Efficient CRISPR/Cas12a-based genome editing toolbox for metabolic engineering in *Methanococcus maripaludis*

Jichen Bao, Silvan Scheller*

Department of Bioproducts and Biosystems, School of Chemical Engineering, Aalto University, FI-02150, Espoo, Finland

*corresponding author: Silvan Scheller (silvan.scheller@aalto.fi)

Abstract

Methanococcus maripaludis is a fast-growing and genetically tractable methanogen. To become a useful host organism for the biotechnological conversion of CO_2 and renewable hydrogen to fuels and value-added products, its product scope needs to be extended. Metabolic engineering requires reliable and efficient genetic tools, in particular for genome editing related to the primary metabolism that may affect cell growth.

We have constructed a genome editing toolbox by utilizing Cas12a from *Lachnospiraceae bacterium* ND2006 (LbCas12a) in combination with the homology-directed repair machinery natively present in *M. maripaludis*.

The toolbox enables gene knock-out with a positive rate typically above 89%, despite *M. maripaludis* being hyper-polyploid. We have replaced the flagellum operon (around 8.9kb) by a β-glucuronidase gene to demonstrate a larger deletion, and to enable quantification of promotor strengths.

The CRISPR/LbCas12a toolbox presented here is currently perhaps the most reliable and fastest method for genome editing in a methanogen.

Introduction

Methanogenic archaea are the methane producers in anaerobic digestion[1], they are the biocatalysts in power-to-gas processes[2], they are crucial players in the global carbon cycle[3], and they are even studied by astrobiologists[4,5]. Hydrogenotrophic methanogens use the reductive acetyl-CoA pathway for CO_2 fixation[6], an energy-efficient route to synthesize organic carbon from CO_2 and hydrogen that is similar to the pathway present in acetogens. There are, however, subtle differences between the acetogenic[7] and the methanogenic way of CO_2 reduction in terms of ATP vs. ion-gradient investment, and in terms of cofactor utilization[8]. It is therefore recommended to consider both, acetogens and methanogens, as possible host organisms for the production of fuels and chemicals from CO_2 as the carbon source.

Methanococcus maripaludis is a promising methanogenic host organism due to its fast growth rate (doubling time = ca. 2h,[9,10], growth at moderate temperature (T = 38 °C), and its ability to fix nitrogen [11,12,13]. The electron donors reported for CO_2 reduction are formate, hydrogen and electrons derived from bioelectric systems [14,15]. To extend the product scope of methanogens beyond methane, metabolic engineering in *M. maripaludis* has been pioneered by extending its mevalonate pathway to produce geraniol from CO_2 and formate[16].

Efficient and reliable genome editing tools are required to promote metabolic engineering in *M. maripaludis*. Marker recycling is prerequisite for multi target engineering. Although a pop-in-pop-out based markerless genome editing technique for *M. maripaludis* has been reported in 2005, there remains an issue of high false positive rate. The positive editing rate is sometimes below 5%[17], especially for modifications that affect cell growth. A CRISPR/Cas system (CRISPR = clustered regularly interspaced short palindromic repeats; Cas = CRISPR associated protein) may solve this issue due to its high efficiency in genome editing.

The CRISPR/Cas9 system has been successfully applied in many organisms for genome editing[18,19,20,21,22] due to its simplicity and high efficiency. Few CRISPR genome editing toolboxes have been developed for archaea[23]. The first Cas9-mediated genome editing system in a methanogen has been reported in 2017 for the model organism *Methanosarcina acetivorans*[24]. The Cas9-based system recognizes and cleaves the target sequence (20nt) which directly has a PAM sequence (PAM = protospacer adjacent motif) of NGG downstream.

Unlike Cas9, Cas12a recognizes a 5'-T-rich PAM sequence. Cas12a is therefore the better option for developing a CRISPR toolbox in microbes with an AT-rich genome. In addition, Cas12a possesses ribonuclease activity, which allows formation of multiple crRNAs from one transcript[25,26].

Considering the low genomic GC content of *M. maripaludis*, we have adopted Cas12a from *Lachnospiraceae bacterium* ND2006 (LbCas12a) and combined it with the intrinsic homology-directed repair machinery to establish a CRISPR genome editing toolbox. We investigated how the length of the repair fragment (RF) and the distance of the RF to the double stranded break (DSB) affects the editing efficiency. To apply our toolbox, we have deleted the flagellum operon and replaced it by a β-glucuronidase.

Results

<u>CRISPR/Cas12a-based introduction of double-stranded breaks</u>

The host strain *M. maripaludis* JJΔupt was used for all experiments, applying the natural transformation protocol recently established [27]. The *Escherichia coli*/*M. maripaludis* shuttle vector pLW40[28] was used as a backbone to construct the final toolbox plasmid pMM002P (Table 1). The schematic diagram of the toolbox plasmid and description of all its components is given in Fig.1a.

The transformation efficiency of pMM002P in JJΔupt was ca. 5 x 10^4 cfu/2µg DNA (Fig. 1b), which is higher than the reported efficiency of the empty plasmid pLW40neo (2.4 x10^3 cfu/µg DNA[27]). The high transformation efficiency indicates that expressing LbCas12a alone is not toxic to the host cell. When one gRNA was co-expressed with LbCas12a, only 3-18 colonies can be observed on the transformation plates. The number of transformants is far less than the cells transformed with pMM002P. When two gRNAs were co-expressed, only 0-3 colonies were formed (Fig. 1b). These results indicated that LbCas12a-crRNA complex can perform a lethal double stranded break in *M. maripaludis*.

