time, which facilitated recombination of short ssDNA donors (40–80 nt) targeted to the lagging strand and enabled us to obtain a recombination efficiency up to 10^3-fold higher than that of endogenous pathways. Finally, by incorporating *Rr*RecT and Cas9 into a single plasmid and then co-transforming cells with sgRNA plasmids and short ssDNA donors, we efficiently achieved gene disruption and base mutation in *R. opacus*, with editing efficiencies ranging from 22 % to 100 %. Simultaneous disruption of double genes was also confirmed, although at a lower efficiency. This effective genome editing tool will accelerate the engineering of *R. opacus* metabolism.

1. Introduction

Rhodococcus opacus PD630, a non-model gram-positive oleaginous bacterium, is of considerable commercial interest with respect to the conversion of lignin to value-added products such as biofuels and muconate [1–3]. *R. opacus* can tolerate aromatic compounds derived from the pre-treatment and depolymerization of lignin [4], and has diverse enzymatic pathways that contribute to the degradation and assimilation of aromatics. In addition, *R. opacus* can accumulate triacylglycerols to high levels of up to 78 % of the cell dry weight [5]. These desirable traits make *R. opacus* an attractive option for lignin conversion and biofuel production, and in this regard, diverse genetic toolkits have recently been developed for this bacterium [6–8], although there remains a necessity for efficient genome editing tools.

Single-crossover integration of non-replicating plasmids is a typical gene disruption technique used for *R. opacus*. Furthermore, to recover antibiotic selection markers from the genome, a two-step crossover can be employed using a counter-selection marker, such as *sacB* or *pheS** [2,3]. However, illegitimate recombination has been reported in *Rhodococcus* in response to the introduction of non-replicating plasmids, resulting in random integration and low editing efficiency [9]. In conjunction with the use of recombinases Che9c60 and Che9c61, derived from a mycobacteriophage [10], a recombination method based on a linear double-stranded DNA (dsDNA) template flanking an

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antibiotic selection marker has been developed for *R. opacus*, which has contributed to enhancing editing efficiency [6]. However, the antibiotic selection marker used in this procedure is non-recyclable, thereby limiting the number of multiple rounds of editing.

The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas) system has been developed as a powerful tool for genome editing in both eukaryotes and prokaryotes [11,12]. The complex of DNA nuclease Cas9 and single-guide RNA sequences (sgRNAs) can introduce DNA double-strand breaks (DSBs) at a target locus with a protospacer adjacent motif (PAM) [11]. Gene editing will be achieved by repairing DSBs through non-homologous end joining (NHEJ) or homologous recombination (HR) [13]. Using a catalytically inactive mutant of Cas9 nuclease (dCas9), CRISPR can be repurposed as an interference system (CRISPRi) for gene repression [14]. However, although a CRISPRi system has previously been developed for *R. opacus* engineering [6], attempts to develop a CRISPR/Cas9 system for gene knockout, which additionally requires efficient pathways for DSB repair, have not been reported yet. Moreover, despite the establishment of a CRISPR/Cas9-mediated dsDNA recombineering system for another *Rhodococcus* species, *R. ruber* [15], this method has proved unviable in the case of *R. opacus*.

With respect to the development of CRISPR/Cas9 systems in prokaryotes, the introduction of recombinases derived from bacteriophages or prophages is usually a necessary prerequisite, owing to the limited efficiency of endogenous NHEJ or HR pathways for DSB repair [12]. Classical recombinases include beta, exo, and gamma proteins of the λ Red system [16], and RecE and RecT of the Rac prophage [17]. However, these proteins are not broadly portable and only function in a small group of phylogenetically related species [18]. To address this problem, a reliable strategy is the screening of recombinases derived from phages or prophages that infect the host of interest [19].

In this study, we initially fine-tuned the expression of the *cas9* gene and enabled the function of the Cas9-sgRNA complex to introduce DSBs in *R. opacus* for counter-selection. To compensate for the inefficient DSB repair pathways in *R. opacus*, we further screened and obtained a novel recombinase *Rr*RecT from an *R. ruber* prophage for single-stranded DNA (ssDNA) recombineering. Using CRISPR/Cas9 for counter-selection, we developed an efficient ssDNA recombineering system for rapid and scarless genome editing in *R. opacus* with editing efficiencies ranging from 22 % to 100 %, and demonstrated its potential utility for one-step multiple gene disruption.

2. Material and methods

2.1. Strains and media

The plasmids and strains used in this study are listed in Table S1. For plasmid construction, we used *Escherichia coli* Top 10. Luria-Bertani (LB) medium (10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter) was used for the cultivation of *E. coli*. Seed medium (20 g glucose, 1 g yeast extract, 7 g tryptone, 0.5 g KH_2PO_4, 0.5 g $K_2HPO_4{\cdot}3H_2O$, 0.5 g $MgSO_4{\cdot}7H_2O$, and 1 g monosodium glutamate per liter, pH 7.5) was used for liquid culture of *R. opacus*, and solid medium (10 g glucose, 3 g yeast extract, 1 g NaCl, 2 g $K_2HPO_4{\cdot}3H_2O$, 0.2 g $MgSO_4{\cdot}7H_2O$, and 15 g agar per liter, natural pH) was used for plate culture. When required, 25 μg/mL kanamycin, 10 μg/mL chloramphenicol, or 12 μg/mL rifampicin were added to the medium.

2.2. Plasmid construction

The plasmids pNV-Pa2-Cas9, pNV-null-Cas9, and pBNVCm-*Bbs*I-sgRNA were constructed in our previous study, in which we developed a CRISPR/Cas9 system for *R. ruber* [15]. For construction of the plasmid pNV-Pniami-Cas9, Pniami promoter was amplified from the *R. ruber* genome using primers *Hin*dIII-Pniami-F and *Xba*I-Pniami-R, and then ligated to plasmid pNV-null-Cas9. The primers used in this study are shown in Table S2, and sequences of the promoters and *cas9* genes are listed in Table S3.

