A RecET-assisted CRISPR–Cas9 genome editing in *Corynebacterium glutamicum*

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**Abstract**

**Background:** Extensive modification of genome is an efficient manner to regulate the metabolic network for producing target metabolites or non-native products using *Corynebacterium glutamicum* as a cell factory. Genome editing approaches by means of homologous recombination and counter-selection markers are laborious and time consuming due to multiple round manipulations and low editing efficiencies. The current two-plasmid-based CRISPR–Cas9 editing methods generate false positives due to the potential instability of Cas9 on the plasmid, and require a high transformation efficiency for co-occurrence of two plasmids transformation.

**Results:** Here, we developed a RecET-assisted CRISPR–Cas9 genome editing method using a chromosome-borne Cas9–RecET and a single plasmid harboring sgRNA and repair templates. The inducible expression of chromosomal RecET promoted the frequencies of homologous recombination, and increased the efficiency for gene deletion. Due to the high transformation efficiency of a single plasmid, this method enabled 10- and 20-kb region deletion, 2.5-, 5.7- and 7.5-kb expression cassette insertion and precise site-specific mutation, suggesting a versatility of this method. Deletion of *argR* and *farR* regulators as well as site-directed mutation of *argB* and *pgi* genes generated the mutant capable of accumulating l-arginine, indicating the stability of chromosome-borne Cas9 for iterative genome editing. Using this method, the model-predicted target genes were modified to redirect metabolic flux towards 1,2-propanediol biosynthetic pathway. The final engineered strain produced 6.75 ± 0.46 g/L of 1,2-propanediol that is the highest titer reported in *C. glutamicum*. Furthermore, this method is available for *Corynebacterium pekinense* 1.563, suggesting its universal applicability in other *Corynebacterium* species.

**Conclusions:** The RecET-assisted CRISPR–Cas9 genome editing method will facilitate engineering of metabolic networks for the synthesis of interested bio-based products from renewable biomass using *Corynebacterium* species as cell factories.

**Keywords:** *Corynebacterium glutamicum*, CRISPR–Cas9, recET-encoded recombinase, Transformation efficiency, Genome editing, 1,2-Propanediol
applicability to facilitate modifying the metabolic network of *C. glutamicum* [4–6].

The traditional approaches dependent on two rounds of homologous recombination require a non-replicating vector with a counter-selectable marker to screen target mutant in *C. glutamicum* ATCC 13032 [7]. The selection markers and vector elements need to be removed via a single crossover event, possibly resulting in undesired restoration of the wild type allele even in the presence of selective pressure. The counter selection by sacB gene leads to a 20–40% false-positive rate due to the spontaneous inactivation of SacB [8]. As another counter-selectable marker, upp encoding a uracil phosphoribosyltransferase is able to decrease the false-positive rate when it is concerted with I-SceI [8]. A toxin counter-selectable cassette regulated by an anti- toxin switch (TCCRAS) system can achieve 100% counter-selectable efficiencies for gene knockout and replacement [9]. To increase the marker eviction efficiency, Cre/ mutant loxP system composed of cre-encoding recombinase and loxP sites are utilized to mediate reciprocal site-specific recombination between the lox71 and lox66 to facilitate easy removal of counter-selectable markers and undesirable vector elements [10, 11]. In spite of high counter-selectable efficiencies, these approaches are still inefficient due to their relatively low frequencies at which homologous recombination take place. To improve homologous recombination efficiency for gene deletion, the exonuclease-recombinase RecE and RecT (RecET) from the Rac prophage of *Escherichia coli* are expressed on a plasmid in *C. glutamicum* to mediate a double-crossover event via a dsDNA fragment containing the counter-selectable markers and homology arms [8, 12]. With the assistance of RecET expression, a linear dsDNA is resected by RecE to expose a 3-ended single-stranded DNA (ssDNA) tail for the binding of RecT, which contributes to promote the annealing of complementary DNA strand, strand exchange and strand invasion [8, 12]. The RecET-Cre/loxP system constructed in *C. glutamicum* ATCC 14067 only requires one round of recombination to obtain the target mutant [12].

The clustered regularly interspaced short palindromic repeat (CRISPR) as a bacterial immune defense system has been developed for genome editing in a wide range of organisms, such as bacteria and eukaryotes [13–17]. The type II CRISPR–Cas9 system from *Streptococcus pyogenes* is widely used for genome editing due to the inherent simplicity and flexibility in sequence requirements for sgRNA [13–15]. Cas9 from *S. pyogenes* (SpCas9) requires a single guide RNA (sgRNA) containing 20 bp complementary sequence of the target region and the SpCas9 binding scaffold to form sgRNA-SpCas9 ribonucleoprotein complex, which generates a double-strand break (DSB) at 20 bp-designated target region after recognizing a protospacer adjacent motif (PAM) sequence NGG [13]. DSB by Cas9 cleavage in the chromosome is lethal to many bacteria deficient in the endogenous non-homologous end joining (NHEJ) mechanism, when DSB is unable be repaired through homologous recombination (HR) [13]. Cas9-mediated killing can help select mutations introduced through HR using a donor DNA as the repair template and eliminate cells carrying the wild-type genotype [16, 17]. So far, CRISPR–Cas9 system has been developed as a powerful tool to genome editing in many bacteria [18–24].

