Guide-directed DNA cleavage by a prokaryotic Argonaute protein induces chromosome recombination in *Escherichia coli*

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Abstract: Emerging evidence supports the argument that some prokaryotic argonautes (pAgos) serve as a defensive system against invasion of viruses and plasmids through guide DNAs (gDNAs) directed DNA cleavage. This DNA-guided DNA interference motivates research to induce genomic mutations via pAgo mediated cleavage. Here we demonstrate that CbAgo, a pAgo from *Clostridium butyricum*, is able to induce chromosomal recombination between direct repeat sequences via its gDNA-directed cleavage in *Escherichia coli* chromosome. We also show that CbAgo targeting can assist Lambda-Red recombineering in RecA-deficient strain. Our study reveals that cleavage by CbAgo in *E. coli* chromosome can be mutagenic and suggests its broader application in genetic manipulation.

Main text:

Prokaryotic argonaute proteins (pAgos) constitute a diverse protein family (*1-2*). Unlike their eukaryotic counterparts, which use small RNA guides to interfere with RNA targets in regulation and defense (*3-4*), many pAgos were reported to cleave DNA targets using small guide DNAs (gDNAs) *in vitro* (*5-18*). *In vivo*, several pAgos were shown to protect bacteria from foreign DNAs (*7, 19-20*), but the defense mechanism, especially whether double stranded DNA break (DSB) is involved, remains elusive. Recently, in-depth insights into the mechanism of pAgo-mediated defense were gained by analyzing CbAgo, a pAgo nuclease from a mesophilic bacterium *Clostridium butyricum*, in *Escherichia coli* as expression host (*21*). In that study, a DNA interference pathway was revealed in CbAgo-mediated protection against invading DNAs. First, CbAgo generates and binds small gDNAs from plasmids or other multicopy genetic elements. Next, gDNA-bound CbAgo introduces DSB at the target sites, including chromosomal regions, and causes DNA degradation in collaboration with *E. coli* exonuclease RecBCD. Such a mechanism enables the host cell to defend invader DNAs such as plasmids and phages.

The observation that CbAgo can be directed to generate DSB in chromosome by plasmid encoded guide sequence (GS) motivates us to leverage such a mechanism to manipulate *E. coli* genome, reasoning DSB generation is the essential step to introduce mutation in genome editing systems such as CRISPR/cas9, TALEN, Zinc-finger nucleases (*22-26*). Till now, mutation induced by guide-directed cleavage of CbAgo or other pAgos in the host genome has never been established.

Here we demonstrate CbAgo, directed by GS on plasmids, is able to induce chromosome recombination between direct repeat sequences in *E. coli*. Other mechanisms that are independent of GS or CbAgo cleavage activity were also observed, but we managed to obtain a recombination system whose dominant mechanism depends on both guide and CbAgo cleavage activity. We further show that RecBCD, exonuclease from *E. coli* cellular DNA repair machinery (*27-28*), plays an essential role in the recombination by CbAgo-GS, confirming the DSB generation during recombination. Besides, guide-directed CbAgo cleavage causes severe growth inhibition in RecA-deficient *E. coli* strain, which can be utilized as counter-selection to assist Lambda-Red recombineering. These findings demonstrate the potential of establishing DNA-directed genome editing system using pAgo.

Previous observation that guide-directed CbAgo cleavage at *E. coli* chromosome efficiently triggers RecBCD activity (*21*) inspires us to hypothesize that pre-established RecBCD-dependent chromosome recombination should be triggered by guide-directed CbAgo cleavage as well. We noticed a report showing DSB introduced by SbcCD cleavage at a 246-bp chromosomal palindrome (pal246) stimulates RecBCD-dependent recombination between two downstream direct repeat sequences (*29*). To determine whether similar recombination can be induced by guide-directed CbAgo cleavage (Fig. 1A, fig. S1), we integrated a recombination cassette at cynX loci on *E. coli* strain DL1777, which is ~6 kb away from the target lacZ loci and

obtained strain nonChikanS (Fig. 1B). This recombination cassette contains a EM7 promoter and a kanamycin resistance gene whose function is abolished by an insertion of a stop codon array and a 270-bp direct repeat sequence. A recombination event between the two direct repeat sequences removes the insertion, restores the gene function and confers kanamycin resistance to the host.

