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An update of the suicide plasmid-mediated genome editing system in *Corynebacterium glutamicum*

Ting Wang,[1,†] Yanjun Li[1,2,3,*,†] Juan Li,[1] Dezhi Zhang,[1] Ningyun Cai,[1] Guihong Zhao,[1] Hongkun Ma,[1] Can Shang,[1] Qian Ma,[1,3] Qingyang Xu[1,3] and Ning Chen[1,3]

[1]*College of Biotechnology, Tianjin University of Science and Technology, Tianjin, 300457, China.*
[2]*Guangdong Provincial Key Laboratory of Fermentation and Enzyme Engineering, Guangzhou, 510006, China.*
[3]*Key Laboratory of Industrial Fermentation Microbiology, Ministry of Education, Tianjin University of Science and Technology, Tianjin, 300457, China.*

Summary

***Corynebacterium glutamicum* is an important industrial microorganism, but the availability of tools for its genetic modification has lagged compared to other model microorganisms such as *Escherichia coli*. Despite great progress in CRISPR-based technologies, the most feasible genome editing method in *C. glutamicum* is suicide plasmid-mediated, the editing efficiency of which is low due to high false-positive rates of *sacB* counter selection, and the requirement for tedious two-round selection and verification of rare double-cross-over events. In this study, an *rpsL* mutant conferring streptomycin resistance was harnessed for counter selection, significantly increasing the positive selection rate. More importantly, with the aid of high selection efficiencies through the use of antibiotics, namely kanamycin and streptomycin, the two-step verification strategy can be simplified to just one-step verification of the final edited strain. As proof of concept, a 2.5-kb DNA fragment comprising *aroG*^{fbr}*pheA*^{fbr} expressing cassettes was integrated into the genome of *C. glutamicum*, with an efficiency of 20% out of the theoretical 50%. The resulting strain produced 110 mg l^{-1} L-tyrosine in shake-flask fermentation. This updated suicide plasmid-mediated genome editing system will greatly facilitate genetic manipulations including single nucleotide mutation, gene deletion and gene insertion in *C. glutamicum* and can be easily applied to other microbes.**

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*For correspondence. E-mail yjli@tust.edu.cn; Tel. +86 022-60601251; Fax +86 022-60602998.
†These authors contributed equally to this work.
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Introduction

Corynebacterium glutamicum, a non-pathogenic Gram-positive soil bacterium, is an important workhorse in industrial biotechnology for the production of several million tons of amino acids annually, especially L-glutamate and L-lysine (Eggeling and Bott, 2015). To date, *C. glutamicum* has been metabolically engineered for the production of a wide portfolio of other compounds of commercial interest, and for the utilization of vastly extended spectrum of substrates. Successes in these regards have rendered this microorganism a versatile microbial platform for a broad range of future biotechnological applications. Despite its importance, the currently available genetic tools, especially those for editing the genome of *C. glutamicum*, remain somewhat limited compared to those for *E. coli*, another important industrial microorganism.

Recently, clustered regularly interspaced short palindromic repeat (CRISPR) technologies associated with either the RNA-guided endonuclease Cas9 or Cas12a (Cpf1) have been reported for the manipulation of the genome of *C. glutamicum*, allowing for gene deletion, insertion or single nucleotide mutation (Cho *et al*., 2017; Jiang *et al*., 2017; Liu *et al*., 2017; Peng *et al*., 2017; Wang *et al*., 2018a). Although CRISPR-based genome editing techniques in *C. glutamicum* have been substantially improved, several drawbacks remain that limit the application of these technologies. First, additional mutations must be introduced in the protospacer and PAM region to avoid Cas9/Cpf1 cutting when generating ssDNA-directed point mutations. Second, the two plasmid-based system currently required involves labour-intensive plasmid construction, slow colony growth, and results in few transformants, especially when the temperature-sensitive plasmid backbone is used for easy curing. Third, due to the low homologous integration efficiency in *C. glutamicum*, the plasmid-borne template is still a mandatory requirement (Wang *et al*., 2018b).

Fourth, editing efficiency is influenced by several factors, including knockout size, overall length of homologous arms, relative length of homologous arm to insert gene, and design of the sgRNA (Becker *et al*., 2018). Consequently, results from different studies vary widely and are not reproducible. Moreover, the currently achievable efficiency for large gene insertion is unacceptably low. For these reasons, CRISPR-based technology is not commonly applied to routine laboratory work, even in the groups with the above-mentioned publications.

Today, genome editing in *C. glutamicum* is predominantly accomplished through use of non-replicating suicide plasmids (Schäfer *et al*., 1994). This method relies on a two-step homologous recombination process that includes a counter-selection system. Specifically, the suicide plasmid pk*mobsacB*, which can be selected via antibiotic screening (usually kanamycin), integrates into the genome during the first recombination event, with the resultant single-cross-over strain being sucrose sensitive due to the expression of lethal levansucrase (*sacB*) (Jäger *et al*., 1992). In the second recombination event, the plasmid backbone is removed for scarless genetic modification, and selection for survival is carried out on sucrose-supplemented medium. Counter selection by *sacB* gene leads to a high false-positive rate due to high spontaneous inactivation of SacB (Hashimoto *et al*., 2003; Ma *et al*., 2015), while the first-cross-over event can be efficiently identified by kanamycin selection. In summary, the need for two-round selection and verification of rare double-cross-over events (Nesvera and Patek, 2011) limits the speed and throughput of strain engineering of *C. glutamicum*.

In this study, we sought an alternative, more rigorous, counter-selectable marker to *sacB*. The small ribosomal protein S12P gene *rpsL* was mutated to confer *C. glutamicum* with resistance to streptomycin. This is shown to be a powerful genetic marker that significantly increases the efficiency of counter selection in suicide plasmid-mediated genome editing. Moreover, the two-step verification procedure can be simplified to one-step verification of the final edited strain, as a result of rigorous antibiotic selection with kanamycin and streptomycin.

