Disrupting Homologous Recombination or Single-Strand Annealing Significantly Hinders CRISPR-Cas12a-Assisted Nonhomologous End-Joining Gene Editing Efficiency in *Mycobacterium abscessus*

Sanshan Zeng,[a,b,c,d] Yanan Ju,[a,b,c,e] Md Shah Alam,[a,b,c,d] Ziwen Lu,[a,b,c,d] H.M. Adnan Hameed,[a,b,c] Lijie Li,[a,b,c,e] Xirong Tian,[a,b,c,d] Cuiting Fang,[a,b,c,d] Xiange Fang,[a,b,c,d] Jie Ding,[a,b,c,f] Xinyue Wang,[g,h] Jinxing Hu,[h] Shuai Wang,[a,b,c,h]* Tianyu Zhang [a,b,c,d,e,f,g] *

[a] State Key Laboratory of Respiratory Disease, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, China

[b] Guangdong-Hong Kong-Macao Joint Laboratory of Respiratory Infectious Diseases, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, China

[c] China-New Zealand Joint Laboratory on Biomedicine and Health, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, China

[d] University of Chinese Academy of Sciences, Beijing, China

[e] School of Basic Medical Sciences, Division of Life Science and Medicine, University of Science and Technology of China, Hefei, China

[f] Institute of Physical Science and Information Technology, Anhui University, Hefei, China

[g] Guangzhou National Laboratory, Guangzhou, China

[h] State Key Laboratory of Respiratory Disease, Guangzhou Chest Hospital, Guangzhou, China

* Corresponding authors: Shuai Wang, E.mail: wshuai1@163.com, Tel: +86-20-32015266; Tianyu Zhang, E.mail: zhang_tianyu@gibh.ac.cn, Tel: +86-20-32015270.

Running title: Influence of HR and SSA on *M. abscessus* Gene Editing Efficiency

ABSTRACT *Mycobacterium abscessus*, a fast-growing, non-tuberculous mycobacterium resistant to most antimicrobial drugs, poses a significant public health challenge because of its ability to cause many types of serious infections in humans. While genetic manipulation tools for *M. abscessus* are still being developed, the clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein (Cas) systems have shown promise in generating highly specific double-strand breaks (DSBs) in its genome. These DSBs can be repaired by the error-prone nonhomologous end joining (NHEJ) mechanism, facilitating targeted gene editing. Here, our study marks a pioneering application of the CRISPR-NHEJ strategy in *M. abscessus*. Moreover, our research uncovered an unexpected finding: contrary to previous observations in *Mycobacterium tuberculosis* and other species, the inhibition of RecA or disruption of key genes in the homologous recombination or single-strand annealing pathways resulted in a significant decrease in NHEJ repair efficiency in *M. abscessus*. This discovery challenges the established perspectives and offers new insights into the interaction among the three DSB repair pathways in Mycobacterium species.

IMPORTANCE There are still very few genetic manipulation tools available for *Mycobacterium abscessus*.. Here we report the successful application of CRISPR-Cas12a-assisted nonhomologous end joining (NHEJ) in efficient gene editing in *M. abscessus*. Contrary to previous research suggesting that homologous recombination (HR) inhibition may enhance such editing efficiency in other Mycobacterium species, our results showed that disruption of either HR or single-strand annealing (SSA) DNA double-strand breaks (DSBs) repair pathways not only failed to enhance but also significantly reduced the gene editing efficiency in *M. abscessus*. This suggests that NHEJ repair in *M. abscessus* may require components from both HR and SSA pathways, highlighting a complex interaction among the DSB repair pathways in *M. abscessus.*

KEYWORDS *Mycobacterium abscessus*, CRISPR-Cas12a, DSB repair, NHEJ

The prevalence of lung infections caused by non-tuberculous mycobacteria (NTM) has been rising annually, posing an escalating threat to public health. Among these NTM, *Mycobacterium abscessus* is one of the primary causative agents, second only to *Mycobacterium avium* complex in both the United States and the humid regions of southern coastal China (1-3). Besides contagious pulmonary infections, *M. abscessus* can infect the skin, soft tissues, central nervous system, eyes, and various other parts of the human body (4). The intrinsic resistance of *M. abscessus* to a broad spectrum of antibiotics not only complicates the treatment, but often requires the use of multidrug regimens and prolonged therapies (5). The threat posed by *M. abscessus* necessitates the urgent development of genetic tools for drug target identification, resistance mechanism elucidation, and vaccine research.