The pBNVCm-sgRNA plasmids were constructed using the Golden Gate Assembly method. For example, to generate pBNVCm-07156-sgRNA, primers 07156-sgRNA-F and 07156-sgRNA-R were annealed to form dsDNA with sticky ends and then ligated to the *Bbs*I-digested pBNVCm-*Bbs*I-sgRNA. For the construction of the plasmid pBNVCm-07156-sgRNA-donor, upstream and downstream homologous arms of the *LPD07156* gene were amplified from the *R. opacus* genome using primers 07156-up-F/R, and 07156-down-F/R, respectively, and then ligated to plasmid pBNVCm-07156-sgRNA via Gibson Assembly. Similarly, the upstream and downstream homologous arms of the *LPD02538* gene were amplified from the *R. opacus* genome using the primers 02538-up-F/R and 02538-down-F/R, respectively, and then ligated to plasmid pBNVCm-02538-sgRNA, to generate pBNVCm-02538-sgRNA-donor. For the construction of pBNVCm-03951-01628-sgRNA, the sgRNA cassette targeting *LPD01628* was amplified from plasmid pBNVCm-01628-sgRNA using the primers *Kpn*I-sgRNA-F and *Eco*RI-sgRNA-R, and ligated to the pBNVCm-03951-sgRNA plasmid via Gibson Assembly.

For the construction of plasmids containing recombinase genes, such as pNV-P_{BAD}-D, we initially amplified the promoter P_{BAD} from pKD46 [16] using the primers *Hin*dIII-P_{BAD}-F and *Xba*I-P_{BAD}-R, and then ligated this to the pNV18.1 plasmid [20] to generate plasmid pNV-P_{BAD}. The gene encoding recombinase D, commercially synthesized by GENEWIZ (Suzhou, China), was amplified using primers *Xba*I-D-F and *Kpn*I-D-R, and subsequently ligated to the pNV-P_{BAD} plasmid to generate pNV-P_{BAD}-D. Furthermore, P_{BAD}-D was amplified from pNV-P_{BAD}-D using primers P_{BAD}-F and D-R2, terminators *rrnBT1* and *rrnBT2* were amplified from pXMJ19 [21] using primers Ter-F and Ter-R, and the two fragments were then ligated with the *Hin*dIII-digested pNV-Pniami-Cas9 via Gibson Assembly, to construct plasmid pNV-P_{BAD}-D-Pniami-Cas9. The sequences of the promoter P_{BAD}, recombinase genes, and terminators are listed in Table S3.

DNA polymerases and T4 DNA ligases were purchased from Vazyme (Nanjing, China), restriction enzymes were purchased from Takara (Shiga, Japan), and Gibson Assembly kits were purchased from Taihe Biotechnology (Beijing, China). Plasmid extraction, DNA purification, and gel extraction kits were purchased from Omega (USA), and DNA sequencing and primer synthesis were performed by GENEWIZ (Suzhou, China). To achieve high cleavage efficiency and reduce potential off-targeting effects, the guide sequences of sgRNAs were designed using a web-based tool, called Cas-Designer [22].

2.3. Transformation protocol for R. opacus

For the preparation of competent cells, *R. opacus* was cultivated in seed medium for 48 h at 28 °C and 200 rpm, and then inoculated into 100 mL of nutrient broth medium (10 g/L peptone, 5 g/L NaCl, and 3 g/L beef extract, pH 7.2) containing 8.5 g/L glycine and 10 g/L sucrose, with an initial OD_{600} of 0.05 [23]. Cells were grown at 28 °C and 200 rpm to an OD_{600} of 0.5, and then centrifuged at 8000×*g* for 20 min at 4 °C. The pellet thus obtained was washed twice with pre-cooled deionized water (4 °C) and finally concentrated 50-fold. For routine transformation of plasmids, 200 μL of competent cells was mixed with 1 μg of plasmids and then loaded into an electroporation cuvette with a 0.2 cm gap. After cooling for 10 min on ice, electroporation was performed using a pulse of 10 kV/cm. Cells were immediately mixed with 800 μL of LBHIS medium (5 g tryptone, 5 g NaCl, 2.5 g yeast extract, 18.5 g brain heart infusion powder, and 91 g sorbitol per liter), incubated at 28 °C and 200 rpm for 4–5 h, and thereafter spread on solid medium containing appropriate antibiotics. Cells were grown at 28 °C for 2–4 days until visible colonies were obtained.

2.4. Screening and optimization of recombinases for ssDNA recombineering

To determine the *rpoB* gene mutations responsible for the rifampicin-resistant phenotype, *R. opacus* was cultivated in seed medium for 48 h, and 200 μL of the culture broth was subsequently spread on solid medium containing rifampicin. The cells were incubated at 28 °C for 3 days, and then four rifampicin-resistant colonies were randomly picked for PCR with primers rpoB-F and rpoB-R. The PCR products were sequenced and aligned with the wild-type *rpoB* gene to determine the mutations resulting in rifampicin resistance.

The plasmids pNV-P_{BAD}-A/B/C/D/E/Che9c61 containing recombinase genes, as well as the empty plasmid pNV-P_{BAD} were transformed to *R. opacus*. For the preparation of competent cells, 10 mmol/L arabinose was added when the OD_{600} of the culture reached 0.25–0.3, and then the recombinases were induced for 3–4 h until attaining an OD_{600} of 0.5. *R. opacus* harboring recombinases was transformed with 5 μg of oligonucleotides S464F–F and S464F-R, recovered in LBHIS medium for 5 h, and spread on rifampicin plates. The colonies thus obtained were counted and compared to determine the recombinase with the highest efficiency. Furthermore, we optimized the protocol for ssDNA recombineering in *R. opacus* (pNV-P_{BAD}-D) with respect to arabinose inducer concentration and the amount and length of ssDNA.

2.5. Protocol of CRISPR/Cas9-based ssDNA recombineering

To combine CRISPR/Cas9 with ssDNA recombineering, we transformed plasmid pNV-P_{BAD}-D-Pniami-Cas9 to *R. opacus*. The resulting strain was induced using 10 mmol/L arabinose during competent cell preparation for the expression of recombinase *Rr*RecT. For genome editing, *R. opacus* (pNV-P_{BAD}-D-Pniami-Cas9) competent cells (200 μL) were transformed with 2 μg of pBNVCm-sgRNA series plasmids and 10 μg of corresponding oligonucleotides, recovered for 5 h, spread on solid medium containing kanamycin and chloramphenicol, and then incubated at 28 °C for 3 or 4 days. Colonies were examined based on PCR analyses using appropriate primers, and when necessary, positive colonies were further sequenced to confirm editing.