Genome editing via CRISPR/LbCas12a by providing a repair fragment

A gRNA was expressed on pMM002P (p002-218) targeting to the gene *flaI* within the flagellum operon. The plasmid containing a functional gRNA is lethal to the host in the absence of a RF. The lethal effect can be relieved by including RFs with various lengths of the homology arms (Fig. 2a). When the 1000 bp long homologous arm was provided, the transformation efficiency reached 2.8 x 10^4 cfu/2 µgDNA. Thus, 1000 bp long homologous arms were used as the standard condition in the following experiments. Notably, the 250 bp long homologous arms are enough to rescue the DSB generated by Cas12a/gRNA complex, albeit with two orders of magnitude lower in transformation efficiency.

Influence of the distance between the RF and the DSB on the transformation efficiency: Distances 25, 500, and 1000 bp have been tested (Fig. 2b). At 500 bp distance, the transformation efficiency was lower than at 25 bp, but there was no significant difference between 500 and 1000 bp.

Determination of the positive rate

A pair of primers were used to amplify the up- and down-stream of the homologous arms on the chromosome. The PCR products were further digested by NotI. The NotI restriction site was pre-designed between the left and the right RF in order to distinguish the wildtype copies and the edited ones (Fig. S1-S3). The system enabled 89%-100% positive rate of genome editing (Fig. 2a, Fig. S1-S5). The results indicate that the CRISPR/Cas12a toolbox performs efficient genome editing in *M. maripaludis*.

Providing the repair fragment as a separate plasmid or as a PCR fragment

The CRISPR/LbCas12a cleavage plasmid was co-transformed with a suicide plasmid containing the RF. We successfully got transformants, but the transformation efficiency was ca. 10-50 fold lower.

When the CRISPR/LbCas12a cleavage plasmid was co-transformed with a PCR fragment as the RF, the efficiency was ca. 100-1000 times lower compared to our optimized method with the full p002-g218-L1000 plasmid.

Although the transformation efficiency decreased by this “quick and dirty” variant of the method, the positive rate appears not to be affected. We made 14 different constructs with suicide plasmid, and randomly tested 3 transformants of each construct. All 42 colonies were correct according to colony PCR results (data not shown).

Heterologous gene expression via CRISPR/Cas12a toolbox

To study whether the CRISPR/Cas12a system could perform an efficient heterologous gene integration, we replaced all the *fla* genes (*flaB1B2B3CDEFGHIJ*, total length 8890 bp) in the flagella operon by *uidA* from *E. coli*, coding for a β-glucuronidase. There was no significant difference in the transformation efficiency when using one gRNA or two gRNAs (Fig. 3). The plasmid with one gRNA generates one lethal DSB on chromosome resulting in two long distance from DSB to the repair fragment, one is 6.4 kbp and the other one is 2.5 kbp, while the plasmid with two gRNAs cut the *fla* genes ORF at two different sites shortening the distance from DSB to the repair fragment, one is 250 bp and the other one is 3.2 kbp. The positive rate was decreased when the distance was 6.4 kbp long (Fig. 3, Fig. S6 and S7), indicating that the positive rate would be affected when the distance is very long.

The CRISPR plasmid in the *uidA* integration strain was removed by streaking on the plate containing 6-azauracil.

Functional expression of the *uidA* gene has been confirmed by measuring the corresponding enzyme activity. Wildtype *M. maripaludis* did not show β-glucuronidase activity (OD_{405}: 0/1 OD_{600}), while the β-glucuronidase activity was detected in the *uidA* integrated strain (OD_{405}: 69.9 ± 1.4/1 OD_{600}).

Discussion

M. maripaludis already had genetic tools and efficient transformation protocols[17,29,27] for standard applications, but sometimes many colonies need to be screened to obtain the desired genotype by the classic pop-in-pop-out genome editing technique[17], especially when the targets to be engineered affect cell growth[30].

As a solution, we have established an efficient CRISPR/Cas12a toolbox in *M. maripaludis* for gene knock-in and knock-out with a high positive rate. Combining all experiments presented in Fig. 2, 63 of 66 colonies were correctly edited, which corresponds to an average positive rate of ca. 95%.

Different from the conventional technique, the CRISPR/Cas system only requires one round of homologous recombination, and no merodiploid is formed, which dramatically lowers the workload of genome editing and increases the success rate. The option of providing the repair fragment separately as a PCR template or as a suicide plasmid may further speed up the editing progress.

An additional advantage of Cas12a is its intrinsic ribonuclease activity, which can process multiplex crRNA in one transcript without extra ribonuclease[25,26]. Therefore, it can be convenient to express two crRNAs in the CRISPR/LbCas12a system, saving time and cost of plasmid construction.

Because *M. maripaludis* JJ strain has intrinsic PstI restriction/modification system, the host cells can digest the foreign DNA containing unmethylated PstI sites, and one PstI site may cause a 1.6-3.4 fold decrease in transformation efficiency[29]. There is one PstI site on the homologous arm on both plasmids (500 bp and 1000 bp), resulting in a lower no. of transformants (Fig 2b). The positive rate was at around 89%-100% (Fig. S1, S4 and S5). Thus, the editing efficiency is not significantly affected when the distance to the DSB is less than 1000 bp.

The CRISPR/LbCas12a toolbox is able to make a large fragment deletion in the genome. However, the positive rate is affected when the distance from RF to DSB is too large. A similar observation has been reported in *M. acetivorans*: The negative effect on the positive rate became significant when the distance to the DSB was larger than 1000 kb[24], and the transformation efficiency decreased with increasing distance[24]. Thus, the concept of using two crRNAs to shorten the distance from RF to DSB

demonstrated here may also improve the positive rate in other methanogenic host organisms for deleting a large fragment.

