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CRISPR-Cas12a–assisted recombineering in bacteria

Mei-Yi Yan[1,3], Hai-Qin Yan[2,3], Gai-Xian Ren[1], Ju-Ping Zhao[1], Xiao-Peng Guo[1], Yi-Cheng Sun[1,*]

[1]MOH key laboratory of Systems Biology of Pathogens, Institute of Pathogen Biology, and Center for Tuberculosis Research, Chinese Academy of Medical Sciences and Peking Union Medical College, 9, Dongdan Santiao, Dongcheng District, Beijing, 100730, China.

[2]Department of Histology and Embryology, Bengbu Medical College, Bengbu, Anhui, 233000, China.

[3]These authors contributed equally to this work.

* To whom correspondence should be addressed. Tel: +86 10 6783 7366; Fax: +86 10 6783 7321; Email: sunyc@ipbcams.ac.cn

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ABSTRACT

Clustered regularly interspaced short palindromic repeats (CRISPR)-Cas12a (Cpf1) has emerged as an effective genome editing tool in many organisms. Here, we developed and optimized a CRISPR-Cas12a assisted recombineering system to facilitate genetic manipulation in bacteria. Using this system, point mutations, deletions, insertions, and gene replacements can be easily generated on the chromosome or native plasmids in *Escherichia coli*, *Yersinia pestis*, and *Mycobacterium smegmatis*. Because CRISPR-Cas12a–assisted recombineering does not require introduction of an antibiotic resistance gene into the chromosome to select for recombinants, it is an efficient approach for generating markerless and scarless mutations in bacteria.

KEY WORDS: Cas12a, recombineering, *Mycobacterium smegmatis*, *Yersinia pestis*.

IMPORTANCE

The CRISPR-Cas9 system has been widely used to facilitate genome editing in many bacteria. CRISPR-Cas12a (Cpf1), a new type of CRISPR-cas system, allows efficient genome editing in bacteria when combined with recombineering. Cas12a and Cas9 recognize different target sites, which allows for more precise selection of the cleavage target and introduction of the desired mutation. In addition, CRISPR-Cas12a assisted recombineering can be used for genetic manipulation of plasmids and plasmid curing. Finally, Cas12a-assisted recombineering in generating of point mutation, deletion, insertion and replacement in bacteria has been systematically analyzed.

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38 Taken together, our findings will guide efficient Cas12a-mediated genome editing in
39 bacteria.
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INTRODUCTION

The CRISPR-Cas system (clustered regularly interspaced short palindromic repeats and CRISPR-associated proteins) is a prokaryotic adaptive immune system that confers resistance to foreign genetic elements (1-3). Class 1 CRISPR systems (comprising types I, III, and IV) typically form multi-subunit protein-crRNA (CRISPR RNA) complexes, whereas class 2 systems (comprising types II, V, and VI) use a single crRNA guided protein for target interference (2). Recently, engineered Cas nucleases, including CRISPR-Cas9 and CRISPR-Cas12a (Cpf1), have been widely adopted as versatile genome editing tools in many organisms (4-6). The CRISPR-Cas9 system, categorized as class 2 type II, consists of a Cas nuclease (Cas9), a trans-activating CRISPR RNA (tracrRNA), and a CRISPR RNA (crRNA) (4, 5, 7). The CRISPR-Cas9 system generates Cas9-mediated double-strand cleavage of the target sequence guided by the crRNA and tracrRNA. Cas12a, which is a type V-A endonuclease of the class 2 CRISPR-Cas system, is a dual nuclease that is involved in crRNA processing, target-site recognition, and DNA cleavage (4, 8). Target DNA binding in most DNA-targeting CRISPR-Cas systems is dependent on the initial recognition of a protospacer adjacent motif (PAM), a short (3-5 bp) DNA sequence adjacent to the protospacer (target) site that is complementary to the crRNA spacer segment (9). Cas12a recognizes thymidine-rich PAM sequences (YTN) and can be guided by a single crRNA without an additional tracrRNA (4, 8, 10). It has been recently used for genome editing in mammals, plants, and bacteria (11-15).

Recombineering, a method based on genetic engineering through homologous

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63 recombination, has provided new ways to manipulate bacterial genomes (16-18).
64 Although the method is effective, antibiotic resistance genes must be used to isolate
65 recombinants. To generate markerless mutations, antibiotic resistance genes must be
66 cured by a technique involving a site-specific recombinase or resolvase (17, 19-21).
67 Typically, these manipulations require multiple steps and create a chromosomal "scar"
68 (e.g., at the LoxP recognition site). Thus, coupling a CRISPR-Cas system with
69 recombineering represents a simple and highly efficient genome editing method in
70 bacteria (6, 22-24). For example, the CRISPR-Cas9 system has been used to assist
71 recombineering in *Escherichia coli* and *Lactobacillus reuteri* (25-27). By coupling the
72 CRISPR-Cas9 system with lambda Red recombineering, highly efficient
73 recombination can be achieved without antibiotic marker using single-stranded DNA
74 (ssDNA) or double-stranded DNA (dsDNA) generated by PCR (25-27). However,
75 Cas9 typically uses a G-rich PAM sequence, such as NGG, which hinders the precise
76 application of CRISPR-Cas9-assisted engineering of point mutations using ssDNA.

77 *Mycobacterium tuberculosis* is the causative agent of tuberculosis (TB), which
78 causes 1.5 million deaths worldwide every year. Genetic manipulation of *M.*
79 *tuberculosis* is limited by the bacterium's slow growth rate, inefficient DNA uptake,
80 and high frequency of illegitimate recombination. Recently, recombineering was
81 successfully applied to the genetic manipulation of mycobacteria (18). Expression of
82 the recombination proteins gp60 and gp61 from the mycobacteriophage Che9c
83 increases the efficiency of recombination and facilitates allelic exchange in both
84 *Mycobacterium smegmatis* and *M. tuberculosis* (18). dCas9 has been used to regulate

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gene expression in mycobacteria (28-30). However, the CRISPR-Cas9-assisted genome editing has not yet been applied to this group of microorganisms.

Thus, we explored whether the CRISPR-Cas12a system could be used to assist recombineering in bacteria, particularly in mycobacteria. Our results show that CRISPR-Cas12a-assisted recombineering can rapidly and efficiently generate point mutations, deletions, and insertions in *Escherichia coli*, *Yersinia pestis* and *Mycobacterium smegmatis*. The technique simplifies the construction of markerless mutations in bacteria, avoids the creation of chromosomal "scars", and is especially effective for the sequential recombineering of multiple genes in bacteria.

