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Journal of Genetics and Genomics 46 (2019) 455–458

Contents lists available at ScienceDirect

Journal of Genetics and Genomics

Journal homepage: www.journals.elsevier.com/journal-of-genetics-and-genomics/

Letter to the editor

A highly efficient *in vivo* plasmid editing tool based on CRISPR-Cas12a and phage λ Red recombineering

Plasmids are useful tools for studying genetic information in living cells, as well as heterologous expression of genes and pathways in cells (Lauritsen et al., 2018). Various methods have been developed for plasmid manipulation both *in vivo* and *in vitro* (Aslanidis and de Jong, 1990; Li and Elledge, 2007; Xia et al., 2018). However, large plasmids, such as P1-based artificial chromosomes (PACs), bacterial artificial chromosomes (BACs), and fosmids, are difficult to manipulate. Recombineering-based plasmid editing approaches have been developed that allow seamless modification in plasmids with precise junctions (Li and Elledge, 2005; Rivero-Muller et al., 2007). However, owing to its low recombination efficiency, selectable markers or counter-selectable cassettes are usually required (Zhang et al., 2000; Lee et al., 2001; Rivero-Muller et al., 2007). In addition, multimeric plasmids or mixtures of recombinant and parental plasmids are formed when plasmids are modified using recombineering, thereby limiting its application, especially in the case of multicopy plasmids (Yu et al., 2000; Thomason et al., 2007). The CRISPR-Cas endonuclease system, which generates programmed double-stranded DNA (dsDNA) cleavage, is an effective genome editing tool (Cong et al., 2013; Yan et al., 2017; Javed et al., 2018). Here, we combined CRISPR-Cas12a and recombineering approaches to establish an efficient plasmid editing system (Fig. 1A) for seamlessly modifying various plasmids utilized by bacteria, viruses, and eukaryotes.

We first constructed an *E. coli* strain SY4539 (Fig. 1B) expressing both the CRISPR-Cas12a and λ Red recombination systems by insertion of the arabinose-inducible *Cas12a* gene into the chromosome and further removal of the *hsdR* gene encoding a type I restriction endonuclease from the chromosome of *E. coli* strain DY331, which contains the temperature-inducible λ Red recombination system (Yu et al., 2000). To test the efficiency of the system, a pUC-derived plasmid pJV53-GFP (Mao et al., 2016), expressing a green fluorescent protein (GFP) reporter, was chosen as the target plasmid. We designed three individual recombinogenic *GFP* disruption oligonucleotides as donor/template DNA to introduce 20- or 25-bp mutations containing a stop codon. The target plasmid pJV53-GFP, crRNA-expressing plasmid pAC-crRNA, and oligonucleotides were electroporated into SY4539 competent cells in which Cas12a and λ Red recombination proteins had been induced. Co-transformation of oligonucleotides and a non-target control crRNA-expressing plasmid (pAC-crRNA-NT) yielded ~10^5 colonies, of which only ~0.20% were GFP-negative (Fig. 1C). By contrast, co-transformation of oligonucleotides and a pAC-crRNA plasmid yielded >90% GFP-negative colonies (Fig. 1C), suggesting this system can be used for plasmid editing. To further assess the efficiency of our system, we designed crRNA-expressing plasmids and oligonucleotides to restore the GFP expression of above three GFP mutants using single-stranded DNA (ssDNA)-mediated recombineering. Results showed that 3.65%, 0.85%, and 2.00% of the transformants were GFP-positive when transformed with the non-target crRNA control, whereas 94%, 84%, and 90% of the transformants were GFP-positive when transformed with target crRNA (Fig. 1D). Twenty GFP-positive colonies from each transformation were picked and confirmed to be the correct recombinants by DNA sequencing (data not shown). We further introduced 1-, 3-, 5-, and 10-bp mutations into the *GFP* gene using this system and yielded >90% GFP-negative colonies (Fig. 1E). PCR and DNA sequencing analysis confirmed that 89% (25 of 28, 1-bp mutation), 91% (32 of 35, 3-bp mutation), 94% (34 of 36, 5-bp mutation), and 93% (26 of 28, 10-bp mutation) of GFP-negative colonies carried the desired mutations in recombinant plasmids. We also tested the system for its ability to perform gene deletions in plasmids using an ssDNA template. We designed oligonucleotides with 40- and 39-bp homology arms to generate 102-, 600-, or 801-bp deletions in the *GFP* gene, respectively. To our surprise, only a small proportion of transformants can grow when they were picked and streaked onto agar plates (Table S1). We hypothesized that these non-culturable transformants might be undergoing continual CRISPR-Cas12a cleavage of original plasmid, and therefore cannot grow when streaked onto a new agar plate. To remove the non-growing transformants, we pre-selected them by culturing the transformants overnight in liquid media. Results showed that 98.3%, 98.0%, and 79.3% of the transformants with 102-, 600-, and 801-bp deletions were GFP-negative, and most of them were confirmed to be the desired recombinants by DNA sequencing (Table S1). Taken together, the above findings demonstrate the successful establishment of an effective plasmid editing system that can be used to generate point mutations or deletions in high-copy plasmids using ssDNA as a repair template.