Proteins can be expressed heterologously in *M. maripaludis* by stable integration of the corresponding genes via the CRISPR/Cas12a toolbox. Utilizing *M. maripaludis* as an expression host may be advantageous for proteins that are difficult to be expressed in *E. coli*, such as formate dehydrogenases[15], methyl-coenzyme M reductases[31], or heterodisulfide reductases[32].

In conclusion, the CRISPR/LbCas12a toolbox can perform efficient genome editing in *M. maripaludis*, requiring only one round of recombination, which provides a solution for editing targets that affect cell growth.

Materials and methods

Strain and plasmids

M. maripaludis JJΔupt[27] and plasmid pLW40[28] are gifts from Prof. Kyle Costa, University of Minnesota. Plasmid pMEV4[16] was kindly provided by Prof. William B Whitman, University of Georgia. *M. maripaludis* S2 was kindly provided by Prof. John Leigh and Dr. Thomas Lie[33]. NEB5α (New England Biolabs) was used for plasmid construction. The plasmid pMM002P was constructed by Gibson assembly method[34]. All the cleavage plasmids were constructed as follow: (1) design the forward primer as "5'-AGAT+(23nt guide sequence)", design the reverse primer as "5'- TATC+(23nt reverse complement guide sequence)" (2) anneal the two primers which contains the gRNA and have 4 nt overhang at 5'-end (3) ligate the annealing product with PaqCI (New England Biolabs) digested pMM002P. The guide sequences were designed on CHOPCHOP (https://chopchop.cbu.uib.no/)[35]. The RF was inserted into the corresponding cleavage plasmid at the MreI restriction site. All the plasmids and strains used in this study are listed in Table S1 and Table S2.

Medium and cultivation conditions

Luria–Bertani medium with 50 mg/L ampicillin was used for plasmid amplification. McC medium was used for *M. maripaludis* strain cultivation with 2.8 bar 80%H_2/20%CO_2 at headspace[27]. 2.5 µg/mL puromycin or 0.25 mg/mL 6-azauracil was added if needed.

M. maripaludis transformation

Natural transformation protocol was used[27]. Briefly, 5 ml of *M. maripaludis* culture was grown for overnight; 2 ug of DNA was add into the culture directly on the next day morning; then flush and pressurize the cultivation tube with 2.8 bar 80%H_2/20%CO_2; incubate the tube on a shaker at 37 degree with 200 rpm for 4 hours; plate the cells on McC with puromycin plates.

β-glucuronidase activity measurements

4-Nitrophenyl β-D-glucuronide (4-NPG) (Sigma-aldrich) was prepared in 50mM sodium phosphate buffer (PBS) (pH=7.0) as a stock solution (10mg/ml). The details procedure was described below: harvest 1 OD of cells by centrifugation; resuspend the cells by 400 µl PBS; add around 30 µl glass beads; vortex the tube for 5 min to break the cells thoroughly; centrifuge the tube and collect the supernatant; dilute the sample to 500 µL by PBS; incubate the sample at 37 degree for 20 minutes; add 40 µL 4-NPG; let the reaction run 10-30 minutes; add 400 µL 200mM Na_2CO_3 to stop the reaction; read the absorbance at 405 nm.

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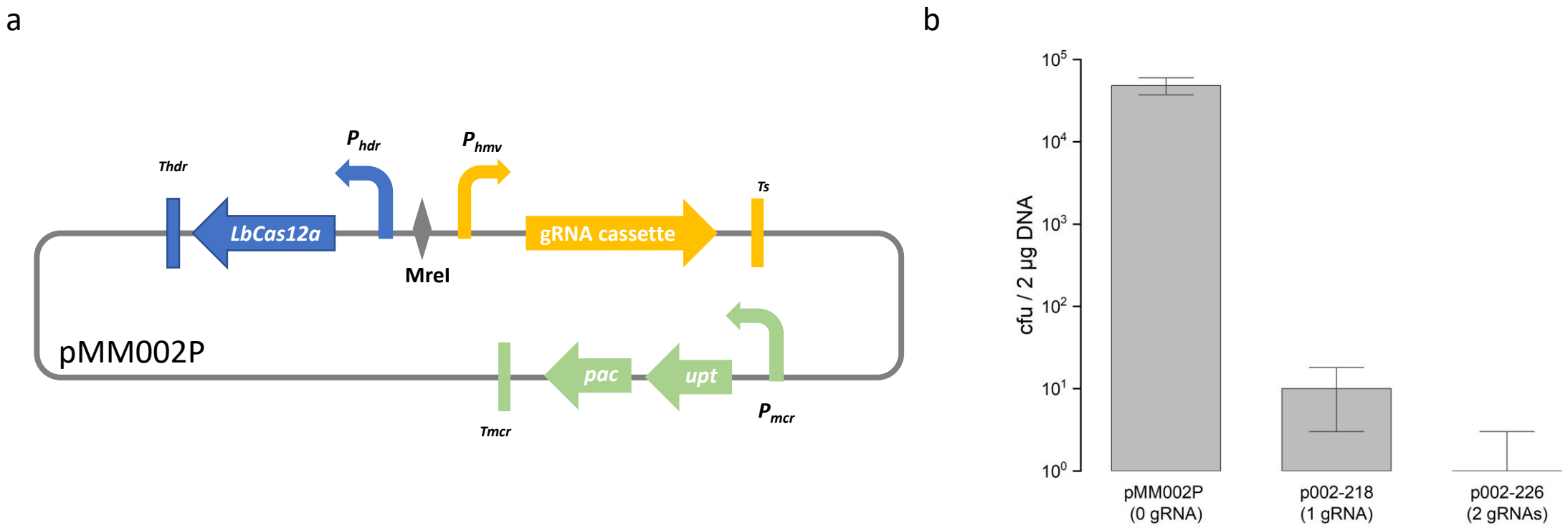