RESULTS

CRISPR-Cas12a-assisted recombineering in *E. coli*

To perform CRISPR-Cas12a assisted recombineering in *E. coli*, we first constructed a system composed of two series of plasmids: the pKD46-Cas12a series, which contains a temperature-sensitive pSC101 replicon (31) and expresses the recombination proteins and FnCpf1 (Cpf1 from *Francisella novicida* (4)), and the pAC-crRNA series, which contains a p15A replicon (32) and a *sacB* gene for counterselection and expresses crRNA (Supplementary Figure S1). To test the efficiency of the system, we selected the *lacZ* gene in MG1655 for gene manipulation, since *lacZ* mutants are easily identified using agar plates supplemented with X-gal for blue-white colony screening. First, we tested this system by attempting to introduce point mutations into the *lacZ* gene using ssDNA oligonucleotide recombination (Figure 1A). We designed recombinogenic *lacZ* disruption oligonucleotides targeting the leading and lagging strands of DNA replication whereby successful recombination

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introduced a stop codon within the *lacZ* ORF (Figure 1B). The oligonucleotides and pAC-crRNA plasmid were electroporated into competent cells harboring pKD46-Cas12a and plated on X-gal. Only 0.19% of the transformants formed white colonies when the oligonucleotide targeting the *lacZ* lagging strand was co-transformed with pcrRNA-ctrl (without CRISPR-Cas12a targeting). In the presence of CRISPR-Cas12a targeting (using pcrRNA-lacZ), approximately 64% of the transformants formed white colonies using an oligonucleotide targeting the leading strand whereas 81% of the transformants formed white colonies using an oligonucelotide targeting the lagging strand (Figure 1C). Colony PCR and sequencing confirmed that 15 of 16 white colonies from the lagging strand transformants and 13 of 16 white colonies from the leading strand transformants were the correct recombinants. Consistent with a previous study that used CRISPR-Cas9 targeting alone (33, 34), more than 20% of the transformants formed white colonies when pcrRNA-lacZ was transformed alone into a strain harboring pKD46-Cas12a (Figure 1C). Colony PCR showed that the *lacZ* gene had been lost in these mutants, suggesting that homologous recombination might be involved in the formation of these mutants (33).

Next, we tested the system for its ability to generate chromosomal deletions in *E. coli*. A 79-nt oligonucleotide with upstream and downstream homology to the area of the deletion was designed to delete 1399 bp from the *lacZ* gene (Figure 1B). The oligonucleotide was transformed together with pcrRNA-lacZ into *E. coli* containing pKD46-Cas12a. Approximately one-half of the transformants formed white colonies on X-gal (Figure 1C), and colony PCR and sequencing confirmed that 5 of 40 white colonies were the correct recombinants. The other 35 white colonies had the *lacZ*

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gene deleted by homologous recombination, as previously reported (33, 34). We also performed an experiment to delete the *aroA* gene (1278 bp) in frame using this system. This experiment yielded approximately 3000 transformants, of which 4% (3/80) were confirmed to have the desired deletions by colony PCR and sequencing.

Finally, we tested the system for its ability to perform gene replacement using a markerless dsDNA PCR product. *gfp* gene PCR products harboring 45 or 500 bp homology arms for the *aroA* gene were used to replace the *aroA* gene in *E. coli*. Approximately 0.4% of the transformants were GFP-positive when the 45-bp homology arms were used whereas approximately 59% of the transformants were GFP-positive when the 500-bp homology arms were used. Collectively, the above results confirm that our two-plasmid system supports efficient recombineering in *E. coli*.

CRISPR-Cas12a-assisted recombineering in *Y. pestis*

To determine whether the CRISPR-Cas12a-assisted recombineering system functions in other bacteria, we applied this system to *Y. pestis*. Point mutations were successfully introduced into two sites (located in *hmsT* and *y4098*) of the chromosome in *Y. pestis* KIM6+ with recombination efficiencies of 83% and 81%, respectively, using ssDNA oligonucleotides. This result suggests CRISPR-Cas12a-assisted recombineering can be used for genetic manipulation of chromosomal DNA *in Y. pestis*.

Native plasmids are usually important for antibiotic resistance, symbiosis, metabolism and virulence in bacteria. To the best of our knowledge, CRISPR-assisted

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recombineering has not been applied for genetic manipulation of native plasmids in bacteria. Wild-type *Y. pestis* harbors three plasmids (pPCP1, pMT1 and pCD1) that are important for the virulence and environmental adaption of the pathogen (35). To test whether genetic manipulation could be conducted on native plasmids, we performed ssDNA oligonucleotide recombination experiments to mutate the *caf1R* gene located in the pMT1 plasmid using the CRISPR-Cas12a-assisted recombineering system. Four arginine codons were mutated to alanine codons individually in the *caf1R* gene with recombination rates of approximately 90% when oligonucleotides targeting the lagging strand were used (Figure 2). Furthermore, the pMT1 plasmid was cured from *Y. pestis* in approximately 40% of transformants when the control oligonucleotide was used (Figure 2), indicating that CRISPR-Cas12a could be used to cure native plasmids (36). We conclude that the CRISPR-Cas12a-assisted recombineering is a useful method for genetic manipulation of chromosomal and plasmid DNA in *Y. pestis*.

Evaluation of Cas12a activity in *M. smegmatis*

The next goal of this study was to establish CRISPR-Cas12a selection in mycobacteria to enable high-efficiency recombineering. To evaluate the feasibility of this goal, we cloned and expressed FnCpf1 in mycobacteria. The gene encoding *Cas12a* was modified to optimize expression in mycobacteria (Supplementary Figure S2). Although Cas12a is an exogenous protein, induced expression of Cas12a (up to 100 ng mL^{-1} ATc) did not strongly affect the growth of *M. smegmatis* (Supplementary Figure S3A). Next, we performed plasmid interference assays to test FnCpf1 activity in mycobacteria (37). In this approach, expression of a Cas endonuclease and a crRNA on a transformed plasmid results in cleavage of the target plasmid, which is

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178 reflected as a decrease in the number of transformants. For this purpose, we
179 constructed a plasmid for co-expression of Cas12a and a crRNA targeting the *gfp* gene
180 (pCpf1-gfp) or a crRNA not targeting the *gfp* gene (pCpf1-ctrl). *M. smegmatis*
181 harboring pCpf1-gfp or pCpf1-ctrl was transformed with pJV53 (without *gfp*) or
182 pJV53-GFP (with *gfp*). Expression of Cas12a and the *gfp*-specific crRNA
183 significantly decreased the number of transformants with pJV53-GFP but not the
184 number of transformants with pJV53, whereas expression of Cas12a and the
185 nonspecific crRNA did not affect the transformation efficiency of either pJV53-GFP
186 or pJV53 (Supplementary Figure S3B). Taken together, these results suggest that
187 FnCpf1 is biologically active and can mediate targeted DNA interference in *M.*
188 *smegmatis*.

189 **Genetic manipulation in *M. smegmatis* using CRISPR-Cas12a–assisted ssDNA**
190 **oligonucleotide recombineering**

191 To determine whether the CRISPR-Cas12a system could be coupled to
192 oligonucleotide recombineering in mycobacteria, we constructed a modified *M.*
193 *smegmatis* strain in which the *gfp* gene was inserted into the chromosome at the
194 *Ms5635-5634* locus. We also constructed a new two-plasmid system (Supplementary
195 Figure 4) as follows: pJV53-Cpf1, which expresses FnCpf1 and the recombination
196 proteins gp60 and gp61 (18), and the pCR series (pCR-Hyg and pCR-Zeo), which
197 contain a temperature-sensitive replicon (38, 39) and express crRNAs. This
198 two-plasmid system was tested for its ability to generate targeted recombinants. For
199 this purpose, we designed recombinogenic *gfp* disruption oligonucleotides (Figure 3A)

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200 targeting the lagging strands of the DNA replication fork; successful recombination
201 with these oligonucleotides would lead to mutation of the PAM sequence and
202 introduce a stop codon within the *gfp* open reading frame (ORF; Figure 3A), which
203 could be easily confirmed by detecting loss of the GFP signal. As shown in Figure
204 3B, co-transformation of pcrRNA-gfp1 and the lagging-strand oligonucleotide
205 generated approximately 10^3 colonies, of which 80% were GFP-negative. Consistent
206 with the previous observation that an oligonucleotide targeting the lagging strand
207 produces more recombinants than an oligonucleotide targeting the leading strand (40),
208 co-transformation of pcrRNA-gfp1 and the leading strand oligonucleotide generated
209 fewer GFP-negative recombinants (Figure 3B). In addition, another mutation in the
210 *gfp* gene was generated with similar efficiency using this system (Figure 3). Finally,
211 we generated two mutations (T49V and C86S) in Ms1521, an essential gene in *M.*
212 *smegamatis*, with 87.5% and 60% recombination rates using CRISPR-Cas12a-assisted
213 ssDNA recombineering.

