Single-Base Genome Editing in *Corynebacterium glutamicum* with the Help of Negative Selection by Target-Mismatched CRISPR/Cpf1

Hyun Ju Kim, Se Youngh Oh, and Sang Jun Lee

**Department of Systems Biotechnology, Chung-Ang University, Anseong 17546, Republic of Korea**

**Introduction**

*Corynebacterium glutamicum*, a gram-positive, aerobic, soil-dwelling bacterium, is generally regarded as safe (GRAS), and is widely used for the industrial production of amino acids [1-3]. Since numerous chemicals, materials, and biofuels can be synthesized from biomass via simple metabolic pathways, *C. glutamicum* has received increasing attention as a robust and versatile cell factory [4, 5]. Owing to the low transformation efficiency of *C. glutamicum* when compared to *E. coli*, genetic or genome engineering of this bacterium is a limitation with respect to its industrial applications [6].

CRISPR technology initially emerged as a prokaryotic adaptive immune system [7-9] and has been recently developed as an efficient in vivo mutagenesis method in various microbial strains including *C. glutamicum* [10, 11]. Cas proteins along with crRNAs can together recognize and cleave target DNAs with a specific protospacer-adjacent motif (PAM) sequence. Upon introduction of mutagenic oligonucleotides to alter the target DNA sequence, the Cas/crRNA complex can cleave the unedited DNA targets, thus rendering genome-edited cells alive; this process is called negative selection [12-14]. Therefore, the ratio of edited cells to unedited cells aided by CRISPR/Cas-mediated negative selection is markedly higher than that of homology-directed repair (HDR) and classical oligo-directed mutagenesis [15-17].

CRISPR/Cas9 cleaves DNA target sequences with its 5′-NGG PAM sequence, yielding blunt ends [18]. *cas9* expression reportedly retards growth of *C. glutamicum* probably owing to the toxicity of gene products [19]. However, it has been known that CRISPR/Cpf1 from *Francisella novicida* displayed lower toxicity in *C. glutamicum* cells [17]. CRISPR/Cpf1 can recognize and cleave the target DNAs with different PAM sequence (5′-TTN), which is useful for engineering of A+T rich region [20-22].

Single oligonucleotide-directed mutagenesis with the coexpression of RecT recombinase has been developed as
a rapid and efficient genome editing tool [23]. Two consecutive nucleotides have been successfully edited in the genome of *C. glutamicum* with very high efficiencies (86 to 100%) [17, 24]. However, single point mutations have been rarely reported in *C. glutamicum*. Single-base genome editing techniques that manipulate promoter strength and alter specific amino acid residues in cellular proteins are essential for future biotechnology. Recently, we reported efficient oligonucleotide-directed single base editing methods in *Escherichia coli*, using target-mismatched sgRNAs (single-molecular guide RNAs) during negative selection via CRISPR/Cas9 [25].

In this study, we tried to use target-mismatched CRISPR/Cpf1 system to change a single nucleotide in the genome of *C. glutamicum*. To monitor base editing through colony color change, we selected the *crtEb* gene that is involved in the biosynthesis of carotenoid pigments in *C. glutamicum*. Our results showed that the target-mismatched CRISPR/Cpf1 negative selection method helped efficient and accurate single-base editing in the *C. glutamicum* genome.

**Materials and Methods**

**Bacterial Strains and Culture Conditions**

The bacterial strains used herein are listed in Table S1. *E. coli* DH5α was cultured in Luria–Bertani (LB) broth at 37°C. *C. glutamicum* ATCC13869 was cultured in brain-heart infusion (BHI) broth at 30°C. Antibiotic concentrations, wherever necessary, were 25 μg/ml kanamycin, 50 μg/ml spectinomycin, and 25 μg/ml chloramphenicol for *E. coli* and 25 μg/ml kanamycin, 75 μg/ml spectinomycin, and 25 μg/ml chloramphenicol for *C. glutamicum*.