Various attempts have been made at genetic manipulation in Mycobacterium species, such as the expression of *gp60* and *gp61* from the mycobacteriophage Che9c to enhance recombination in *Mycobacterium smegmatis, Mycobacterium tuberculosis* (6), and *M. abscessus* (7). CRISPR-assisted recombineering has further enabled the effectual construction of marker-free recombinants in Mycobacterium spp. After successful implication in *M. smegmatis* (8), this technique has recently been applied successfully for the first time by us in *M. abscessus* (9). Additionally, nonhomologous end joining (NHEJ), another DNA double-strand break (DSB) repair mechanism, presents the potential for genetic manipulation together with CRISPR, owing to its template-independent and error-prone repair characteristics (10, 11). The combined use of CRISPR and NHEJ for genetic manipulation has been proven effective in *M. smegmatis* and *M. tuberculosis*, with inhibition of the host's homologous recombination (HR) system by overexpressing RecX or disrupting *recA* gene (12). RecX, known to antagonize RecA, impedes the HR process by negatively regulating the array of biochemical reactions facilitated by RecA (13). However, attempts to apply these strategies to *M. abscessus* have not been reported so far. Here, we report that gene editing by the CRISPR-NHEJ strategy was successful and efficient in *M. abscessus*. Interestingly, on the contrary to previous findings, gene editing efficiency significantly decreased in *M. abscessus* once RecX was overexpressed or key genes in HR or single-strand annealing (SSA) were knocked-out.

We tried to apply the CRISPR-NHEJ strategy to *M. abscessus,* and used *M. smegmatis* as a control. The pNHEJ-Cpf1 plasmid containing *Mycobacterium marinum* NHEJ elements (*ligD*, *ku* and *nrgA*) as well as *cpf1* (*cas12a*) or the pNHEJX-Cpf1 plasmid additionally expressing $recX^{Msm}$ (*recX* gene of *M. smegmatis*, Fig. S1) were transduced into *M. abscessus* GZ002 (14, 15) and *M. smegmatis* MC^2 155 by electroporation, respectively. Transformants were selected on kanamycin-containing 7H10 plates. Strains carrying the corresponding plasmids were cultured in 3 mL of 7H9 medium supplemented with 10% OADC, 0.2% glycerol, 0.05% Tween 80, and 100 μg/mL kanamycin for *M. abscessus* or 50 μg/mL for *M. smegmatis*. Then 1 mL of the starter culture was inoculated into a 125 mL shake flask containing 40 mL of 7H9 complete broth, and incubated at 37°C until OD_{600}=0.8-1.0. After that, the bacteria were harvested for preparation of competent cells as previously described (16). About 500 ng of the plasmid expressing crRNA (primers for constructing crRNA are listed in Table S1) was mixed with 100 μL of competent cells, transferred to a 2 mm electroporation cuvette, and incubated on ice for 10 min. Then the cuvette was placed into an electroporation chamber for electroporation at a voltage of 2,500 V, a capacitance of 25 μF, and a resistance of 1,000 Ω. After electroporation, 2 mL of 7H9 medium was added to the electroporation cuvette to resuspend the bacteria, which was then transferred to a 50 mL tube and incubated at 30°C for 4 to 5 hours. Then, cultures were plated separately on 7H11 agar supplemented with the appropriate antibiotics and 200 ng/mL anhydrotetracycline (ATc) for *M. abscessus* or 50 ng/mL for *M. smegmatis*. The editing efficiency was determined by the ratio of colonies with altered target sequences to the total number of randomly picked colonies. For each assessment, at least 20 colonies were randomly selected for PCR (primers are shown in Table S1) and sequencing analysis. The colonies that either lacked the target bands in PCR or showed sequencing results deviating from the wild-type sequence were identified as colonies that had undergone gene editing.

Here, we observed a surprising phenomenon. In the *M. abscessus* transformed with pNHEJ-Cpf1 group, the gene editing efficiency of the randomly selected nonessential gene *MAB_3513c* was 63.35 ± 4.738 % (Fig. 1A). However, the gene editing efficiency in the *M. abscessus* transformed with pNHEJX-Cpf1 group was only 18.35 ± 2.333 %. The editing efficiency in the pNHEJ-Cpf1 group was

122 approximately threefold higher than that in the strain with additional *recX*^{Msm}
123 expression (Fig. 1A, $P = 0.0068$), which is completely opposite to a previous research
124 in *M. smegmatis* showing that it is necessary either to introduce additional *recX*^{Msm}
125 gene or to knock-out the *recA* gene to suppress HR to edit gene efficiently when using
126 the CRISPR-NHEJ strategy (12). Consequently, researchers hypothesized that in *M.*
127 *smegmatis*, HR and NHEJ are competing pathways (12). To verify this hypothesis, we
128 repeated their study by editing the *MSMEG_1946* gene in *M. smegmatis* to compare
129 the editing efficiency with or without expressing additional *recX*^{Msm} gene. Although
130 the gene editing efficiency remained high at 85 ± 7.071% in *M. smegmatis* containing
131 pNHEJX-Cpf1 with overexpressed *recX*^{Msm}, in the group containing pNHEJ-Cpf1
132 without additional *recX*^{Msm}, the gene editing efficiency was still 78.35 ± 11.31%. We
133 did not observe an obvious decrease in editing efficiency (Fig. 1A, $P = 0.5650$). These
134 results were clearly different from the earlier report (12).

