Applied Microbiology and Biotechnology

Development of a highly efficient base editing system for *Lactobacilli* to improve probiotics and dissect essential functions

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Running Head: Base editing by Target-AID in *Lactobacilli*

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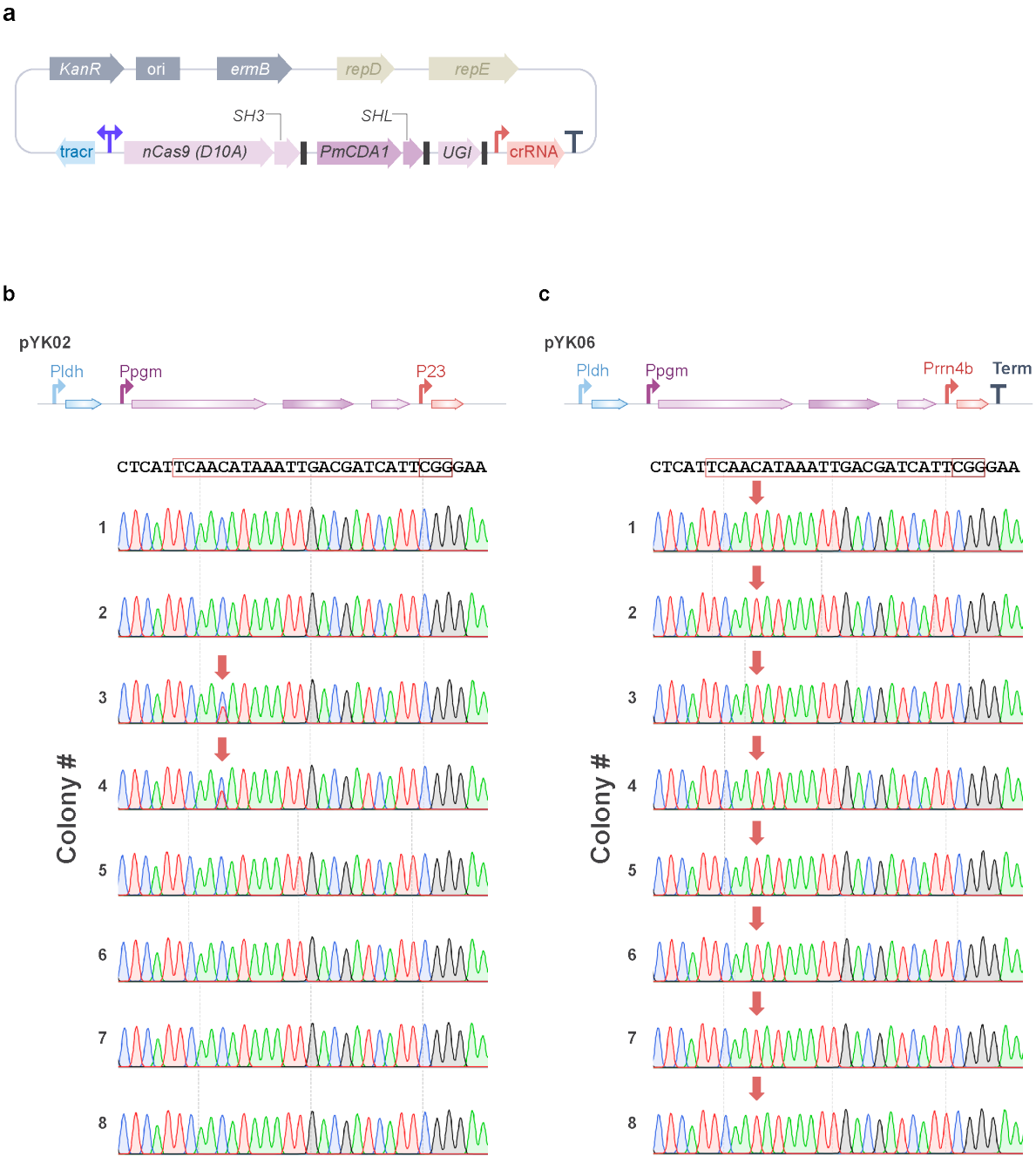
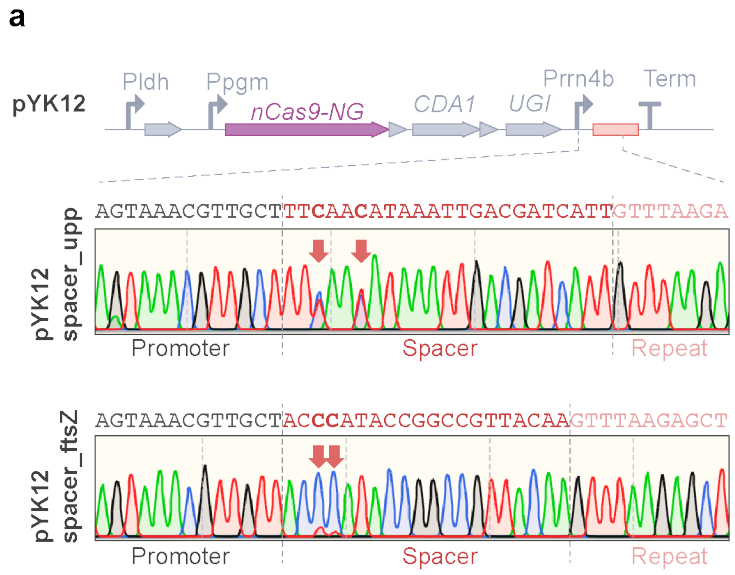
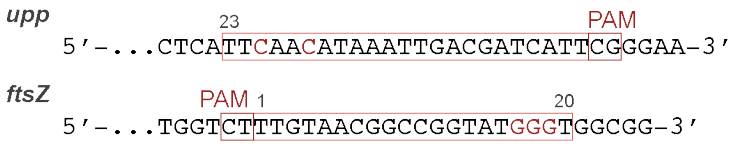
*Hitoshi Mitsunobu and Yudai Kita contributed equally to this work.