In recent studies regarding CRISPR–Cas9 assisted genome editing of *C. glutamicum*, several attempts to transform the plasmid harboring the native codon-encoded SpCas9 under different inducible promoters failed to obtain any colony, which might be attributed to a negative effect of its strong expression on cell viability [25, 26]. To decrease the translation level, attempts to utilize the codon-optimized cas9 gene and different ribosomal binding sequences succeeded to express plasmid-borne Cas9 protein [26–28]. Two plasmids-based CRISPR–Cas9 editing system consisted of inducible expression cassette of Cas9 in one plasmid and sgRNA expression cassette together with a repair template in the other plasmid [27, 28]. However, the transformation with two plasmids harboring CRISPR–Cas9 system yielded the abnormally large colonies in which their cas9 genes were deleted from pCas9 plasmid, indicating the instability of pCas9. Furthermore, the manipulation procedure is relatively complicated due to the construction of two plasmids harboring genetic elements and transformation efficiency is low for the co-occurrence of the introduction of Cas9-sgRNA and repair template [double-stranded or single-stranded DNA (ssDNA)], resulting in a high requirement for transformation efficiency. The plasmid-borne dsDNA template can directly repair the DSB for gene deletion via HR in NHEJ-deficient *C. glutamicum*; however, it is necessary to express recombinase RecT to incorporate ssDNA to repair DSB [26], indicating that HR is a key factor in CRISPR–Cas9 editing system. Compared to Cas9, Cpf1 that utilizes a T-rich PAM site has less editing targets in the GC-rich *C. glutamicum* genome and only reaches 5–15% of editing efficiencies for gene deletion and insertion [25]. These editing methods have disadvantage in modifying endogenous genes or integrating heterologous genes to construct synthetic pathways. Hence, it is of great importance to develop an easy and efficient CRISPR–Cas9 system to implement an extensive genome editing including gene deletion, integration and mutation in *C. glutamicum* for academic studies and industrial applications.
In this study, to simplify the editing elements construction and minimize the physiological impacts of Cas9 on cells, we developed a CRISPR–Cas9 genome editing method in *C. glutamicum* by integrating *cas9* into chromosome and constructing a single plasmid harboring a sgRNA and repair DNA templates. Through introducing *recET*-encoded recombinase to increase the probability of homologous recombination, this genome editing method enables the deletion and integration of large DNA fragments, and accomplishes the precise site-directed mutations in the chromosome at a high efficiency. It was successfully applied to modify multiple genes for increasing 1,2-propanediol production. This developed genome editing method will facilitate engineering of native metabolic network or introducing novel pathways in *C. glutamicum* to synthesize the desired bio-based products.

**Results**

**Optimization of CRISPR–Cas9 system**

To decrease the effect of Cas9 expression on cell viability, *cas9* gene under the control of a weak promoter *P* _hom_ and *rrnB* terminator was introduced into the wild-type *C. glutamicum* ATCC 13032 genome at the transposase (Cgl1066) locus, giving rise to strain WT:*P* _hom_*cas9* (Fig. 1a and Additional file 1: Figure S1). To test the lethality of Cas9-induced DSB, the sgRNA targeting on *upp* gene (encoding uracil phosphoribosyltransferase) was designed and inserted into pXMJ19ts under the control of strong promoter *P* _glvA_, resulting in plasmid psgRNA上诉. Transformation with psgRNA上诉 yielded ~10^5 cfu/μg plasmid DNA (Fig. 1b), indicating a high escape rate that is inadequate for eliminating wild-type cells. We assumed that the amount of Cas9 protein might be not enough to cleave target DNA effectively. Therefore, a strong promoter *P* _tuf_ was used to increase the transcription of chromosomal *cas9* gene. The mRNA level of *cas9* with *P* _tuf_ control increased by 4.93-fold compared to that under the control of *P* _hom_ (Fig. 1c), leading to a decrease in escape rate (Fig. 1b). Then, two ribosome binding sequences (RBS1 and RBS2) with increased translational initiation efficiencies were inserted into the front of the start codon of *cas9* gene, resulting in WT:*P* _tuf* _rbs1*-cas9 and WT:*P* _tuf* _rbs2*-cas9 strains (Fig. 1a). This led to the great decreases in the numbers of transformants (Fig. 1b), demonstrating the feasibility of CRISPR/Cas9-based counter-selection. The plasmid-borne Cas9 expression hindered cell growth of *C. glutamicum* in the previous report [27], therefore, we investigated the effect of chromosome-borne Cas9 expression on cell viability. When these strains harboring different expression cassettes of *cas9* gene were cultivated in BHIS medium, no difference in growth rate was observed (Fig. 1d). Furthermore, the expression level of chromosome-borne Cas9 was visibly lower than those of plasmid-borne IPTG-inducible Cas9 by Western blotting analysis, suggesting that the decreased expression of chromosome-borne Cas9 has little impact on cell growth (Fig. 1e).

To assess the editing efficiencies using sgRNA and different Cas9 expression cassettes for gene deletion in *C. glutamicum*, the *upp* gene encoding uracil phosphoribosyltransferase was chosen as the target gene. Since its inactivation led to the failure of conversion from 5-fluorouracile (5-FU) to a toxic product for cell growth and generated a 5-FU-resistant phenotype for convenient screening [8]. To delete *upp* gene, two homologous arms as repair templates were inserted into psgRNA上诉 to construct pHASgRNA上诉. When transformed with pHASgRNA上诉 into WT:*P* _hom_*cas9*, 21.79 ± 5.88% of the colonies (7/26, 4/26, 6/26) were confirmed by colony PCR to correctly edit *upp* gene (Fig. 1f and Additional file 1: Figure S2) and correspondingly gained the 5-FU resistance (Fig. 1g). For the next round genome editing, nearly all of colonies after an overnight incubation had lost the plasmid pHASgRNA上诉, indicating the convenience of a single-plasmid-based CRISPR–Cas9 system for genome editing in *C. glutamicum*. When transformed with pHASgRNA上诉 into WT:*P* _tuf* _rbs1*-cas9 and WT:*P* _tuf* _rbs2*-cas9, the numbers of transformants decreased (Fig. 1f); however, the editing efficiency improved from 43.59 ± 4.44 to 60.26 ± 5.88% (Fig. 1f and Additional file 1: Figures S3–S5), indicating that the lethal efficiency of CRISPR/Cas9-based counter selection has a significant effect on editing efficiency for gene deletion.