Insertion or deletion of a gene by plasmid recombineering is usually performed using linear dsDNA substrates containing a selectable marker. To engineer a seamless genetic mutation in a plasmid, a two-step selection/counter selection method is generally employed (Zhang et al., 2000; Lee et al., 2001; Rivero-Muller et al., 2007). However, multiple rounds of genetic manipulations are time-consuming. To investigate whether our system can be used for dsDNA-mediated plasmid editing, a series of dsDNA fragments consisting of the *GFP* gene with 20-, 30-, 40-, 100-, 200-, and 500-bp flanking homologous arms respectively were transformed into bacteria with pUC19 using our system. 0.50%, 1.55%, 3.88%, 5.10% and 9.15% GFP-positive colonies were obtained when dsDNA fragments flanked with 30-, 40-, 100-, 200-, and 500-bp

https://doi.org/10.1016/j.jgg.2019.07.006

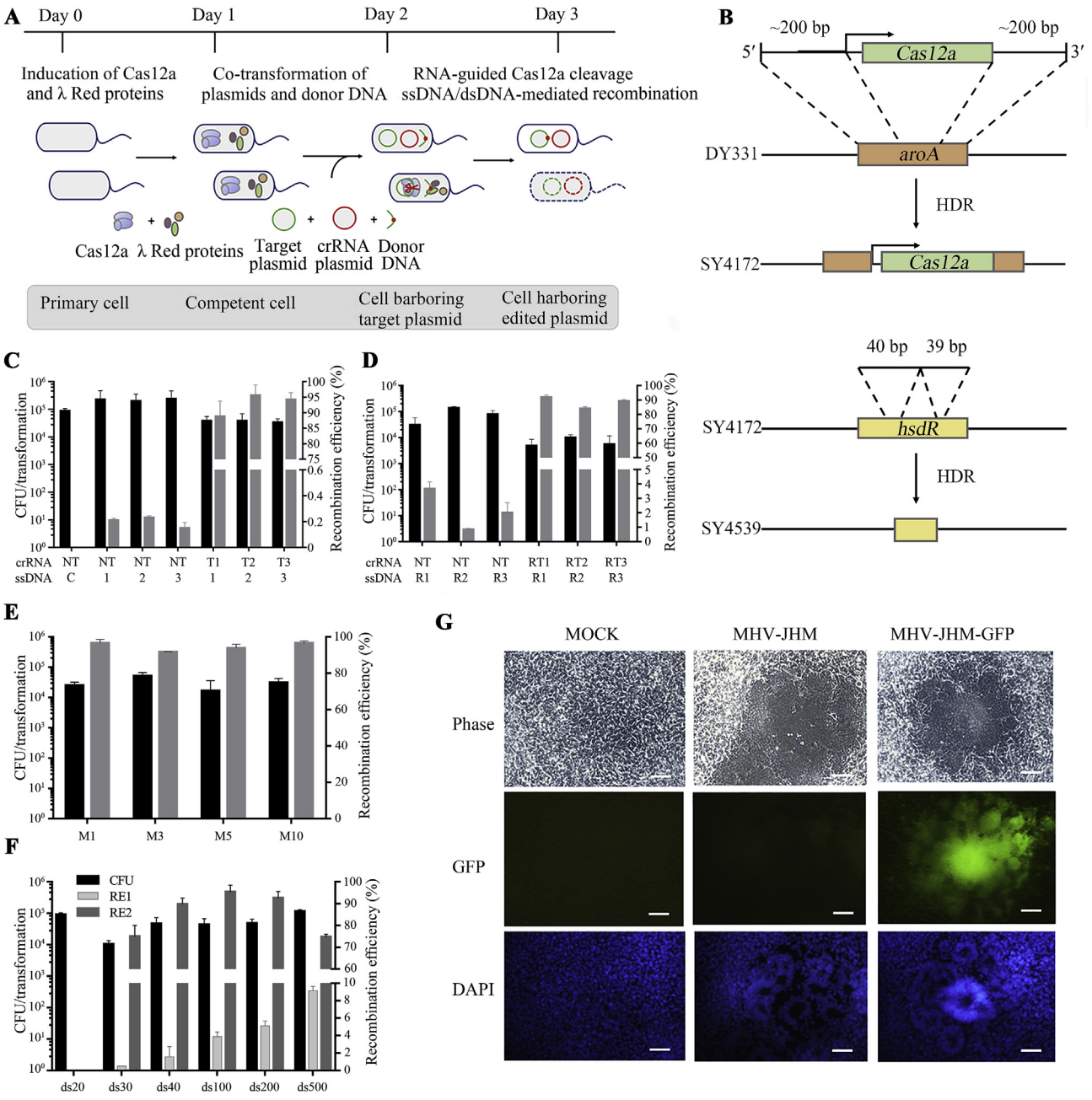

Fig. 1. The design, construction and application of plasmid editing tool based on CRISPR-Cas12a and phage λ Red recombineering. **A**: Schematic illustration of the combined CRISPR-Cas12a and recombineering system for plasmid editing *in vivo*. Cas12a was integrated into the chromosome (as shown in **B**) and induced by arabinose under the control of the *pBAD* promoter, and λ Red proteins were expressed by shifting the temperature to 42 °C for 15 min. The target plasmid, crRNA-expressing plasmid, and donor/template DNA (ssDNA or dsDNA) were co-transformed into competent SY4539 cells. Under screening pressure from RNA-guided DNA cleavage by Cas12a, cells harboring the target plasmid died, whereas cells harboring the edited plasmid survived. Subsequently, the crRNA-expressing plasmid was cured on LB plates supplemented with 7% sucrose. **B**: Schematic