135 Unlike HR, NHEJ repair was characterized by deletions of base pairs of random
136 lengths (Fig. S2). To further validate the broad applicability of this strategy, we tried
137 to edit other *M. abscessus* genes only using pNHEJ-Cpf1 without additional
138 expression of *recX*^{Msm}. To date, we have successfully knocked-out over 30 genes
139 (partially listed in Table S2) in *M. abscessus* using this method. A limitation of this
140 study is that we did not edit more genes to compare the efficiencies with and without
141 additional expression of the *recX*^{Msm} widely. However, the overall efficiency range of
142 editing different genes by different researchers using this method in *M. abscessus* was
143 30% - 90% which was generally sufficient for a common gene editing. This strategy
144 has significantly improved our efficiency in acquiring mutants.

145 The surprising results spurred our further investigation. On the one hand, we
146 tried the gene editing in the HR-disrupted *M. abscessus* strain as reported in *M.*
147 *smegmatis* (11). On the other hand, though it was reported that the inhibition of SSA
148 can enhance HR (17), but no study has been reported on whether disruption of SSA
149 would affect the NHEJ-dependent gene editing in Mycobacteria, so, we tried to
150 investigate weither blocking SSA or disrupting HR and SSA both would impact the
151 NHEJ-dependent gene editing efficiency in *M. abscessus*.

152 We constructed selection-marker free, in-frame deletion knock-out strains of
153 *recA*, *recC* or *recO* gene in *M. abscessus* (unpublished) using the CRISPR-assisted

recombineering method as described in our previous study (9). The RecA plays a key role in HR, while the RecBCD complex composed of RecC plays an important role in SSA independently of the RecA-dominated HR. In addition, it was shown that deletion of the *recO* gene would result in defective repair of both HR and SSA (18-20). Next, in these HR and SSA repair-deficient strains, we repeated the gene editing experiments performed in the wild-type strain as described above to observe the potential impact of HR (*recA*) or SSA (*recC*) or both (*recO*) repair defects on the gene editing efficiency.

Our results showed that the gene editing efficiencies were 3.33 ± 2.877 %, 17.5 ± 3.536 % and 7.5 ± 3.536 % in the *recA*, *recC* or *recO* knock-out strains, respectively (Fig. 1B). As expected, the gene editing efficiency in the *recA* gene knock-out strain was much lower than that in the wild-type strain (P = 0.0004) and even significantly lower than that in the wild-type strain overexpressing $recX^{Msm}$ gene (P = 0.009, Fig. 1A and Fig. 1B). Similarly, the gene editing efficiencies in the *recC* or *recO* gene knock-out strains were significantly lower than that in the wild-type strain (P = 0.0082 and P = 0.0056, respectively, Fig. 1B). All these results supported that disruption of the HR or SSA repairing pathway would decrease the CRISPR-Cas12a-assisted NHEJ gene editing efficiency.

Interestingly, the ranking of editing efficiencies in the knock-out strains was *recA* (HR) ≤ *recO* (HR&SSA) ≤ *recC* (SSA). The significant difference in editing efficiencies between two of the three knock-out strains was only observed between *recA* and *recC* (P = 0.0156, Fig. 1B). This indicated that the gene editing efficiency was influenced differently by the different genes in the HR or SSA DSB repair pathways, and suggested that those in the HR pathway or gene playing a key role in the two pathways might have a stronger influence than that only affects SSA. Even though *recO* may influence both HR and SSA, it only affects a branch pathway of HR unlike RecA which plays a key role in HR (21). However, the influence of each gene in either HR or SSA pathways on the gene editing efficiency needs further investigation.

Previous studies largely indicated that the three DSB repair mechanisms might be competitive with each other for gene editing efficiency (12, 17), which contrasts sharply with our findings that they cooperate for the completion of gene editing. Here

we report for the first time the successful application of the NHEJ-CRISPR strategy for the efficient gene editing but without inhibiting the HR pathway in *M. abscessus*, and also reveal a potential synergistic interaction between HR or SSA and NHEJ within this bacterium. For *M. smegmatis*, a study showing that disruption of HR would not enhance the DSB repair efficiency through the NHEJ pathway supports what we found here that disruption of HR did not affect the CRISPR-Cas12a-assisted NHEJ gene editing efficiency (Fig. 1A) (22). Our novel finding offers a new perspective for further exploration of the interrelationships between DSB repair mechanisms in mycobacteria and paves the way for future enhancement of specific repair pathway efficiencies by manipulating these relationships. More refined mechanism research of the relationship among HR, SSA, and NHEJ and their impact on the CRISPR-Cas12a-assisted NHEJ gene editing efficiency requires further investigation.