Results

Workflow of rpsL counter selection in suicide plasmid-mediated genome editing

The pk18*mobrpsL*-mediated genome editing workflow in *C. glutamicum* is illustrated in Fig. 1. As a prerequisite for using *rpsL* as a counter-selection marker, the chromosomal *rpsL* gene from the wild-type strain must be mutated to confer streptomycin resistance. The non-replicating plasmid pk18*mobsacB* is then modified to pk18*mobrpsL*, which expresses the streptomycin-sensitive wild-type *rpsL* gene under the strong constitutive promoter P*tuf*. As an example, we constructed plasmid pk18*mobrpsL-gfp*, in which the green fluorescent protein gene was designed to integrate at the locus of *iolD* (*cgl0162*), which is involved in myo-inositol catabolism. The pk18*mobrpsL-gfp* plasmid is electroporated into cells that exhibit streptomycin resistance attributing to a mutated chromosomal *rpsL* gene, for integration into the genome via single cross-over with kanamycin resistance (kan^R) selection. The additional wild-type allele of *rpsL* provided by the integrated plasmid is dominant over the *rpsL* mutant in the genome, thereby causing streptomycin sensitivity ($strep^S$) in the single-cross-over strain. Subsequently, streptomycin pressure ($strep^R$) is applied, and only cells in which the plasmid has been removed from the genome following the second-cross-over event can survive. Meanwhile, the second-cross-over strain is kanamycin sensitive (kan^S). Finally, correct integration of *gfp* in $strep^R kan^S$ colonies is confirmed by colony PCR and sequencing.

RpsL mutants enable C. glutamicum resistance to streptomycin

Streptomycin is a broad-spectrum aminoglycoside that raises missense error levels in bacterial translation by increasing the affinity of the ribosome to non-cognate tRNAs and interfering with the proofreading of the ribosome (Pelchovich *et al*., 2013). In the majority of cases, resistance to streptomycin is acquired as a consequence of mutations in *rpsL*, the gene encoding ribosomal protein S12 (Bottger and Springer, 2008). Mutations conferring streptomycin resistance were intensively studied in *Mycobacterium smegmatis* (Kenney and Churchward, 1994) and *E. coli* (Tubulekas *et al*., 1991; Timms *et al*., 1992), as well as in *Salmonella typhimurium* (Tubulekas *et al*., 1991), *Haemophilus influenza* (Stuy and Walter, 1992) and *Micrococcus luteus* (Salles *et al*., 1992). We aligned partial *rpsL* sequences from these bacteria containing highly mutated nucleotides. The amino acid alterations (Fig. 2A) show that the gene/protein sequences of *rpsL* are highly conserved in bacteria, and that substitution of the lysine at position 43 (K43) with arginine (R), threonine (T), asparagine (N), isoleucine (I) or aspartate (D) results in resistance to high concentrations of streptomycin.

The K43 codon of *rpsL* in *C. glutamicum* ATCC 13032 is AAG, and streptomycin resistance can be conferred by altering this codon to ACG, AGG, AAT (or AAC) by single base mutation, resulting in amino acid exchange of K43T, K43R and K43N respectively (Fig. 2A). Therefore, we rationally mutated this lysine codon to ACG, AGG and AAT by electroporating DNA fragments containing the corresponding mutations, and selecting with

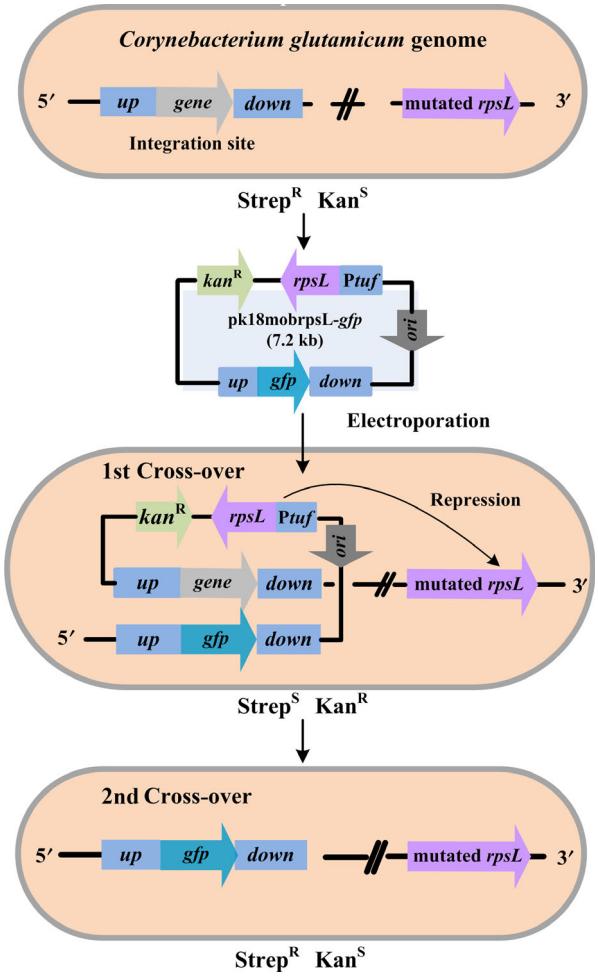

Fig. 1. Diagram of the updated genomic editing technology in *C. glutamicum*. Prerequisite for the used strain is a chromosomal resistance against streptomycin conferred by a mutation in *rpsL*. The pk18*mobrpsL* backbone, harbouring *kan*^{R} and the wild-type *rpsL* expression cassettes, is used for constructing gene deletion/insertion plasmids, and the *gfp* gene is herein designed for chromosomal integration. Initially, the starting strain with a mutated *rpsL* is resistant to streptomycin (strep^R) and sensitive to kanamycin (kan^S). After electroporation, pk18*mobrpsL-gfp* can be integrated into the genome via single cross-over with kanamycin resistance (kan^R) selection. Owing to the high expression of the plasmid-derived wild-type allele of *rpsL* (under the strong constitutive promoter P*tuf*), which represses the streptomycin resistance of the *rpsL* mutant, the strain becomes sensitive to streptomycin (strep^S). Subsequently, streptomycin pressure (strep^R) is applied to select the second-cross-over strain, which becomes kan^S due to elimination of the plasmid. Finally, correct integration of *gfp* is confirmed by colony PCR and sequencing.

streptomycin. The resulting strains were named *C. glutamicum rpsL*^{K43T}, *rpsL*^{K43R} and *rpsL*^{K43N} respectively.

