Journal of Bioscience and Bioengineering
VOL. 128 No. 3, 283–289, 2019

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# Establishment of a transient CRISPR-Cas9 genome editing system in *Candida glycerinogenes* for co-production of ethanol and xylonic acid

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Received 27 January 2019; accepted 12 March 2019
Available online 6 April 2019

***Candida glycerinogenes*, an industrial yeast with excellent multi-stress tolerance, has been applied to glycerol production for decades. However, its genetic manipulation was limited by the absence of meiosis, the diploid genome, and the lack of molecular tools. We described here the implementation of a transient CRISPR-Cas9 system for efficient genome editing in *C. glycerinogenes*. By targeting the counterselectable marker genes (*TRP1*, *URA3*), single and double gene knock-outs were achieved and the auxotroph obtained can be used as a background for targeting other gene (*HOG1*) at a mutation efficiency of 80%. Further, a xylonic acid producing *C. glycerinogenes* strain was constructed by knock-in of the xylose dehydrogenase gene, which produced up to 28 g/L ethanol and 9 g/L xylonic acid simultaneously from simulated lignocellulosic hydrolysate (contained 70 g/L glucose and 24 g/L xylose). These results indicated that the CRSIPR-Cas9 system developed here can facilitate the study of gene functions and metabolic pathways in *C. glycerinogenes*.**



[**Key words:** *Candida glycerinogenes*; CRISPR-Cas9; Genome editing; Ethanol; Xylonic acid]

*Candida glycerinogenes* is an industrial diploid yeast with excellent multi-stress tolerance, has been regarded as an attractive organism for fundamental and applied researches (1–3). Due to some properties including the remarkable osmotolerance, rapid growth, overproducing and high yield of extra-cellular glycerol, *C. glycerinogenes* has been applied to commercial scale production of glycerol for decades in China (4). Beyond that, *C. glycerinogenes* is a potential cell factory for the production of value-added biochemicals. Nevertheless, the absence of meiosis, the diploid genome, and limited selectable markers made it laborious to generate gene disruption by double-crossover homologous recombination, which impeded the broader exploitation of *C. glycerinogenes* in biotechnology.

Over the last years, Clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated gene 9 (Cas9) system has been widely applied as a powerful molecular tool for genome engineering in multiple organisms, ranging from bacteria to plants and animals (5–7). Most CRISPR-Cas9 systems for genome editing require two components: (i) the endonuclease Cas9; (ii) a single synthetic guide RNA (sgRNA) (8). The attached requirement is that the target DNA region should be followed by an NGG protospacer adjacent motif (PAM). The sgRNA comprises both a 20 nucleotide RNA guide sequence complementary to the target DNA adjacent to the NGG, and an ~80 nucleotide trans-activating RNA which facilitates binding to Cas9. During CRISPR-Cas9 based genome editing process, the Cas9 endonuclease is recruited to the target DNA region by sgRNA, directing DNA double-strand break (DSB). Following that, the DSB will be repaired either via non-homologous end-joining (NHEJ) resulting in indels, or homology-directed repair (HDR) used to precise editing, such as gene knock-out, knock-in and point mutation (9). Although CRISPR-Cas9 genome editing systems have been established for use in several *Candida* species (10–14), its application has not yet been validated in *C. glycerinogenes*.

Bioethanol is an ideal substitute for non-renewable fossil fuel, which can be obtained from low-costing starting feedstock like lignocellulose (15). *C. glycerinogenes* is an ideal candidate for lignocellulose-based bioethanol production owing to its superior resistance to high temperature, acetic acid and furfural (3). However, bioethanol production by *C. glycerinogenes* mainly consumes glucose of lignocellulose hydrolysate rather than xylose, causing most xylose remained. Bioconversion of xylose is a strategy to improve commercial value of lignocellulose hydrolysate. Xylonic acid can be obtained from xylose by microbial conversion and is a high-value chemical with applications of aerospace industry, pharmaceutical and agriculture (16). However, due to lack of specific genes like *xylB* which encoded $NAD^+$-dependent xylose dehydrogenase (17), the wild type strain of *C. glycerinogenes* was incapable of producing xylonic acid from xylose.

In this study, we developed a transient CRISPR-Cas9 system for efficient gene modifications in *C. glycerinogenes*, which successfully generated mutations with high efficiency in counterselectable marker genes (*TRP1*, *URA3*) and other gene (*HOG1*). Using this

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1389-1723/$ – see front matter 
https://doi.org/10.1016/j.jbiosc.2019.03.009