illustration of the strain SY4539 construction. An arabinose-inducible *Cas12a* gene was integrated into the chromosome of DY331, and *hsdR* was further deleted from above strain SY4172. *aroA*, 5-enolpyruvylshikimate-3-phosphate synthetase encoding gene; *hsdR*, Type I restriction enzyme encoding gene; HDR, homology-dependent repair. **C**: ssDNA-mediated mutation of the *GFP* gene. The *GFP* gene in the target plasmid pJV53-GFP was mutated at three different sites (T1–T3) using our CRISPR-Cas12a recombineering system. NT, the non-target crRNA control. Transformation efficiency was defined as the total colony-forming unit (CFU) generated per transformation, and recombination efficiency was calculated based on the proportion of GFP-negative colonies. **D**: Restoration of the mutated *GFP* genes described in **C**. Recombination efficiency was calculated directly from the percentage of green colonies. **E**: Various nucleotide substitutions were generated in the *GFP* gene of plasmid pJV53-GFP. Recombination efficiency was calculated based on the proportion of GFP-negative colonies. **F**: Insertion of dsDNA fragments comprising the *GPF* gene and flanking homologous arms of pUC19 of various lengths into pUC19 using our combined CRISPR-Cas12a and recombineering system. Recombination efficiency was calculated based on the proportion of GFP-positive colonies. RE1, recombination efficiency without pre-selection; RE2, recombination efficiency with pre-selection. The graphs in **C**–**F** show the results from two independent experiments. **G**: Infection of DBT cells by MHV-JHM and MHV-JHM-GFP viruses produced by modified plasmids. The images presented are a representative experiment performed independently at least three times. Scale bar, 100 μm.