**Plasmid Constructions**

All plasmid constructs used herein are listed in Table S1. Furthermore, the primers and oligonucleotides used herein are listed in Table S2. Genomic DNA was extracted using the Wizard Genomic DNA purification kit (Promega A2611, USA). Plasmids and PCR products were extracted using the NucleoSpin Plasmid EasyPure kit (Macherey-Nagel, Germany, Cat No. 740727) and the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Cat No. 7400609), respectively. KOD FX polymerases were used for PCR (Toyobo, Japan, Cat No. KFX-101). DNA fragments were amplified and assembled to generate various plasmids, using the Gibson assembly mix (NEB, Hitchin, UK, Cat No. E2611).

To generate the cpf1 integration pHK487 plasmid (10.3 kb), we assembled three DNA fragments amplified as follows: first, pK18mobSacB [26] was used as a template to amplify a 5.7-kb DNA fragment, using P1/P2 primers. Second, a homologous region (0.5 kb) of *C. glutamicum* ATCC13869 genome (cg1211) was amplified using *C. glutamicum* ATCC13869 genomic DNA as a template and P3/P4 primers. The third DNA fragment (4.0 kb) of cpf1 under the control of the PlacM promoter was obtained through PCR amplification of pYS1Ptac plasmid with P5/P6 primers.

To generate the cpf1 integration pHK487 plasmid (10.3 kb), we assembled three DNA fragments amplified as follows: first, pK18mobSacB [26] was used as a template to amplify a ~1.5-kb DNA fragment, using pBL ts-ori and P1/P2 primers. Second, a ~0.7-kb region of the chromosomal cpf1 resistance gene was amplified using pLS360 [27] as a template and P3/P4 primers. These two fragments (~7.4 and 0.7 kb) were purified and recovered for isothermal assembly.

To introduce temperature-sensitive origin of replication in the crRNA expression vector, pYS1Ptac plasmid was used as a template to amplify a ~1.5-kb fragment of pBL ts-ori using P13/P14 primers. A ~2.9-kb crRNA backbone was amplified using pYS2_crtYF plasmid as a template and P15/P16 primers. These two fragments were purified and recovered for isothermal assembly to generate pHK473.

To construct perfect-matched and mismatched crRNA expression plasmids targeting *crtEb*, pHK473 was used as a template to amplify a ~1.9-kb fragment and a ~2.5-kb fragment. These two fragments were digested with DpnI and purified for isothermal assembly to generate pHK493. Other crRNA expression vectors (pHK494–pHK499) were generated using the same method as that used for pHK493 and confirmed through Sanger sequencing, using P35.

To generate crRNA-deleted plasmid (pHK475) for analyzing the transformation efficiency of HK1220/pHK489 competent cells, pHK473 was used as a template to amplify a ~8.0-kb fragment using 5′-phosphorylated P17/P18 primers. This fragment was treated with DpnI and self-ligated to construct pHK473. Thereafter, a ~7.4-kb fragment was amplified using pHK493 as a template and P9/P10 primers. A ~0.7-kb fragment of the chloramphenicol resistance gene was amplified using pLS360 [27] as a template and p11/P12 primers. These two fragments (~7.4 and 0.7 kb) were purified and recovered for isothermal assembly.

To introduce temperature-sensitive origin of replication in the crRNA expression vector, pYS1Ptac plasmid was used as a template to amplify a ~1.5-kb fragment of pBL ts-ori using P13/P14 primers. A ~2.9-kb crRNA backbone was amplified using pYS2_crtYF plasmid as a template and P15/P16 primers. These two fragments were purified and recovered for isothermal assembly to generate pHK473.

To construct perfect-matched and mismatched crRNA expression plasmids targeting *crtEb*, pHK473 was used as a template to amplify a ~1.9-kb fragment and a ~2.5-kb fragment. These two fragments were digested with DpnI and purified for isothermal assembly to generate pHK493. Other crRNA expression vectors (pHK494–pHK499) were generated using the same method as that used for pHK493 and confirmed through Sanger sequencing, using P35.

To generate crRNA-deleted plasmid (pHK475) for analyzing the transformation efficiency of HK1220/pHK489 competent cells, pHK473 was used as a template to amplify a ~8.0-kb fragment using 5′-phosphorylated P17/P18 primers. This fragment was treated with DpnI and self-ligated to construct pHK475.