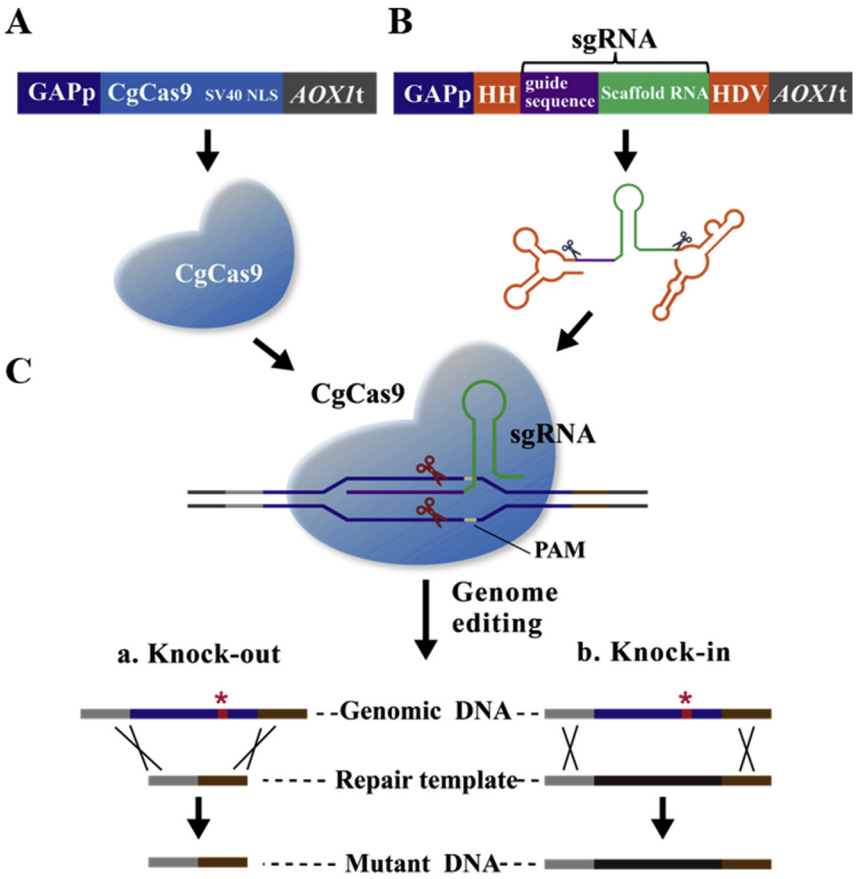


FIG. 1. Diagram of transient CRISPR-Cas9 system in *C. glycerinogenes* and schematic of genetic constructs. CgCas9 gene contains 3×SV40 NLS at the 3′ end and the constitutive GAP promoter ensured maximal expression (A). The single guide RNA (sgRNA), which guides Cas9 to identify the target loci, is composed of a scaffold RNA structure and a 20-bp guide sequence specific to the target loci. The sgRNA expression is driven by GAP promoter combined with the 5′ hammerhead (HH) and 3′ hepatitis delta virus (HDV) ribozymes. Functional sgRNA is released by the self-cleavage of ribozyme structures (B). Supplemented with the suitable repair template, efficient genome editing such as gene knock-out and knock-in can be achieved in *C. glycerinogenes* (C).

system, biosynthesis of xylonic acid was achieved in *C. glycerinogenes* by *xylB* gene knock-in.

## MATERIALS AND METHODS

**Strains, media and culture conditions** All *C. glycerinogenes* strains used in this study (Supplementary Table S1) were routinely cultivated at 30°C in YPD medium (10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose) or synthetic dextrose (SD) medium (6.7 g/L yeast nitrogen base without amino acids plus 20 g/L glucose) supplemented with the appropriate auxotrophic requirements. For counterselection, 2 g/L 5-fluoroanthranilic acid (FAA) or 3 g/L 5-fluoroorotic acid (FOA) were supplemented to the growth media. All the plasmids used in this study (Table S1) were propagated in *Escherichia coli* JM109 by culturing in Luria–Bertani medium containing 100 μg/ml ampicillin at 37°C. Agar at 2% was supplemented for plates.

**Construction of the helper plasmid pMY** The helper plasmid pMY was constructed for gene expression, containing glycerladehyde-3-phosphate dehydrogenase (GAP) promoter, multiple cloning site (MCS), and *AOX1* terminator (Fig. S1). The constitutive GAP promoter was amplified from *C. glycerinogenes* genomic DNA using primers CgGAPp_F and CgGAPp_R (Table S2), and then was ligated into the plasmid pMD19-T (Takara, Dalian, China) generating the vector pMD19-T-$P_{CgGAP}$ by TA-cloning. In particular, the 5′ end of the primer CgGAPp_R contains MCS for the entry of exogenous genes. The *Pichia pastoris AOX1* terminator was amplified from the plasmid pPIC9K using primers PpAOX1t_F and PpAOX1t_R (Table S2), which includes *Xba* I or *Xho* I recognition sites respectively. The *PpAOX1* terminator was then cloned into pMD19-T-$P_{CgGAP}$ cut with the indicated enzyme, yielding the helper plasmid pMD19-T-$P_{CgGAP}$-MCS-$T_{PpAOX1}$ (designated as pMY).