Streptomycin resistance of the three strains was preliminarily checked by test tube cultivation (data not shown). Afterwards, the gradient brain heart infusion agar (BHIA) plates were used for determining the minimum inhibitory concentrations (MICs) of streptomycin, which were estimated to be 9200, 4400 and 7600 μg ml^{-1}, respectively, for *C. glutamicum rpsL*^{K43N}, *rpsL*^{K43R} and *rpsL*^{K43T} (Fig. 3A). During the genome editing process illustrated in Fig. 1, the single-cross-over strain cultured on agar plate should be sensitive to streptomycin, while the starting strain or the second-cross-over strain is streptomycin resistant. We determined the streptomycin resistance of these strains spread on gradient BHIA plates. As expected, the single-cross-over strains were sensitive to streptomycin, and the MICs for *C. glutamicum rpsL*^{K43N}-pk18*mobrpsL-gfp*, *rpsL*^{K43R}-pk18*mobrpsL-gfp* and *rpsL*^{K43T}-pk18*mobrpsL-gfp* were detected to be approximately 1.7, 0.5 and 0.4 μg ml^{-1} respectively (Fig. 3B). In the process of screening double-cross-over strains, only those colonies derived from the single-cross-over strain accomplishing homologous recombination (with low rate) could survive on agar plate supplemented with streptomycin. In order to simulate this process, the overnight cultivations of these mutant strains and their corresponding single-cross-over strains, diluted by 10^5 times, were spread onto BHIA with different concentrations of streptomycin. The results indicated that the starting strains survived well with the addition of 500 μg ml^{-1} streptomycin (Fig. 3A), while the single-cross-over strains were sensitive to 10 μg ml^{-1} streptomycin (Fig. 3B). Based on these findings, we chose *rpsL*^{K43N} as the starting strain and used a streptomycin concentration of 50 μg ml^{-1}, which prevents growth of the first-cross-over strain but allows growth of the second-cross-over strain comparable to that on solid medium without streptomycin.

Application of rpsL counter selection under different cultivation conditions

The application of this counter-selection method was testified taking *gfp* integration as an example. At the same time, its integration mediated by pk18*mobsacB* was carried out as control. First, the plasmid pk18*mobsacB-gfp* was constructed and integrated into the genome of *C. glutamicum* 13032 by the first recombination. For the second-cross-over selection, the single-cross-over strains were subjected to a variety of culture conditions (Fig. 4). For *sacB* counter selection, we performed two-step culture, first in brain heart infusion broth (BHIB), followed by plating on BHIA, with both media supplemented with 1.5% sucrose. In addition, CGXII minimum medium (Eggeling and Bott, 2005) containing 3% sucrose as the sole carbon source was utilized with the aim of increasing selection efficiency. For *rpsL* counter selection, four cultivation modes were compared as follows: (i) first in BHIB with kanamycin, second on BHIA with streptomycin; (ii) first in BHIB, second on BHIA, both with streptomycin; (iii) first in BHIB without

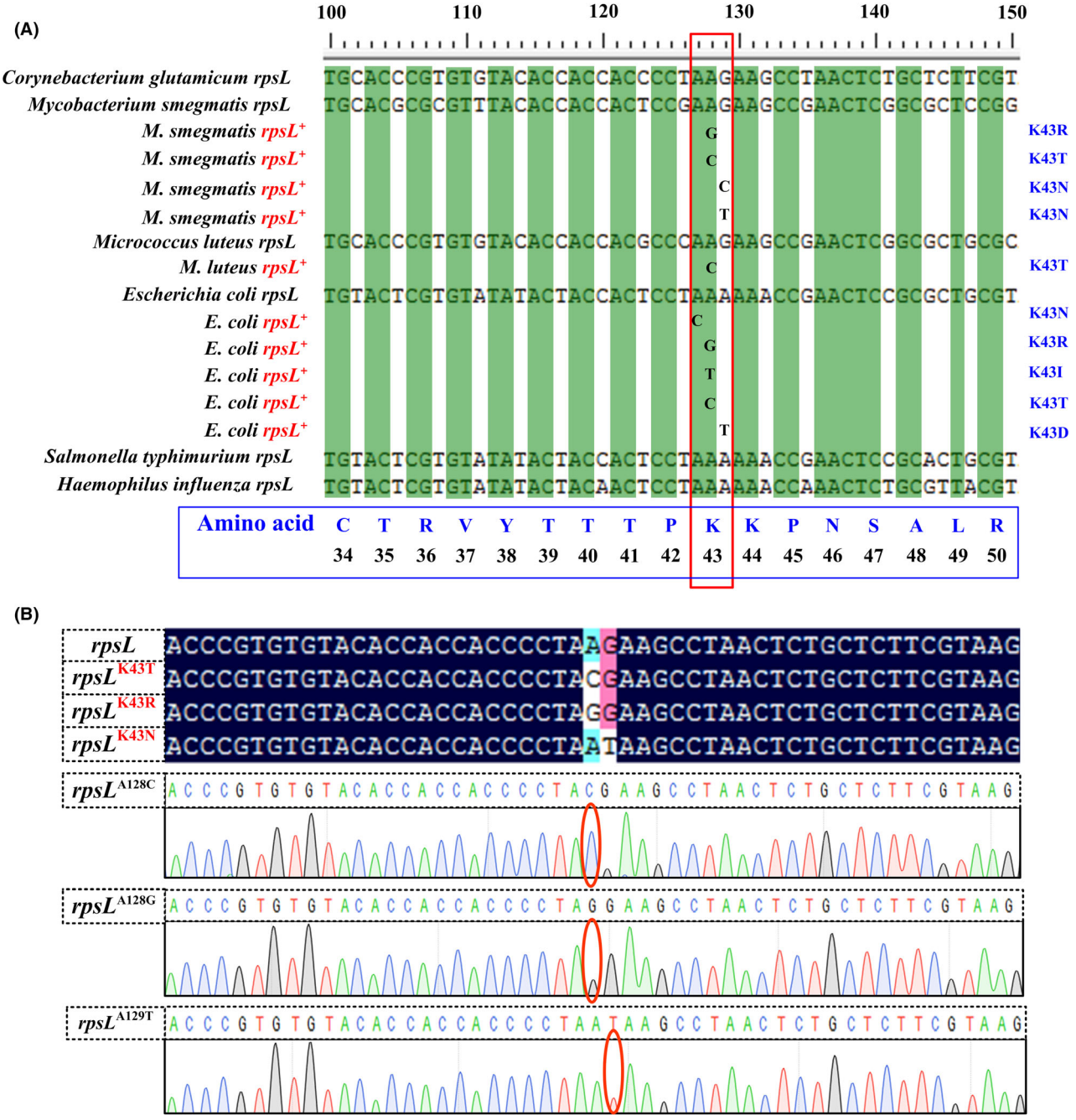

Fig. 2. *RpsL* mutations in *C. glutamicum* conferring streptomycin resistance. Alignment of partial *rpsL* sequences from several bacteria with the lysine43 codon mutations reported for streptomycin resistance (A), designed mutations of *C. glutamicum rpsL*, and the sequencing maps (B). *rpsL*$^+$ represents mutants that confer streptomycin resistance.