2.6. Curing of plasmids

For multiple rounds of genome editing, previously introduced sgRNA plasmids must be cured prior to the introduction of a further plasmid. To cure sgRNA plasmids, edited colonies were cultivated for 2 days at 28 °C in seed medium containing kanamycin, and then spread on solid medium containing kanamycin. After incubation at 28 °C for 2 days, colonies were inoculated onto plates containing chloramphenicol. Those colonies unable to grow on chloramphenicol were identified as those lacking pBNVCm-sgRNA, and they were selected for the subsequent round of editing. Having completed the final round of editing, the plasmids pNV-P_{BAD}-D-Pniami-Cas9 and pBNVCm-sgRNA could be simultaneously cured when cells were cultured and spread on plates lacking antibiotics.

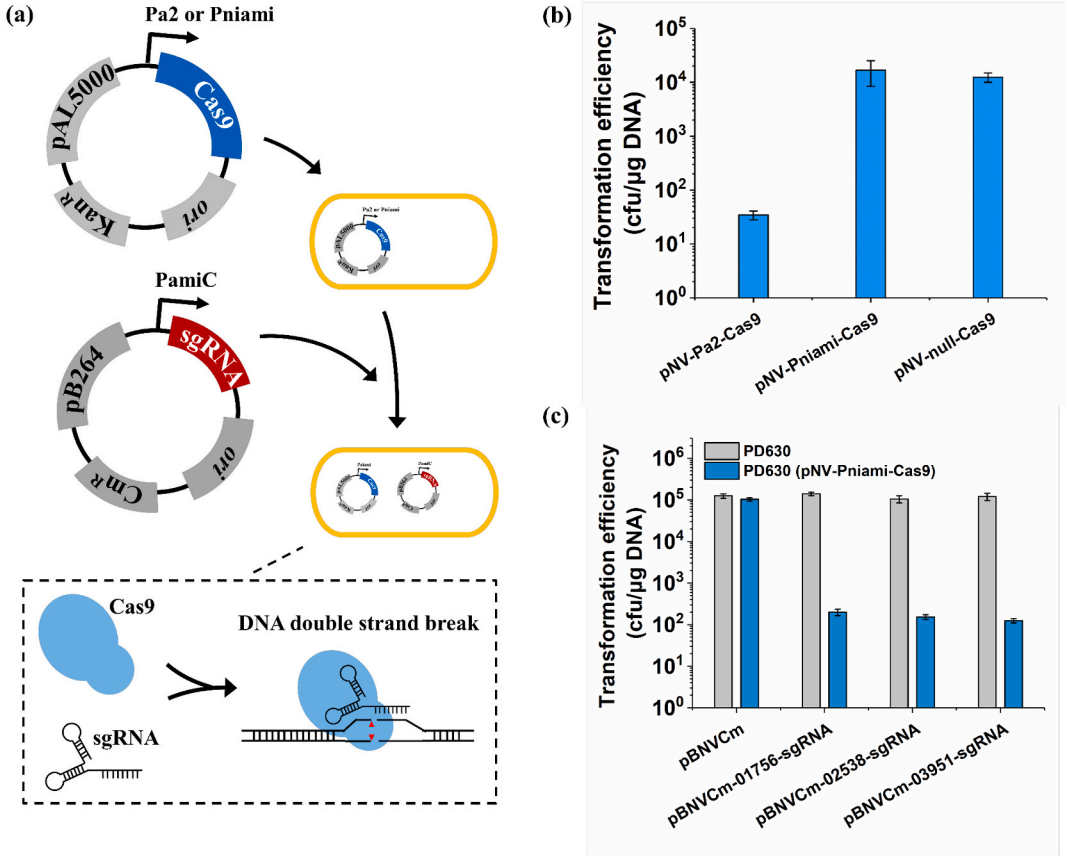

Fig. 1. Verification of the cleavage function of the Cas9-sgRNA complex in *R. opacus*. (a) The experimental scheme used to generate a Cas9-sgRNA complex designed to introduce double-strand breaks in the target genomic locus in *R. opacus*. (b) The transformation efficiency of Cas9 plasmids in which Cas9 was driven by different promoters. (c) The transformation efficiency of sgRNA plasmids to *R. opacus* with or without plasmid pNV-Pniami-Cas9. Plasmids pBNVCm-07156-sgRNA, pBNVCm-02538-sgRNA and pBNVCm-03951-sgRNA contained sgRNAs targeting genes *LPD07156*, *LPD02538*, and *LPD03951*, respectively. The sgRNA-deficient plasmid pBNVCm was used as a control and experiments were performed in triplicate.

3. Results

3.1. Fine-tuning of Cas9 expression for efficient function in R. opacus PD630

In order to develop a CRIPSR/Cas9 genome editing system applicable to *R. opacus* PD630, we initially needed to enable the function of the Cas9-sgRNA complex to introduce DSBs in the target genomic locus. To this end, we designed a two-plasmid system, one for Cas9 expression and the other for sgRNA transcription, to examine the function of the Cas9-sgRNA complex, as shown in Fig. 1a.

Previously, we constructed the pNV-Pa2-Cas9 plasmid [15], in which a codon-optimized *cas9* gene was driven by the urea-inducible Pa2 promoter [24], for genome editing in *R. ruber*. However, we found the Pa2 promoter acted as a strong constitutive promoter in *R. opacus*. This can be attributed to the fact that although transcriptional regulators of Pa2 are located in the *R. ruber* genome, they are not present in that of *R. opacus*. The strong constitutive overexpression of Cas9 driven by Pa2 would conceivably be lethal to *R. opacus* and prevent pNV-Pa2-Cas9 transformation. We accordingly found that the transformation efficiency of pNV-Pa2-Cas9 was 345-fold lower than that of pNV-null-Cas9 lacking a promoter for Cas9 (as shown in Fig. 1b). Subsequently, colony PCR of the resulting colonies failed to generate the intact *cas9* gene fragment (see Fig. S1). Given these observation, we therefore replaced the Pa2 promoter with a 20-fold weaker promoter, Pniami [24], and constructed a new plasmid, pNV-Pniami-Cas9, which was successfully transformed to *R. opacus* at high efficiency (see Fig. 1b).

The sgRNA cassette was transcribed under the control of a strong constitutive promoter, PamiC, and inserted into the temperature-sensitive plasmid pBNVCm [15]. To verify the function of the Cas9-sgRNA complex, the pBNVCm-sgRNA series of plasmids were introduced into *R. opacus* strains both with and without pNV-Pniami-Cas9. In the present study, we selected several genes for sgRNA design, namely, *LPD07156* encoding a benzoate transport protein, *LPD02538* encoding a restriction enzyme, and *LPD03951* encoding a DNA repair protein, the functions of which are assumed to be non-essential in *R. opacus*. Sequences of these genes were obtained from GenBank P003949.1, which contains the whole genome sequences of *R. opacus* PD630. Compared with wild-type *R. opacus*, we found that the transformation efficiencies of the pBNVCm-sgRNA plasmids to *R. opacus* harboring Cas9 were reduced by ~10^3 fold, as shown in Fig. 1c, thereby indicating that the Cas9-sgRNA complex functions efficiently in *R. opacus* for counter-selection.