Fig. 1 The feature of CRISPR/LbCas12a toolbox. a) the schematic diagram of pMM002P. The uracil phosphoribosyltransferase (*upt*) from *M. maripaludis* S2 as a counter-selective marker was co-expressed with the codon-optimized puromycin N-acetyltransferase (*pac*) under P_{mcr} promoter (16). The promoter P_{hdr} from *Methanococcus voltae* A3 was used to drive the expression of LbCas12a (25). The histone promoter P_{hmv} from *M. voltae* A3 was used to express crRNA. Two PaqCI sties were placed between the direct repeat sequence and the synthetic terminator in the opposite direction for spacer fusion. The crRNA of the CRISPR tool plasmid pMM002P containing two PaqCI sites cannot target onto the native chromosome. An MreI restriction site was assigned between the promoters, P_{hdr} and P_{hmv}, for RF insertion if needed. b) cleavage test of pMM002P.

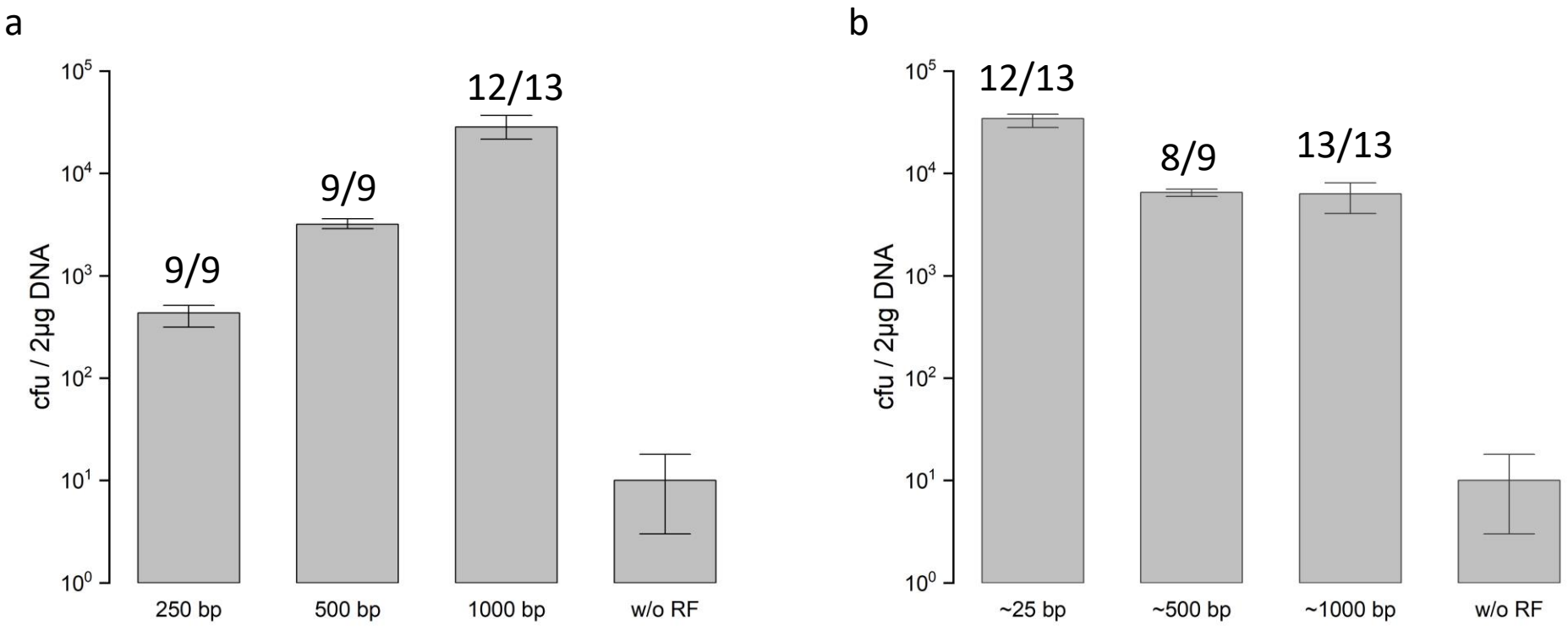

Fig. 2 Length and distance test of CRISPR/LbCas12a genome editing toolbox in *M. maripaludis*. a) the transformation efficiency of different length of the RF, and the distance from the RF to DSB is 25 bp. b) the transformation efficiency of different distance from the RF to the DSB, and the length of the RF is 1000 bp. The numbers over the bars the indicates the positive rate from colony PCR result.