streptomycin, second on BHIA with streptomycin; and (iv) first in BHIB with streptomycin, second on BHIA without streptomycin. Colonies appearing on the plates were inoculated on streptomycin- and kanamycin-containing BHIA, in parallel. Counter-selection efficiency is calculated as the ratio of colonies that cannot grow on BHIA (kanamycin) divided by those grow on BHIA (streptomycin). As seen in Fig. 4, selection efficiency with sucrose in BHI medium was 13.3%, reaching 28.9% in CGXII medium, revealing that the utilization of minimum medium can indeed facilitate *sacB*-related sucrose selection. Fortunately, the selection efficiencies with streptomycin were extraordinarily high, exceeding 90%. Notably, the selection efficiency of the first culture mode

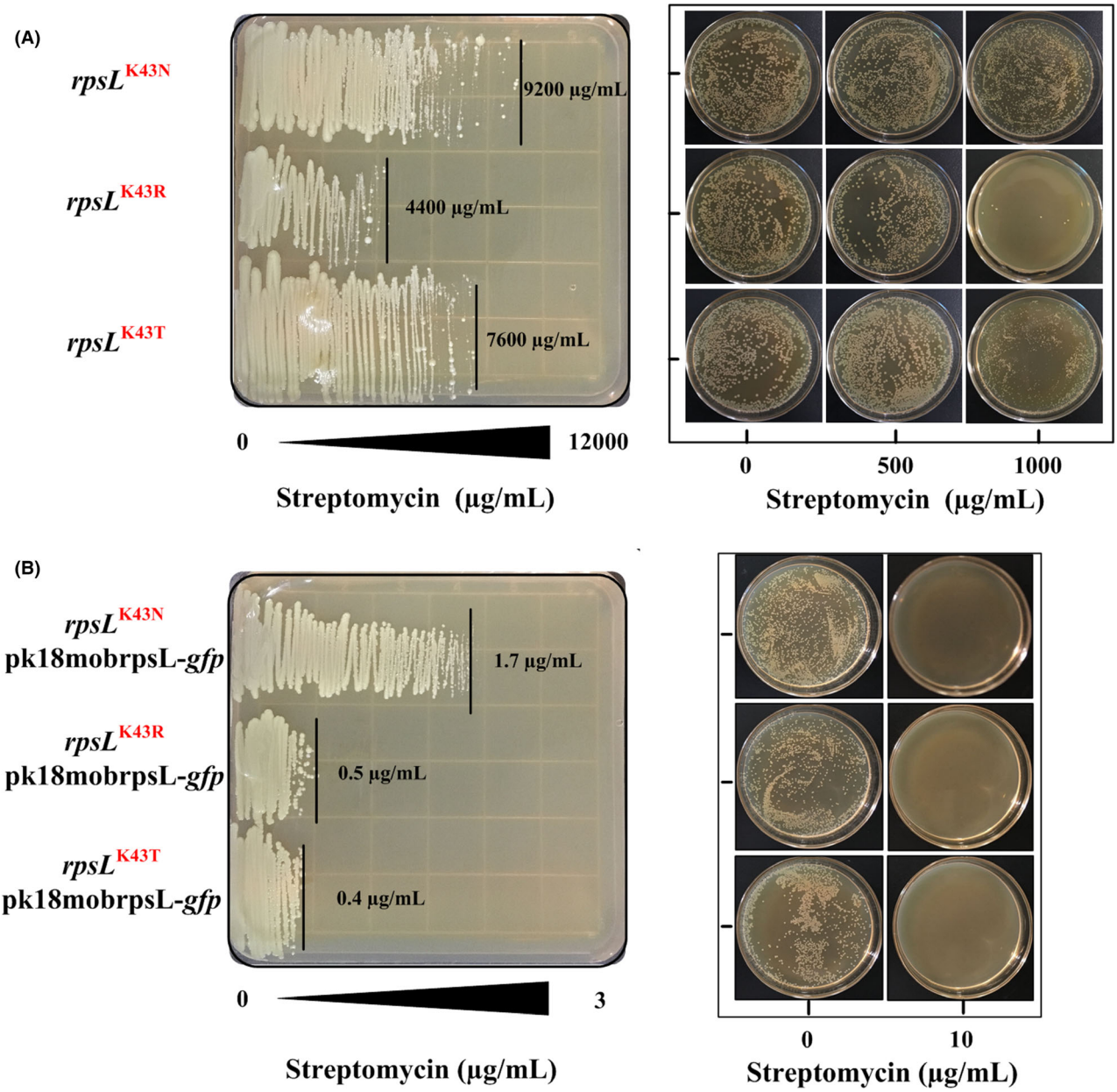

Fig. 3. Streptomycin resistance determination of *C. glutamicum* strains with *rpsL* mutations and the corresponding single-cross-over strains. The left figure represents growth of strains with mutated *rpsL* in the gradient agar plate, in which streptomycin concentration is ranged from 0 μg ml^{-1} (left-end of plate) to 12 000 μg ml^{-1} (right-end of plate), and the streptomycin MICs for each strain are marked; the right figure represents growth of strains with diluted cell density under different concentrations of streptomycin (A), the left figure represents growth of the single-cross-over strains in the gradient agar plate, in which streptomycin concentration is ranged from 0 μg ml^{-1} (left-end of plate) to 3 μg ml^{-1} (right-end of plate), and the MICs for each strain are marked; and the right figure represents growth of strains with diluted cell density under different concentrations of streptomycin (B).

reached 100%, making streptomycin selection a fast and highly efficient approach.