**Introduction of RecET increases the editing efficiency of CRISPR–Cas9**

The edited mutants would be generated from double-crossover HR; therefore, the HR ability of *C. glutamicum* became a key factor in the editing efficiency of CRISPR/Cas9 system. To increase the probability of HR occurrence, *recET* from the defective Rac prophage under the control of inducible *P* _prep_ were introduced into the chromosome at the downstream of *cas9* locus, giving rise to the WT:*P* _tuf* _rbs2*-cas9:*P* _prep* _recET* (Fig. 2a and Additional file 1: Figure S6). As the inducible expression of RecET in the presence of propionate, the transformation with pHASgRNA上诉 produced 2.1 × 10^3 cfu/μg plasmid DNA, which was a 70% increase compared to WT:*P* _tuf* _cas9* (Fig. 2b). About 82.05 ± 8.88% of selected colonies were identified as the expected deletion of *upp* by colony PCR (Fig. 2c and Additional file 1: Figure S7). Two RB5s (RBS3 and RBS4) were inserted in the front of the start codon of *recET* to improve its translation initiation rate. The numbers of obtained colonies showed no significantly difference (Fig. 2d). As expected, editing
efficiencies improved to 83.33 ± 2.22% in WT::Ptuf-rbs2-cas9::Prtp-rbs2-recET and 92.31 ± 0.00% in WT::Ptuf-rbs2-cas9::Prtp-rbs4-recET (Fig. 2e and Additional file 1: Figures S8, S9), respectively. These results suggested that introduction of recET gene increased the HR efficiency of C. glutamicum, resulting in a high editing efficiency. For convenience, WT::Ptuf-rbs2-cas9::Prtp-rbs4-recET strain was referred as EDT (Editable Type) in the following text. A detailed experimental protocol of the RecET-assisted CRISPR–Cas9 method had been shown in Additional file 2.
Applicability of CRISPR–Cas9 editing method in *C. pekinense*

To determine whether CRISPR–Cas9 system is applicable for other *C. glutamicum*-related bacteria, Cas9 expression cassette instead of RecET–Cas9 expression cassette was used to integrate into the chromosome due to the low frequency of integrating the 8.5-kb expression cassette of RecET–Cas9 using two rounds of homologous recombination in the chromosome. *C. pekinense* 1.563, a lysine producer screened by random mutagenesis was chosen as a candidate strain for genome editing [29]. Two sgRNA targeting argR encoding a regulator and farR encoding a global repressor were designed and used to construct pHASgRNA<sub>argR</sub> and pHASgRNA<sub>farR</sub> for deleting these two genes. Transformation with pHASgRNA<sub>argR</sub> produced 2.6 × 10<sup>3</sup> cfu/μg plasmid DNA (Fig. 3a). The editing efficiency for argR deletion in *C. pekinense* 1.563 was 30.3%, which was similar to that in *C. glutamicum* ATCC 13032 (Fig. 3b and Additional file 1: Figure S10).

And the deletion of farR was accomplished with a transformation efficiency of 2.4 × 10<sup>3</sup> cfu/μg plasmid DNA and an editing efficiency of 30% in *C. pekinense* 1.563, respectively (Fig. 3a, c and Additional file 1: Figure S11). Therefore, our results indicated that the CRISPR–Cas9 system could be available for genome editing in other *Corynebacterium* species.

**Gene deletion and integration using RecET-assisted CRISPR–Cas9 method**

The method was further tested for efficiencies of gene deletion at different loci. Firstly, the inactivation of lactate dehydrogenase was performed by deleting *ldh* gene. Using the same sgRNA as that in the two-plasmid-based CRISPR/Cas9 method [27] for deleting *ldh* gene and the donor templates with 500 and 1000 bp HAs, pHA500sgRNA<sub>ldh</sub> and pHA1000sgRNA<sub>ldh</sub> were constructed and transformed into EDT, respectively (Fig. 4a). The editing efficiency with 1000 bp HAs (78.21 ± 2.22%)
was higher than that with 500 bp HAs (65.38 ± 3.85%) (Additional file 1: Figures S12, S13), which was consistent with the effects of repair arm sizes on gene editing efficiency in the previous report [28]. To validate its efficiencies for deleting the fragments with variant sizes, CGP3, a 220 kb prophage [30], was chosen as the target to delete 1-, 10-, 20- and 200-kb regions using the same sgRNA and different upstream and downstream HAs in EDT (Fig. 4b). The transformation with pHAsgRNA_{ACGP3-10kb} and pHAsgRNA_{ACGP3-20kb} decreased by 43% and 70%; and the deletion efficiencies of 10- and 20-kb regions were 35.90 ± 2.22 and 26.92 ± 3.85%, respectively (Additional file 1: Figures S15, S16). Unfortunately, the transformation with pHAsgRNA_{ACGP3-200kb} only yielded ~10 colonies and the 200-kb deficient mutant was not obtained by colony PCR screening.

For gene insertion, 717-bp gfpmut3a gene as a candidate was inserted in upp, CGP1, CGP2 and CGP3 loci, respectively. The insertion of gfp at upp locus reached an efficiency of 69.23 ± 3.85% (Fig. 4c and Additional file 1: Figure S17). In contrast, the insertion of gfp at CGP1, CGP2 and CGP3 loci only showed editing efficiencies below 10.0% (Fig. 4c and Additional file 1: Figures S18–S20). Due to the effects of gRNA performance on the cutting effectiveness of the Cas9-sgRNA complex [31–33], we designed nine sgRNAs to target the different PAM sites locating in CGP1. However, the numbers of colonies harboring psgRNA series vectors were significantly higher than those of colonies harboring the psgRNA_{upp} vector (data not shown), indicating that gRNA performance might be a key factor to affect the editing efficiency of CRISPR–Cas9 system. Using the sgRNA targeting upp locus, a 2.5-kb P_{tuf}-hom-thrB and 5.7-kb P_{tuf}-hom-thrB-P_{glyA}-lysC-thrC expression cassettes were inserted with the editing efficiencies of 7.14 and 2.22%, respectively (Fig. 4c and Additional file 1: Figure S21). Furthermore, only a positive mutant with a 7.5-kb P_{trp}-trpEG-P_{glyA}-trpDC-P_{sod}-trpBA expression cassette insertion at the upp locus was obtained through screening 61 transformants by colony PCR (Fig. 4c and Additional file 1: Figure S21). Using two different sgRNAs targeting the same locus between cgl0900 and cgl0901, the 3.6-kb lacZ expression cassette was inserted into the genome with the efficiency of 16.67 ± 2.22 and 20.51 ± 2.22% (Additional file 1: Figure S22), respectively. Taken together, the differences in genomic locus and the length of inserted fragment had an obvious effect on the insertion efficiency.