**Electrocompetent Cells**

Electrocompetent *C. glutamicum* cells were generated as previously described with minor modifications [17]. *C. glutamicum* ATCC13869 and its derivatives were cultured on BHI agar. A single colony from each strain was inoculated into 15 ml of BHI supplemented with 50 mM sorbitol and 10 g/l glucose (BHISG), and cultured overnight at 30°C with agitation at 200 rpm. If needed, chloramphenicol (25 μg/ml) was added in the culture. From this pre-culture, 10 ml was inoculated into 200 ml of BHISG supplemented with 1 ml/l Tween 80 and 4 g/l glycine. Cells harboring pHK489 were treated with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for inducing RecT recombinase. Cells were harvested and rendered electrocompetent when the OD₆₀₀ approached ~1.0. Cells were chilled on ice for 20 min and harvested through centrifugation at 3,500 rpm and 4°C for 20 min and washed thrice with 50 ml of ice-chilled 10% glycerol. Competent cells were resuspended 2 ml of 10% glycerol, and 100-μl aliquots were stored at −80°C.
Plasmids (~2 μg) and oligonucleotides (1 μg) were added to the electrocompetent cells thawed on ice and then transferred into pre-cooled electroporation cuvettes and covered with 100 μl of 10% glycerol. Electroporation was performed at 25 μF, 200 Ω, and 2.5 kV, using 4°C precooled electroporation cuvette (width, 2 mm). Cells were immediately transferred to 800 μl of BHISG medium and heat-shocked for 6 min at 46°C. The cells were then allowed to recover for 3 h at 30°C with agitation at 180 rpm. Thereafter, recovered cells were spread on BHI containing chloramphenicol or spectinomycin and incubated for 72 h at 30°C. Pink colonies on agar plates were enumerated to determine the base editing efficiency, and nine colonies per plate were selected for Sanger sequencing.

**Results**

**Construction of a CRISPR/Cpf1- and RecT-Mediated Scarless Genome Editing System**

To stably express *cpf1* and generate a scarless genome editing system, we electroporated non-replicating plasmid pHK487 harboring *cpf1*, and confirmed the integration of pHK487 in the chromosome of *C. glutamicum* ATCC13869 strain, which was named the HK1220 strain (Fig. 1A). Subsequently, *recT*-expressing plasmid pHK489 was transformed into HK1220 cells, which were cultured, induced with IPTG, and harvested for electroporation of crRNA plasmids and mutagenic oligonucleotides (Fig. 1B). After obtaining genome-edited cells negatively selected using the CRISPR/Cpf1 system, plasmids were cured through culturing at high temperatures and *cpf1* was eliminated via the *sacB* (encoding levansucrase) counter-selection system.
Oligonucleotide-Directed Genome Editing of *crtE* in *C. glutamicum*

*crtE* in the carotenoid biosynthesis operon was selected as a target gene for base editing in the genome of *C. glutamicum*, the disruption of which resulted in the conversion of yellow to pink cells owing to lycopene accumulation in the impaired carotenoid synthesis pathway (Fig. 2) [28]. We introduced a stop codon (Y50Z) in the middle of *crtE*, through oligonucleotide mutagenesis and subsequent negative selection via the CRISPR/Cpf1 system, and we then estimated the base editing efficiency by enumerating the pink colonies among all surviving cells after transformation of crRNA plasmids and mutagenic oligonucleotides.

The crRNA plasmid pHK493 was electroporated with four different oligonucleotides inducing single (150T to G), double (150TA to GC), triple (150TAA to GCC), and quadruple (150T AAC to GCCA) base mutations in the genome, where a T150G transversion can cause a nonsense mutation (Y50Z) in *crtE* (Fig. 3A). Consequently, without mutagenic oligonucleotides, no pink cells were obtained. Double-, triple-, and quadruple-base-edited pink cells were obtained with very high editing efficiencies of 95.3, 91.5, and 92.0%, respectively (Fig. 3B). However, single-base-edited pink cells were rarely observed with a low editing efficiency of 0.6%, where the shape and size of transformant colonies were not homogeneous (Fig. S1), indicating that CRISPR/Cpf1-mediated negative selection was not efficient with single-base mutagenic oligonucleotides, presumably because single base-edited target DNA sequences can be also cleaved as a target by the Cpf1/crRNA complex, which is known as mismatch tolerance (Fig. 3C) [29]. When more than two-mismatched base pairs were present between edited DNA target and crRNA, the target seems not to be recognized by Cpf1/crRNA complex.