**Generation of CgCas9 expression cassette** Taking the CaCas9 gene which was codon-optimized for expression in *Candida albicans* as the reference (10), the Cas9 gene (CgCas9) with three SV40 nuclear localization signals (3×SV40 NLS) at the 3′ end was synthesized by Synbio Technology (Suzhou, China). The CgCas9 fragment and pMY digested by *Apa* I and *Not* I were ligated to generate pMY-CgCas9 (pMYC9). The CgCas9 expression cassette $P_{CgGAP}$-CgCas9-$T_{PpAOX1}$ was amplified from pMYC9 by primers CgGAPp_F and PpAOX1t_R (Table S2). The CgCas9 cassette contains the CgGAP promoter, CgCas9 coding sequence (CDS) and *PpAOX1* terminator (Fig. 1A).

**Generation of sgRNA expression cassettes** All sgRNAs were designed to target *TRP1*, *URA3* and *HOG1* genes by using 20-bp guide sequences immediately followed by a PAM sequence (Table S3). The 20-bp guide sequences were analyzed according to the target genes with the help of bioinformatics tool sgRNACas9 (18). The GC content of the guide sequence was set within 40–60%. sgRNA genes with length of 223 bp were synthesized by Synbio Technology, including the predesigned 20-bp guide sequence and the sgRNA scaffold sequence flanked by the self-cleaving hammerhead (HH) and hepatitis delta virus (HDV) ribozyme (Fig. 1B, Table S4). The six nucleotides at the 5′ end of the HH ribozyme must be complementary to the first six nucleotides of the guide sequence. The sgRNA fragments involved the HH-sgRNA-HDV sequence were integrated into pMY by restriction sites *Nhe* I and *Kpn* I. The recombinant plasmids pMY-HH-sgRNA-HDV (pMYsgRNA) are listed in Table S1. Using primers CgGAPp_F and PpAOX1t_R, the sgRNA cassettes were amplified from the above plasmids (Table S5).

**Construction of repair templates** The repair templates targeting counter-selectable marker genes were designed to remove sequences between start and stop codon of the target gene. Long repair templates (100–1000 bp arms) were generated using overlapping PCR of upstream and downstream flanking the target gene (Fig. S2A). Short repair templates (30 or 50 bp arms) were generated by primer extension from two oligonucleotide primers with 20 bp overlaps at the 3′ ends (Fig. S2B). The repair template for *HOG1* gene knock-out containing the CDS of

*TRP1* gene were generated by 70-bp oligonucleotide primers including 50-bp of the upstream and downstream flanking regions of the target gene (Fig. S2C). The repair template for *xylB* gene (codon-optimized for *Saccharomyces cerevisiae*, GenBank accession number: LT897784.1) knock-in was amplified from the pGUKd-xylB by 70-bp oligonucleotide primers containing 50-bp homology arms flanking the *TRP1* gene (Fig. S2D). All primers for repair template construction are given in Table S2.

**Yeast transformation** The repair template (5 μg) were co-transformed with the CgCas9 cassette (2 μg) and sgRNA cassette (2 μg), using the LiAc/SS carrier DNA/PEG transformation method (19). After transformation, cells were plated onto selective medium to identify desired transformants. Transformant genomic DNA was extracted and verified by diagnostic PCR and sequencing analysis. Mutation frequency was calculated as the ratio of the number of colonies on selective medium divided by the number on nonselective YPD medium.

**Molecular biology techniques** Restriction enzymes, DNA polymerase, and T4 DNA ligase were purchased from Takara (Dalian, China). The genomic DNA was extracted from a single colony using TIANamp Yeast DNA Kit (Tiangen, Beijing, China). Plasmid DNA was extracted using SanPrep Column Plasmid Mini-Preps Kit (Sangon Bioth, Shanghai, China). The DNA fragments from restriction enzyme digestions and polymerase chain reactions (PCRs) were purified using SanPrep Column PCR Product Purification Kit (Sangon Bioth). The primer synthesis and DNA sequencing were performed by Synbio Technology (Suzhou, China).

**Co-production of ethanol and D-xylonic acid from simulated lignocellulosic hydrolysate** Media employed to simulate lignocellulosic hydrolysate was based on YPD with additional components: 50 g/L D-glucose, 24 g/L D-xylose, 6 g/L acetic acid and 1 g/L furfural, pH was adjusted to 5.5. Experiments for determining production were stared with an aliquot of the culture in YPD medium, inoculating 250 mL shake flasks with 100 mL of simulated lignocellulosic hydrolysate medium at a starting $OD_{600}$ of 0.1. The fermentation consisted of two phases: (i) the culture was firstly incubated in a horizontal shaking incubator under the micro-aerobic condition at 37°C, 100 rpm for ethanol production; (ii) after the glucose was consumed, the culture was transferred to the aerobic condition at 37°C, 200 rpm for xylonic acid production. All experiments were performed at least in triplicate.

**Analysis methods** Biomass was measured using the optical density at 600 nm ($OD_{600}$) after appropriate dilutions. Glucose, ethanol, xylose and xylonic acid concentrations were analyzed by using high-performance liquid chromatography (HPLC; Dionex, Sunnyvale, CA, USA) equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) and an RI-101 refractive index detector (Shodex, Tokyo, Japan). Mobile phase was 5 mM $H_2SO_4$, with a flow rate of 0.6 ml/min at 60°C. The data are shown as the mean values ±SD of three independent experiments.