3.2. The endogenous HR and NHEJ pathways were insufficiently efficient for DSB repair in R. opacus PD630

To verify the editing efficiency of the CRISPR/Cas9 system, we selected gene *LPD07156* encoding a benzoate transport protein for knockout. This gene has been reported to be non-essential, based on deletion using an alternative method [25]. The homologous arms of *LPD07156* (750 bp each side) were cloned into plasmid pBNVCm-07156-sgRNA to construct pBNVCm-07156-sgRNA-donor, which was introduced into *R. opacus* PD630 (pNV-Pniami-Cas9) for gene knockout. However, we found that the homologous recombination (HR) efficiency of *R. opacus* was insufficient for DSB repair, and the editing efficiency via HR was 0/23 (0 %), as shown in Fig. 2. Knockout of a further gene, *LPD02538*, encoding a restriction enzyme, using plasmid pBNVCm-02538-sgRNA-donor, proved similarly ineffective with an editing efficiency of 0/23, as shown in Fig. S2. These findings regarding the low efficiency of DSB repair via endogenous HR pathways in *R. opacus* are consistent our observations on *R. ruber* [15].

Interestingly, when sequencing the target site of the *LPD07156* gene recognized by the sgRNA, we found that three of the 10 randomly selected colonies showed deletions of different lengths ranging from 34 to 310 bp, as shown in Fig. 2. We speculate that this phenomenon can probably be attributed to the NHEJ pathway in *R. opacus*. In prokaryotes, the NHEJ pathway typically comprises two core elements, namely, Ku, a DNA end-joining protein, and the ATP-dependent DNA ligase LigD [26]. BLAST search results using Ku and LigD from *Mycobacterium smegmatis* [27] as queries revealed the presence of the NHEJ pathway in *R. opacus* PD630, as shown in Fig. S3. However, when we examined the effects of more sgRNAs, we found that the efficiency of DSB repair mediated by NHEJ was dependent to a considerable extent on the nature of the target site, as summarized in Table 1. For most sgRNAs, we were unable to detect either insertions or deletions, which thus tended to indicate that similar to the HR pathway, the endogenous NHEJ pathway in *R. opacus* was insufficiently efficient for DSB repair. Most of the surviving colonies were escapers that mutated the *cas9* gene or sgRNA cassette, thereby blocking the cleavage function of Cas9-sgRNA complex [15].

3.3. Screening of Rhodococcus phage- or prophage-derived recombinases for ssDNA recombineering

Given that we found the endogenous recombination pathways in *R. opacus* to be inefficient for DSB repair, we further attempted to

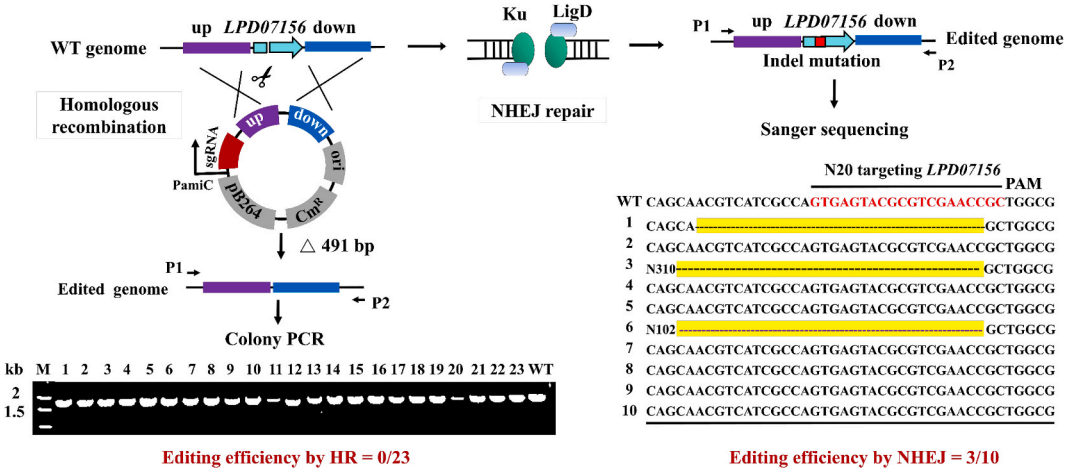

Fig. 2. Double-strand break repair pathways in *R. opacus* for gene knockout using the CRISPR/Cas9 system. If the DSB of *LPD07156* was repaired by homologous recombination (HR), 491 bp would be deleted from the genome. When using primers P1 and P2 for PCR, DNA fragments of 1698 bp and 1207 bp should be amplified from the wild-type and edited colonies, respectively. To verify potential DSB repair mediated by non-homologous end joining (NHEJ), 10 colonies were randomly selected for PCR using primers P1 and P2, and the products were sequenced and aligned to the *LPD07156* gene.

Table 1
Editing efficiency for assessed genes based on endogenous NHEJ.

Gene ID[a]	Guide sequence of sgRNA[b]	Editing efficiency by NHEJ[c]
LPD07156	GTGAGTACGCGTCGAACCGCTGG	3/10
LPD02538	CCTCCTCTGGCCAGCGCTTCAGG	0/24
LPD03951	GAAGAGTTACTTCCTCGAACCGG	3/11
LPD01620	CCGCTTCCACCGTCTCCTGCCGG	0/16
LPD01622	CAGGATCGTCAGCGCATTGCAGG	0/16
LPD01628	ATCTGCAACGGTTCCCGGACGGG	0/16

[a] *LPD01620* encodes a subunit of gramicidin synthase and *LPD01628* encodes an ATP-dependent DNA ligase.
[b] The guide sequence was designed using a web-based tool [22].
[c] Number of edited colonies/number of examined transformants.

introduce an exogenous recombinase to facilitate recombination in *R. opacus*. In this regard, DeLorenzo et al. have reported a dsDNA recombineering method for gene knockout in *R. opacus* based on the use of the mycobacteriophage recombinases Che9c60 and Che9c61 [6]. In our previous study, we similarly utilized these two recombinases to develop a CRISPR/Cas9-based dsDNA recombination system for *R. ruber* [15]. However, we found that the same method was not applicable for *R. opacus*, as the recombination efficiencies obtained using Che9c60 and Che9c61 differed notably between *R. ruber* and *R. opacus*. Comparatively, whereas approximately 200 colonies were obtained for *R. ruber* [15] when using Che9c60 and Che9c61 to facilitate recombineering of dsDNA templates flanking antibiotic resistance markers, only 2 to 4 colonies could be obtained for *R. opacus* [6]. Such low dsDNA recombineering efficiency using Che9c60 and Che9c61 would be insufficient for DSB repair in *R. opacus*, and consequently it will be necessary to identify a more efficient system for engineering *R. opacus*.