In the case of single-base oligonucleotides, 3.0 × 10^2 (CFU/μg DNA of pHK493) of transformant cells survived among the electrocompeent cells, with a transformation efficiency of ~10^6 CFU/μg DNA of pHK475 (Fig. 3B). Even without mutagenic nucleotides, 2.4 × 10^2 (CFU/μg DNA of pHK493) of transformant cells survived owing to failed negative selection with the CRISPR/Cpf1 system.

**Single-Base Genome Editing by Target-Mismatched crRNAs**

Since double-, triple-, and quadruple-base mutations were successfully obtained through negative selection, target-mismatched crRNAs were designed to cleave unedited DNA without cleaving a single-base-edited DNA sequence. One- to three-base-mismatched crRNA plasmids along with single-base mutagenic oligonucleotides were electroporated into IPTG-induced HK1220/pHK489 cells for negative selection of single-base-edited DNA sequences (Fig. 4A). Consequently, in cases of single-base-mismatched crRNAs (pHK494 and pHK497), pink colonies were obtained with efficiencies of 14.9 and 99.7%, respectively. When double-mismatched crRNA (pHK495) were used, we obtained single-base edited pink colonies with an editing efficiency of 91.5%. At higher editing efficiencies, the shape and size of transformant colonies were more homogeneous (Fig. S3). When another double-mismatched (pHK498) and two triple-mismatched crRNAs (pHK496 and pHK499) were used, no pink colonies were observed at higher transformation efficiencies (~10^6 CFU/μg DNA), indicating that those crRNAs cannot recognize even unedited DNA target sequences.

Furthermore, pink colonies were randomly selected from negatively selected pink colonies (pHK493, pHK494, pHK497, and pHK495), and Sanger sequencing was carried out for edited regions in *crtE* (Fig. 5). Consequently, in case of perfectly matched crRNA (pHK493), two of nine regions were accurately altered. In the case of single-
mismatched pHK494, three of nine regions were perfectly edited. In the case of pHK497 (single-mismatched) and pHK495 (double-mismatched), all T-to-G single-base edits were successful, as intended. These results indicated that the use of target-mismatched crRNA is not only an efficient but also an accurate negative selection method for single-base genome editing using the CRISPR/Cpf1 system.

**Discussion**

Since bacterial cells synthesize valuable metabolites as encoded by their genomes, precise editing of microbial genomes is indispensable for the design of microbial cell factories. CRISPR/Cas9 (or Cpf1) technologies have been recently developed to edit genome sequences in numerous cellular platforms including *C. glutamicum* [30]. The
PAM sequence (5′-NYTV) of CRISPR/Cpf1 [31] does not restrict editing of the genome of industrial C. glutamicum strains with G+C contents of 53.8% [32]. Moreover, owing to the potential toxicity of CRISPR/Cas9, the CRISPR/Cpf1 system of C. glutamicum has received increasing attention [11, 17, 33, 34].

Herein, we integrated cpf1 in the C. glutamicum genome because frequent transformations were laborious for introducing a genome editing tool into C. glutamicum with a low transformation efficiency. The intergenic region between cg1121-cg1122 has been often used as an integration site of foreign genes in C. glutamicum genome [35, 36], which might not affect cellular growth and/or physiology of C. glutamicum. First, cpf1 was introduced into the cg1121-cg1122 intergenic region of C. glutamicum ATCC13869 genome via a single-crossover to generate strain HK1220 (Fig. 1A).

Negative selection using CRISPR/Cpf1 facilitates the survival of genome-edited cells; however, unedited cells are eliminated through double-stranded breaks at target DNA sequences. Therefore, CRISPR/Cpf1 can increase the editing efficiency of surviving cells [38]. After transformation of IPTG-induced HK1220 cells harboring the
RecT plasmid (pHK489) with single-stranded mutagenic oligonucleotides and crRNA plasmids, the surviving cells putatively harboring the desired mutations were obtained through negative selection (Fig. 3A). The use of double, triple, and quadruple mutagenic oligonucleotides successfully introduced the TAG stop codon in the middle of \(crtEb\) with an editing efficiency (i.e., the proportion of pink colonies) of 91.5–95.3%. However, we rarely obtained pink colonies (0.6%) when using single-base-mutagenic oligonucleotides (Fig. 3B). Moreover, Sanger sequencing revealed successful single-base edits in 2 of 9 selected pink colonies generated through target-matched crRNA (pHK493) (Fig. 5), probably owing to mismatch tolerance, whereby the Cpf1/crRNA complex can recognize and cleave both single-base-edited and unedited targets (Fig. 3C), which have been assessed to resolve off-target effects [29, 39, 40].