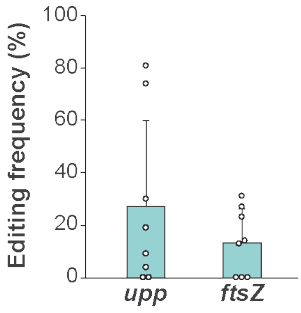
Supplementary Materials



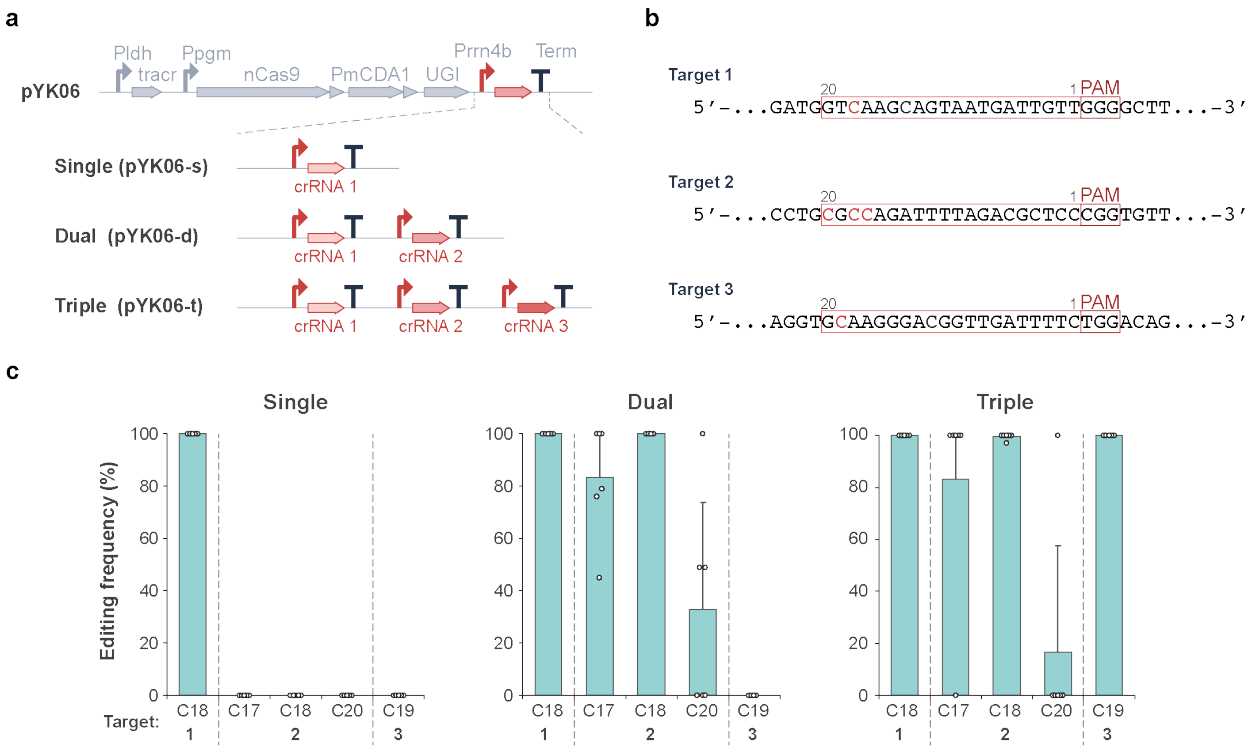
Supplementary Fig. 1



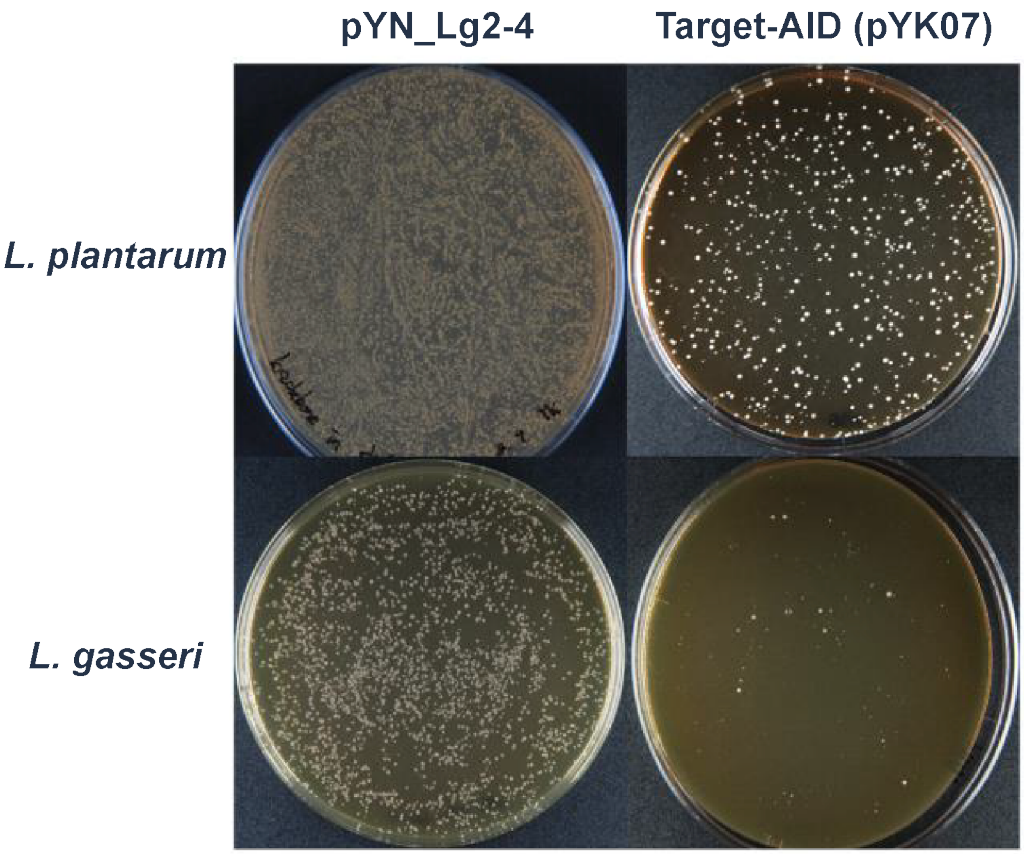




Supplementary Fig. 2

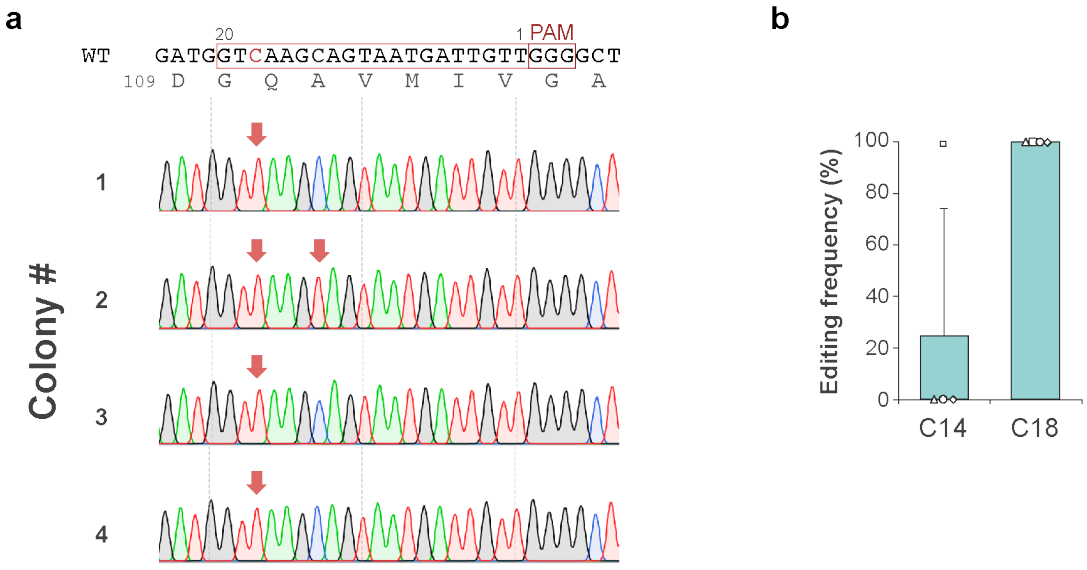


Supplementary Fig. 3

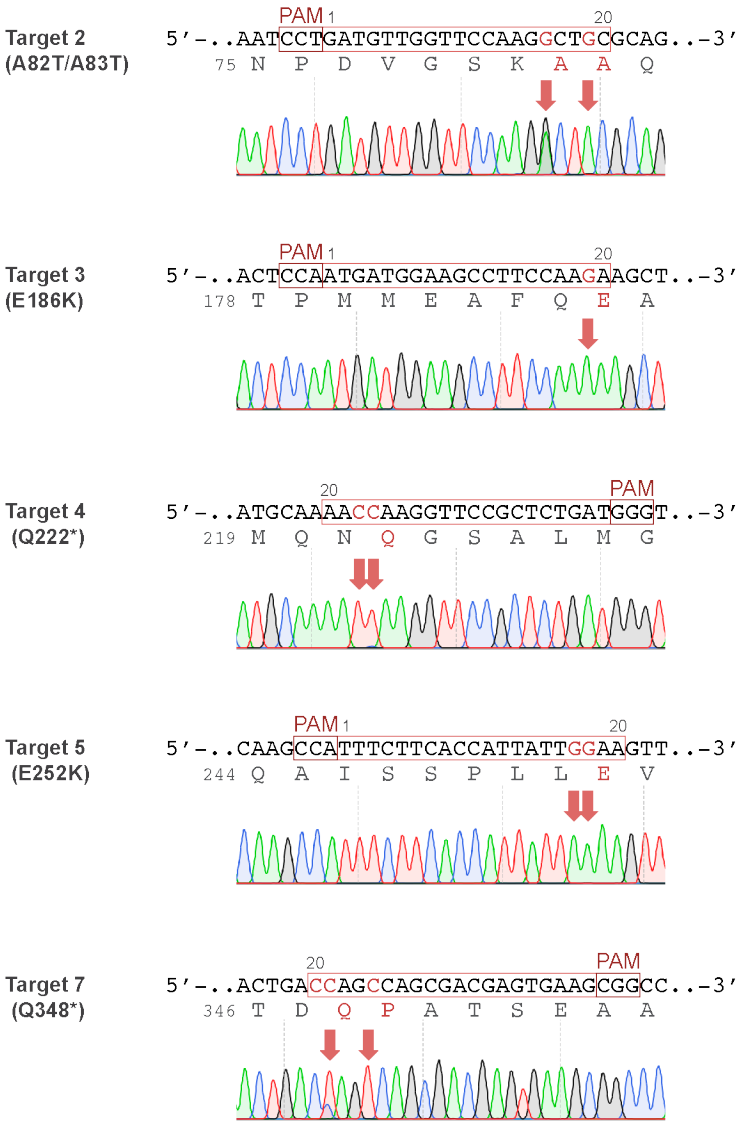


28 **Supplementary Fig. 4**

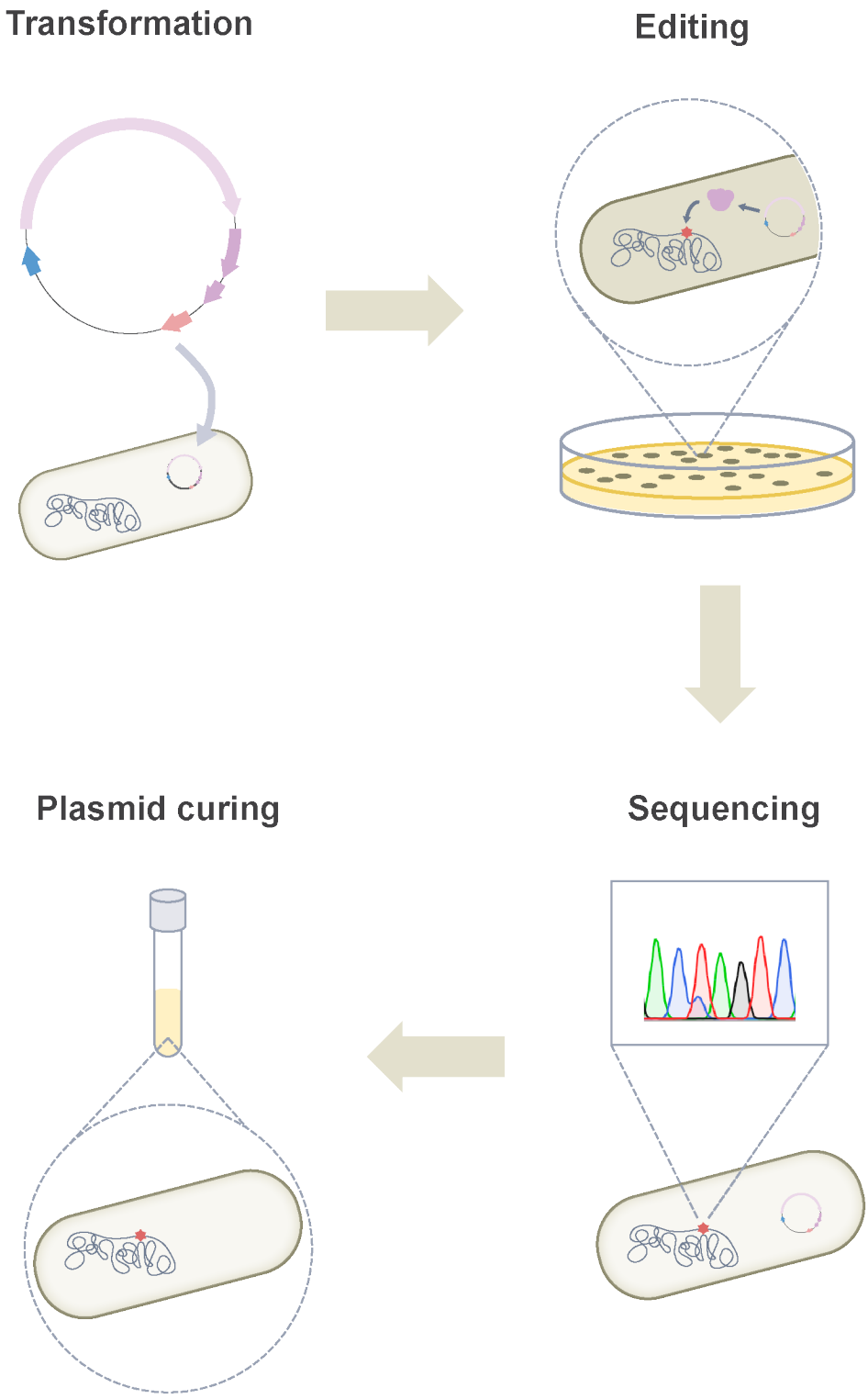
29



Supplementary Fig. 5



Supplementary Fig. 6



Supplementary Fig. 7

Supplementary Fig. 1

The architecture of a plasmid with the bidirectional promoter and independent sanger sequencing data for the representative plasmids. (a) A bidirectional promoter is located between the nCas9 and tracrRNA