Compared with dsDNA, recombineering of ssDNA provides a more convenient means of performing genome modification, as it requires only short oligonucleotides as templates and eliminates the need for PCR amplification of long homologous arms. To facilitate highly efficient ssDNA recombineering in *R. opacus*, we searched the UniProt database (https://www.uniprot.org/) for novel recombinases specifically derived from *Rhodococcus* phages and prophages. We accordingly identified five potential recombinases containing a RecT family domain as summarized in Table 2, and synthesized the corresponding genes based on the codon preference of *R. opacus* for further assessment, together with the previously reported Che9c61. The protein sequence alignment results are presented in Fig. S4.

It has been reported that certain mutations in the β subunit of RNA polymerase can result in rifampicin resistance of some bacteria [19]. This phenomenon has also been utilized for the assessment of ssDNA recombineering efficiency, which involves mutating the RNA polymerase gene (*rpoB*) with desgined single-stranded oligonucleotides and counting the rifampicin-resistant colonies [19]. To determine the mutations responsible for rifampicin resistance, we sequenced the *rpoB* genes of four spontaneous resistant *R. opacus* colonies, and thereby established that all were characterized by a C·G to T·A base mutation resulting in mutation of the 464th residue from a Ser to Phe, as shown in Fig. 3a. On the basis of these observations, we designed and synthesized two 80-nt single-stranded oligonucleotides (S464F–F and S464F-R) as ssDNA donors (complementary to the leading and lagging strand of the *rpoB* gene but containing a mutation in the central sequence) for the assessment of ssDNA recombineering efficiency.

Table 2
The recombinases charaterized in this study.

Recombinase	Entry	Length (aa)	Source
A	A0A260VZP4	339	*Rhodococcus* sp. 14-2470-1a
B	A0A2U8UJ13	292	*Rhodococcus* phage Jace
C	A0A285E014	379	*Rhodococcus* sp. OK270
D	A0A098BJR0	618	*R. ruber*
E	A0A515MHE2	387	*Rhodococcus* phage Sleepyhead
Che9c61	Q854T9	353	*Mycobacterium* virus Che9c

The five recombinase genes, along with the *che9c61* gene, were cloned into the pNV18 plasmid under the control of the P_{BAD} arabinose-inducible promoter [16], which were transformed to *R. opacus*. Having induced the resulting recombinant strains using arabinose, the oligonucleotides S464F–F and S464F-R were introduced to mutate the *rpoB* gene, followed by spreading on rafampicin-containing medium for colony counting. As shown in Fig. 3c, the highest recombination efficiency was obtained using recombinase D derived from an *R. ruber* prophage with oligonucleotide S464F-R as the ssDNA donor. Contrastingly, the empty vector control bacteria dependent on endogenous pathways showed no evidence of recombination. Furthermore, compared with S464F-R, the S464F–F oligonucleotide proved relatively ineffective for ssDNA recombineering, thereby indicating a strong bias toward ssDNA targeted to the lagging strand. On the basis of these observations, we thus used the novel recombinase D (which we named *Rr*RecT) in combination with the oligonucleotide S464F-R for further optimization of ssDNA recombineering.

With regards to optimizing the newly developed system, we examined the effects of arabinose concentration for *Rr*RecT induction during the preparation of competent cells, the amount of ssDNA used for electropoation, and ssDNA length, the results of which are shown in Fig. 3d, 3e, and 3f, respectively. On the basis of these assessments, we used an arabinose concentration of 10 mM and an ssDNA amount and length of 25 μg and 60–80 nt, respectively, for optimized evaluations. As a consequence of optimization, we obtained an *Rr*RecT-mediated ssDNA recombineering efficiency that was 10^3-fold higher than that obtained based on endogenous pathways.

3.4. *RrRecT-mediated ssDNA recombination with CRISPR/Cas9 counter-selection for gene knockout*

We subsequently assessed the efficacy of ssDNA recombineering combined with the CRISPR/Cas9 system for genome editing in *R. opacus*. As an initial step, we constructed the pNV-P_{BAD}-D-Pniami-Cas9 plasmid harboring the *cas9* gene and the recombinase *Rr*RecT gene, and used this to transform *R. opacus*. For the purposes of gene knockout, we subsequently introduced pBNVCm-sgRNA series plasmids and the corresponding oligonucleotides, as shown in Fig. 4a. The recombinase can facilitate the annealing of oligonucleotides to the target sites as primers for Okazaki fragments during replication, thereby resulting in the loss of sgRNA binding or PAM sequences. Moreover, the Cas9-sgRNA complex can recognize and cleave the target genomic locus in unedited genomes, thus implementing counter-selection.

To verify the efficiency of the system, we selected the *LPD07156* gene as a target gene for knockout. We designed a single-stranded oligonucleotide that would delete two base pairs in the PAM sequence of *LPD07156*, which was used to introduce a frame shift mutation in the gene. The editing efficiency of ssDNA recombineering was accordingly found to be 8/23 (35 %), as shown in Fig. 4b, thereby confirming the efficacy of the system for precise genome editing. We also examined the knockout of several other genes, for which we achieved editing efficiencies ranging from 22 % to 75 % (see Table 3), thereby demonstrating the robustness of the system for genome editing. In addition, we investigated the effect of deletion length on editing efficiency, and accordingly found that the efficiency obtained for a 2-bp deletion in *LPD03951* reached 75 %, whereas efficiencies decreased to 25 % for 100- and 500-bp deletions, and to 0 % for a 1000-bp deletion. We also examined the effect of a single base mutation in *LPD03951* (alteration of the PAM site from CGG to CCG to avoid recognition), and accordingly achieved an editing efficiency up to 100 %. These observations thus indicate that this system is effective for single-base mutations and short fragment deletions, but is less efficient when longer fragment are deleted.