One-step verification of the final genome-edited strain

Because selection efficiencies with kanamycin and streptomycin are very high, we hypothesized that it might be possible to simplify the verification procedure from two to one step for the final second-cross-over strain (Fig. 5). To this end, *C. glutamicum rpsL*^{K43N} competent cells were electroporated with pk18*mobrpsL-gfp*, allowed to recover for 2 h and transferred to a test tube containing BHIB supplemented with kanamycin. After cultivation for about 12 h, the

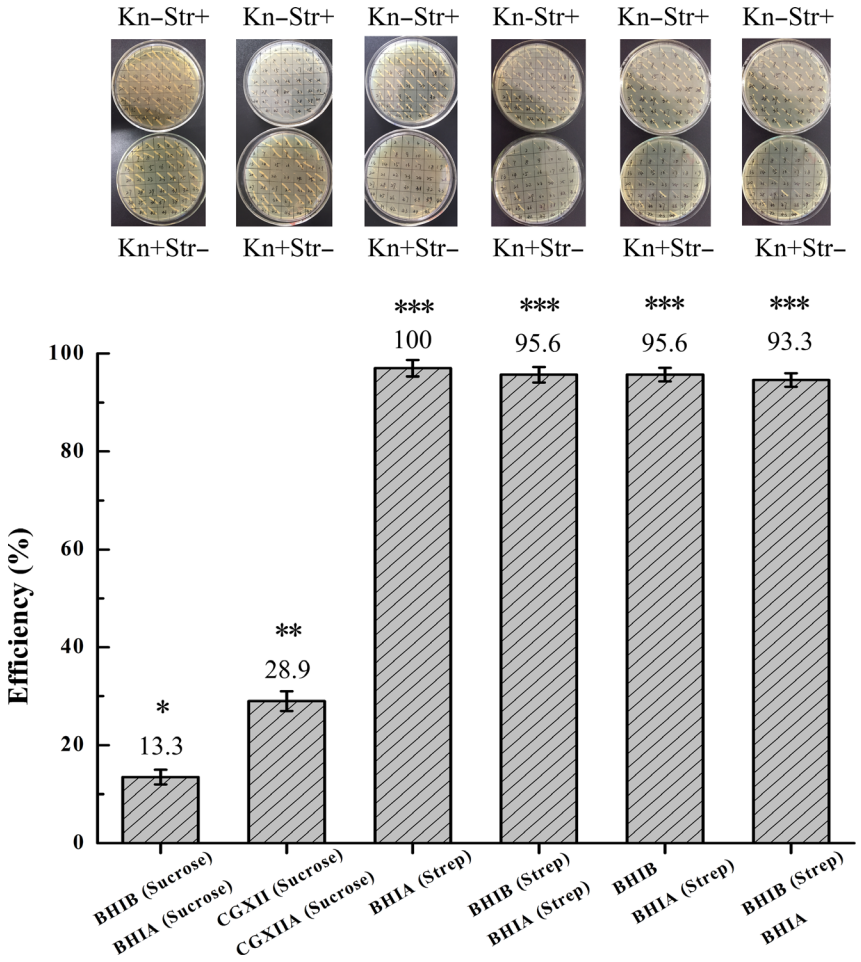

Fig. 4. Efficiencies of second-cross-over event selection by *sacB*-mediated sucrose counter selection or *rpsL*-mediated streptomycin counter selection. BHIB: brain heart infusion broth, BHIA: brain heart infusion agar, CGXII: minimum medium for *C. glutamicum* culture, CGXIIA: CGXII agar. Experiments were repeated at least three times, and values are presented as mean ± SD. Different numbers of asterisks indicate significant differences ($P < 0.05$).

bacterial suspension was plated directly on BHIA with streptomycin. Colonies appeared after approximately 24 h, and correct integration of *gfp* at the *iolD* locus was verified by colony PCR. Meanwhile, colonies on BHIA (streptomycin) created by single cross-over were also verified. The *gfp* integration efficiencies resulting from one-step and two-step verification strategies are compared in Fig. 6. The efficiency of one-step verification reached 26.5%, which is acceptable as the theoretical editing efficiency is 50% using this method. The integration efficiency following two-step verification was 45%. The entire one-step verification process can be completed in 2 days if one works extended hours. Otherwise, genotyping by colony PCR and electrophoresis can be accomplished within 2 h on the third day. By comparison, the two-step verification strategy takes three and a half days to complete.

Application of the method to integrate genes in the C. glutamicum *genome*

The simultaneous overexpression of feedback inhibition resistant *aroG* (*aroG*^{fbr}) and *pheA*^{fbr} in *E. coli* W3110, encoding the 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase and the bifunctional enzyme chorismate mutase/prephenate dehydratase, respectively, has been reported to result in L-phenylalanine overproduction (Liu *et al*., 2013). In order to test the applicability of *rpsL* counter-selection-assisted one-step verification method developed in the present study, we attempted to integrate the *E. coli*-derived *aroG*^{fbr} (Ger *et al*., 1994) and *pheA*^{fbr} (Liu *et al*., 2013) expressing cassettes together at the locus of *cgl1675*, which encodes a hypothetical protein. In the *C. glutamicum* synthetic pathway for L-phenylalanine, DAHP synthase,