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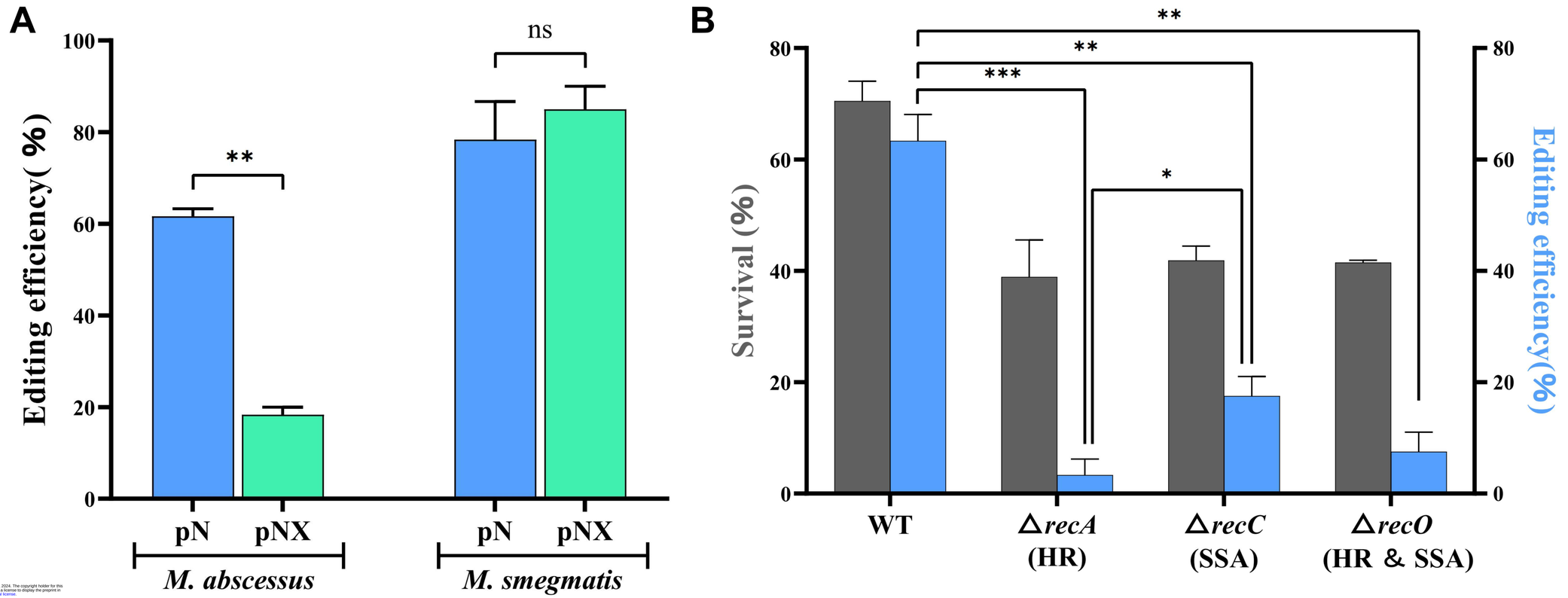

FIG 1 CRISPR-Cas12a-NHEJ-assisted gene editing in *M. abscessus* and *M. smegmatis*. (A) Overexpression of the *recX*^{Msm} gene has different effects on the editing efficiency of *M. smegmatis* and *M. abscessus*. The plasmid expressing crRNA targeting *MSMEG_1946* or *MAB_3513c* was introduced into *M. smegmatis* or *M. abscessus* strains harboring pNHEJ-Cpf1 or pNHEJX-Cpf1, respectively. pN: pNHEJ-Cpf1, pNX: pNHEJX-Cpf1. (B) CRISPR-Cas12a-NHEJ gene editing efficiency in *M. abscessus* and its derivatives. Knocking out the *recA* (disruption of HR), *recC* (disruption of SSA), *recO* (disruption of HR and SSA) genes all resulted in a significant decrease in gene editing efficiency in *M. abscessus*. Editing efficiency was calculated by measuring the proportion of colonies with mutations at the target site among the total randomly selected clones and survival rate was defined as the ratio of the number of CFUs obtained from plates with ATc to the number of CFUs obtained from plates without ATc. ns, not significant. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$.