To verify the feasibility of double gene knockout, we introduced the plasmid pBNVCm-03951-01628-sgRNA (see Fig. S5) containing tandem sgRNAs targeting the genes *LPD03951* and *LPD01628*, along with the

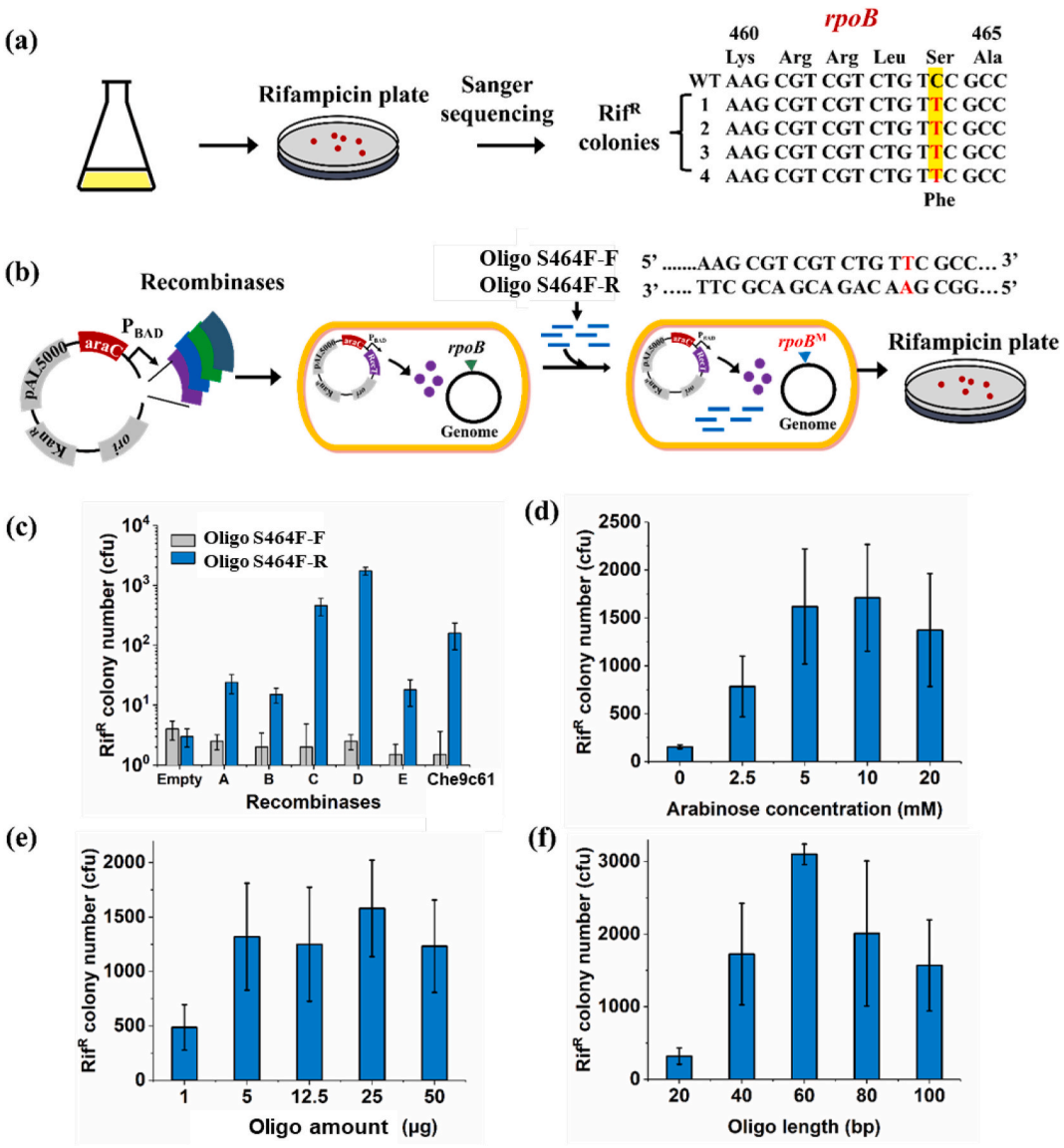

Fig. 3. Screening of recombinases and optimization of the ssDNA recombineering protocol. (a) Determination of the *rpoB* gene mutations responsible for rifampicin resistance. (b) The experimental scheme to assess the ssDNA recombineering efficiency of recombinases. (c) Selection of exogenous recombinases for ssDNA recombination. *R. opacus* harboring recombinases were induced with 10 mM arabinose for 3–4 h during competent cell preparation. Oligonucleotides S464F–F and S464F-R with a length of 80 nt and amount of 5 μg were introduced. (d) Optimization of arabinose concentration for the induction of recombinase *Rr*RecT. The amout and length of oligonucleotide S464F-R was 5 μg and 80 nt, respectively. (e) Optimization of ssDNA amount. The arabinose concentration was 10 mM, and the length of oligonucleotide S464F-R was 80 nt. (f) Optimization of ssDNA length. The arabinose concentration was 10 mM, and the amount of oligonucleotide S464F-R was 25 μg. The sequences of oligonucleotides were shown in Tables S4. Experiments were performed in triplicate.

corresponding oligonucleotides oligo-03951-1 and oligo-01628. As the editing of *LPD03591* or *LPD01628* was affected by the introduction of an additional sgRNA cassette, their editing efficiencies in the double gene knockout experiment were lower than that in single knockout experiments. The editing efficiencies for *LPD03951* and *LPD01628* were accordingly found to be 12/72 (16.7 %) and 3/72 (4.2 %), respectively, as shown in Fig. S6. Three of the 72 colonies obtained showed simultaneous disruption of the two genes, thus indicating the potential of the CRISPR/Cas9-based ssDNA recombineering system for one-step multiple gene inactivation. At present, however, the efficiency of this double gene knockout system is insufficient for practical purposes, and will thus necessitate further optimization.

3.5. An iterative genome editing protocol for R. opacus

For the purpose of conducting multiple rounds of genome editing, we further developed an iterative protocol for *R. opacus* (as shown in Fig. 5), which entails curing of the sgRNA plasmid after successive rounds of editing. In our design, the pB264 replicon used in construction of the pBNVCm-sgRNA plasmids is temperature-sensitive and can be readily cured when *R. opacus* is cultured at 37 °C in the absence of chloramphenicol. At this temperature, however, *R. opacus* PD630 has a significantly lower rate of growth than at 28 °C, as shown in Fig. S7. Thus, we assessed the effect of curing sgRNA plasmids at 28 °C. Unexpectedly, we found that the plasmids were also unstable at 28 °C in the absence of chloramphenicol, with a curing rate of up to 16/16 (100 %) after a round of seed culture and solid plate screening. The edited cells free of pBNVCm-sgRNA could thus be used for subsequent rounds of gene editing.