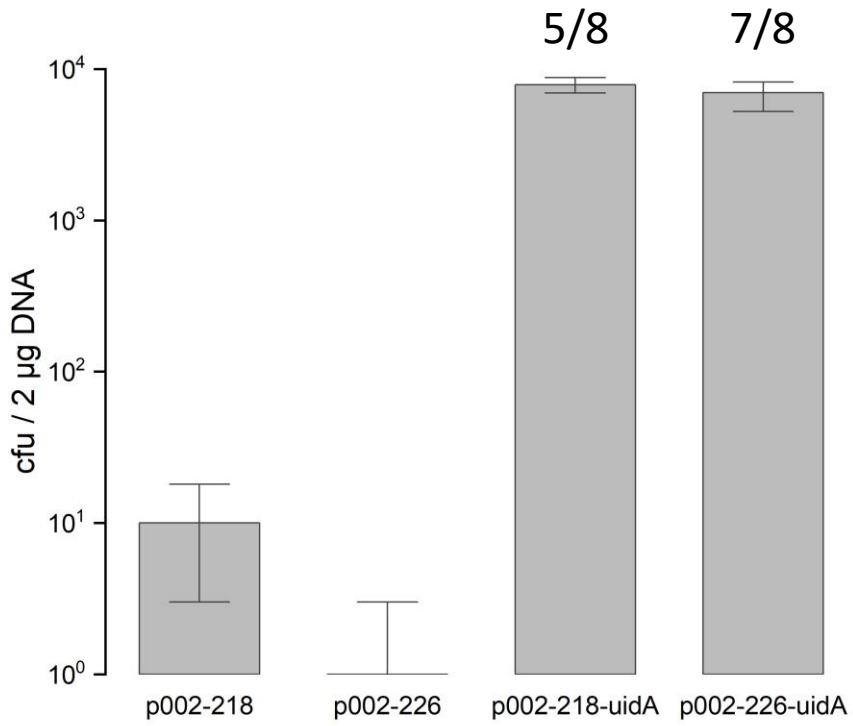

Fig. 3 Replacing the whole ORF of flagellum operon with *uidA* from *E. coli*. p002-218 expresses one gRNA, p002-226 expresses two gRNAs. “uidA” indicated if the RF is fused to the corresponding plasmid. The numbers over the bars indicates the positive rate from colony PCR result.

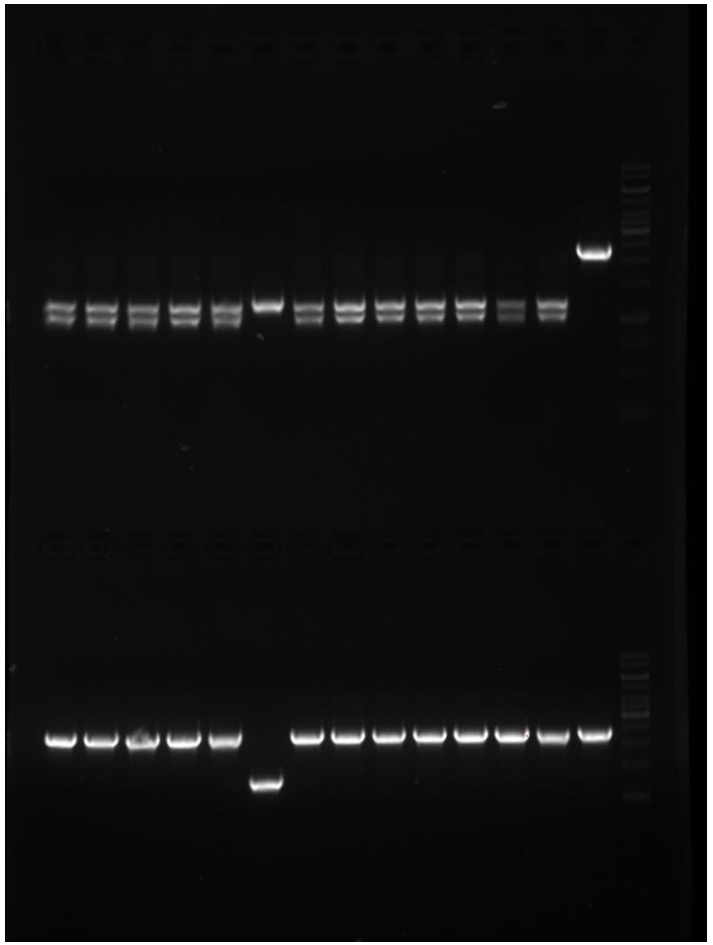

Fig. S1 Colony PCR result of p002-218-D25 (p002-218-L1000) edited colonies. Col. 1-13 are samples; Col. 14 is the wildtype genome. Col. 15 is the DNA ladder (Thermoscientific GeneRuler 1 kb DNA ladder #SM0311). The first row is the NotI digested PCR products; the second row is the PCR products. The primers amplify from 225 bp upstream and 69 bp downstream of the RF on the genome. The expected size of the PCR products of the edited transformants and wildtype genome is 2302 bp and 2350 bp. The NotI digested PCR products. The edited transformants should have two bands: 1227 bp and 1075 bp. The PCR product of the wildtype genome is not digestible by NotI.