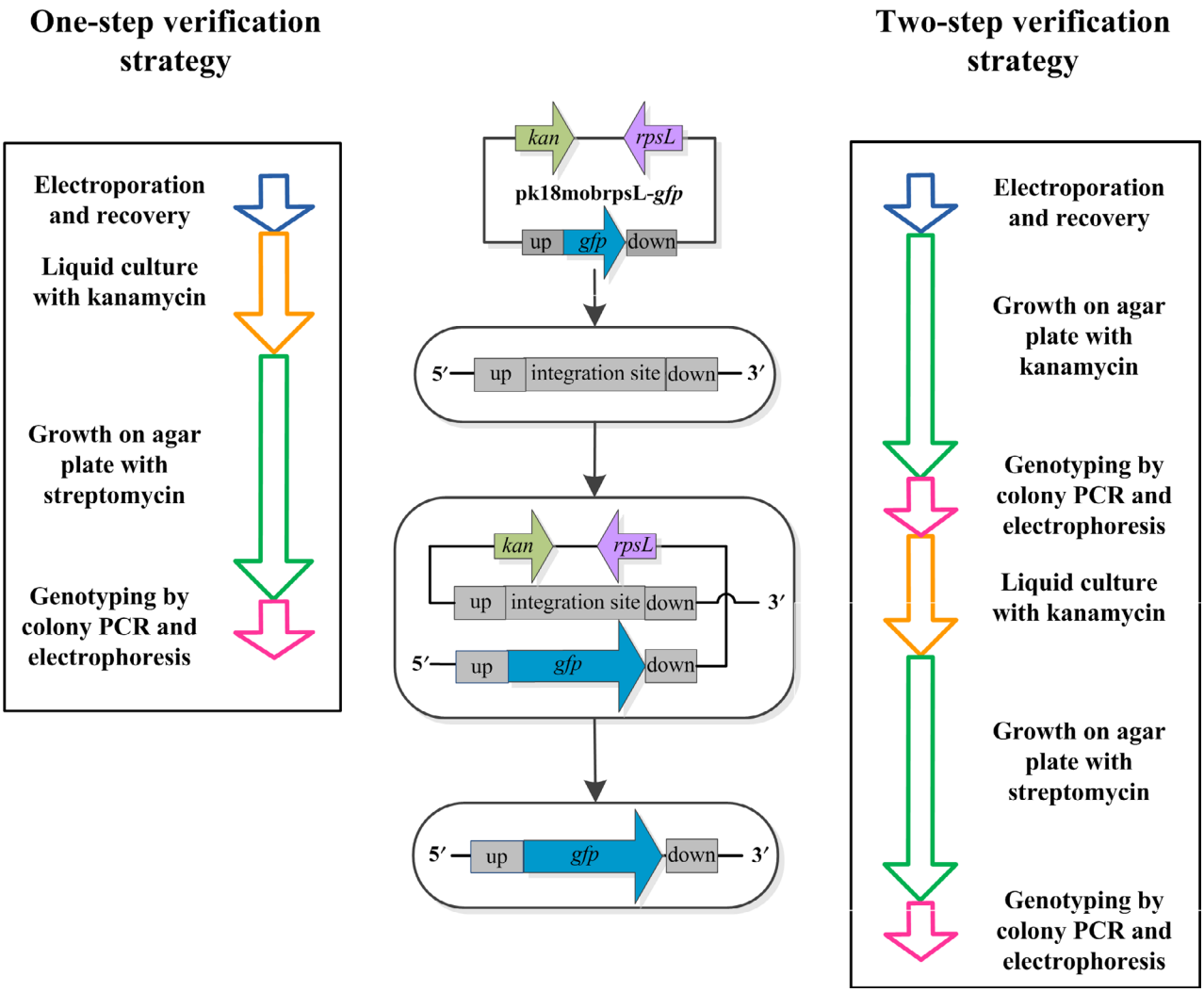

Fig. 5. Workflows of one-step and two-step verification strategies applied in the updated genomic editing system using antibiotics (kanamycin and streptomycin) for selection.

chorismate mutase and prephenate dehydratase are feedback-inhibited by L-phenylalanine and/or L-tyrosine (Ikeda, 2006) (Fig. 7A). A 2.5-kb DNA fragment containing *aroG*^{fbr} and *pheA*^{fbr} driven by P*tuf* and P*sod*, respectively, was constructed, flanked by ~ 500 bp upstream and downstream sequences of *cgl1675*, and ligated into plasmid pk18*mobrpsL*. Using the technology developed in this study, the integration efficiency of this 2.5-kb fragment reached 20% (Fig. 7B). Shake-flask fermentations of the resulting strain, *C. glutamicum* AP (*cgl1675*::*aroG*^{fbr}*pheA*^{fbr}), and the starting strain, *rpsL*^{K43N}, were performed. To our surprise, this strain produced 110 mg l^{-1} L-tyrosine and only 10 mg l^{-1} L-phenylalanine at 24 h (Fig. 7C). However, the L-phenylalanine titre was much higher than that of L-tyrosine at 12 h. The reason for the reduction in L-phenylalanine production is unknown owing to the lack of information regarding catabolic pathways for aromatic amino acids in *C. glutamicum* (Shen *et al.*, 2012). However, the hydroxylation of L-phenylalanine to L-tyrosine is the first step in the reported homogentisate pathway by which some bacteria degrade L-phenylalanine (Arias-Barrau *et al.*, 2004). Meanwhile, the titres of L-phenylalanine and L-tyrosine were low compared to the values reported in *C. glutamicum* following similar genetic manipulations (Liu *et al.*, 2004; Zhang *et al.*, 2013), mostly because CGXII minimum medium was used here.

Discussion

Corynebacterium glutamicum, originally discovered as an L-glutamate-secreting microorganism, is widely used for the industrial production of various amino acids. It has received increasing attention as a novel cell factory

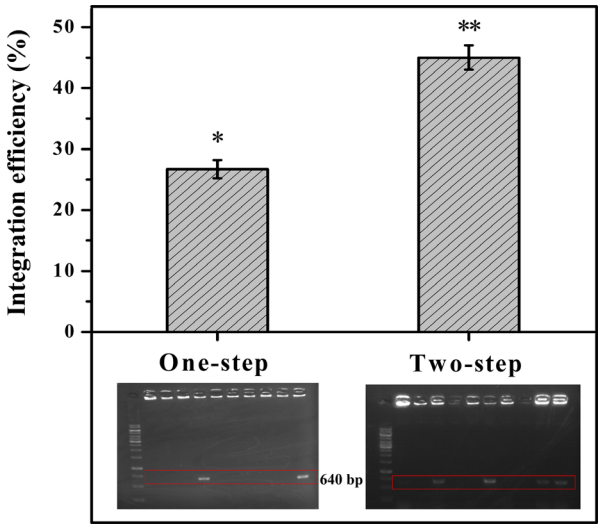

Fig. 6. Comparison of gene integration efficiencies by one-step and two-step verifications. The representative colony PCR results are given, where a 640-bp DNA fragment was designed to verify the correct integration of *gfp*. The DNA ladder used was Thermo Scientific GeneRuler 1 kb DNA Ladder (0.25–10 kb). The means ± SDs from three experiments with good repeatability are shown. Different numbers of asterisks indicate significant differences ($P < 0.05$).

due to its great potential to produce many desirable metabolites, and to utilize alternative non-food feedstocks (Becker and Wittmann 2012). However, compared to other model microorganisms such as *E. coli*, the availability of facile genetic tools has lagged. Despite great advances regarding CRISPR-related technologies, the current prevailing genome editing method for *C. glutamicum* is still based on non-replicating plasmids of the pk*mobsacB* series (Schäfer *et al*., 1994). In this study, this approach has been updated to a simple and efficient method that enables multiple genetic modifications in the chromosome of *C. glutamicum*, by virtue of harnessing the antibiotic streptomycin as a powerful counter-selection agent.