genes for driving the effector proteins and tracrRNA in concert. The orientation of tracrRNA with the bidirectional promoter is opposite from the one with a separated promoter. (b) Independent sanger sequencing data of the random 8 colonies of the transformants with the representative plasmids (pYK02 and pYK06) were shown

Supplementary Fig. 2

PAM-flexible Target-AID with NG-Cas9 vector and its self-targeting effect. (a) pYK12 was developed based on pYK6 by replacing coding sequence for Cas9. Sanger sequencing spectra of the constructed plasmids containing targeting sequences (*upp* (top) and *ftsZ* (bottom)) showed mixed populations at the crRNA region. (b) The target sequences for *upp* and *ftsZ* on the genome of *L. plantarum*. (c) The editing frequencies are determined by sequencing eight randomly selected colonies. The highest frequency among the edited base positions in each colony was plotted as its representative value. The mean editing frequencies are shown as bar graphs, with error bars representing the standard deviation.

Supplementary Fig. 3

Multiplex Target-AID base editing in *L. plantarum.* (a) pYK06-s (single), -d (dual), and -t (triple) were constructed based on pYK06 by inserting in tandem each crRNA expression cassette containing a promoter, a crRNA with a target sequence and a terminator. (b) Three target sequences are selected from *urdA* gene and editable cytosines are shown in red. (c) The editing

frequencies at the three sites are determined by sequencing six randomly selected colonies and plotted at each editable base position. The mean editing frequencies (bars) are shown with standard deviation (error bars)

Supplementary Fig. 4

Transformation efficiencies of *Lactobacillus plantarum* and *Lactobacillus gasseri* with plasmids pYN_Lg2-4 and pYK07. Plates show transformations of *L. plantarum* WCFS1 (top) and *L. gasseri* ATCC 33323 (bottom) with pYN_Lg2-4 (Left) and pYK07 (Right). Plates were incubated at 30°C for 3 for *L. plantarum* and 7 days for *L. gasseri*