To target lacZ loci, we created a targeting plasmid pTet_CbAgo-GS encoding a CbAgo expression cassette under control of a tetracycline inducible promoter (pTet) and a 1000-bp GS homologous to lacZ gene (Fig. 1C). Importantly, GS is the only sequence on the plasmid that has homology in genome, assuring only the lacZ loci will be targeted. For controls, plasmids with no GS, no CbAgo gene, or neither, were created and tested. To determine the dependence of CbAgo cleavage activity, we created plasmids encoding a CbAgo mutant (dCbAgo: CbAgo D541A-D611A) that contains mutations of two catalytic residues in its active site which was previously shown to abolish its endonuclease activity *in vitro* (*12*-*13*) and DSB generation activity *in vivo* (*21*).

Initially, we transformed the obtained plasmids into strain nonChikanS, induced CbAgo expression and recovered induced cells to measure recombination rates, which was calculated as the fraction of ampicillin-resistant cells that became resistant to kanamycin (kanamycin-resistant and ampicillin-resistant colony-forming units (cfu)/ampicillin-resistant cfu), because only the recombinants have restored functional kanamycin resistance gene. Unexpectedly, only the cell transformed with pTet_dCbAgo-GS exhibited remarkable recombination (Fig. 1D), with a rate being ~tenfold higher than the one bearing pTet_CbAgo-GS. This result suggests there is a recombination pathway that is mediated by non-cleavage function of CbAgo and is outperformed by the cleavage-dependent pathway in the presence of cleavage site. Although the exact mechanism remains unknown, this dCbAgo-mediated recombination pathway should be

independent of DSB and RecBCD because dCbAgo-GS was previously shown not able to introduce DSB or trigger RecBCD activity *in vivo* (*21*).

Considering the introduction of Chi site, an 8-base 5'-GCTGGTGG-3' motif recognized by RecBCD (*27*, *30*-*31*), has been shown to stimulate RecBCD recombination (*29*), we incorporated a varied number of Chi sites adjacent to the recombination cassette (Fig. 1B and D). Incorporation of one Chi site (corresponding strain ChikanS) resulted in remarkable increase of recombination rate by CbAgo-GS (Fig. 1D). Increasing the number of Chi sites to three (corresponding strain 3×ChikanS) did not further increase the recombination rate by CbAgo-GS, but decreased recombination rates in dCbAgo groups. 3×ChikanS cell transformed with pTet_CbAgo-GS exhibited fivefold higher recombination rate than the ones expressing dCbAgo and approximately 30-fold higher over the rest control groups. The actual contribution of dCbAgo-mediated recombination to the total recombination events in 3×ChikanS/pTet_CbAgo-GS should be much smaller than one-fifth as it should be largely outperformed by cleavage-dependent pathway under this condition. Further increasing the number of Chi sites to six (corresponding strain 6×ChikanS) did not yield considerable effects. Together, these findings reveal a novel chromosome recombination that is induced by guide-directed CbAgo cleavage. Its Chi site dependence implicates DSB formation and RecBCD processing during recombination.

To demonstrate the reliability of our recombination system, we tested CpAgo and IbAgo, two recently identified pAgos exhibiting moderate double stranded DNA (dsDNA) cleavage activity at mesophilic temperatures *in vitro* (*14*) and their catalytic mutants (dCpAgo: CpAgo D544A-D614A and dIbAgo: IbAgo D517A-D586A) in strain 3×ChikanS. The cell transformed with pTet_CpAgo-GS exhibited ~tenfold higher recombination rate than the one with pTet_IbAgo-GS (Fig. 2A, B). This result correlates well with the *in vitro* data showing CpAgo is more active than IbAgo in dsDNA cleavage (*14*). We also tested engineered DSB in our system