Even upon transformation of only crRNA plasmids into cells without oligonucleotides, we still observed numerous surviving cells (~10^2 CFU/μg DNA of pHK493), probably owing to null \(cpf1\) mutations or the repair of double-strand breaks in target DNA sequences. Furthermore, heterogeneity in colony shape and size was observed primarily in cases of failed negative selection (Figs. S1 and S2). Accurately edited colonies were larger than unedited colonies in our Cpf1-mediated study, while larger colonies were false-positive during Cas9-mediated genome editing of \(C.\) glutamicum [33]. Therefore, colony size does not reflect successful genome editing on using CRISPR-mediated negative selection.

To differentiate single-base-edited targets from unedited targets, mismatched crRNAs were designed and used for precise CRISPR/Cpf1-mediated negative selection (Fig. 4A). With single-base-mutagenic oligonucleotides, different target-mismatched crRNA plasmids were transformed for single-base editing of T150G (i.e.,

| crRNA       | Target   |
|-------------|----------|
| pHK493 (0.6%) | TTTCTTTATCCC-GTAT-AACATGGCCATGAT |
| pHK494 (14.9%) | TTTCTTTATCCC-GTAT-AACATGGCCATGAT |
| pHK497 (99.7%) | TTTCTTTATCCC-GTAT-AACATGGCCATGAT |

Fig. 5. Sequence alignment of single-base-edited target regions in \(crtEb\). The PAM sequence of Cpf1 was underlined. Dots and bars indicate perfectly aligned nucleotides and gaps, respectively, in comparison with the target DNA sequence. The gray-shaded nucleotides indicate undesirable mutations. The black-shaded G indicate single-base-edited nucleotides (T150G) after genome editing. E01–E23 show precise single-base changes, and U01–U13 show undesirable substitutions and indels proximal to the edited target region. Parenthesis indicate the proportion of pink colonies among the surviving colonies.
introduction of TAG stop codon in crtEb. In cases of single-mismatched crRNAs (from pHK494 and pHK497), and one of double-mismatched crRNAs (from pHK495), 14.9%, 99.7%, and 91.5% of surviving colonies were pink owing to intracellular lycopene accumulation (Fig. 4B). Subsequently, pink colonies were randomly selected from each agar plate and their genomes were subjected to Sanger sequencing. Three of nine colonies were correctly edited among 14.9% pink colonies. The DNA sequences were accurately edited in all colonies among 99.7% and 91.5% of surviving colonies (Fig. 5), indicating that even if mutants harboring the desired phenotypes were obtained among the colonies obtained through negative selection, using the CRISPR/Cpf1 system, the efficiency of harboring a genotype that is precisely altered to the base sequence can be much lower.

The transformation efficiencies reflected between $10^2$ and $10^6$ CFU/μg crRNA plasmid among genome-edited cells. However, in one case of double- and two cases of triple-mismatched crRNAs (from pHK498, pHK496, and pHK499), no pink colonies were observed. Moreover, the number of surviving colonies increased to $10^5–10^6$ cells. However, in one case of double- and two cases of triple-mismatched crRNAs (from pHK498, pHK496, and pHK499), no pink colonies were observed. Moreover, the number of surviving colonies increased to $10^5–10^6$ (CFU/μg crRNA plasmid). The increased number of surviving colonies indicates that Cpf1/target-mismatched crRNAs could not accurately recognize the targets, and consequently, improper negative selection facilitated the survival of all transformants on agar plates. As applicable design rules for target-mismatched sgRNAs in CRISPR/ Cas9 system have been provided [25], further studies should address how to design mismatched crRNAs in CRISPR/Cpf1 for single base editing in microbial genomes.