In the case of the pNV-P_{BAD}-D-Pniami-Cas9 plasmid, although no temperature-sensitive replicons were available, the plasmid had a low copy number and was unstable in the absence of kanamycin owing to the

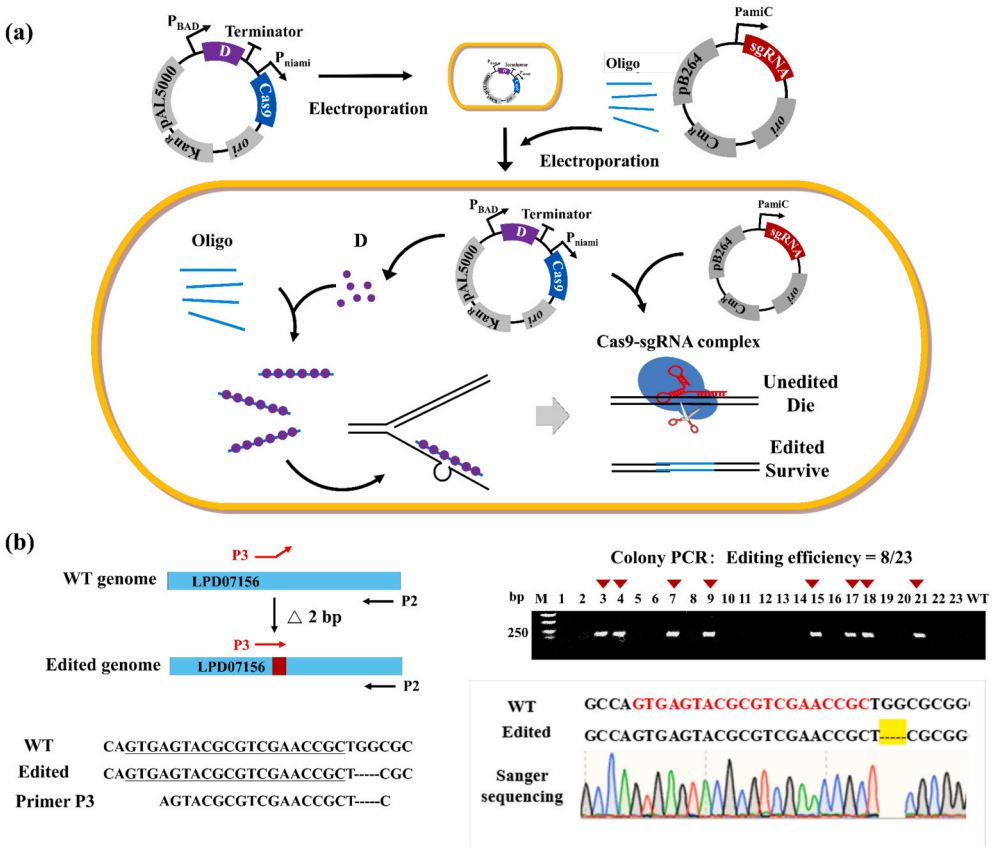

Fig. 4. The CRISPR/Cas9-based ssDNA recombineering system for genome editing in *R. opacus*. (a) A schematic diagram illustrating the mechanism of the system. Plasmid pNV-P_{BAD}-D-Pniami-Cas9 harboing the recombinase *Rr*RecT and Cas9 was initially introduced into *R. opacus*, and cells were then co-transformed with a series of pBNVCm-sgRNA plasmids and olignonucleotides. (b) Gene knockout of *LPD07156*. Edited genomes would lose 2 base pairs in the PAM site of *LPD07156* targeted by sgRNA. Primers P3 matched the altered sequence in the edited genome although not the wild-type sequence. Primers P3 and P2 could amplify a fragment of 197 bp from the edited genome. The deletion was also confirmed by Sanger sequencing of the fragments.

Table 3
Gene editing using the CRISPR/Cas9-based ssDNA recombineering system.

Gene	Guide sequence of sgRNA	Oligonucleotide[a]	Deletion length	Editing efficiency
LPD07156	GTGAGTACGCGTCGAACCGCTGG	Oligo-07156	2	8/23 (35 %)
LPD02538	CCTCCTCTGGCCAGCGCTTCAGG	Oligo-02538	2	5/16 (31 %)
LPD01620	CCGCTTCCACCGTCTCCTGCCGG	Oligo-01620	2	5/23 (22 %)
LPD01628	ATCTGCAACGGTTCCCGGACGGG	Oligo-01628	2	11/23 (48 %)
LPD03951	GAAGAGTTACTTCCTCGAACCGG	Oligo-03951-1	2	15/20 (75 %)
		Oligo-03951-2	20	23/31 (74 %)
		Oligo-03951-3	100	5/20 (25 %)
		Oligo-03951-4	500	5/20 (25 %)
		Oligo-03951-5	1000	0/20 (0 %)
		Oligo-03951-6	Point mutation	36/36 (100 %)

[a] Sequences of the single-stranded oligonucleotides are listed in Table S4.

burden caused by the recombinase and Cas9. Both pBNVCm-sgRNA and pNV-PBAD-D-Pniami-Cas9 could be simultaneously cured at 28 °C to obtain the final edited strain free of plasmids.

4. Discussions

The PD630 strain of *R. opacus* represents a promising vehicle for lignin bioconversion, and in this respect has attracted considerable attention in recent years [28]. However, despite the development of CRISPR-based interference systems for gene repression in *R. opacus*, a CRISPR/Cas9 system for gene knockout in this strain has yet to be reported. Moreover, although a CRISPR/Cas9-based dsDNA recombineering system has been effectively established for another *Rhodococcus* species (*R. ruber*) [15], this procedure proved to be untransferable to *R. opacus*, owing to the cytotoxicity of Cas9, as well as limited dsDNA recombineering efficiency of recombinases Che9c60 and Che9c61.