**Site-directed mutation mediated by RecET-assisted CRISPR–Cas9 method**

To evaluate the efficiency of site-directed mutation, the start codon of pgi gene were mutated from ATG to GTG to downregulate its expression, which led to the increase in the flux distribution towards pentose phosphate pathway [2]. Eleven colonies were identified to be correct edited by PCR amplification and DNA sequencing with an efficiency of 55.0% (Fig. 5a). When the mutation from CGG to CAG at the PAM was introduced into the repair template, the efficiency for pgi mutation increased to 95%. Then, this method was applied to introduce
double amino acid mutations of A26V and M31V into the chromosomal ArgB to release the feedback inhibition by L-arginine [2]. The sgRNA targeting the sequence from 90th to 110th bp of argB gene and HA containing 10 bp mismatches were designed (Fig. 5b). Thirteen colonies were randomly selected for colony PCR identification. Ten colonies contained the total mutated sites in the argB* with an editing efficiency of 77% (Fig. 5b).

| N20 | NGG | Efficiency |
|-----|-----|------------|
| ATATCCTGACCTACGCAGTG | TGG  | 17/26; 18/26; 16/26 |
| ATATCCTGACCTACGCAGTG | TGG  | 20/26; 20/26; 21/26 |

**Fig. 4** Gene deletion and insertion using the RecET-assisted CRISPR–Cas9 method. **a** Deletion of ldh gene using pHA500sgRNA <sup>ldh</sup> and pHA <sup>1000</sup>sgRNA <sup>ldh</sup>. **b** Deletion of 1-, 10- and 20-kb regions in CGP3 locus using pHAsgRNAΔCGP3–1kb, pHAsgRNAΔCGP3–10kb and pHAsgRNAΔCGP3–20kb. **c** Gene insertion in upp, CGP1, CGP2, CGP3 loci. For upp locus, both gfp, P<sub>ptuf</sub>-hom-thrB and P<sub>ptuf</sub>-hom-thrB-P<sub>glyA</sub>-lysC-thrC expression cassettes were chosen as the insertion fragments. For CGP1, CGP2 or CGP3 loci, gfp was chosen as the insertion gene.
Interestingly, one colony showed eight point mutations without A26V mutation, indicating that the double-crossover event happened at the downstream sites of A26 to repair the DSB.

Due to the potential instability of plasmid-borne Cas9, we further performed consecutive multiplex gene modifications in EDT to detect the stability of chromosome-borne Cas9. The deletion of argR showed an editing efficiency of 83.33% in EDT. The efficiency for farR deletion in EDTΔargR was 75.00%. Two amino acid mutations of ArgB* was also performed in EDTΔargRΔfarR with an editing efficiency of 77.06%. Shake flask cultivation showed that EDTΔargRΔfarRargB* accumulated 1.98 ± 0.26 g/L.
l-arginine. As knockdown of pgk expression in EDTΔargRΔfarRargB*, the growth and glucose consumption rates of EDTΔargRΔfarRargB*pgiΔGTG were comparable to those of other strains (Fig. 5c), and l-arginine production reached 2.77 ± 0.19 g/L (Fig. 5c). These results indicated the stability of chromosome-born Cas9 for iterative genome editing in C. glutamicum.

**Application of RecET-assisted CRISPR–Cas9 method to modify multiple genes for biosynthetic production of 1,2-propanediol**

*Corynebacterium glutamicum* have been engineered for 1,2-propanediol production by expressing the heterologous genes mgsA encoding methylglyoxal synthase, gldA encoding glycerol dehydrogenase and yqhD encoding alcohol dehydrogenase from *E. coli* [34, 35]. Genetic modification strategy for increasing 1,2-propanediol production have been predicted by iCW773 in our previous study [36]. The deletion of hdpA encoding dihydroxyacetone phosphate phosphatase and ldh encoding lactate dehydrogenase could decrease the formation of byproducts, glycerol and lactate, to increase the 1,2-propanediol production (Fig. 6a). Downregulation of gap, pgk and gpmA could decrease the split-flow of glyceraldehyde phosphate and drive more carbon fluxes towards 1,2-propanediol synthesis. Among three genes, pgk encoding phosphoglycerate kinase is essential for growth of *C. glutamicum* on carbon sources requiring glycolysis and gluconeogenesis, and a major control point in the glycolytic pathway during growth on glucose [37]. The effects of decreased Pgk flux on cell metabolism and 1,2-propanediol synthesis were simulated by iCW773 using FBA. The fluxes toward MgsA was significantly enhanced in the relative Pgk flux, and the maximum extracellular 1,2-propanediol production was achieved when the relative Pgk flux was decreased by 30% compared with WT (Fig. 6b). Therefore, these genes were modified by RecET-assisted CRISPR–Cas9-mediated method to regulate the metabolic network for 1,2-propanediol synthesis (Fig. 6a).

Firstly, expression of mgsA, gldA, and yqhD genes in an artificial operon under the P_{sec} control from the plasmid pXMJ19-mgsA-gldA-yqdd gave rise to 1.69 ± 0.09 g/L 1, 2-propanediol accumulation with a yield of 0.10 mol/mol in a shake flask cultivation of PT (Fig. 6c). The efficiencies of hdpA and ldh deletion were 66.67 and 50.00%, respectively (Additional file 1: Figures S23, S24). Deletion of hdpA gene resulted in a 94.08% decrease in the extracellular glycerol accumulation in PTΔhdpAΔldh (Fig. 6c). The lactate was almost undetectable in PTΔhdpAΔldh that accumulated 4.95 ± 0.24 g/L 1,2-propanediol with a 2.0-fold increase in the product yield (0.30 mol/mol).

The byproduct acetate was accumulated (2.99 ± 0.15 g/L), which was consistent with the previous report [34, 35]. To control the competing pathway for glyceraldehyde phosphate, two weak promoters, P_{hom} and P_{dapa}, were used to construct pHASgRNA_{hom} and pHASgRNA_{dapa} for the promoter replacements to decrease the transcriptional level of pgk gene. The efficiencies of P_{hom} and P_{dapa} replacement were 66.67 and 83.33%, resulting in 22.66 and 74.41% decreases in the mRNA level of pgk, respectively (Fig. 6d). Meanwhile, the mRNA level of gapX decreased and the mRNA level of tpiA increased, which contributed to drive the flux toward the 1,2-propanediol synthetic pathway. However, no significant changes were observed in the mRNA levels of fda, gpmA, gldA and acn. When the mRNA level of pgk decreased, there were no significant differences in the growth and glucose consumption (Fig. 6d). PTΔhdpAΔldhP_{hom}pgk and PTΔhdpAΔldhP_{dapa}pgk produced 6.75 ± 0.46 and 5.57 ± 0.36 g/L 1,2-propanediol, respectively, which were 36.36 and 12.53% increase compared to PTΔhdpAΔldh. Meanwhile, the precursor acetol showed the increasing trend similar to 1,2-propanediol and the byproduct acetate decreased by 25.42 and 13.71%, respectively (Fig. 6c). Finally, the downregulation of pgk improved the product yield to 0.41 mol/mol glucose, which was higher than the deletion of hdpA and ldh genes in the previous engineered *C. glutamicum* [34, 35]. For industrial applications, the cas9–recET expression cassettes were deleted from the chromosome of EDTΔldhΔhdpAP_{hom}pgk using pOUT-cas9–recET plasmid to obtain a final engineered strain for 1,2-propanediol production (Additional file 1: Figure S25).