214 To bind a target gene, Cas12a recognizes thymidine-rich PAM sequences and the
215 targeting crRNA. Consequently, point mutations can be introduced into the PAM and
216 crRNA targeting regions using CRISPR-Cas12a–assisted ssDNA oligonucleotide
217 recombineering (26, 41, 42). We sought to determine the sites susceptible to mutation
218 by testing the recombination efficiency of a series of lagging-strand oligonucleotides
219 designed to introduce a stop codon in the *gfp* gene by mutation of one to three
220 nucleotides (Figure 4A). A two- or three-base pair mutation was successfully
221 introduced into the PAM or crRNA targeting region (Figure 4B). Consistent with a

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222 previous report showing that a 16-nt guide sequence is required to achieve detectable
223 DNA cleavage, and a 18-nt guide sequence is required to achieve efficient DNA
224 cleavage (4), a 1-bp mutation was introduced at position 13 of the spacer region but
225 not at position 19 (Figure 4B). Additionally, consistent with a previous report that the
226 crRNA of Cas12a is 24 nt (4, 8), mutation of several base pairs was achieved at
227 positions 19–21 or 22–24 but not at positions 25–30 (Figure 4B). To ascertain
228 site-specific susceptibility to mutation, we designed a series of lagging-strand
229 oligonucleotides to introduce a frameshift mutation into the *gfp* gene by insertion or
230 deletion of one nucleotide (Figure 4C). We found that a 1-bp insertion or deletion was
231 efficiently generated at positions 1–18 of the protospacer sequence but not at positions
232 19 and 22 (Figure 4D). Taken together, these experiments suggest that our system can
233 efficiently introduce point mutations into PAM and crRNA targeting regions in *M.*
234 *smegmatis*.

235 Next, we tested whether CRISPR-Cas12a–assisted recombineering could generate
236 gene deletions in mycobacteria. For this purpose, we designed 59- or 79-mer
237 oligonucleotides, which yielded a 5-, 10-, 20-, 418-, or 1000-bp deletion when
238 incorporated into the chromosome, thereby disrupting the protospacer sequence in the
239 *gfp* gene (Figure 5A). Deletions of 5, 10, or 20 bp were easily generated using 59-mer
240 oligonucleotides, and 70–80% of the transformants were recombinants (Figure 5B).
241 However, the recombineering efficiency decreased as the size of the deleted fragment
242 increased. Deletion of a 1000-bp fragment could not be achieved using the 59-mer
243 oligonucleotide, although the efficiencies of deletion of 418 and 1000 bp fragments

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244 from the *gfp* gene were 17.4% and 8.2%, respectively, when 79-mer oligonucleotides
245 were used. These results demonstrate that CRISPR-Cas12a–assisted ssDNA
246 recombineering is a feasible method for the construction of gene deletions in *M.*
247 *smegmatis*.

248 Next, we investigated whether CRISPR-Cas12a–assisted recombineering
249 allowed the isolation of oligonucleotide-mediated chromosome insertions. A series of
250 oligonucleotides were designed to yield 5-, 10-, or 20-bp insertions and to disrupt the
251 protospacer sequence in the *gfp* gene (Figure 5A). The efficiency of the
252 oligonucleotide-mediated chromosome insertion was strongly affected by the size of
253 the insertion fragment. Approximately 27% of the transformants contained 5-bp
254 insertions and 10% had 10-bp insertions, whereas only 3.1% had a 20-bp insertion
255 (Figure 5B). Together, the results presented in this section show that this system can
256 generate short insertions using ssDNA oligonucleotide recombineering in *M.*
257 *smegmatis*.

258 **Markerless gene manipulation in *M. smegmatis* using CRISPR-Cas12a–assisted**
259 **dsDNA recombineering**

260 We next attempted to use the Cas12a-assisted recombineering system to perform
261 chromosomal gene deletion in *M. smegmatis* with a markerless dsDNA template. We
262 targeted the same *gfp* gene region used in the oligonucleotide recombineering
263 experiments described above. Four dsDNA fragments containing 2-, 392-, 1000-, or
264 4000-bp deletions in the *gfp* region were constructed and co-transformed with
265 pcrRNA-gfp1. As shown in Figure 6, markerless gene deletions up to 4 kb were

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obtained with high efficiency. We also investigated whether dsDNA PCR products could be used in this system for gene manipulation in *M. smegmatis*. The *gfp* gene was successfully replaced by *Ms5635–5634* and the *hyg* antibiotic resistance gene using PCR products as homology recombination fragments (Figure 6). Taken together, these experiments suggest that CRISPR-Cas12a–assisted dsDNA recombineering is an efficient method for markerless gene manipulation.

The introduction of multiple mutations is often necessary to study the functions of redundant genes. For example, four pairs of toxin–antitoxin (TA) genes (*Ms1277–1278*, *Ms1283–1284*, *Ms4447–4448*, and *Ms5635–5634*) are present in *M. smegmatis*. To sequentially delete these genes, crRNA sequences targeting *Ms1278* (pYC1009) and *Ms4447* (pYC983) were cloned into pCR-Hyg, while crRNA sequences targeting *Ms1283* (pYC1010) and *Ms5635* (pYC1011) were cloned into pCR-Zeo. Recombineering DNA fragments were also constructed for the in-frame deletion of the TA genes and transformed in combination with the corresponding crRNA-expressing plasmid to sequentially delete the four TA genes. The resulting transformants were selected and verified by PCR at each step (Supplementary Figure S6). The recombineering efficiencies for the deletion of the four TA genes were 62%, 53%, 45%, and 47%, respectively. The four in-frame scarless deletions were obtained in approximately 5 weeks, compared to that more than 10 weeks normally required using suicide vector-based allelic replacement (43). Taken together, these results demonstrate that our novel system is a time-efficient approach for the construction of multiple markerless and scarless mutations in *M. smegmatis*.

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DISCUSSION

Recombineering is a powerful method that was developed in the last decade to introduce precise genetic changes into bacterial genomes. Recently, CRISPR-Cas9 was used to facilitate recombineering that simplifies genetic manipulation methods and abolishes reliance on antibiotic markers (22-27, 44). Genetic manipulation in mycobacteria is time-consuming, especially if multiple changes are needed. Therefore, more efficient molecular tools are required to meet the increasing need for more diverse types of genetic manipulation. In this study, we described the development of a system that couples CRISPR-Cas12a genome editing with recombineering to allow efficient genetic manipulation in *E. coli*, *Y. pestis*, and *M. smegmatis*.

ssDNA recombineering has been used to generate point mutations, deletions, and small insertions in *E. coli* (45), as well as to introduce mutations into mycobacteria (40). However, the recombination efficiency is low, and isolating the recombinants is difficult, which hinders the practical application of this method. CRISPR-Cas9 has been successfully used in ssDNA recombineering to isolate recombinants in *E. coli* and *L. reuteri* (25-27). Very recently, Jiang and colleagues showed that CRISPR-Cas12a-assisted genome editing can be used in *Corynebacterium glutamicum* (15). We showed here that ssDNA recombineering assisted by the CRISPR-Cas12a system can efficiently generate point mutations and deletions in *E. coli*, *Y. pestis* and *M. smegmatis* (Figures 1-4). More interestingly, this method can be used to insert DNA fragments of up to 20 bp into the chromosome (Figure 5). Thus, Cas12a-assisted ssDNA recombineering could be used to insert a tag-encoding

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310 sequence, such as DAS+4 (12 bp, Ref.(46)) or His_6 (18 bp), into a specific gene on the
311 chromosome. Because this method does not require the construction of a dsDNA
312 fragment for recombination, Cas12a-assisted ssDNA recombineering is time-efficient
313 and cost-effective for bacterial genome editing.