We found that overexpression of Cas9 driven by the same Pa2 promoter had markedly differing effects on the growth of *R. opacus* and *R. ruber*, thereby indicating the substantial genetic differences between these two *Rhodococcus* species. The cytotoxic effect of overexpressed Cas9 on *R. opacus* in the absence of sgRNA is consistent with the previous findings of studies on *C. glutamicum* [29], *Halomonas* spp. [30], and *Mycobacterium smegmatis* [27]. Strategies including fine-tuning the expression of Cas9 and selecting alternative Cas effectors, such as Cas12a, have been proposed to eliminate this problem [27,31]. However, given that Cas12a utilizes T-rich PAM sites [32], compared with Cas9, there are fewer potential editing targets in *R. opacus*, in which the genome GC content can be up to 67 %. Consequently, we prioritized Cas9 when developing a CRISPR system for *R. opacus*, and subsequently established that driving the expression of Cas9 with a weak promoter is an effective strategy for reducing cytotoxicity.

To compensate for the limited endogenous DSB repair pathway of prokaryotes, bacteriophage recombinases have often been introduced to enhance the efficiency of recombination. On the basis of our screening of candidate recombinases, we identified a novel recombinase, *Rr*RecT, derived from an *R. ruber* prophage, the use of which facilitated the

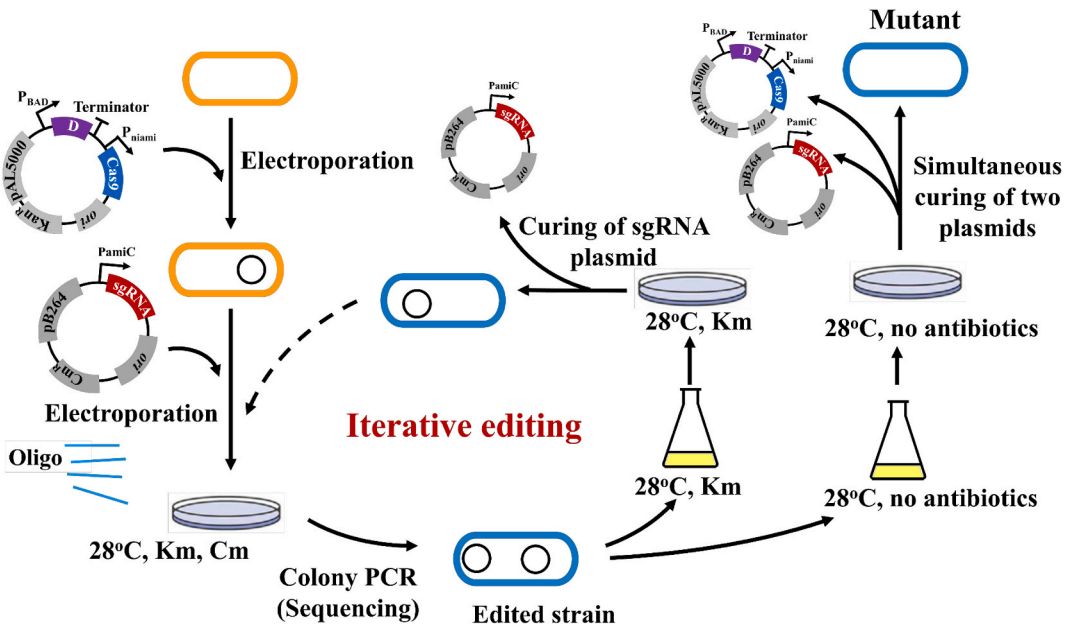

Fig. 5. A schematic diagram showing the iterative genome editing protocol for *R. opacus*. After a round of genome editing, selected cells were cultured in seed medium and spread on plates containing kanamycin to cure plasmid pBNVCm-sgRNA for the next round of editing. The final edited colonies were grown on antibiotic-free plates to simultaneously cure the two plasmids.

incorporation of short ssDNAs (as short as 60 nt) to generate recombinants in *R. opacus*. A strong bias toward ssDNA targeting the lagging strand was also observed. Compared with the previously reported Che9c61 recombinase derived from mycobacteriophages, the efficiency of *Rr*RecT was 10-fold higher, indicating that it is a reliable strategy to screen recombinases from the phages or prophages of phylogenetically related species. Even so, however, compared with Redβ or RecT used for *E. coli* [16], the efficiency of *Rr*RecT was still not high enough with respect to *R. opacus*. Therefore, we anticipate that more effective recombinases will be identified or that engineering of the *Rr*RecT based on directed evolution/rational design could be applied to further enhance the efficiency of ssDNA recombineering in *R. opacus*.

By coupling ssDNA recombineering with CRISPR/Cas9-based counter-selection, we succeeded in developing a two-plasmid genome editing system for *R. opacus*. Compared with the triple-plasmid CRISPR/Cas9-based dsDNA recombination system used for *R. ruber* [15], the two-plasmid system proved to be more convenient, by virtue of simplifying the procedure of plasmid transformation and requiring only short oligonucleotides (60–80 nt). The system was found to be efficient with respect to gene disruption and point mutation, with editing efficiencies ranging from 22 % to 100 %. We also established the feasibility of single-step double gene disruption, albeit at a relatively low efficiency (approximately 4 %). Unfortunately, the system is inefficient for long fragment deletion or integration. To address this problem, a dsDNA recombineering system might be established in the future by introducing the corresponding exonuclease *Rr*RecE complementary to *Rr*RecT.

5. Conclusions

In this study, we revealed that the cytotoxicity of Cas9 and inefficient pathways for DNA double-strand break repair represent major obstacles with respect to developing a CRISPR/Cas9 genome editing system for use in *R. opacus*. By fine-tuning the expression of Cas9 using a weak promoter, we succeeded in reducing its cytotoxicity and established CRISPR/Cas9-mediated counter-selection in *R. opacus*. Furthermore, by screening candidate recombinases, we identified the novel recombinase *Rr*RecT, which could be applied in ssDNA recombineering and effectively enhance the recombination efficiency of *R. opacus*. Finally, by coupling *Rr*RecT-assisted ssDNA recombineering with CRISPR/Cas9 counter-selection, we successfully developed an efficient and scarless genome editing system for *R. opacus*. We anticipate that this system will facilitate the engineering of *R. opacus* to yield superior strains for the production of value-added biochemicals and biofuels from renewable biomass.

CRediT authorship contribution statement

Youxiang Liang: Methodology, Investigation, Writing – original draft. **Yuwen Wei:** Methodology, Resources. **Song Jiao:** Methodology, Resources. **Huimin Yu:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2021.08.001.

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