## RESULTS

**Developing a transient CRISPR-Cas9 system in *C. glycerinogenes*** We first synthesized a codon-optimized gene Cas9 (CgCas9), which was incorporated 3×SV40 nuclear localization signal (SV40 NLS) sequence into the 3′ end. The sgRNA genes (sgRNAs) contained the guide sequences were also synthesized and flanked by self-cleaving hammerhead (HH) and hepatitis delta virus (HDV) ribozymes. Both CgCas9 and sgRNAs were assembled into a helper plasmid (pMY) and expressed transiently by a strong RNA pol II GAP promoter from *C. glycerinogenes* (Fig. 1A and B).

To test the feasibility of this system, we first targeted the counterselectable marker gene *TRP1*, the disruption of which conferred the resistance to FAA (20). Three sgRNAs were designed by sgRNAcas9 software, targeting different alleles of *TRP1*: sgTRP1.1, sgTRP1.2 and sgTRP1.3 (Fig. 2A, Table S3). Transformation of the WT strain with the CgCas9 cassette and the sgTRP1 expression cassette yielded no colony on YPD+FAA medium, but the *trp1* mutants occurred at a frequency of $2.6–3.6\times10^{-6}$ when offered a repair template with 1-kb arms homologous flanking the *TRP1* coding region (Fig. 2B, Table S6). Further diagnostic PCR and DNA sequencing analysis confirmed the preplanned deletion of whole *TRP1* coding region in randomly selected colonies (Fig. 2C) and no integration of CgCas9 and sgRNA cassettes occurred in them (data not shown). On the other hand, no colonies were produced from co-transformations of the repair template with the CgCas9 cassette (Fig. 2B, Table S6). These results indicate that all CRSIPR components (CgCas9, sgRNA, and repair template) are necessary for gene knock-out and CRISPR-mediated DSB repair relies entirely on homology-directed recombination in *C. glycerinogenes*.

Next, a series of repair templates with homology arms of varied length (30 bp to 1 kb) were used to evaluate their effect on

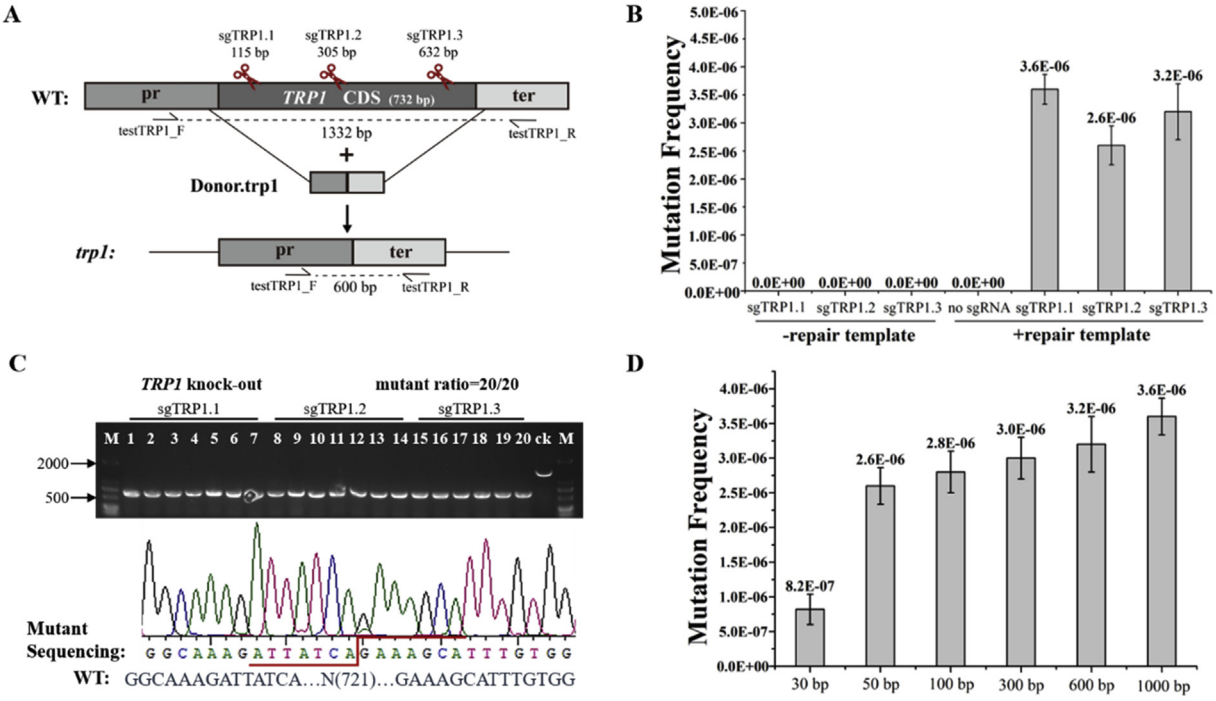


FIG. 2. CRISPR-Cas9 mediated knock-out of counterselectable marker gene *TRP1*. (A) Schematic representation of *TRP1* gene editing. The primers for diagnostic PCR and DNA sequencing of transformants are indicated. (B) Diagnosis of 20 randomly selected colonies of the above transformation. Lane ck is a PCR product of 1332 bp consistent with the wild-type allele. All 20 transformants (lanes 1–20) showed a PCR product of 600 bp consistent with the mutant allele. The result was confirmed by DNA sequencing. (C) *TRP1* mutation frequency in different combinations of the repair template and sgRNA cassettes. (D) Mutation frequency of *TRP1* gene mediated by repair templates with different size homology arms. Each transformation experiment was repeated three time. Error bars represent standard deviation (SD) among three experiments.