by integrating a pal246 into the lacZ loci on the genome of strain 3×ChikanS and its *sbcCD* knockout mutant and measuring their recombination rates (Fig. 2C, fig. S2, see supplementary materials and methods). The sbcCD$^+$, lacZ::pal246 strain yielded ~100-fold increase of recombination rate compared to sbcCD$^+$, lacZ$^+$ strain and *sbcCD*, lacZ::pal246 strain. The bigger fold change stimulated by SbcCD-pal246 over CbAgo-GS is consistent with previous observation that SbcCD-pal246 is more efficient in DSB generation than CbAgo-GS *in vivo* (*21*). These findings indicate a strong correlation between pAgo DNA cleavage activity, DSB generation efficiency and recombination rate in our system.

We sought to determine the involvement of *E. coli* endogenous DNA repair machinery RecA and RecBCD in the recombination by creating and testing *recA* and *recBCD* knockout mutants of strain 3×ChikanS. The viability of *recA* strain transformed with pTet_CbAgo-GS was extremely low upon anhydrotetracycline addition, while cells in other genetic contexts exhibited much milder growth inhibition comparing to wildtype (Fig. 3A). Similar results were obtained from the cell expressing LacZ (fig. S3B), which has similar molecular weight as CbAgo but does not interact with DNA, suggesting growth inhibition from protein overexpression was negligible. The fatality of CbAgo targeting in *recA* strain can be explained by 'reckless' DNA degradation where without the protection of RecA, continuously introduced DSB triggers extensive DNA degradation by RecBCD, causing enormous loss of chromosomal DNA and subsequent cell death (*32-34*). From the other perspective, the highly efficient RecA repairing supports our model that CbAgo-induced DSB is dependent on replication, because during replication multiple copies of chromosome are produced and can serve as templates for RecA repairing (*35*).

However, despite of its low viability, *recA* strain bearing pTet_CbAgo-GS had an incredibly high recombination rate close to 0.1 (Fig. 3B), indicating those recombinants were not merely escapers from 'reckless' DNA degradation with poor CbAgo expression, instead they

were still expressing functional CbAgo and survived DSB generation in their chromosome. This suggests RecA is not essential in the recombination induced by guide-directed CbAgo cleavage even though it actively repairs DSB generated during the process. The higher recombination rate by CbAgo-GS in *recA* strain than in recA$^+$ strain further suggests RecA-dependent DSB repair is a competing mechanism against recombination. These findings prompt us to modify our model that the final step of recombination (Fig. 1A, step iii) should be independent of RecA, reminiscent of the RecA-independent, replication arrest-induced deletion (*36*-*37*).

In *recBCD* strain, recombination rate by CbAgo-GS had no difference with the empty plasmid control, representing a profound change from the result using recBCD$^+$ strain (Fig. 3B), indicating CbAgo-GS induced recombination is dependent on RecBCD. Since RecBCD works closely with DSB (*38*), this again strongly suggests DSB generation in CbAgo-GS induced recombination. On the other hand, dCbAgo-GS induced recombination seems to be promoted in the absence of RecBCD, suggesting a fundamentally different pathway.

The observation that cfu of *recA* strain was reduced by three orders of magnitude when its genome is targeted by CbAgo (Fig. 3A) is very intriguing, as it supports the strategy to leverage CbAgo-GS targeting as counter-selection to facilitate recombineering (Fig. 4A). For comparison, self-targeting CRISPR-Cas9 system was reported to reduce cfu by three orders of magnitude in *E. coli* (*39*). Co-expressing CRISPR-Cas9 system to eliminate unedited cells, Lambda-Red recombineering achieved an increase of efficiency by ~10^4 fold and a 65% overall mutation rate. We sought to combine CbAgo targeting system with Lambda-Red recombineering by introducing CbAgo expression plasmids into strain SIJ488_ΔrecA, which is RecA-deficient and has arabinose inducible Lambda-Red recombineering genes integrated into its genome. We first performed standard recombineering procedure with dsDNA donor encoding kanamycin resistance cassette flanked by 1000-bp homology arms to replace genomic lacZ gene. The

recombineering efficiency was 1.2×10^{-4}, calculated from the fraction of cells that became kanamycin resistant. Then we performed recombineering in CbAgo-plasmids contained cells, induced CbAgo expression and recovered the cells to characterize the proposed counter-selection effect. The cell transformed with pTet_CbAgo-GS had a mutation rate of 2.3×10^{-2}, representing ~100-fold increase of efficiency from standard recombineering (Fig. 4B). Other control groups did not yield improvement, therefore the increased proportion of edited cell population depends on guide-directed CbAgo cleavage.