**Discussion**

CRISPR–Cas9 systems have been employed to be efficient biotechnology tools for genome editing in many bacteria [18–24]. Two-plasmid-based CRISPR–Cas9 editing methods available for *C. glutamicum* require a high transformation efficiency to guarantee the co-occurrence of two plasmids transformation, resulting in their editing efficiencies comparable to those of other methods relying on two rounds of homologous recombination [26–28]. In this study, we developed a RecET-assisted CRISPR–Cas9-genome editing method for *C. glutamicum*. This approach employed a single plasmid to transform EDT, which enables transformation and plasmid curing procedures to be more simple and convenient than two-plasmid-based CRISPR–Cas9 method [27, 28]. Introduction of RecET increased the editing efficiency of this method, which is applicable for comprehensive genome editing, such as deletion of large DNA fragments, integration of multiplex gene expression cassettes,
and promoter replacements as well as site-directed mutations.

In two-plasmid-based CRISPR–Cas9 methods, only the decreased expression level of SpCas9 under the control of inducible $P_{tac}$ was successful to obtain transformants due to the potential toxicity of SpCas9 on cell viability [26–28]. Even so, the transformants harboring pCas9 grew at a lower rate than the wild-type strain [28]. Moreover, plasmid-borne Cas9 occurred spontaneous mutation, resulting in editing failure [27]. To decrease the copies of cas9 gene, it was integrated into the chromosome under the control of $P_{tuf}$, whose strength was lower than that of $P_{tac}$ [38]. Western blotting analysis testified that the expression of chromosome-borne Cas9 maintained at the relative lower level compared to plasmid-borne Cas9, which might be the reason for no impacts of chromosome-borne Cas9 expression on cell growth. Furthermore, no off-target mutations could be detected in C. glutamicum expressing plasmid-borne Cas9 protein by genome sequencing [28], indicating that
the chromosome-borne Cas9 might generate a very low probability of off target.

The induction of DSB on chromosome by functional Cas9-sgRNA complex is lethal to some bacteria lacking NHEJ pathway like eukaryotes [14, 39, 40]. For C. glutamicum, the co-transformation of CRISPR–Cas9 system with either dsDNA with different HA lengths or ssDNA as editing templates had been testified to be insufficient to repair the DSB in the absence of RecT [26]. The dsDNAs on the plasmids was unable to repair the DSB unless expressing the phage-derived recombinases (RecET) in E. coli [18], indicating that homologous recombination efficiency is a key factor that affects the editing efficiency of CRISPR–Cas9 system. However, plasmid-borne dsDNA could repair DSB in C. glutamicum expressing the chromosome-borne Cas9, suggesting that the introduction of DSB by CRISPR–Cas9 system increased the HR of the damaged DNA as reported elsewhere [18]. Introduction of exogenous RecET into C. glutamicum chromosome promoted the frequency of double-crossover event between chromosomal DSB region and repair templates [8, 25, 26, 41–43], which contributed to genome editing mediated by CRISPR–Cas9.

Compared to other CRISPR–Cas9 genome editing methods, our method has some advantages in transformation efficiency and multiple genetic manipulation, indicating a promising application in iterative genome editing of C. glutamicum. A high transformation efficiency is necessary for two-plasmid-based CRISPR–Cas9-coupled recombineering system to co-transform single-stranded oligodeoxyribonucleotides (ssODN) and Cas9-sgRNA vector into C. glutamicum [26]. A one-step electro-transformation strategy to co-transform pCas9 and pgRNA had been adopted to reduce false positives formation [27]. The low frequency of co-transformation led to less transformants. In contrast, transformation with a single-plasmid harboring sgRNA and HAs produced 10^3 cfu/μg plasmid DNA. This transformation efficiency could be achieved easily and reproducibly for C. glutamicum at laboratory level, suggesting a universal application of a single-plasmid-based CRISPR–Cas9 system. Moreover, both pCas9 and pgRNA plasmids need to be removed in the absence of antibiotic selection pressure for several subcultures and then be co-transformed together for the next round of genome editing [27]. In contrast, a single-plasmid is easy to be cured by overnight cultivation and another new editing plasmid can be efficiently transformed for the next round of genome editing, indicating the convenience of our method for iterative genome editing. As for gene deletion, the efficiencies of ldh deletion using this method are slightly higher than that using two-plasmid-based CRISPR–Cas9 method (Additional file 1: Table S1) [27]. However, the deletion of 200-kb fragment was unsuccessful, which might be attributed to the decreased efficiency for gene deletion with the increase in gene length as reported previously [9]. Furthermore, CRISPR–Cas9 method relied on a double-crossover event, which simultaneously occurred between the DSB region and donor DNA template after the cleavage of Cas9-sgRNA complex. DSB was generated in the middle part of 200-kb fragment, while the strand exchange of HR occurred between the two sides of 200-kb fragment and plasmid-borne HAs. Therefore, a long spacing between DSB and HAs gave rise to a rare frequency of HR relying on a double-crossover event. However, TCCRAS method could achieve the deletion of 179.8-kb fragment in our previous report, due to a little high probability in the successive occurrence of a single-crossover event between the chromosomal homologous regions and plasmid-borne HAs under the screening pressure. Despite the numbers of lacZ insertion transformants (13 colonies) obtained by this method were higher than those (4 colonies) screened by two-plasmid-based CRISPR–Cas9 method, the insertion efficiency using this method was relatively low. It attributed to the differences in transformation efficiency and lethality of Cas9 between two methods. A large number of colonies were obtained as transformation with a single plasmid compared to the co-transformation with two plasmids. Meanwhile, the abundance of chromosomal-borne Cas9 maintained at a relatively lower level than that of plasmid-borne Cas9, resulting in the low lethality of chromosomal-borne Cas9 and the high numbers of false positive colonies. Furthermore, the integration efficiencies mediated by CRISPR–Cas9 at different chromosomal position in E. coli were variable due to the differences of cutting effectiveness of the Cas9-sgRNA complex [44]. It has also been demonstrated that different sgRNA can generate a great impact on editing efficiency in C. glutamicum. Therefore, the sgRNA less than 60% GC content is recommended to design for increasing the editing efficiency of CRISPR–Cas9 system in C. glutamicum [28].