recombination efficiency when targeting TRP1.1 locus. As shown in Fig. 2D, the mutation frequency increased with the length of homology arms. The mutation frequency with 50 bp arms was $2.6\times10^{-6}$, which was only 27% lower than the one with 1 kb arms, but the frequency with 30 bp arms dropped rapidly to $8.2\times10^{-7}$. These data suggest that 50 bp homology arms are ideal choices when considering the complexity of repair template construction.

**Targeting counterselectable marker genes for knock-out analysis** Another counterselectable marker gene *URA3*, which encoded products conferring sensitivity to FOA (21), was targeted by co-transforming two separate sgRNA cassette into the WT strain with the same repair template and the CgCas9 cassette (Fig. 3A, Table S3). Following PCR and sequence analysis in colonies on YPD+FOA medium, the mutation frequency in the *URA3* gene was measured at $2.3-2.7\times10^{-6}$ (Fig. 3B and C).

Then, *TRP1* and *URA3* were selected for simultaneous gene knock-out to explore the possibility of multiplex genome engineering. Co-transforming sgTRP1.1 and sgURA3.1 with the corresponding repair templates, the desired mutants (*trp1-ura3*) were obtained on YPD+FAA+FOA medium with the mutant frequency of $8.7\times10^{-7}$, which was about 64% lower than the frequency of single gene knock-out (Fig. 3C). Further auxotrophic phenotype analysis showed that *trp1-ura3* mutants were constructed with the expected auxotroph (Fig. 3D).

**Expanding CRISPR-Cas9 system for non-counterselectable marker gene knock-out** As a non-counterselectable marker gene, *HOG1* gene which involved in tolerance of the osmotic stress (1), was targeted by two sgRNAs (Fig. 4A, Table S3). The *trp1* strain obtained above was set as the transformation background, and the repair template Donor.hog1 was constructed to contain the *TRP1* gene as a selection marker and complement the tryptophan auxotroph. PCR analysis of randomly chosen *TRP1* transformant colonies on SD medium showed that 16 of 20 carried only Δ*hog1*:*TRP1* alleles, meaning the proportion of *hog1* mutants in transformants was about 80% (Fig. 4B). The result was further confirmed by phenotypic validation of the representative colonies (Fig. 4C).

**Constructing xylonic acid producing strains by gene knock-in** To realize xylonic acid production, a repair template containing the *xylB* gene (711 bp) with 50-bp homologous arms, was constructed for homologous integration at the *TRP1* locus (Fig. 5A). PCR analysis confirmed the integration of the *xylB* gene in the colonies on YPD+FAA medium (Fig. 5B). Then the production of xylonic acid by this engineered strain (XB) was tested after the bioethanol fermentation. As shown in Fig. 6, the biomass growth and ethanol productivity of the XB strain lagged behind the WT strain a little, but ethanol produced by two strains reached the same maximum concentration of 28−30 g/L at 36 h. After conversion to aerobic fermentation, only the XB strain can produce xylonic acid which eventually was accumulated at a concentration of 9.1 g/L at 96 h. These results indicate that the xylonic acid producing strain was successfully constructed by the CRSIPR system.

## DISCUSSION

The expression of Cas9 and sgRNA is the core issue of CRISPR-Cas9 system implementation. Although the expression of CRISPR components by stable episomal plasmids is widely used in well-studied organisms (14,22−24), it normally requires ARS sequences and selection markers which are lacking in *C. glycerinogenes*. The second choice is integrating CRISPR