Compared to classical chromosomal modification based on the insertion of drug resistance-selectable markers, counter-selectable markers allow for straightforward construction of unmarked mutants. They are powerful tools for editing target genes without affecting other parts of the chromosome. Initially, we tested other counter-selectable markers for possible application in *C. glutamicum* genome editing. One of these, the *upp* gene-encoded uracil phosphoribosyltransferase, can convert the pyrimidine analog 5-fluorouracil (5-FU) into 5-fluorouridine monophosphate (5-FUMP). This is ultimately converted to fluorodeoxyuridine, a potently toxic product, by the uracil biosynthetic pathway (Boeke *et al*., 1984). The deletion of *upp* thus confers 5-FU resistance, which is widely used as a counter-selectable marker in bacteria such as *Bacillus subtilis* (Shi *et al*., 2013), *Lactobacillus acidophilus* (Goh *et al*., 2009) and *C. glutamicum* (Ma *et al*., 2015). Orotidine monophosphate decarboxylase, encoded by the *pyrF* gene in bacteria, converts 5-fluoroorotic acid (5-FOA) to 5-FUMP. Mutants of *pyrF* are resistant to 5-FOA, which can be used in counter selection for the mutant allele. The *pyrF* marker was successfully applied in *Caldicellulosiruptor* species (Chung *et al*., 2013), but has not be attempted in *C. glutamicum*.

At first, the 5-FU resistance of *C. glutamicum* 13032 was tested and it was found that the spontaneous mutation rate of strains resistant to 5-FU was high in comparison with that of streptomycin-elicited mutations. To confer 5-FOA resistance, the *pyrF* gene needs to be deleted, leading to pyrimidine nucleotides auxotrophy. Although the addition of uridine to the medium restored the growth of *C. glutamicum*, its growth rate decreased significantly. Because the toxic product fluorodeoxyuridine kills growing cells that are synthesizing uracil (Chung *et al*., 2014), the basal media for 5-FU and 5-FOA are needed (Ma *et al*., 2015). This presents a significant disadvantage, as slow growth of the bacteria greatly prolongs the genome editing process, also raising the possibility of spontaneous mutations. In contrast to the use of 5-FU or 5-FOA to apply selective pressure, selection for streptomycin-resistant clones is carried out on complex medium agar plates, which allows clones to grow within 1–2 days of incubation. In comparison with other genetic markers, antibiotic selection is more convenient, rigorous and efficient.

Although genetic manipulation of *C. glutamicum* has been greatly facilitated through the use of the counter-selectable marker *sacB* from *B. subtilis*, this marker's high false-positive rate remains an obstacle (Eggeling and Bott, 2005). On one hand, the mutation rate of *sacB* is extremely high (Hashimoto *et al*., 2003). Ma *et al*. (2015) found that just 50% of the colonies after selection of the second-cross-over were sensitive to kanamycin, and the rate is even lower in our routine practice. Therefore, mutant selection following second recombination is rather time-consuming. On the other hand, the expression level of *B. subtilis sacB* gene in *C. glutamicum* is low, because it is often cloned together with a 463-bp upstream region (Steinmetz *et al*., 1985). The *sacB* promotor is rather far from the beginning of *sacB*, and it has been shown that expression of *sacB* with its native upstream region is low in *C. glutamicum* (Tan *et al*., 2012). The authors replaced the native promoter with PF104, P*neo* and P*lacM*, finding that P*lacM*-driving expression resulted in the highest levansucrase activity. Similarly, the *B. subtilis sacB* was expressed under P*tac* or P*trc* to increase its expression level in *C. glutamicum* (Inui *et al*., 2004; Zhu *et al*., 2013). However, it is difficult to optimize the expression level of *sacB* to a high lethal dose (Ma *et al*., 2015), which might be due to the