**Conclusions**

In this study, we developed a RecET-assisted CRISPR–Cas9 genome editing method for C. glutamicum. This method took advantage of the chromosome-borne Cas9 to reduce the instability of plasmid harboring Cas9 and utilized RecET to increase the frequency of homologous recombination. Transformation efficiency was improved by using a single-plasmid harboring a sgRNA and repair template, resulting in high efficiencies in gene deletion, insertion and site-specific mutation. In silico model-predicted target genes were modified using this method,
and the final engineered strain produced 6.75 ± 0.46 g/L 1, 2-propanediol. This method will boost the metabolic engineering of C. glutamicum to produce the desired bio-based products from renewable biomass.

**Methods**

**Bacterial strains, plasmids and cultivation conditions**

Corynebacterium glutamicum ATCC 13032 (American Type and Culture Collection, Manassas, VA, USA) was used as the parental strain for genome editing. The E. coli strain EC135 [45] was used as the cloning host for plasmid construction. The pK18mobsacB was used for the introduction of cas9 and recET genes into the chromosome. The pXMJ19ts with a temperature-sensitive replication origin [46] was applied to express sgRNA cassette and carry the DNA repair template for gene deletion, insertion and site-directed mutation. All of the strains and plasmids used in this study are listed in Additional file 1: Tables S2 and S3. E. coli strains were cultured in Luria–Bertani medium at 37 °C. C. glutamicum strains were cultured in heart brain infusion medium at 30 °C. When necessary, antibiotics were added at the following concentrations: 50 μg/mL kanamycin or 20 μg/mL chloramphenicol for E. coli and 25 μg/mL kanamycin, 10 μg/mL chloramphenicol for C. glutamicum.

**DNA manipulations**

DNA manipulations were performed using the standard cloning techniques [47], and all primers for plasmids construction are shown in Table S4. Polymerase chain reaction (PCR) amplifications were performed with Q5 High-Fidelity DNA Polymerase (NEB) and Taq DNA Polymerase (Tiangen) for cloning and colony PCR identification, respectively. Gibson Assembly were conducted with NEBuilder Master Mix (NEB). DNA gel Extraction Kit (Tiangen) was used to concentrate the DNA fragment and NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific) was used to determine the concentration of DNA fragment for Gibson Assembly [48].

**Insertion of Cas9 and RecET expression cassettes into chromosome**

To introduce cas9 gene into the chromosome, the cas9 gene was amplified using pCas as the template with primers P5/P6. P_hom was the upstream homologous arms (HA), and downstream HA were amplified using C. glutamicum ATCC 13032 genome as template with primers P3/P4, P1/P2 and P9/P10, respectively. The terminator rnb was amplified from pXMJ19 with primers P7/P8. These fragments together with linearized pK18mobsacB were purified and recovered for Gibson assembly to construct pIN-P_hom-cas9. To construct pIN-P_tuf-cas9, pIN-P_tuf-rbs1-cas9 and pIN-P_tuf-rbs2-cas9, the same procedures were applied using the indicated primers shown in Additional file 1: Table S4. Then, pIN-P_hom-cas9, pIN-P_tuf-cas9, pIN-P_tuf-rbs1-cas9 and pIN-P_tuf-rbs2-cas9 were transformed to the competent cells of C. glutamicum ATCC 13032 by electroporation, respectively. Screening for the first and second recombination events were performed as described previously [7]. Kanamycin-sensitive and sucrose-resistant clones were verified by colony PCR and DNA sequencing to obtain WT::P_hom-cas9, WT::P_tuf-cas9, WT::P_tuf-rbs1-cas9, WT::P_tuf-rbs2-cas9 strains (Additional file 1: Figure S1).

To integrate recET gene into the chromosome, E. coli W3110 genome was used as a template to amplify recET(anti) with primers P33/P34. WT::P_hom-Cas9 genome was used as a template to amplify cas_tail_rrnB with primers P31/P32. WT::P_hom-Cas9 genome was used as a template to amplify P_prp(anti) and down HA with primers P35/P36 and P37/P38. These fragments together with linearized pK18mobsacB were purified and recovered for Gibson assembly to construct pIN-P_prp-recET. The same procedures were performed to construct pIN-P_prp-rbs3-recET and pIN-P_prp-rbs4-recET using the indicated primers in Additional file 1: Table S4. Then, pIN-P_prp-recET, pIN-P_prp-rbs3-recET and pIN-P_prp-rbs4-recET were transformed to WT::P_tuf-rbs2-cas9 by electroporation. Screening for the first and second recombination events were performed as described previously [7]. Kanamycin-sensitive and sucrose-resistant clones were verified by colony PCR and DNA sequencing to obtain WT::P_tuf-rbs2-cas9::P_prp-recET, WT::P_tuf-rbs2-cas9::P_prp-rbs3-recET and WT::P_tuf-rbs2-cas9::P_prp-rbs4-recET strains (Additional file 1: Figure S6).