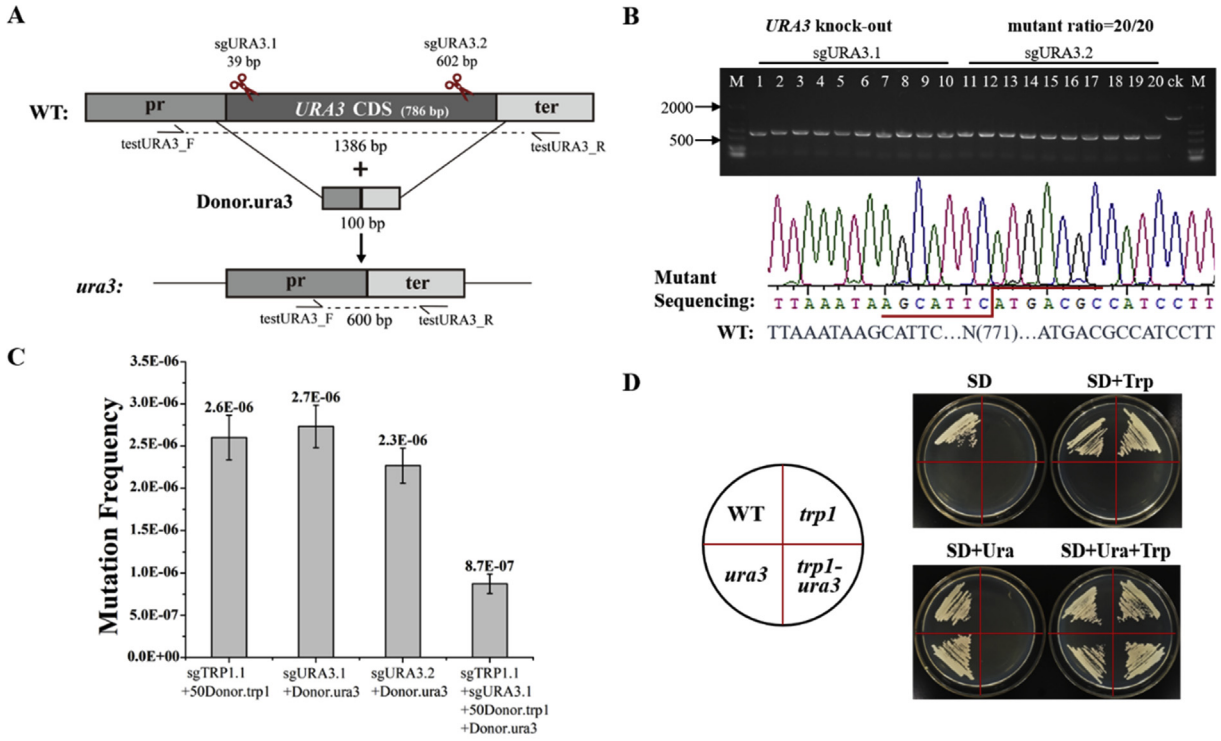


FIG. 3. Counterselectable marker *URA3* gene knock-out and simultaneous double gene knock-out. (A) Schematic representation of *URA3* gene editing. The primers for diagnostic PCR and DNA sequencing of transformants are indicated. (B) Diagnosis of 20 randomly selected colonies of the above transformation. Lane ck is a PCR product of 1386 bp consistent with the wild-type allele. All 20 transformants (lanes 1−20) showed a PCR product of 600 bp consistent with the mutant allele. The result was confirmed by DNA sequencing. (C) Mutation frequency of single gene (*TRP1*, *URA3*) and double genes (*TRP1* and *URA3*) by transformations of CgCas9 cassette with corresponding sgRNA cassettes and repair templates. (D) Verification of the phenotype of auxotrophic strains. The WT and auxotrophic strains were cultured in YPD medium until saturation, and then streaked on agar plates of synthetic dextrose (SD) medium with auxotrophic supplements. The genotypes of the strains are shown in the pie chart.