314 Consistent with a previous report showing that Cas12a provides highly specific
315 gene targeting in human and murine cells (47-49), Cas12a was also highly specific in
316 mycobacteria. Cas12a is sensitive to mismatched crRNA nucleotides at positions 1–24
317 of the targeting region, but not to mismatches at positions 25–31. This finding is
318 supported by the observation that 2- or 3-bp mutation can be introduced into the PAM
319 or crRNA targeting region (positions 1–24), whereas mutations cannot be introduced
320 into positions 25–31 (Figure 4). Additionally, we did not detect any mutations in the
321 absence of ssDNA when both Cas12a and crRNA were expressed (Figure 3B). Taken
322 together, these results suggest that Cas12a-assisted recombineering is highly specific
323 and confirm that non-homologous end joining (NHEJ) mutants are unlikely to be
324 generated in *M. smegmatis* using this method.

325 Cas12a-assisted genome editing has broadened the application of the
326 CRISPR-Cas system in bacteria. Firstly, Cas12a is an alternative to Cas9 when Cas9
327 is toxic in an organism (15). Secondly, Cas9 recognizes a NGG PAM sequence,
328 whereas Cas12a recognizes an YTN (CTN or TTN) PAM sequence (10). Therefore,
329 Cas12a and Cas9 recognize different target sites, which allows for more precise
330 selection of the cleavage target; this is especially important for the generation of point
331 mutations in the genome. Finally, the Cas12a cleavage site is 18 and 23 positions

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distal to the PAM sequence; thus, an indel resulting from NHEJ would not disrupt the PAM and the resulting sequence could be cleaved, which could increase the likelihood of recovering homologous recombinants and decrease the likelihood of recovering NHEJ-mediated mutants. This is potentially important given that an NHEJ is present and functional in *M. smegmatis* (23, 50, 51). However, we did not detect any NHEJ in *M. smegmatis* using Cas12a-assisted recombineering.

Our method is especially useful for the introduction of multiple genetic mutations into bacteria. In this regard, it has several advantages: 1) following recombination, the helper plasmids can be easily cured because they contain either a temperature replicon or the *sacB* counterselection gene; 2) crRNA plasmids containing different resistance cassettes can be used in alternation when multiple genes must be deleted sequentially, thereby decreasing the time required to cure the crRNA plasmid in multiple gene deletions; 3) the use of an antibiotic gene as a selection marker, whose removal usually requires an additional step, is unnecessary; and 4) the creation of chromosome scar sites (e.g., LoxP recognition sites) is avoided. The presence of multiple scars in the chromosome can lead to chromosomal rearrangements or deletions resulting from recombination between scars.

Overall, the approach described in this report demonstrates that coupling the recombination system with the CRISPR-Cas12a system simplifies the construction of scarless genome mutations, especially multiple mutations, in bacteria. Given that the Che9c recombination system has been successfully used in *M. tuberculosis* (18), we postulate that this methodology could also be applied to other mycobacteria, including

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354 *M. tuberculosis*, although this still needs to be tested.

355 **MATERIALS AND METHODS**

356 **Strains, media, and growth conditions.** *E. coli* MG1655, *Y. pestis* KIM6+ and
357 *M. smegmatis* mc^2155 were used in this study. *E. coli* and *Y. pestis* were grown in LB
358 medium supplemented with appropriate antibiotics (25 μg/mL kanamycin, 100 μg/mL
359 ampicillin, or 30 μg/mL chloramphenicol). *M. smegmatis* was grown in Middlebrook
360 7H9 broth (Difco) supplemented with 0.05% Tween 80 and 0.2% glycerol or on
361 Middlebrook 7H10 agar supplemented with the appropriate antibiotic (25 μg/mL
362 kanamycin, 50 μg/mL hygromycin, or 50 μg/mL zeocin). Appropriate concentrations
363 of anhydrotetracycline (ATc) were added to the *M. smegmatis* cultures when
364 necessary. To facilitate the screening of recombinants in *M. smegmatis*, a GFP
365 reporter gene was inserted into the *Ms5635–5634* locus of the chromosome using
366 recombineering (52).

367 **Plasmids.** The *FnCpf1* open reading frame (ORF) sequence was cloned into
368 pKD46(13) using Gibson cloning to yield pKD46-Cas12a series plasmids
369 (Supplementary Figure S1A). The pKD46-Cas12a series plasmids, which contain a
370 temperature-sensitive replicon, can be cured at 42°C. The pre-crRNA cassette
371 containing the *gfp* gene was commercially synthesized and cloned together with the
372 *sacB* gene into the modified pACYC184 vector to yield pAC-crRNA series plasmids
373 (Supplementary Figure S1). The *gfp* gene, which is flanked by *Bpm*I and *Bsa*I
374 restriction enzyme sites, was used as a selection marker for protospacer cloning
375 (Supplementary Figure S1). Two complementary oligonucleotides containing the

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target sequence adjacent to 5'-YTN-3' were synthesized, annealed to yield a protospacer cassette with *Bpm*I or *Bsa*I overhangs at the 5' and 3' ends, respectively, and then cloned into the pAC-crRNA plasmid (Supplementary Figure S1). The research presented in this study was conducted using the pKD46-Cas12a-Amp and pAC-crRNA-Cm plasmids. pKD46-Cas12a-Cm and the other pAC-crRNA series plasmids were also tested and yielded similar results to those obtained using npKD46-Cas12a-Amp and pAC-crRNA-Cm plasmids(data not shown).

A codon-optimized *FnCpf1* ORF sequence (Optimized with JCat) (53) and Supplementary Figure S2) under the control of the P_{myc1}*tetO* promoter was commercially synthesized (GENEWIZ) and cloned into pMV261 and pJV53 to yield pMV261-Cpf1 and pJV53-Cpf1, respectively (Supplementary Figure S4A). The pre-crRNA cassette was commercially synthesized and cloned into pSL003(54) to yield pCR-Zeo (Supplementary Figure S4B). The *Hyg*-resistance gene was amplified by PCR from pSL002 and cloned into pCR-Zeo to replace the *Zeocin*-resistance gene, yielding pCR-Hyg (Supplementary Figure S4B). The *Cas12a* gene was amplified by PCR from pMV261-Cpf1, and then ligated into pcrRNA-ctrl and pcrRNA-gfp1 digested with *Kpn*I and *Not*I, to yield pCpf1-ctrl and pCpf1-gfp, respectively. To mutate a particular gene in mycobacteria, two complementary oligonucleotides containing the target sequence adjacent to 5'-YTN-3' were synthesized, annealed to yield a protospacer cassette with *Bpm*I and *Hin*dIII overhangs at the 5' and 3' ends, respectively, and then cloned into pCR-Zeo or pCR-Hyg. All plasmids constructed in this study are listed in Supplementary Table S1. The oligonucleotides used in this

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study are listed in Supplementary Table S2.