homologous arms were used, and the percentages of GFP-positive colonies were further increased to 75%–95% with pre-selection in liquid media (Fig. 1F). 30-bp homologous arms in the dsDNA template were sufficient for gene insertion in the plasmid, suggesting that dsDNA template can be easily generated using PCR products, which further simplifies the procedures for genetic manipulation of plasmids.

To further test the robustness of our system, we attempted to manipulate different types of high-copy plasmids used for research on bacteria and viruses such as chikungunya virus (Mainou et al., 2013; Zhang et al., 2019). These plasmids are large and do not contain appropriate restriction enzyme sites, making them difficult to manipulate using regular methods, but they can be easily manipulated by our developed system to generate point mutations, deletions, and insertions with efficiencies from 33% to 100% (Table S2).

Given the high efficiency of our CRISPR-Cas12a-assisted plasmid editing system, we investigated whether chemically competent cells could be used for plasmid editing. Competent cells containing plasmid pJV53-GFPm1 were prepared using the classic $CaCl_2$ method, and these were then transformed with pAC-crRNA-RT1 and ssDNA template R1 (the same crRNA-expressing plasmid and ssDNA used in Fig. 1D). Although <0.1% of cells were GFP-positive when transformed with non-target crRNA control, >70% of cells were GFP-positive when transformed with pAC-crRNA-RT1 (Fig. S1). This result suggests that our system can be used for plasmid editing in chemically competent cells, facilitating the application of our methods in labs that do not have access to an electroporator.

To investigate whether our Cas12a recombineering system could be used for low-copy large plasmids, a Bacmid pBAC-JHMV^{IA} (Fehr et al., 2015) containing the genome of mouse hepatitis virus JHM Iowa strain (MHV-JHM^{IA}) was chosen as the target plasmid. MHV-JHM is a widely utilized model for studying coronaviruses. We first decided to replace the *ORF4* gene, encoding an accessary protein of MHV-JHM in pBAC-JHMV^{IA}, with the *GFP* gene to generate an MHV variant expressing GFP. Two slightly different constructs were made: in the first construct (pBAC-JHMV^{IA}-GFP1), the coding region of the *ORF4* gene from the first ATG codon to the stop codon was replaced by the *GFP* gene; in the second construct (pBAC-JHMV^{IA}-GFP2), a CACC Kozak sequence was also introduced into pBAC-JHMVIA-GFP1 to replace the original AGTT sequence preceding the first ATG. Among 24 pBAC-JHMV^{IA}-GFP1 colonies picked, 14 contained the *GPF* gene, and the CACC sequence was found in seven out of nine pBAC-JHMV^{IA}-GFP2 colonies examined. We subsequently chose four pBAC-JHMV^{IA}-GFP1 clones and two pBAC-JHMV^{IA}-GFP2 clones and sequenced their entire viral genomes (~31.5 kb), and the results showed that no unwarranted mutations, deletions, insertions, or recombination events occurred during the process (data not shown). Mouse delayed brain tumor (DBT) cells were then transfected with the above pBAC-JHMV^{IA}-GFP2 plasmid to generate viruses. Compared with wild-type virus (MHV-JHM), infection by pBAC-JHMV^{IA}-GFP2-derived viruses (MHV-JHM-GFP) induced not only the formation of large syncytia in DBT cells but also high expression of GFP protein (Fig. 1G), as expected. To further evaluate the efficiency of our system for bacmid manipulation, seven additional point mutations, five deletions, and one insertion were engineered into pBAC-JHMV^{IA}. A minimum of 22.5% mutation efficiency was achieved with slight variations among individual mutations (Table S2). These results show that our plasmid editing system proves high efficiency for low-copy plasmid manipulation.