In summary, single-base genome editing is indispensable for repairing errors in nucleotide sequences in microbial cell factories. Moreover, useful genotypes representing evolved phenotypes can be introduced directly into new backgrounds through precise base editing methods. For example, promoter strength and/or transcriptional regulatory sequences can be altered, and codons of interest in the structural gene can be also edited. The target-mismatched crRNA method is an efficient negative selection tool for elaborate single base editing in C. glutamicum, which could be extended to other platform microbial cells.

**Acknowledgments**

This study was supported by CJ Cheiljedang Institute of Biotechnology (CG-20-17-01-0002), and the Chung-Ang University Research Grants in 2017.

**Conflict of Interest**

H.J.K., S.Y.O., and S.J.L. have filed a patent application based on this work.

**References**

1. Keilhauer C, Eggeling L, Sahn H. 1993. Isoleucine synthesis in Corynebacterium glutamicum: molecular analysis of the ilvB-ilvN-ilvC operon. J. Bacteriol. 175: 5595-5603.

2. Georgi T, Rittmann D, Wendisch VF. 2005. Lysine and glutamate production by Corynebacterium glutamicum on glucose, fructose and sucrose: roles of malic enzyme and glucose-1,6-bisphosphatase. Metab. Eng. 7: 291–301.

3. Kinosita S, Udaoka S, Shimanono M. 2004. Studies on the amino acid fermentation. Part I. Production of L-glutamic acid by various microorganisms. J. Gen. Appl. Microbiol. 50: 331-343.

4. Lee JT, Na YA, Kim E, Lee HS, Kim P. 2016. The Actinobacterium Corynebacterium glutamicum, an Industrial Workhorse. J. Microbiol. Biotechnol. 26: 807–822.

5. Becker J, Giesselsmann G, Hofmann SL, Wittmann C. 2018. Corynebacterium glutamicum for sustainable bioproduction: from metabolic physiology to systems metabolic engineering. Adv. Biochem. Eng. Biotechnol. 162: 217–263.

6. Ruan Y, Zhu L, Li Q. 2015. Improving the electro-transportation efficiency of Corynebacterium glutamicum by weakening its cell wall and increasing the cytoplasmic membrane fluidity. Biotechnol. Lett. 37: 2445-2452.

7. Jansen R, Embden JD, Gaastra W, Schols LM. 2002. Identification of genes that are associated with DNA repeats in prokaryotes.

8. Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S. et al. 2007. CRISPR provides acquired resistance against viruses in prokaryotes. Science 315: 1709-1712.

9. Mojica FJ, Diez-Villasenor C, Garcia-Martinez J, Soria E. 2005. Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. J. Mol. Evol. 60: 174-182.

10. Lone BA, Karna SKL, Ahmad F, Shahi N, Pokharel YR. 2018. CRISPR/Cas9 System: A Bacterial Tailor for Genomic Engineering. Genet. Res. Int. 2018: 3797214.

11. Peng F, Wang X, Sun Y, Dong G, Yang Y, Liu X et al. 2017. Efficient gene editing in Corynebacterium glutamicum using the CRISPR/Cas9 system. Microb. Cell Fact. 16: 201.

12. Brouns SJ, Jore MM, Lundgren M, Westra ER, Slijkhuis RJ, Sijnders AP et al. 2008. Small CRISPR RNAs guide antiviral defense in prokaryotes. Science 321: 960-964.

13. Marraffini LA, Sontheimer EJ. 2008. CRISPR interference limits horizontal gene transfer in viruses in prokaryotes.

14. Zerbini F, Zanella I, Fraccascia D, Konig E, Irene C, Frattini LF. 2018. Initiation of homologous recombination at DNA nicks.