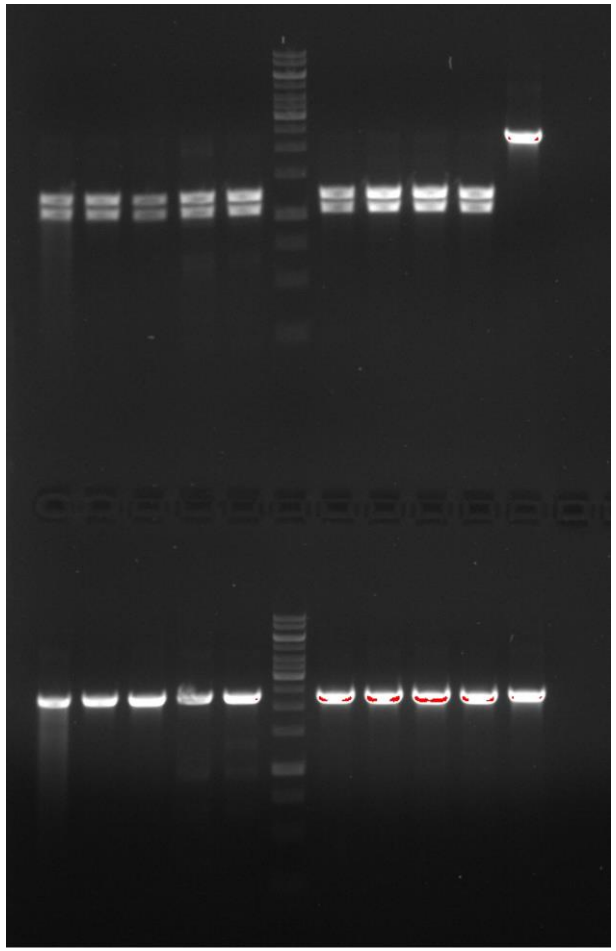

Fig. S2 Colony PCR result of p002-218-L500 edited colonies. Col. 1-5, 7-10 are samples; Col. 11 is the wildtype genome. Col. 6 is the DNA ladder (Thermoscientific GeneRuler 1 kb DNA ladder #SM0311). The first row is the NotI digested PCR products; the second row is the PCR products. The primers amplify from 725 bp upstream and 569 bp downstream of the RF on the genome. The expected size of the PCR products of the edited transformants and wildtype genome is 2302 bp and 2350 bp. The NotI digested PCR products. The edited transformants should have two bands: 1227 bp and 1075 bp. The PCR product of the wildtype genome is not digestible by NotI.

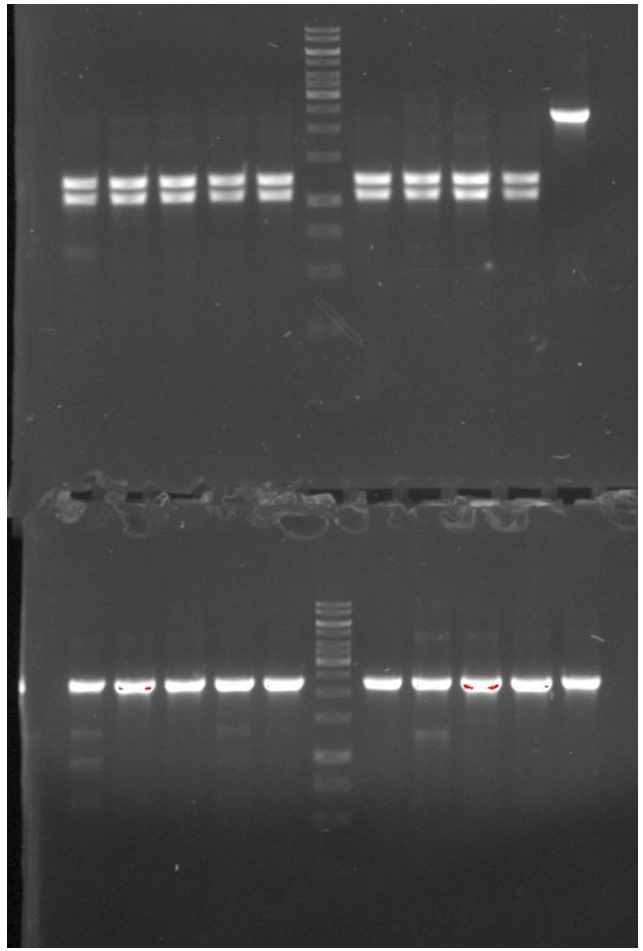

Fig. S3 Colony PCR result of p002-218-L250 edited colonies. Col. 1-5, 7-10 are samples; Col. 11 is the wildtype genome. Col. 6 is the DNA ladder (Thermoscientific GeneRuler 1 kb DNA ladder #SM0311). The first row is the NotI digested PCR products; the second row is the PCR products. The primers amplify from 975 bp upstream and 819 bp downstream of the RF on the genome. The expected size of the PCR products of the edited transformants and wildtype genome is 2302 bp and 2350 bp. The NotI digested PCR products. The edited transformants should have two bands: 1227 bp and 1075 bp. The PCR product of the wildtype genome is not digestible by NotI.

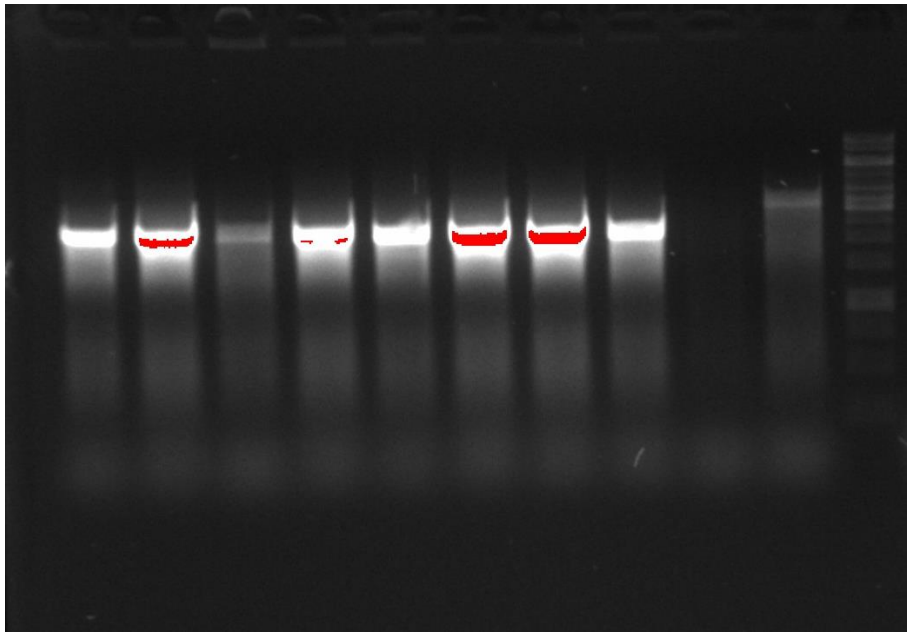

Fig. S4 Colony PCR result of p002-218-D500 edited colonies. Col. 1-9 are samples; Col. 10 is the wildtype genome. Col. 11 is the DNA ladder (Thermoscientific GeneRuler 1 kb DNA ladder #SM0311). The primers amplify from 215 bp upstream and 57 bp downstream of the RF on the genome. The expected size of the PCR products of the edited transformants and wildtype genome is 2280 bp and 3274 bp.