Our study here demonstrates the combination of CbAgo and plasmid encoded GS is able to induce mutations in *E. coli* chromosome, via guide-directed CbAgo cleavage of target DNA, activation of DNA repair mechanism and subsequent chromosome recombination. This strategy may apply to other organisms, if chromosomal DSB can be introduced and necessary cellular repair machinery can be triggered. Besides, we also demonstrate the potential of pAgo targeting to assist recombineering in RecA-deficient strains as another genome editing strategy. This method may extend to RecA-active strains, if RecA activity can be efficiently inhibited by, for example, expressing RecA inhibitor (*40*). A recent study also reported NgAgo-assisted recombineering (NgAgo, pAgo from *Natronobacterium gregoryi*), but the fold change was smaller than 2 and the enhancement of editing was not dependent on NgAgo endonuclease activity (*41*).

The mechanism of guide-directed recombination by dCbAgo in our system remains unexplored, although this pathway appears to be inhibited by RecBCD and independent of RecA. Since dCbAgo is also able to load gDNAs from plasmids *in vivo* (*21*, fig. S1), there is a possibility that dCbAgo may play a role in target recognition and following recruitment of *E. coli* endogenous nuclease or recombinase for DNA cleavage or recombination. This speculation is supported by similar findings in other pAgo researches (*42, 43*) and the fact that many pAgo

genes have been found associated with a variety of genes including nuclease and helicase (*1*, *19*). These observations suggest the promiscuity of pAgos and raise the possibility to improve pAgo endonuclease activity via protein engineering. To this end, our recombination system presented here can potentially serve as a selection platform, which links DSB generation by pAgo cleavage to the development of antibiotic resistance. Therefore, mutant with enhanced DNA cleavage activity is likely to exhibit higher survival rate and be selected. Highly active pAgo nuclease, once obtained, should have bigger potential in DNA-guided genome engineering *in vivo*, and alternatively, may serve as a versatile restriction enzyme *in vitro* with the capability of targeting theoretically any DNA sequence using small oligos as gDNAs, providing unique advantage over commercial ones (*44*).