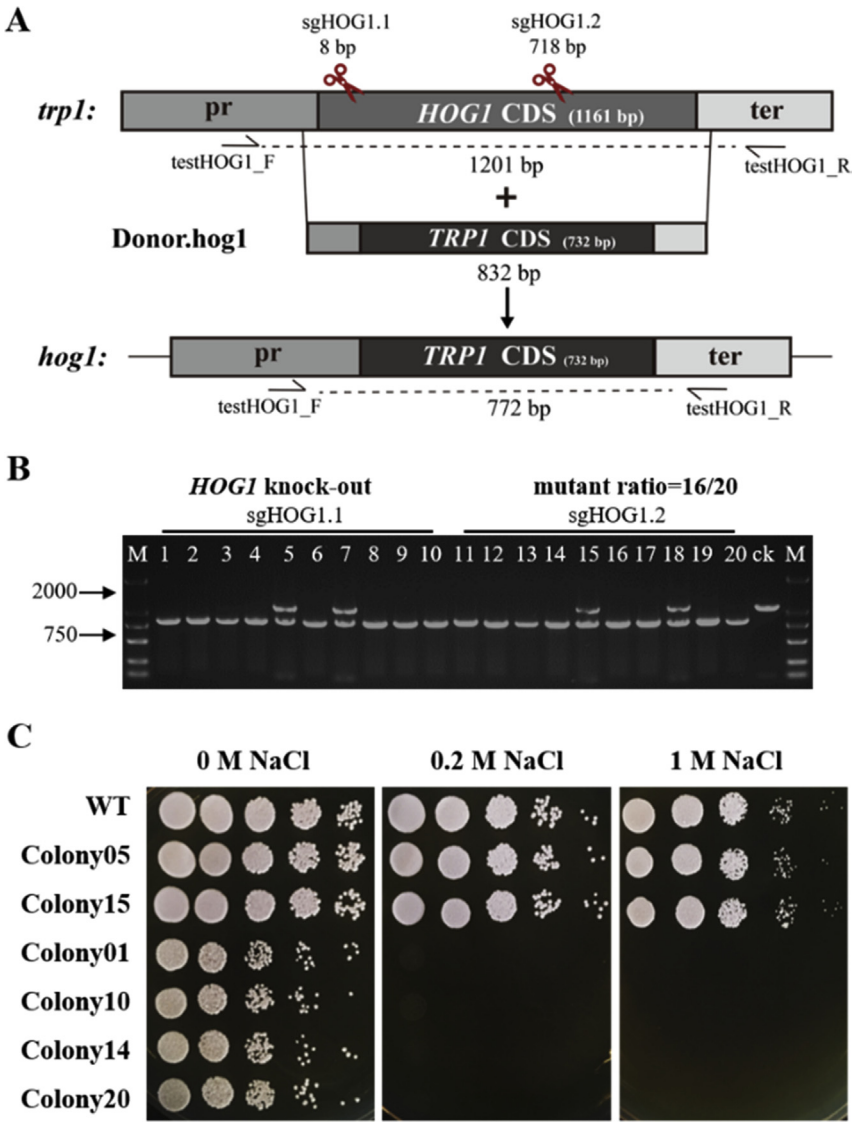


FIG. 4. Non-counterselectable marker *HOG1* gene knock-out. (A) Schematic representation of *HOG1* gene editing. The primers for diagnostic PCR of transformants are indicated. (B) Diagnosis of 20 randomly selected colonies of the above transformation. Lane ck is a PCR product of 1201 bp consistent with the wild-type allele. Four transformants (lanes 5, 7, 15 and 18) showed a mixed PCR product of 1201 bp *HOG1* fragment and 772 bp Δ*hog1*:*TRP1* fragment. The other 16 transformants showed a PCR product of 772 bp consistent with the mutant allele. The efficiency for *HOG1* knock-out was 16/20. (C) Verification of the phenotype of WT strain and representative mutants. Cell suspensions diluted tenfold serially were spotted on YPD plates with 0 M, 0.2 M or 1 M NaCl, and then were cultured at 30°C for 2 days.

expression cassettes into the chromosome (10,14). However, it has potential off-target risks (18) and is not suitable for *C. glycerinogenes* in which homologous recombination efficiency with exogenous DNA remains low. Introducing CRISPR components transiently is another choice (13,25). Normally, RNA pol III promoters are used for sgRNA transient expression, but they have not been identified in *C. glycerinogenes* so far. Introduction of Cas9-sgRNA protein complex is also a CRISPR-based genome editing method used in some yeasts, however it is more demanding and high-cost than the previous three methods (14). Taking all these into account, we developed a transient CRISPR-Cas9 system for efficient genome editing in *C. glycerinogenes*, in which both Cas9 and sgRNA were transiently expressed by an RNA pol II promoter. The ribozymes flanking sgRNA prevent the interference of 5′ cap and 3′ poly-A tail transcribed by RNA pol II promoters (14), and function effectively in our transient system.

Counterselectable marker genes (*TRP1*, *URA3*) which are also commonly used as auxotrophic markers were first targeted, because the mutant colonies recovered by counterselection had visible phenotype. The feasibility and efficiency of the system can be evaluated by easy statistical analysis. Thus, it delivers the dual benefit of verifying system efficiency and obtaining the marker-less auxotroph mutant. We found that the efficiency varied with different target genes and sgRNAs. The repair of the CRISPR-mediated DSB by NHEJ in *C. glycerinogenes* was also found to be rare, thus it is necessary to supplement homologous recombination templates for the repair of toxic DSB in *C. glycerinogenes*. Although a higher mutation frequency can be obtained by long homologous arms (1 kb), 50 bp homology arms are enough for the homology-directed DNA repair in *C. glycerinogenes*. In addition, this system proved to be feasible in multiple genome editing of *C. glycerinogenes* by simultaneous double-gene knockout with a lower mutation frequency.

As an added bonus, the marker-less auxotroph obtained above can expand this system for knock-out of non-counterselectable marker gene at a high mutation efficiency of 80% (*HOG1*) and

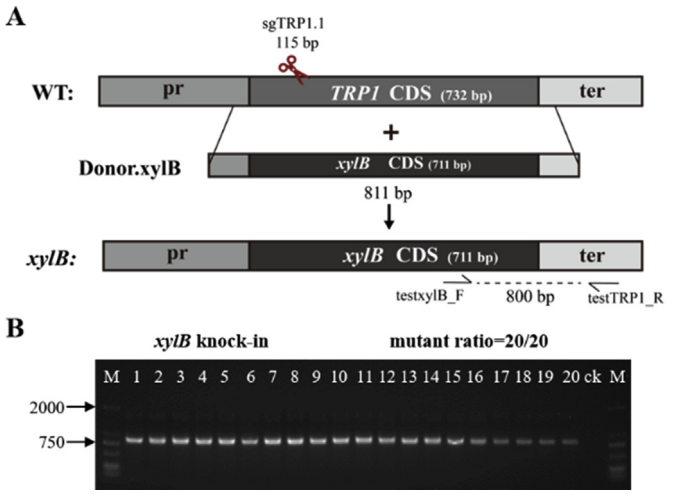


FIG. 5. CRISPR-Cas9 mediated *xylB* gene knock-in. (A) Schematic representation of *xylB* gene knock-in. The primers for diagnostic PCR of transformants are indicated. (B) Diagnosis of 20 randomly selected colonies of the above transformation. All transformants (lanes 1–20) showed a PCR product of 800 bp consistent with the mutant allele. No PCR product was detected in WT strain (lane ck).