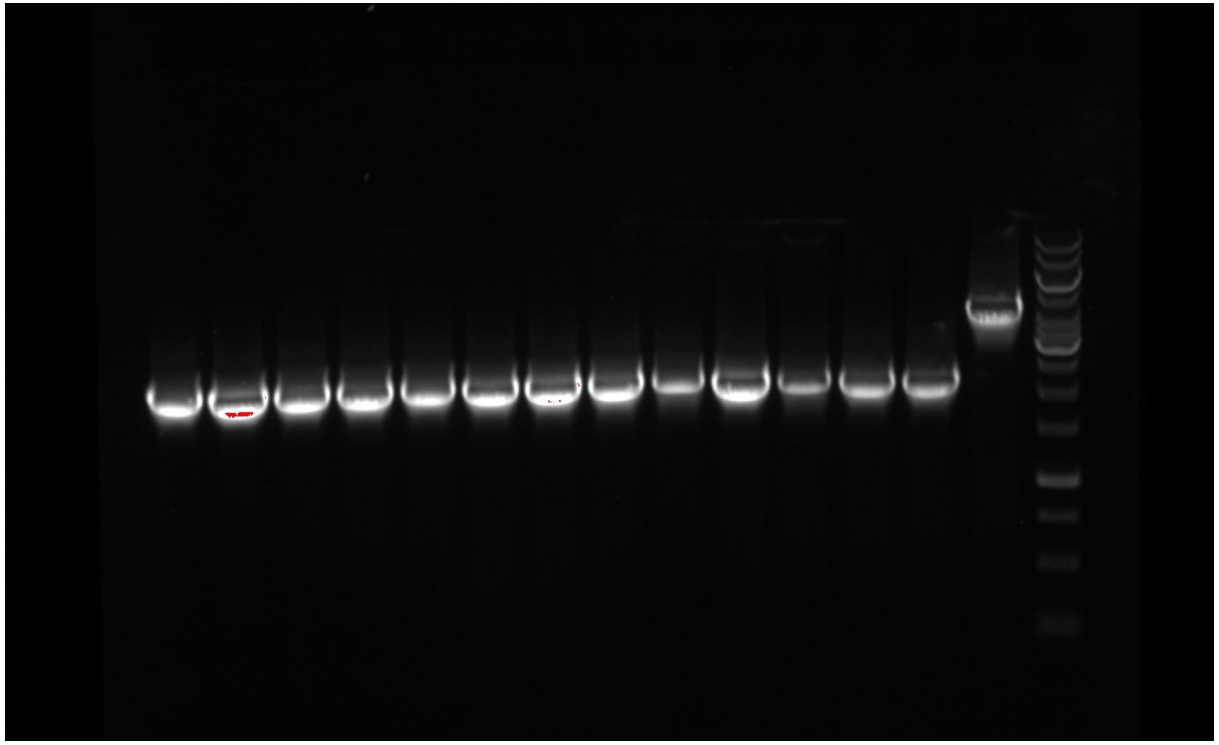

Fig. S5 Colony PCR result of p002-218-D1000 edited colonies. Col. 1-13 are samples; Col. 14 is the wildtype genome. Col. 15 is the DNA ladder (Thermoscientific GeneRuler 1 kb DNA ladder #SM0311). The primers amplify from 64 bp upstream and 102 bp downstream of the RF on the genome. The expected size of the PCR products of the edited transformants and wildtype genome is 2174 bp and 4078 bp.

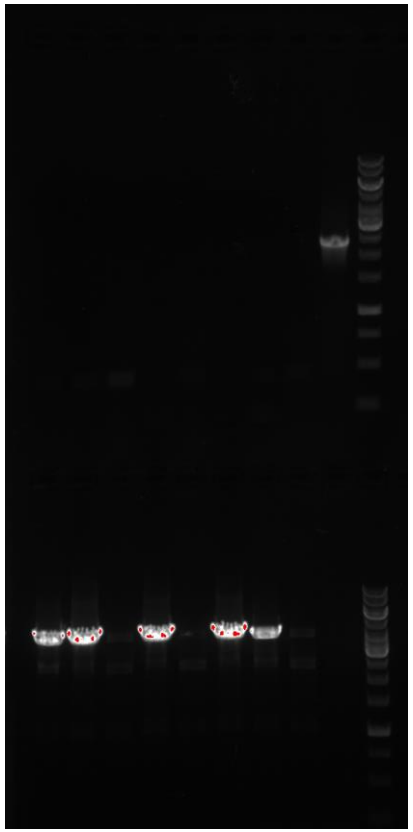

Fig. S6 Colony PCR result of p002-218-uidA edited colonies. Col. 1-8 are samples; Col. 9 is the wildtype genome. Col. 10 is the DNA ladder (Thermoscientific GeneRuler 1 kb DNA ladder #SM0311). Two pairs of primers were used to check whether the whole flagellum ORF were replaced with uidA. The first row is the amplicon of the part of the flagellum ORF. The expected size of the amplicon of the edited transformants and wildtype genome is no band and 2350 bp. The second row is the amplicon from 64 bp upstream and 86 bp downstream of the RF on the genome. The expected size of the PCR products of the edited transformants and wildtype genome is 3905 bp and 10983 bp. Unfortunately, our polymerases are not able to amplify a huge fragment (larger than 8 kb).