***M. smegmatis* growth assay.** *M. smegmatis* cells harboring pMV261 or pMV261-Cpf1 were inoculated into 3 mL of 7H9 broth supplemented with kanamycin and grown overnight with shaking at 37°C. The overnight cultures were diluted to OD_{600} = 0.02 in 50 mL of 7H9 broth supplemented with kanamycin and appropriate concentrations of ATc. These cultures were grown overnight with shaking at 37°C, and samples were taken at appropriate time points to determine the OD_{600}.

Plasmid interference assay. The plasmid interference assay was carried out as previously reported with minor modifications (37). Briefly, *M. smegmatis* mc^2155 harboring pCpf1-ctrl or pCpf1-gfp was transformed with pJV53 or pJV53-GFP, respectively. The transformants were plated onto 7H10 medium supplemented with or without 50 ng/mL ATc, and then grown for 3 days at 37°C. The colonies were counted to calculate colony forming units (CFU).

Preparation of recombinogenic DNA. For the ssDNA oligonucleotide recombineering experiments, the recombinogenic oligonucleotides were synthesized and mutations were introduced into the middle of the oligonucleotide sequences with at least 25 nt of sequence identity on both sides of the mutation site. The leading and lagging strands of the bacterial chromosomes were determined using cumulative skew diagrams (55).

For the dsDNA recombineering experiments, the *gfp* gene was amplified from pAcGFP1 vector using primers with 45-nt homology regions of *aroA* to generate recombinogenic dsDNA products for *aroA* replacement in *E. coli*. To generate

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dsDNA homologous arms for the deletion of 2, 392, or 1000 bp from the *gfp* gene in *M. smegmatis*, the *Ms5635–5634*::*gfp* cassette with flanking regions was amplified by PCR and inserted into pUC19 to yield the plasmid pYC847. Then, pYC847 was used as a template for inverse PCR with appropriate primer sets to generate plasmids containing dsDNA homologous arms. To generate dsDNA homologous arms for the 4000-bp deletion, a 539-bp DNA fragment downstream of *Ms5634* and a 596-bp DNA fragment upstream of *Ms5635* were amplified by PCR and cloned into pUC19 to yield pYC848. These plasmids were digested with *Kpn*I and *Sph*I, and the digested fragments were gel purified. pYC710, pYC711, pYC799, and pYC738 were used as templates to generate pYC984, pYC985, pYC986, and pYC987, which contain the dsDNA homologous arms for the toxin–antitoxin systems *Ms1277–1278*, *Ms1283–1284*, *Ms4447–4448*, and *Ms5635–5634*, respectively. The dsDNA homologous arms generated from these new plasmids were digested with *Hin*dIII and *Kpn*I. *Ms5635–5634*, the *hyg*-resistance gene, and their flanking regions were amplified by PCR from the chromosomes of wild-type *M. smegmatis* and the *M. smegmatis Ms5635–5634*::*hyg* mutant respectively, and used as recombinogenic fragments for the replacement of the *gfp* gene. The primers used in this study are listed in Supplementary Table S2.

Cas12a-assisted genome editing. Competent cells of *E. coli*, *Y. pestis* and *M. smegmatis* were prepared as previously described.(17, 52, 56) For ssDNA oligonucleotide recombineering, approximately 500 ng of recombinogenic or non-recombinogenic oligonucleotides and 100 ng of the crRNA expression plasmid

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were mixed and electroporated into competent cells. For dsDNA gene deletion, 700 ng of gel-purified restriction-digested product or PCR product and 100 ng of the crRNA-expressing plasmid were mixed and electroporated into competent cells. For *E. coli* and *Y. pestis*, the electroporated cells were plated and grown on LB agar supplemented with appropriate antibiotics overnight at 30°C. Recombination of *lacZ* was assessed by examining for the formation of white colonies, and then confirmed by PCR and sequencing analysis. Recombination in *Y. pestis* was analyzed and verified by PCR amplification and sequencing. Plasmid-free colonies were obtained by incubating the cells in LB culture medium supplemented with sucrose at 42°C. For *M. smegmatis*, cells were recovered after incubation in 1 mL of 7H9 broth with 10 ng/mL ATc for 4 h at 30°C at 200 rpm and then plated on 7H10 agar supplemented with the appropriate antibiotics and 50 ng/mL ATc. After growth for 4 days at 30°C, the plates contained normal size and tiny transformant colonies. The normal colonies were counted and used to calculate the transformation and recombination efficiencies. Recombination of *gfp* was assessed by examining for the loss of the GFP signal, and then each time at least 5 GFP negative recombinants were picked for PCR and sequencing analysis and all of the tested colonies were confirmed to be desired recombinants. Recombination involving other genes was assessed by PCR and sequencing. To cure the helper plasmids from the *M. smegmatis* recombinant, the right recombinant colony was picked and grown overnight at 37°C in 7H9 media without antibiotic. The overnight culture was diluted 1:500 into 7H9 media and grown for 24 hours at 37°C. The resultant culture were diluted, plated, and grown for 3 days

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on 7H10 plates and then tested for the loss of plasmids using replica streaking on the plate with or without appropriate antibiotics.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at *Applied and Environmental Microbiology* online.

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615 **Figure legends**

616 **FIG 1** CRISPR-Cpf1-assisted genome editing in *E. coli*. (A) Schematic of
617 CRISPR-Cpf1 coupled with the λ-Red system *in E.coli*. First, Cpf1 and recombinase
618 were expressed in bacteria. Then, the crRNA expressing plasmid and ssDNA (or
619 dsDNA) were transformed into the cell. When the crRNA targets the *E.coli lacZ* locus,
620 wild-type *E. coli* dies or forms a blue colony, whereas the mutant forms a white
621 colony on the X-gal plate. (B) Schematic showing the crRNA and oligonucleotides
622 used for editing of the *E.coli lacZ* locus. Cleavage sites are indicated by red arrows.
623 The oligonucleotides lacZ.lag (59 nt) and lacZ.lead (59 nt, reverse and
624 complementary sequence of lacZ.lag) were designed to mutate the PAM sequence and
625 introduce a stop codon within the *lacZ* open reading frame. LacZ.del was designed to
626 delete 1399bp from the lacZ gene. (C) The number and percentage of white colonies
627 of transformants from the electroporation of the indicated pcrRNA plasmids and
628 oligonucleotides into *E. coli* MG1655 expressing recombinase and FnCpf1. The
629 transformation efficiency was defined as the total number of CFU generated per
630 transformation. The transformants were plated on X-gal plates for blue-white
631 screening. Results are the average of at least two independent experiments, and the
632 error bars depict the standard deviations.

633

634 **FIG 2** CRISPR-Cpf1–assisted plasmid editing in *Y. pestis*. Generation of *caf1R*
635 mutations in the pMT plasmid using CRISPR-Cpf1–assisted ssDNA oligonucleotide
636 recombineering. The 59-nt recombinogenic oligonucleotides targeting the lagging

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637 strand or the leading strand of DNA replication were utilized for mutation. The
638 transformation efficiency (A), the recombination efficiency (B) and the loss
639 percentage of plasmid (C) are are the average of two independent experiments. ctrl, a
640 control oligonulceotide with no homology to the genome of *Y. pestis* was used. 20
641 colonies from each transformation were picked to test for plasmid loss and
642 recombination by colony PCR and sequencing.