Previous studies demonstrated that plasmid recombineering has to suffer from a tendency to form multimeric plasmids (Yu et al., 2000; Thomason et al., 2007). To investigate whether multimeric plasmids also existed in our recombineering system, we analyzed the migration of recombinant plasmids using agarose gel electrophoresis; repair of a *GFP* mutation with a 20-bp replacement would not change the size of the plasmid, whereas multimers would migrate more slowly than the parental plasmid. When recombinant plasmids were purified and analyzed by agarose gel electrophoresis, we observed the formation of plasmid multimers following a standard recombineering experiment, but did not observe the formation of higher molecular weight plasmid species when recombineering was combined with the CRISPR-Cas12a system (Fig. S2A). In accordance with the reports that recombinant and parental plasmids are usually present as a mixture after recombineering (Yu et al., 2000; Thomason et al., 2007), DNA sequencing analysis revealed that parental and recombinant plasmids coexisted in transformants generated by standard recombineering, while the recombinant plasmids individually existed in transformants generated by recombineering combined with the CRISPR-Cas12a system (Fig. S3). A similar phenomenon was observed when the *GFP* gene was inserted into the pUC19 vector (Fig. S2B). Taken together, these results suggest that editing using our system did not result in the formation of multimeric plasmids or plasmid mixtures.

In summary, we developed a simplified, reliable, and efficient plasmid editing technique by combining the CRISPR-Cas12a and λ Red recombineering systems. This approach allowed us to conveniently generate seamless deletions, point mutations, and insertions in the plasmids that are difficult to be edited by other methods. Therefore, the method described herein could prove useful for efficient modification of various plasmids used for studying bacteria, viruses, and eukaryotes.

Acknowledgments

We would like to thank Dr. Donald L. Court (National Cancer Institute, USA) for kindly providing the DY331 strain. The work was funded by the National Basic Research Program of China (973 Program) (2015CB554200), the National Natural Science Foundation of China (31870067, 31670139, and 31800120), and the CAMS Initiative for Innovative Medicine (2016-I2M-1-013) and Fundamental Research Funds (2018RC310016).

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgg.2019.07.006.

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Yiman Geng[1]
NHC Key Laboratory of Systems Biology of Pathogens, Institute of Pathogen Biology, Center for Tuberculosis Research, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, 100730, China

Haiqin Yan[1]
Department of Basic Medical Sciences, Anhui Key Laboratory of Infection and Immunity, Bengbu Medical College, Bengbu, 233030, China

Pei Li, Gaixian Ren, Xiaopeng Guo, Peiqi Yin, Leiliang Zhang, Zhaohui Qian[*], Zhendong Zhao[*], Yi-Cheng Sun[*]
NHC Key Laboratory of Systems Biology of Pathogens, Institute of Pathogen Biology, Center for Tuberculosis Research, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, 100730, China

[*] Corresponding authors.
E-mail addresses: zqian2013@sina.com (Z. Qian), timjszzd@163.com (Z. Zhao), sunyc@ipbcams.ac.cn (Y.-C. Sun).

10 March 2019
Available online 10 September 2019

[1] These authors contributed equally to this work.