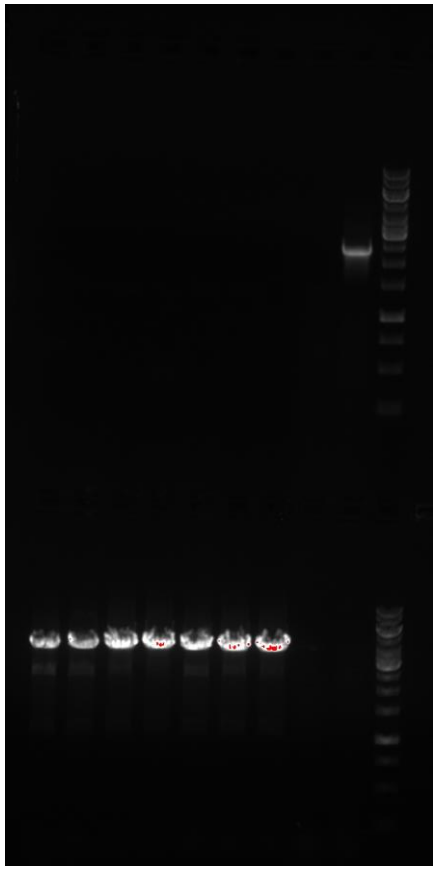

Fig. S7 Colony PCR result of p002-226-uidA edited colonies. Col. 1-8 are samples; Col. 9 is the wildtype genome. Col. 10 is the DNA ladder (Thermoscientific GeneRuler 1 kb DNA ladder #SM0311). Two pairs of primers were used to check whether the whole flagellum ORF were replaced with uidA. The first row is the amplicon of the part of the flagellum ORF. The expected size of the amplicon of the edited transformants and wildtype genome is no band and 2350 bp. The second row is the amplicon from 64 bp upstream and 86 bp downstream of the RF on the genome. The expected size of the PCR products of the edited transformants and wildtype genome is 3905 bp and 10983 bp. Unfortunately, our polymerases are not able to amplify a huge fragment (larger than 8 kb).

Table S1 Plasmid list

Plasmid	Description	Reference
pLW40	*E. coli*/*M. maripaludis* shuttle vector; the backbone of pMM002P	28
pMEV4	*E. coli*/*M. maripaludis* shuttle vector, only replicates in *M. maripaludis* S0001[36], carrying codon-optimized *pac*	16
pY016	Nucleic acid sequence source of LbCas12	25
pMM002P	CRISPR/LbCas12a toolbox plasmid	This study
p002-218	pMM002P containing a guide sequence 5'-CCCACAGATGAATATACTCATTCC targeting to *flaI*	This study
p002-226	pMM002P containing guide sequences 5'-GATATACCCGACACAATGATATCG and 5' -CTGTTCCCACTCTACTAATTAAAA targeting to *flaJ* and *flaD*	This study
p002-218-L1000	p002-218, 1000 bp long homologous arms on each side, 25 bp from the RF to DSB	This study
p002-218-L500	p002-218, 500 bp long homologous arms on each side, 25 bp from the RF to DSB	This study
p002-218-L250	p002-218, 250 bp long homologous arms on each side, 25 bp from the RF to DSB	This study
p002-218-D500	p002-218, 1000 bp long homologous arms on each side, 500 bp from the RF to DSB	This study
p002-218-D1000	p002-218, 1000 bp long homologous arms on each side, 1000 bp from the RF to DSB	This study
p002-218-uidA	p002-218, P_{fla}-*uidA*-T_{fla}; the 1000 bp homologous arms on each side covers the promoter and the terminator of the flagellum operon	This study
p002-226-uidA	p002-226, P_{fla}-*uidA*-T_{fla}; the 1000 bp homologous arms on each side covers the promoter and the terminator of the flagellum operon	This study

Table S2 Strain list

Strain	Genotype	Remarks	Reference
S2	wildtype	Used as PCR template for *upt* gene	33
JJΔupt	Δ*upt*		27
JL1000	Δ*upt* Δ*flaI(849-904)::gcggccgc* with p002-218-L1000	JJΔupt was transformed with p002-218-L1000	This study
JL500	Δ*upt* Δ*flaI(849-904)::gcggccgc* with p002-218-L500	JJΔupt was transformed with p002-218-L500	This study
JL250	Δ*upt* Δ*flaI(849-904)::gcggccgc* with p002-218-L250	JJΔupt was transformed with p002-218-L250	This study
JD500	Δ*upt* Δ*flaI(378-1379)::gcggccgc* with p002-218-D500	JJΔupt was transformed with p002-218-D500	This study
JD1000	Δ*upt* Δ*flaH(621-693)IJ(1-179)::gcggccgc* with p002-218-D1000	JJΔupt was transformed with p002-218-D1000	This study
J218U	Δ*upt* Δ*flaB1B2B3CDEFGHIJ::uidA*	JJΔupt was transformed with p002-218-uidA, plasmid was removed afterwards.	This study
J226U	Δ*upt* Δ*flaB1B2B3CDEFGHIJ::uidA*	JJΔupt was transformed with p002-226-uidA, plasmid was removed afterwards.	This study