643

644 **FIG 3** CRISPR-Cpf1–assisted single-stranded oligonucleotide recombineering in *M.*
645 *smegmatis*. (A) Schematic showing the sequence of the *gfp*-targeting crRNA and
646 oligonucleotides used for recombineering. This region corresponds to nucleotides
647 65–249 of the *gfp* ORF. The 60-nt recombinogenic oligonucleotides targeting the
648 lagging strand (gfp1.lag and gfp2.lag) or targeting the leading strand (gfp1.lead) of
649 DNA replication were utilized to disrupt the *gfp* gene by introducing changes in five
650 consecutive base pairs (shown in brown) to generate two consecutive in-frame stop
651 codons (lowercase and italic). The green and top-lined sequences represent the PAM.
652 (B) Transformation and *gfp* recombination efficiency resulting from electroporation of
653 the indicated pcrRNA plasmids and oligonucleotides into *M. smegmatis* expressing
654 recombinase and FnCpf1. The transformation efficiency was defined as the total
655 number of CFU generated per transformation, and the recombination efficiency was
656 measured by determining the proportion of GFP-negative colonies. ATc (50 ng/mL)
657 was added to the agar to induce Cpf1 expression. Results are the average of at least
658 two independent experiments, and the error bars depict the standard deviations.

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659

660 **FIG 4** Generation of subtle gene mutations using CRISPR-Cpf1–assisted ssDNA
661 oligonucleotide recombineering *M. smegmatis*. (A) Schematic showing the sequences
662 of the point mutations generated in the *gfp* gene. Mutated sequences are shown in red.
663 (B) Generation of point mutations described in (A) using CRISPR-Cpf1–assisted
664 ssDNA oligonucleotide recombineering. (C) Schematic showing the sequences of
665 1-bp frameshifts generated in the *gfp* gene. Inserted sequences are shown in red, and
666 the dashed line indicates the deleted sequence. (D) Generation of 1-bp insertions or
667 deletions described in (C) using CRISPR-Cpf1–assisted ssDNA oligonucleotide
668 recombineering. Transformation efficiency was defined as the total number of CFU
669 generated per transformation, and recombination efficiency was measured by
670 determining the proportion of GFP-negative colonies. Results are the average of at
671 least two independent experiments, and the error bars depict the standard deviations.

672

673 **FIG 5** Gene deletion and insertion using CRISPR-Cpf1–assisted single-stranded
674 oligonucleotide recombineering in *M. smegmatis*. (A) Schematic of gene deletions
675 and insertions generated in the *gfp* gene using CRISPR-Cpf1–assisted single-stranded
676 oligonucleotide recombineering in *M. smegmatis*. (B) Generation of deletions and
677 insertions shown in (A) using CRISPR-Cpf1–assisted single-stranded oligonucleotide
678 recombineering in *M. smegmatis*. Introduction of 5-, 10-, 20-, 418-, or 1000-bp
679 deletion or 5-, 10-, or 20-bp insertion in the *gfp* gene using CRISPR-Cpf1–assisted
680 single-stranded oligonucleotide recombineering. The transformation efficiency was

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defined as the total number of CFU generated per transformation, and recombination efficiency was measured by determining the proportion of GFP-negative colonies. Results are the average of at least two independent experiments, and error bars depict standard deviations.

FIG 6 Double-stranded DNA recombineering assisted by CRISPR-Cpf1. Deletions of 2-, 392-, 1000-, or 4000-bp were introduced into *M. smegmatis* chromosomal DNA using approximately 1 kb double-stranded DNA fragments with induced Cpf1. The *gfp* gene was replaced with a dsDNA PCR fragment containing the Hyg-resistance gene or *Ms5635–5634* and its flanking region with induced Cpf1. Diagrams of the above gene deletions and replacements are shown in Supplementary Figure S5. Transformation efficiency was defined as the total number of CFU generated per transformation, and recombination efficiency was measured by determining the proportion of GFP-negative colonies. Results are the average of at least two independent experiments, and error bars depict standard deviations.