Supplementary Fig. 5

urdA editing with Target-AID in *L. plantarum*. (a) Sanger sequencing spectra of the four independent colonies edited at *urdA*. (b) The editing frequencies are plotted at each editable base position. The mean editing frequencies (bars) are shown with standard deviation (error bars) from four randomly selected colonies

Supplementary Fig. 6

Sanger sequencing analysis of *ftsZ* targets edited by Target-AID. Sequencing data for the five successfully edited targets are shown, with the wild-type sequences are shown above each chromatogram. The target and PAM sequences are highlighted in boxes. The edited bases are indicated by arrows

Supplementary Fig. 7

Experimental workflow for editing and establishing edited strains in *Lactobacillus*. Target-AID expressing vectors are introduced into cells via electroporation. The transformants with the plasmid are selected on the plate, and randomly selected colonies undergoes sequencing to investigate editing at the target sites. Edited colonies are cultured in antibiotic-free medium to remove the Target-AID plasmid, and plasmid-free strains are confirmed by PCR and sequencing to verify the absence of plasmid and the presence of the desired mutations

Supplementary Note 1

pYN_Lg4

Teal: Pldh(Lc) promoter

Blue: tracrRNA

Green: Ppgm(La) promoter

Lime: P23 (Ll) promoter

Maroon: crRNA direct repeat

Lavender: pIB184-derived seq (*repD*, *repE*, and *ermB*)

Cyan: ColE1 ori

Yellow: KanR

Uppercase: ORF

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Supplementary Note 2

pYK01

Blue: tracrRNA

Green: gyrA-ldh (Sm) bidirectional promoter

Pink: nCas9(D10A)

Brown: SH3

Red: PmCDA1

Apricot: SHL

Purple: UGI

Lime: P23 (Ll) promoter

Maroon: crRNA direct repeat

Lavender: pIB184-derived seq (*repD*, *repE*, and *ermB*)

Cyan: ColE1 ori

Yellow: KanR

Uppercase: ORF

Underline: gRNA spacer sequence (upp)

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267 ATTGAACGCATGACAAACTTTGATAAAAATCTTCCAAATGAAAAAGTACTACCAAAACATAGTT

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308 AGGTGACGGTGGAGGAGGTTCTGGAGGTGGAGGTTCTGCTGAGTATGTGCGAGCCCTCTTTGAC

309 TTTAATGGGAATGATGAAGAGGATCTTCCCTTTAAGAAAGGAGACATCCTGAGAATCCGGGATA

310 AGCCTGAGGAGCAGTGGTGGAATGCAGAGGACAGCGAAGGAAAGAGGGGGATGATTCCTGTCCC

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430 gttccgcgacc

431

Supplementary Note 3

pYK06

Teal: Pldh(Lc) promoter

Blue: tracrRNA

Green: Ppgm(La) promoter

Pink: nCas9(D10A)

Brown: SH3

Red: PmCDA1

Apricot: SHL

Purple: UGI

Lime: rrn4a(Lp) promoter

Maroon: crRNA direct repeat

Magenta: rrnB(Ec) terminator

Lavender: pIB184-derived seq (*repD*, *repE*, and *ermB*)

Cyan: ColE1 ori

Yellow: KanR

Uppercase: ORF

Underline: gRNA spacer sequence (upp)

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Supplementary Note 4

Distinct elements from pYK01 and pYK06 in the other vectors

Prrn4b (Lp)

attggattaattgttgacagtttcataactggctggtatattagtaaacgttgct

Prrn3a (Lp)

ttataaaaagatgttgacagcttgttctgatgatgataaactttaatagttgcg

rrnA (Ls) terminator for tracrRNA

aaaaaggttgaaccttagggttcagccttttt

rrnB T1 (Ec) terminator for crRNA

ataaaacgaaaggctcagtcgaaagactgggcctttcgttttat

dCas9

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NG-nCas9

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