15. Maizels N, Davis L. 2018. Adaptive immunity in bacteria.

16. Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P et al. 2015. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. Cell 163: 759-771.
21. Wright AV, Nunez JK, Doudna JA. 2016. Biology and applications of CRISPR systems: harnessing nature’s toolbox for genome engineering. Cell 164: 29-44.
22. Komor AC, Badran AH, Liu DR. 2017. CRISPR-based technologies for the manipulation of eukaryotic genomes. Cell 168: 20-36.
23. Binder S, Siedler S, Marienhagen J, Bott M, Eggeling L. 2013. Recombineering in Corynebacterium glutamicum combined with optical nanosensors: a general strategy for fast producer strain generation. Nucleic Acids Res. 41: 6360-6369.
24. Krumbach K, Sonntag CK, Eggeling L, Marienhagen J. 2019. CRISPR/Cas12a Mediated genome editing to introduce amino acid substitutions into the mechanosensitive channel MscCG of Corynebacterium glutamicum. ACS Synth. Biol. 8: 2726-2734.
25. Lee HJ, Kim HJ, Lee SJ. 2020. CRISPR-Cas9-mediated pinpoint microbial genome editing aided by target-mismatched sgRNAs. Genome Res. 30: 768-775.
26. Schafer A, Tauch A, Jager W, Kalinowski J, Thierbach G, Puhler A. 1994. Small mobilizable multi-purpose cloning vectors derived from the Escherichia coli plasmids pK18 and pK19: selection of defined deletions in the chromosome of Corynebacterium glutamicum. Gene 145: 69-73.
27. Park S-D, Lee S-N, Park I-H, Choi J-S, Jeong W-K, Kim Y, et al. 2004. Isolation and characterization of transcriptional elements from Corynebacterium glutamicum. J. Microbiol. Biotechnol. 14: 789-795.
28. Heider SA, Peters-Wendisch P, Wendisch VF. 2012. Carotenoid biosynthesis and overproduction in Corynebacterium glutamicum. BMC Microbiol. 12: 198.
29. Anderson EM, Haupt A, Schiel JA, Chou E, Machado HB, Strezoska Z, et al. 2015. Systematic analysis of CRISPR-Cas9 mismatch tolerance reveals low levels of off-target activity. J. Biotechnol. 211: 56-65.
30. Shen J, Chen J, Jensen PR, Solem C. 2017. A novel genetic tool for metabolic optimization of Corynebacterium glutamicum: efficient and repetitive chromosomal integration of synthetic promoter-driven expression libraries. Appl. Microbiol. Biotechnol. 101: 4737-4746.
31. Zhang J, Yang F, Yang Y, Jiang Y, Huo YX. 2019. Optimizing a CRISPR-Cpf1-based genome engineering system for Corynebacterium glutamicum. Microb. Cell Fact. 18: 60.
32. Nishio Y, Nakamura Y, Kawarabayasi Y, Usuda Y, Kimura E, Sugimoto S, et al. 2003. Comparative complete genome sequence analysis of the amino acid replacements responsible for the thermostability of Corynebacterium efficiens. Genome Res. 13: 1572-1579.
33. Liu J, Wang Y, Lu Y, Zheng P, Sun J, Ma Y. 2017. Development of a CRISPR/Cas9 genome editing toolbox for Corynebacterium glutamicum. Microb. Cell Fact. 16: 205.
34. Wang B, Hu Q, Zhang Y, Shi R, Chai X, Liu Z, et al. 2018. A RecET-assisted CRISPR-Cas9 genome editing in Corynebacterium glutamicum. Microb. Cell Fact. 17: 63.
35. Tung QN, Loi VV, Busche T, Nerlich A, Milse J, et al. 2019. Stable integration of the Mrx1-roGFP2 biosensor to monitor dynamic changes of the mycothiol redox potential in Corynebacterium glutamicum. Redox Biol. 20: 514-525.
36. Santamaria R, Gil JA, Mesas JM, Martin JF. 1984. Characterization of an endogenous plasmid and development of cloning vectors and transformation system in Brevibacterium lactofermentum. Microbiology 130: 2237-2246.
37. Huang Y, Li L, Xie S, Zhao N, Han S, Lin Y, et al. 2017. Recombineering using RecET in Corynebacterium glutamicum ATCC14067 via a self-excisable cassette. Sci. Rep. 7: 7916.
38. Ronda C, Pedersen LE, Sommer MO, Nielsen AT. 2016. CRMAGE: CRISPR Optimized MAGE Recombineering. Sci. Rep. 6: 19452.
39. Fu BX, St Onge RP, Fire AZ, Smith JD. 2016. Distinct patterns of Cas9 mismatch tolerance in vitro and in vivo. Nucleic Acids Res. 44: 5365-5377.
40. Zheng T, Hou Y, Zhang P, Zhang Z, Xu Y, Zhang L, et al. 2017. Profiling single-guide RNA specificity reveals a mismatch sensitive core sequence. Sci. Rep. 7: 40638.