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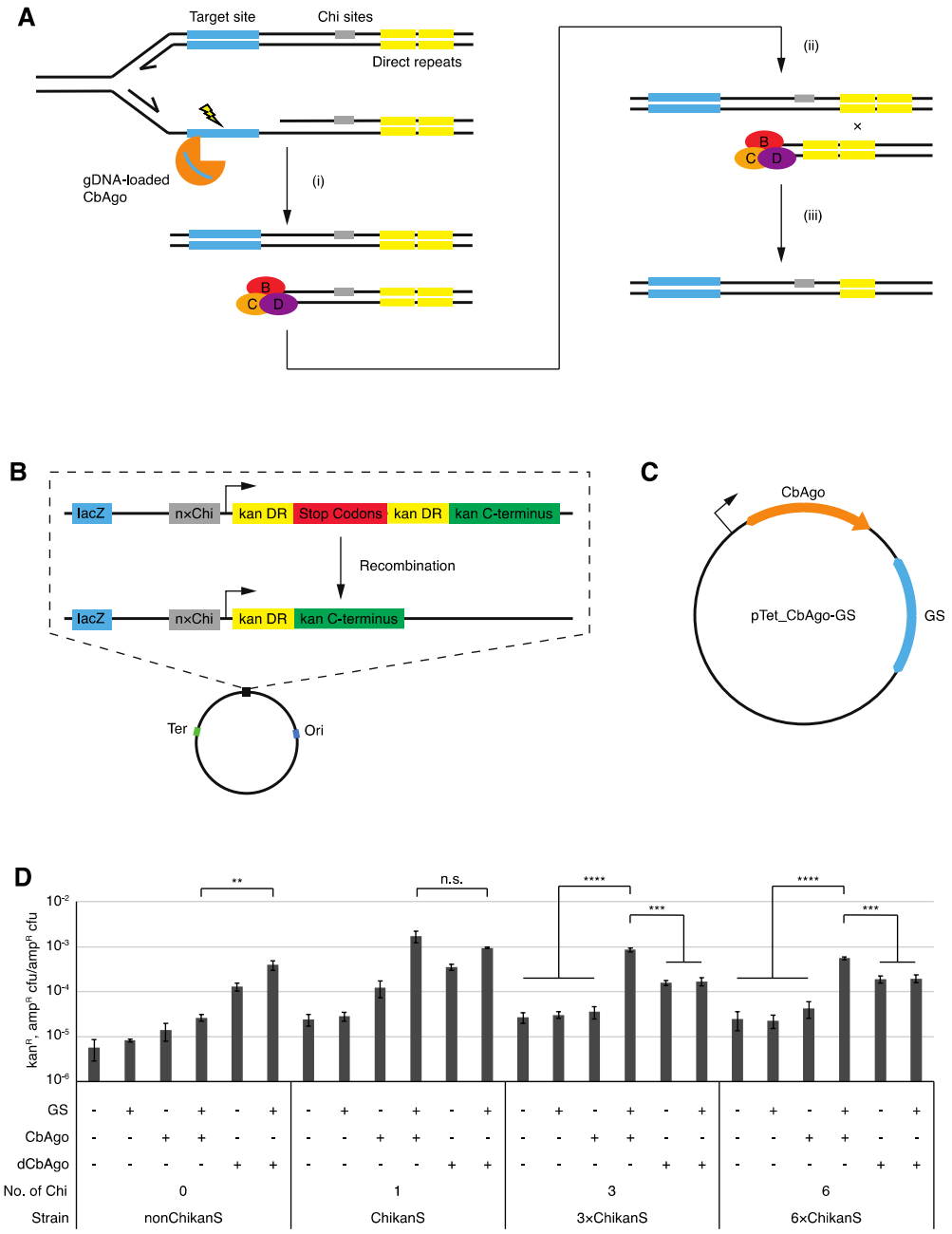

Fig .1. Guide-directed CbAgo cleavage stimulates chromosome recombination. (**A**) Proposed mechanism for guide-directed CbAgo cleavage and RecBCD-dependent recombination. (i) Guide-directed CbAgo cleavage of lagging strand during chromosome replication generates DSB and (ii) triggers RecBCD binding and processing of chromosome DNA. (iii) Recognition of Chi sites by RecBCD attenuates its DNA degradation activity and

facilitates recombination between sister chromosomes via RecA. (**B**) Structure of the recombination cassette. See supplementary materials and methods for DNA sequence. kan, kanamycin resistance gene. DR, direct repeat. (**C**) Structure of the CbAgo targeting plasmid. (**D**) Recombination rates in different genetic contexts. Error bars, mean ± s.d. for three independent experiments. n.s. $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