avoid the use of resistance markers which are not suitable for industrial applications. *HOG1* gene was chosen in this study because its loss will cause cells to be sensitive to salt (1), and this visible phenotypic change helps to determine the success of the knock-out. Interestingly, there was still one copy of *HOG1* gene remained in 20% of $Trp^+$ transformants because of *C. glycerinogenes* diploid genome and imperfect CRISPR editing efficiency, but this also means homozygous and heterozygous deletion can be achieved simultaneously by this system. Besides, this expanded system theoretically can target any gene and achieve sequential gene knock-out.

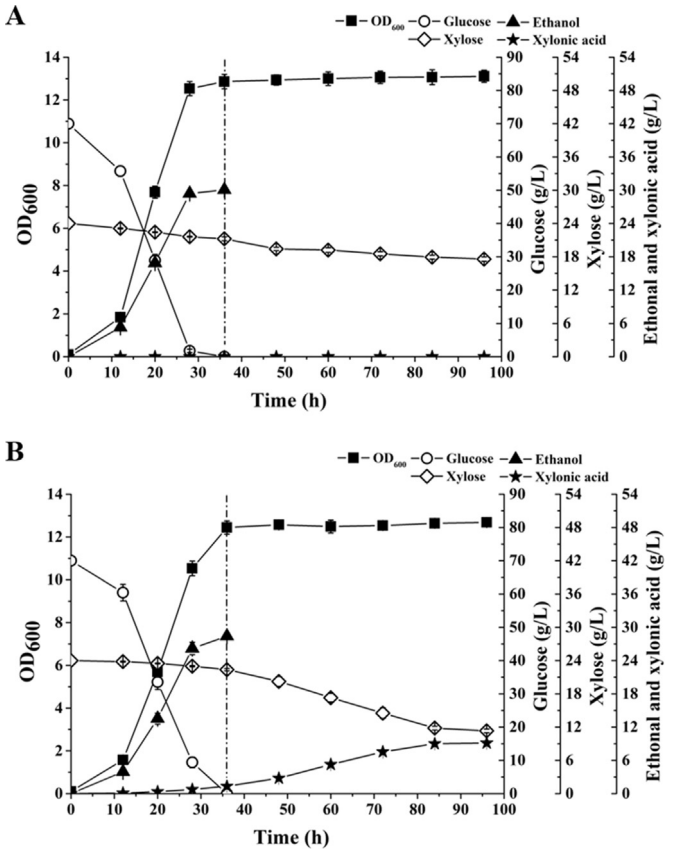


FIG. 6. Co-production of ethanol and xylonic acid by *C. glycerinogenes* WT strain (A) or engineered XB strain (B) from simulated lignocellulosic hydrolysate. The culture started with an $OD_{600}$ of 0.1 and under the micro-aerobic condition (37°C, 100 rpm). The aerobic condition (37°C, 200 rpm) was set when glucose was consumed at 36 h. Squares, biomass; circles, glucose; diamonds, xylose; triangles, ethanol; pentagrams, xylonic acid. All data are shown as the mean ± SD of three independent replicates.

Furthermore, the feasibility of gene knock-in was verified by inserting *xylB* gene to create a metabolic pathway from xylose to xylonic acid in *C. glycerinogenes*. Laboratory scale fermentation results indicate that the engineered strain can efficiently continue to produce xylonic acid after ethanol fermentation from the simulated lignocellulosic hydrolysate. The only pity is that about half of the xylose still was not utilized at the end of the fermentation, which can be optimized by improving *xylB* gene expression level or xylose transport efficiency in the future. Currently, two strains are usually required for the industrial co-production of ethanol and xylonic acid from lignocellulose feedstock: *S. cerevisiae* is employed in the early ethanol fermentation, and later *Gluconobacter oxydans* is used to oxidize xylose to xylonic acid (26,27). Compared to the above method, the use of single engineered *C. glycerinogenes* can simplify processing and lower the cost, which might be a promising application for the industrial co-production of ethanol and xylonic acid.

In summary, the transient CRISPR-Cas9 system developed in this study will make the genetic modification of *C. glycerinogenes* more convenient and accelerate the research on gene functions, metabolic engineering and fermentation engineering. The strategy we adopt might contribute to unlock the application of the CRISPR-Cas9 genome editing system in more organisms.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2019.03.009.

## ACKNOWLEDGMENTS

This work was funded by the National Natural Science Foundation of China (Nos. 31601456, 31570052, 21708016), and national first-class discipline program of Light Industry Technology and Engineering (LITE2018-01).

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