**Construction of sgRNA expression plasmids**

To construct sgRNA expression plasmid, the 20 bp spacer sequence specific for each target was synthesized in primers and the sgRNA scaffold was amplified by PCR using pTargetF as the template. The P_gfp, for sgRNA transcription and the upstream and downstream HAs for DSB repair were amplified using genome of C. glutamicum ATCC 13032 as the template. The pXMJ19 was used as a template to amplify mutated pXMJ19ts using primers P11/P12. The PCR product was digested with DpnI as a template to amplify mutated pXMJ19ts using primers P31/P32. The PCR product was treated with anti-Cas9 genome was used as a template to amplify P_prp(anti) and down HA with primers P35/P36 and P37/P38. These fragments together with linearized pK18mobsacB were purified and recovered for Gibson assembly to construct pIN-P_prp-recET. The same procedures were performed to construct pIN-P_prp-rbs3-recET and pIN-P_prp-rbs4-recET using the indicated primers in Additional file 1: Table S4. Then, pIN-P_prp-recET, pIN-P_prp-rbs3-recET and pIN-P_prp-rbs4-recET were transformed to WT::P_tuf-rbs2-cas9 by electroporation. Screening for the first and second recombination events were performed as described previously [7]. Kanamycin-sensitive and sucrose-resistant clones were verified by colony PCR and DNA sequencing to obtain WT::P_tuf-rbs2-cas9::P_prp-recET, WT::P_tuf-rbs2-cas9::P_prp-rbs3-recET and WT::P_tuf-rbs2-cas9::P_prp-rbs4-recET strains (Additional file 1: Figure S6).
CRISPR/Cas9–assisted genome editing in C. pekinense

A lysine-producing C. pekinense 1.563 screened by random mutagenesis was used to verify the application of the CRISPR/Cas9 system. To insert cas9 expression cassette, the non-replicated pNn-P tua-RBS2-cas9 plasmid was electroporated into the competent cells of C. pekinense 1.563 to obtain 1.563::P tua-RBS2-cas9 strain by two rounds of recombination events. To delete argR and farR genes, 100 ng of the pHasgRNA agR and pHasgRNA farR plasmids were then transformed by electroporation into 1.563::P tua-RBS2-cas9, respectively. Cells were recovered in the resuscitation medium, spread on BHIS plates supplemented with 10 μg/mL chloramphenicol and then incubated for 2 days. The procedures for transformant screening and plasmid curing were performed as above described in C. glutamicum ATCC 13032.

RNA preparation and quantitative RT-PCR

In order to determine the level of cas9 of two cas9-expressing strain, i.e. WT::P hom-cas9 and WT::P tua-cas9, quantitative reverse transcription PCR (qRT-PCR) was performed using total RNA samples. Total RNA was isolated from the cells with the RNAprep Pure Cell/细菌 Kit (Tiangen, China). The cDNA from approximately 300 ng of RNA was prepared using the specific primers listed in Additional file 1: Table S4 and the FastQuant RNA Preparation and Quantitative RT-PCR Kit (Tiangen, China). The rpoB gene, which encodes RNA polymerase β subunit, was used as the reference gene [51]. The primers used for RT-PCR were listed in Additional file 1: Table S4. The qRT-PCR was performed with the GoTaq qPCR master mix (Promega, USA) in a 20-μL mixture using the LightCycler® 96 Real-Time PCR System (Roche, Switzerland). The data analyses were performed using the LightCycler® 96 software (Roche, Switzerland).

Western blotting

Corynebacterium glutamicum cells were resuspended in 1 mL of phosphate-buffered saline (PBS) and disrupted using an Ultrasonic Cell Disruptor. The supernatants were collected by centrifugation at 12,000 rpm at 4 °C for 30 min. Protein samples were separated by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% running gel and 5% stacking gel and transferred to a nitrocellulose membrane (PVDF) by electroblotting. The proteins were probed with rabbit
polyclonal antibody against Cas9. The blots were visualized with a peroxidase-coupled goat anti-rabbit secondary antibody and an ECL color development reagent (GE, USA).

Modification of L-arginine synthetic network in C. glutamicum
The strain WT::P_{far\_rbs2-cas9::prp\_rbs4-recET} (EDT) was used to construct the engineered strains for L-arginine production. The plasmid pHAsgRNA_{argR} and pHAsgRNA_{farR} were iteratively transformed by electroporation to delete argR and farR genes encoding two transcriptional regulator as the above-described procedure, resulting in the strains EDT\Delta argR and EDT\Delta argR\Delta farR. pHAsgRNA_{argB} was transformed by electroporation into the strain EDT\Delta argR\Delta farR for site-directed mutation of chromosomal argB to release the feedback inhibition by L-arginine. The plasmid pHAsgRNA_{pgk} was transformed by electroporation into the strain EDT\Delta argR\Delta farR\Delta argB* to change the start codon ATG to GTG for pgk knockdown, resulting in the strain EDT\Delta argR\Delta farR\Delta argB*pgk_{GTG}.

Modification of 1,2-propanediol synthetic network in C. glutamicum
The strain WT::P_{far\_rbs2-cas9::prp\_rbs4-recET} (EDT) was used to construct the engineered strains for 1,2-propanediol production. The plasmid pHAsgRNA_{ldh} and pHAsgRNA_{dapA} were iteratively transformed by electroporation to delete ldh and dapA as the above-described procedure, resulting in the strain EDT\Delta ldh\Delta dapA. pHAsgRNA_{pholino} and pHAsgRNA_{pgk} were transformed by electroporation into the strain EDT\Delta ldh\Delta dapA separately for promoter replacement of pgk to increase the flux towards 1,2-propanediol, generating the strains EDT\Delta ldh\Delta dapA_{pholino}pgk and EDT\Delta ldh\Delta dapA_{pgk}. pXMJ19- mgsA-gldA-yqhD was transformed by electroporation into the strains EDT, EDT\Delta ldh\Delta dapA, EDT\Delta ldh\Delta dapA_{pholino}pgk and EDT\Delta ldh\Delta dapA_{pgk}, giving rise to the strains PT, PT\Delta ldh\Delta dapA, PT\Delta ldh\Delta dapA_{pholino}pgk and PT\Delta ldh\Delta dapA_{pgk}, respectively. To obtain a final engineered strain for 1,2-propanediol production, the plasmid pOUT-cas9-recET was transformed into the strain EDT\Delta ldh\Delta dapA_{pholino}pgk by electroporation to plug out the cas9-recET expression cassette in the chromosome to get a final engineered 1,2-PDO strain.

Shake flask cultivation
For L-arginine fermentation, C. glutamicum strains were precultured in the CGIII seed medium [52] at 30 °C and 220 rpm until the OD_{600} reached 10. One milliliter of seed culture was inoculated in 500-mL baffled shake flasks containing 30 mL CGXII medium supplemented with 40 g/L glucose and 2 g/L yeast extract. The cells were grown at 30 °C, 220 rpm and the pH was maintained at 7.0–7.2 by supplementation with ammonia.