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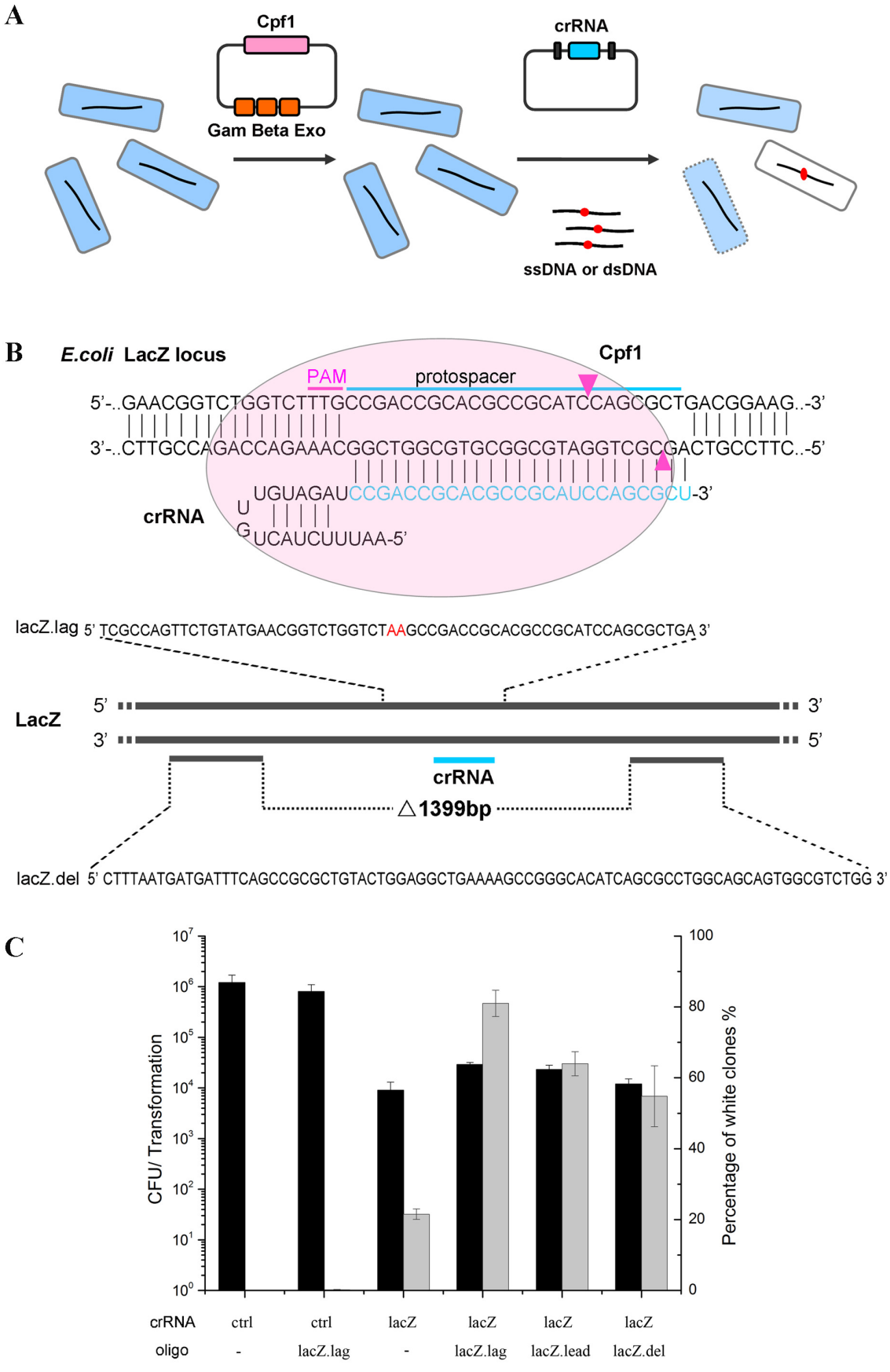
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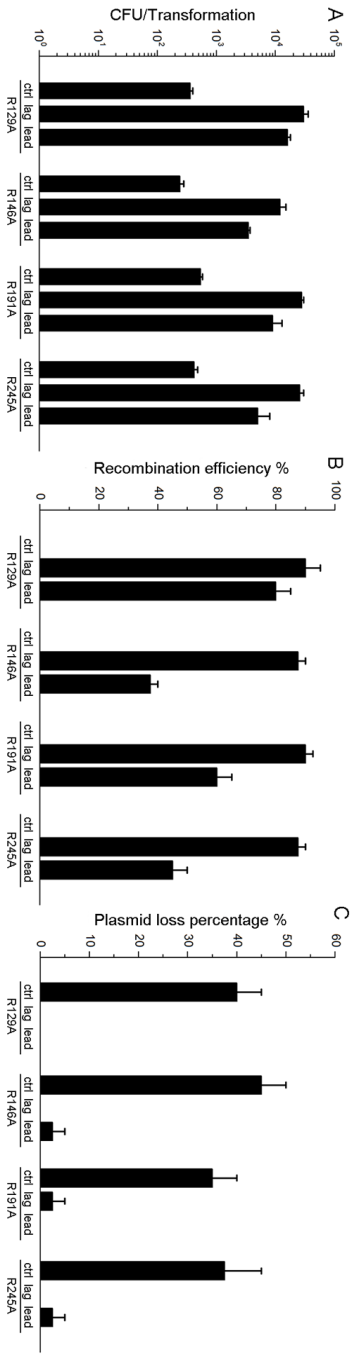
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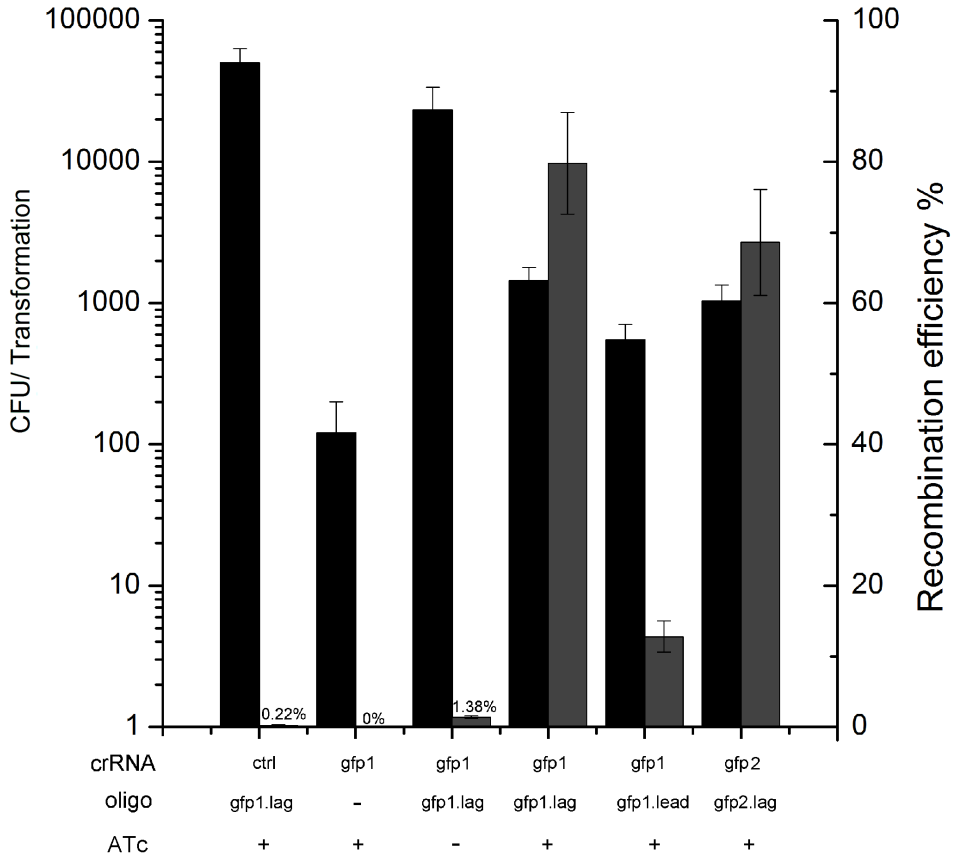