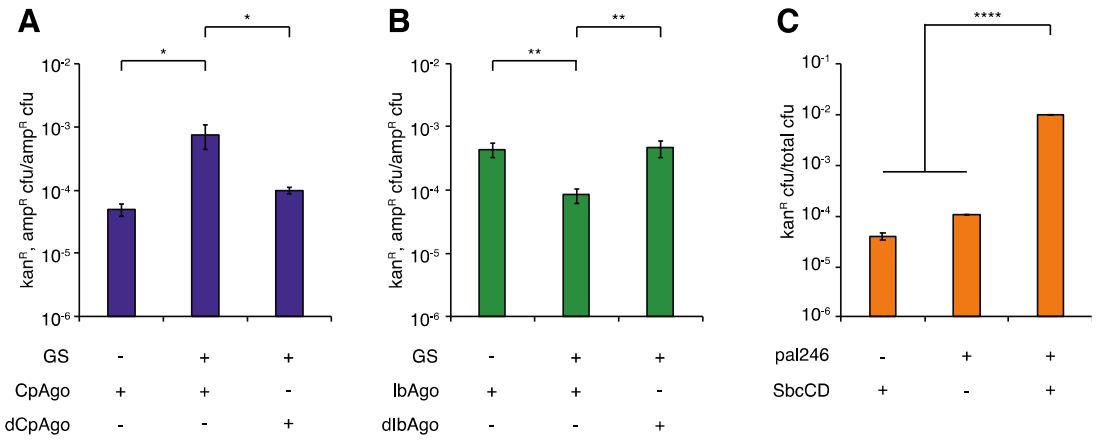

Fig. 2. Other pAgos and engineered DSB stimulate chromosome recombination. (**A**) Recombination rate using pTet_CpAgo-GS and control plasmids in 3×ChikanS. (**B**) Recombination rate using pTet_IbAgo-GS and control plasmids in 3×ChikanS. (**C**) Recombination rate using sbcCD$^+$/ΔsbcCD, lacZ$^+$/lacZ::pal246 strains. Strains used here were 3×ChikanS, 3×ChikanS_pal246_ΔsbcCD and 3×ChikanS_pal246. See fig. S2 for genetic structure of engineered DSB. Error bars, mean ± s.d. for three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