For 1,2-propanediol fermentation, C. glutamicum strains were precultured in CGIII seed medium. The cells of an overnight preculture were inoculated into 40-mL fermentation cultures in 500-mL baffled flasks by transferring appropriate volume for a final optical density of 1.0. Fermentation medium contained (g/L): glucose 40, yeast extract 2, MgSO_{4}·7H_{2}O 0.25, (NH_{4})_{2}SO_{4} 10, KH_{2}PO_{4} 1, K_{2}HPO_{4} 1, FeSO_{4}·7H_{2}O 0.01, MnSO_{4}·H_{2}O 0.01, ZnSO_{4}·7H_{2}O 0.001, CuSO_{4}·0.0002, NiCl_{2}·6H_{2}O 0.00002, biotin 0.0002, thiamine 0.0002 and 3-(N-morpholino) propanesulfonic acid (MOPS) 42. The cells were grown at 30 °C and 160 rpm. The expressions of mgsA, gldA, and yqhD genes were induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 6 h. The pH was maintained at 7.0–7.2 by supplementation with ammonia.

Analytical methods
The glucose and lactate concentrations were assayed using an SBA-40D biosensor analyzer (Institute of Biology of Shandong Province Academy of Sciences, Shandong, China). The cell concentration was determined by measuring the absorbance at 600 nm (OD_{600}) using a spectrophotometer (V-1100D; Mapada Instruments, Shanghai, China). To quantify L-arginine, high-pressure liquid chromatography (HPLC) was performed using an Agilent 1260 series chromatography system equipped with a ZORBAX Eclipse AAA column (4.6 mm × 150 mm, 5 μm; Agilent) at 40 °C. Fluorescence detection was carried out after automatic pre-column derivatization with O-phthalaldehyde (OPA) using a variable wavelength detector (VWD) at 338 nm according to the instruction manual. The 1,2-propanediol, acetal, glycerol and acetate in the shake flask were analyzed using a Gas Chromatograph Mass Spectrometer (GCMS-QP2010 Ultra, Shimadzu, Japan) connected to an AOC-20i Auto-sample using a TG-WAXMS (length: 30 m; I.D.: 0.25 mm; film: 0.25 μm) (Thermo Scientific, USA). The samples were directly diluted 1:10 with methanol and centrifuged by 12,000×g for injection. The operating set up followed as the previous report [35]. The concentrations of 1,2-propanediol, acetal, glycerol and acetate were determined according to a calibration curve with an external standard. The peaks were identified by retention time and quantified using the intensity of one specific m/z value.
Additional files

**Additional file 1: Figure S1.** Colony PCR verification of cas9 integration. 
**Figure S2.** Colony PCR verification of upp deletion in WT::P\_recET
**Figure S3.** Colony PCR verification of upp deletion in WT::P\_recET
**Figure S4.** Colony PCR verification of upp deletion in WT::P\_recET
**Figure S5.** Colony PCR verification of upp deletion in WT::P\_recET
**Figure S6.** Colony PCR verification of reET integration. 
**Figure S7.** Colony PCR verification of upp deletion in WT::P\_recET
**Figure S8.** Colony PCR verification of upp deletion in WT::P\_recET
**Figure S9.** Colony PCR verification of upp deletion in WT::P\_recET
**Figure S10.** Colony PCR verification of upp deletion in WT::P\_recET
**Figure S11.** Colony PCR verification of upp deletion in WT::P\_recET
**Figure S12.** Colony PCR verification of upp deletion in WT::P\_recET
**Figure S13.** Colony PCR verification of upp deletion in WT::P\_recET
**Figure S14.** Colony PCR verification of upp deletion in WT::P\_recET
**Figure S15.** Colony PCR verification of upp deletion in WT::P\_recET
**Figure S16.** Colony PCR verification of upp deletion in WT::P\_recET
**Figure S17.** Colony PCR verification of upp deletion in WT::P\_recET
**Figure S18.** Colony PCR verification of upp deletion in WT::P\_recET
**Figure S19.** Colony PCR verification of upp deletion in WT::P\_recET
**Figure S20.** Colony PCR verification of upp deletion in WT::P\_recET
**Figure S21.** Colony PCR verification of upp deletion in WT::P\_recET
**Figure S22.** Colony PCR verification of upp deletion in WT::P\_recET
**Figure S23.** Colony PCR verification of upp deletion in WT::P\_recET
**Figure S24.** Colony PCR verification of upp deletion in WT::P\_recET
**Figure S25.** Colony PCR verification of upp deletion in WT::P\_recET
**Figure S26.** Colony PCR verification of upp deletion in WT::P\_recET
**Figure S27.** Colony PCR verification of upp deletion in WT::P\_recET
**Figure S28.** Colony PCR verification of upp deletion in WT::P\_recET
**Figure S29.** Colony PCR verification of upp deletion in WT::P\_recET
**Figure S30.** Colony PCR verification of upp deletion in WT::P\_recET

**Additional file 2:** Standard protocol of a ReET-assisted CRISPR-Cas9 genome editing in Corynebacterium glutamicum.

**Abbreviations**
TCCRS: toxin-counter-selectable cassette regulated by an antitoxin switch; CRISPR: clustered regularly interspaced short palindromic repeat; sgRNA: single guide RNA; DSB: double-strand break; PAM: protospacer adjacent motif; NHEJ: non-homologous end joining; HR: homologous recombination; ssDNA: single-stranded DNA; RBS: ribosome binding sequences; EDT: editable type; ssODN: single-stranded oligodeoxyribonucleotides; PCR: polymerase chain reaction; HA: homologous arm; UHA: upstream homologous arm; DHA: downstream homologous arm; qRT-PCR: quantitative reverse transcription PCR; PBS: phosphate-buffered saline; OD\_\_400 optical density at 600 nm; HPLC: high-pressure liquid chromatography; OPA: O-phthalaldehyde; WVD: variable wavelength detector; GC\_M5: gas chromatograph mass spectrometer; IPTG: isopropyl-β-D-thiogalactopyranoside.

**Authors' contributions**
TW and YZ conceived the study. TW, YZ, BW, and QH designed the study. BW, QH, YZ, and XZ performed the experiments. BW, QH, YZ, and XZ performed all data analysis. YZ, BW, and TW wrote the paper. All authors read and approved the final manuscript.

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**Competing interests**
The authors declare that they have no competing interests.

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