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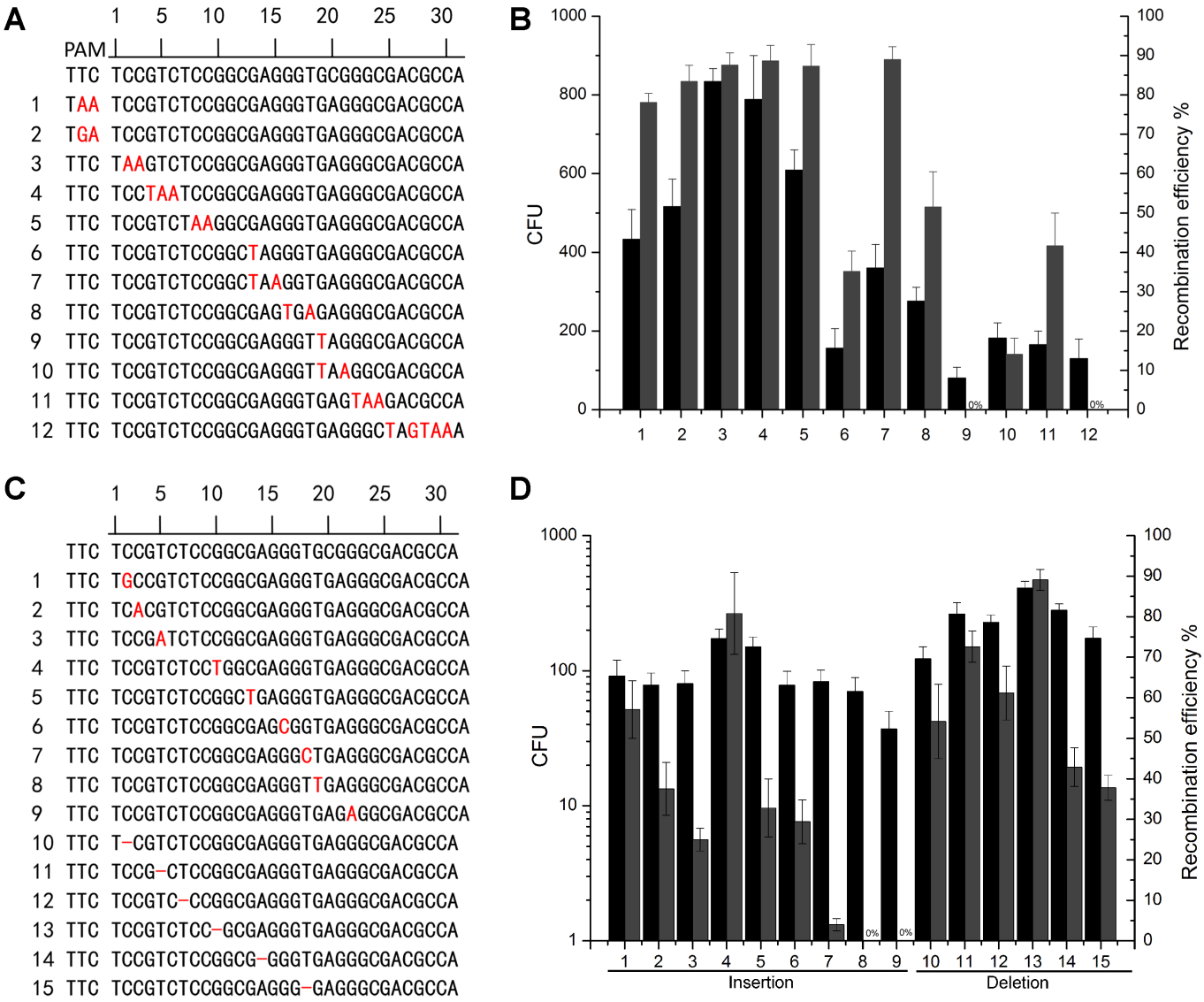
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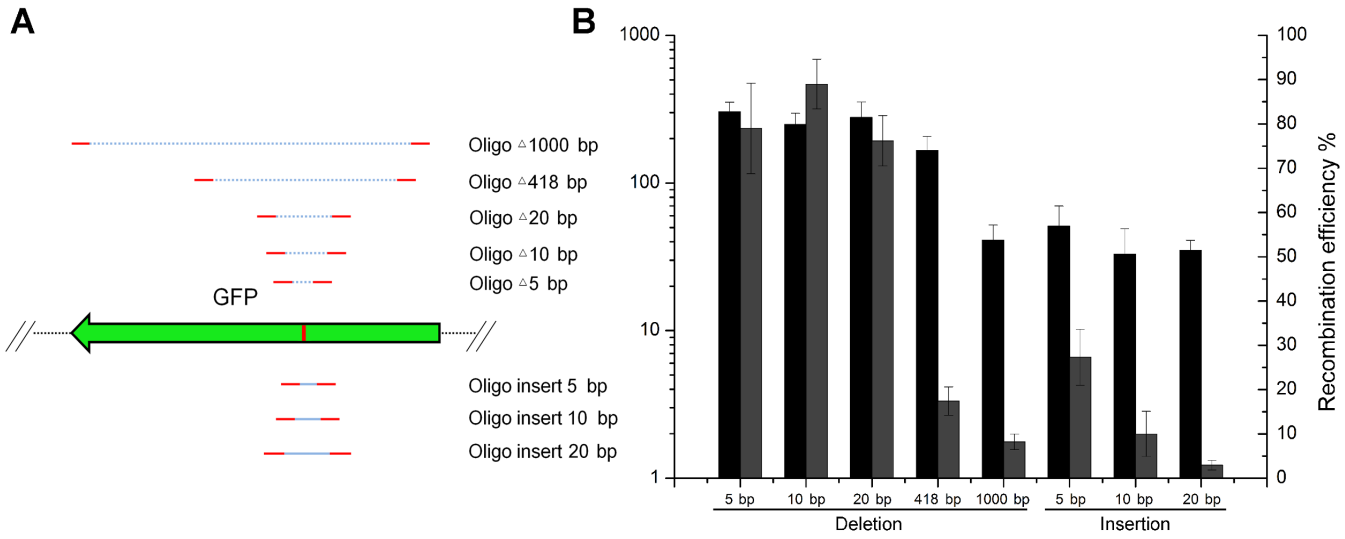
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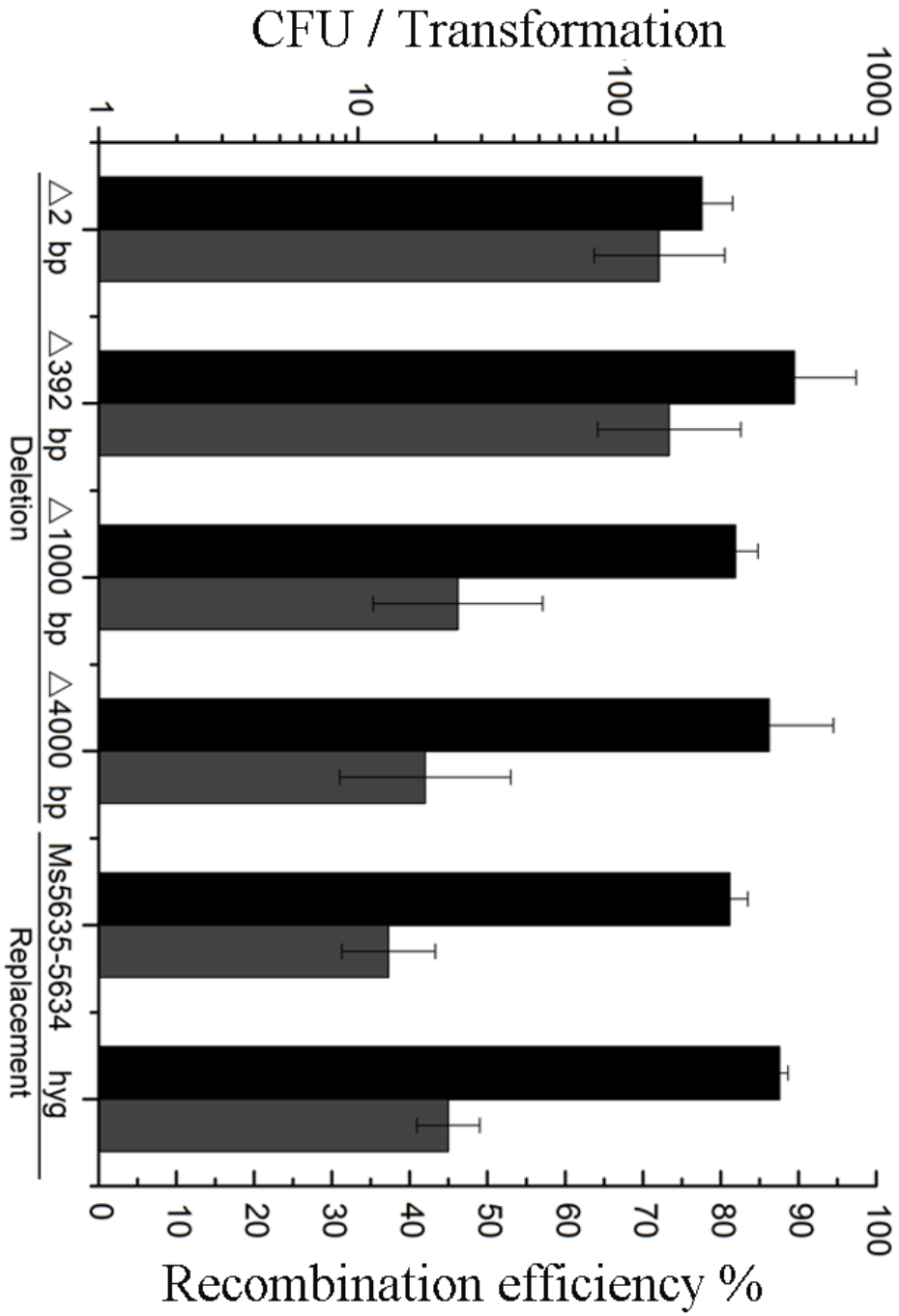
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