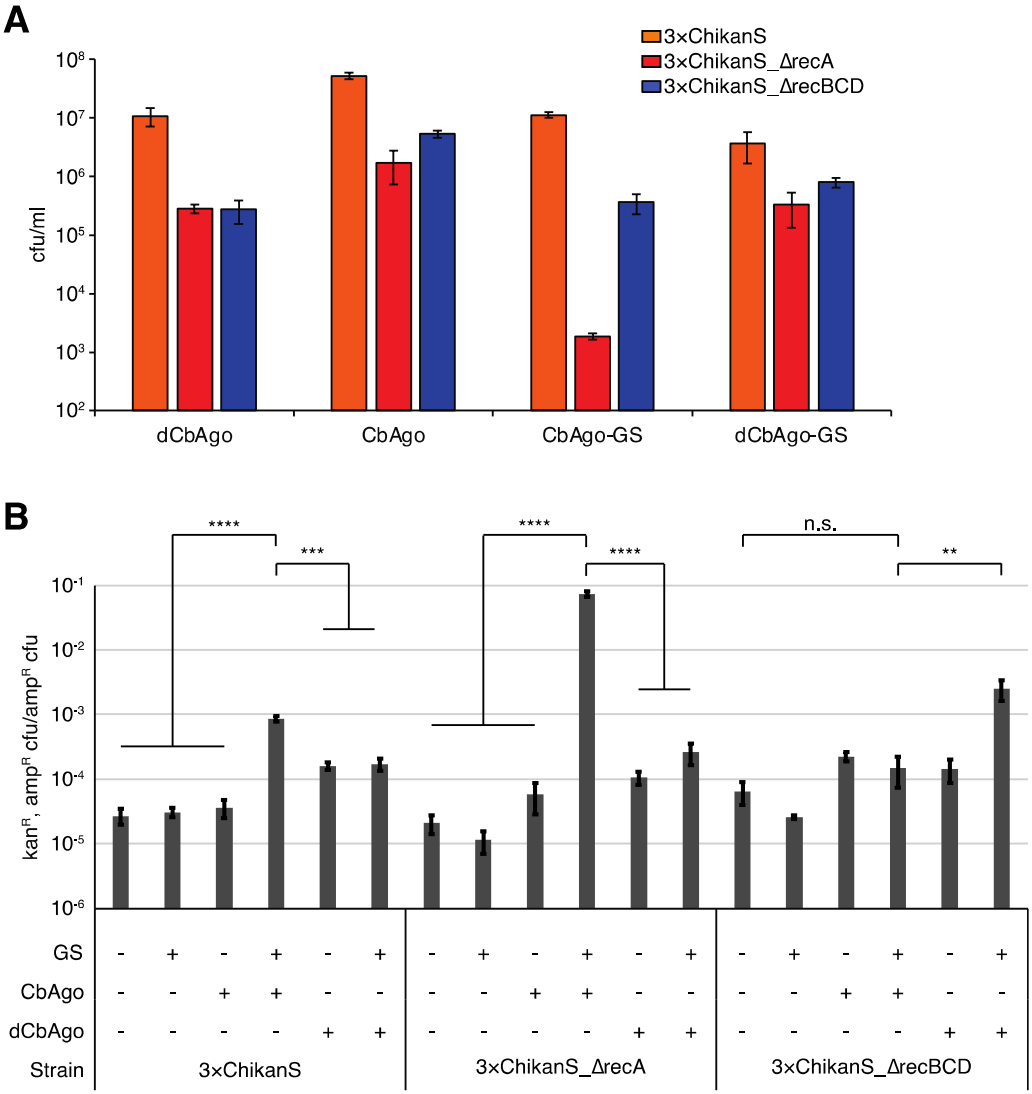

Fig. 3. Behavior of RecBCD and RecA-deficient strains. (**A**) Viability of 3×ChikanS or its *recA*, *recBCD* strains upon anhydrotetracycline addition. (**B**) Recombination rates in different genetic contexts. Error bars, mean ± s.d. for three independent experiments. n.s. P > 0.05, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

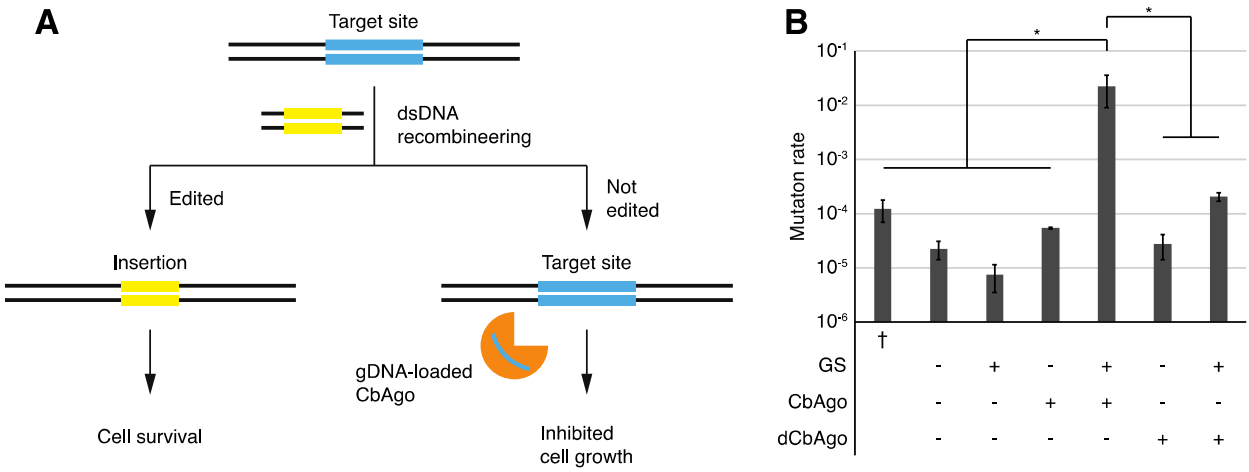

Fig. 4. Targeted CbAgo cleavage assists Lambda Red-mediated recombineering in RecA-deficient *E. coli* strain. (A) Scheme of CbAgo-assisted recombineering. After recombineering, growth of unedited cell will be suppressed by CbAgo cleavage and subsequent DNA degradation, while successfully edited cell will be resistant to CbAgo cleavage and exhibit kanamycin resistance. Yellow, kanamycin resistance cassette. Blue, lacZ gene. (**B**) Mutation rates using strain SIJ488_ΔrecA in different genetic contexts. †Standard recombineering procedure was applied using plasmid-free cells. See supplementary materials and methods for details. Error bars, mean ± s.d. for three independent experiments. *P < 0.05.