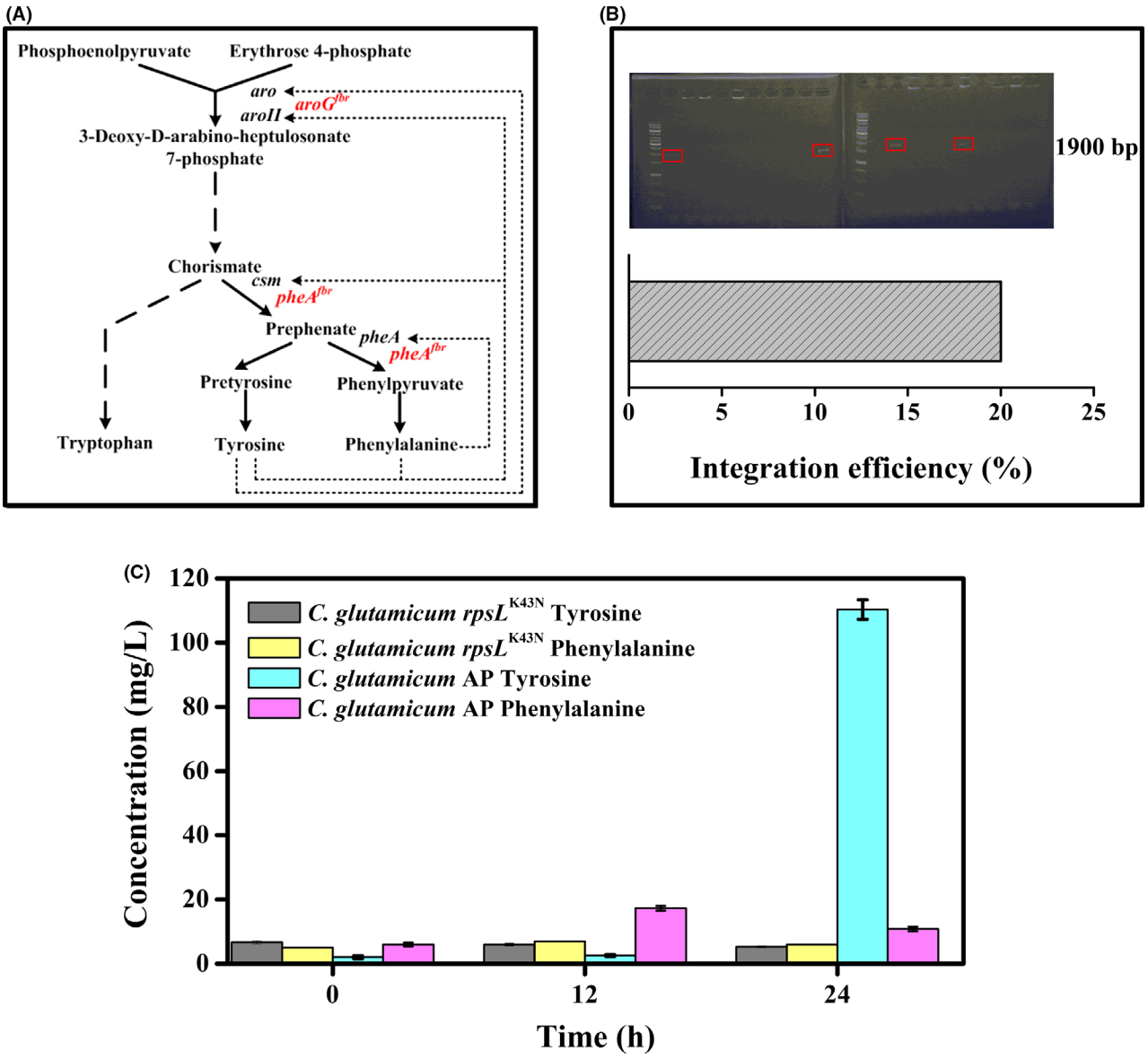

Fig. 7. Application of *rpsL* counter-selection one-step verification genome editing system using the production of aromatic amino acids as example. Simplified synthetic pathways of L-tyrosine and L-phenylalanine, dotted lines represent feedback inhibition. Feedback-resistant *aroG* and *pheA* are marked (A), chromosomal integration efficiency of *aroG*^{fbr}*pheA*^{fbr} expressing cassettes (B), production of L-tyrosine and L-phenylalanine by *C. glutamicum rpsL*^{K43N} and AP in shake-flask fermentations (C). The shake-flask experiments were conducted in triplicate, and values are presented as mean ± SD.

different characteristics of bacteria. The Gram-positive bacteria *C. glutamicum* possesses a periplasm-like space, in which the accumulation of levan could potentially cut off substance transportation, signal transduction and energy metabolism between the bacterial cell and its external environment. In the present study, we also found that the utilization of minimum medium with sucrose as sole carbon source increased the lethal efficiency of *sacB* (Fig. 4), but delayed bacterial growth.

In comparison, according to the results of this study and long-term experience in our group, streptomycin counter selection is highly efficient and the *rpsL* marker is less mutated. Due to the low selection efficiency of sucrose lethality, the colonies need to be streak-inoculated in parallel onto solid medium supplemented with kanamycin or sucrose in order to perform 'phenotype verification' prior to 'genotype verification' by colony PCR and sequencing. When streptomycin is used for counter selection, colonies appearing on agar plates can be directly subjected to genotype verification. In addition, the *rpsL* gene is small, only 372 bp, and can therefore be easily manipulated *in vitro* for genetic analysis.

Taking advantage of the high-efficiency selection with kanamycin and streptomycin, the two-step verification procedure can be simplified to just one verification step. Using this strategy, transformant plating after recovery and genetic confirmation of the first-cross-over strain are skipped, significantly condensing the process from 3 days and a half to just 2 days. The final editing efficiency of the one-step verification strategy is, however, lower than that of the two-step verification strategy (Fig. 6). The reason might be that although very few, there are cells lacking pk18*mobrpsL* integration which escape kanamycin selection, and which are carried over into subsequent cultures, lowering the editing efficiency. Nevertheless, the overall editing efficiency is high enough to attain the desired mutant.

Based on the same principle, *rpsL* counter selection can be applied for ssDNA-mediated nucleotide mutations and dsDNA-mediated gene deletions, or other small alterations, using a two-step transformation procedure. Sung *et al*. (2001) used a 1.3-kb cassette consisting of a kanamycin resistance marker and a counter-selectable *rpsL* marker to create silent mutations and deletions or other gene replacements in *S. pneumoniae*. In combination with Red/ET recombination, *rpsL* counter selection was used to introduce single point mutations in the *E. coli* chromosome (Heermann *et al*., 2008). The homologous recombination capability of *C. glutamicum* is low, but can be significantly improved through the expression of RecET recombinases. We are currently developing ssDNA- and dsDNA-mediated gene modification systems in *C. glutamicum*.

Experimental procedures

Strains, media and growth conditions

Bacterial strains used in this study are listed in Table 1. Wild-type *C. glutamicum* ATCC 13032 was the original strain and was cultured in brain heart infusion broth (BHIB, Oxoid) medium (37 g l^{-1}) at 30°C. *E. coli* DH5α was used for the construction and maintenance of plasmids and was cultured in Luria–Bertani (LB) medium at 37°C. BHIS medium containing 37 g l^{-1} BHI and 91 g l^{-1} sorbitol was used to prepare *C. glutamicum* competent cells. Where necessary, culture media were supplemented with antibiotics at final concentrations of 50 μg ml^{-1} kanamycin (for *E. coli*), and 10 μg ml^{-1} kanamycin, or 50 μg ml^{-1} streptomycin (for *C. glutamicum*).

For detection of streptomycin resistance in strains *rpsL*^{K43N}, *rpsL*^{K43R} and *rpsL*^{K43T}, and in the corresponding single-cross-over strains integrated with pk18*mobrpsL-gfp*, gradient BHIA plates, and BHIA supplemented with varying concentrations of streptomycin, were used. The BHIB and BHIA supplemented with 50 μg ml^{-1} of streptomycin or 1.5% (w/v) sucrose, as necessary, were used to select second-cross-over strains. In addition, the minimum media CGXII (Eggeling and Bott, 2005) and CGXII agar (CGXIIA) supplemented with 3% sucrose were also used for *sacB*-mediated counter selection.

Construction of plasmids and strains

The plasmids and primers used in this study are listed in Table 1 and Table S1 respectively. The pk18mob DNA fragment was amplified by reverse PCR using pK18mobsacB as template, digested with XhoI, and self-ligated to generate plasmid pK18*mob*. For construction of other plasmids, ligation by homologous recombination was accomplished using the ClonExpress II One Step Cloning Kit (Vazyme Biotech Co., Ltd, Nanjing, China). The *rpsL* gene was amplified using genomic DNA of *C. glutamicum* 13032 as template, and inserted into pXTuf linearized by digestion of HindIII and EcoRI to generate pXTuf-*rpsL*. Next, the P*tuf-rpsL* fragment was amplified from pXTuf-*rpsL*, and inserted into pK18*mob* linearized by XhoI digestion, generating plasmid pK18*mobrpsL*. The *gfp* gene was amplified using pEGFP-N1 as template and inserted into pXTuf linearized by HindIII and KpnI, generating pXTuf-*gfp*. For *gfp* integration at the genomic locus of *iolD* (*cgl0162*), the upstream and downstream arms of *iolD* and the Ptuf-*gfp-rnnB* fragment were first separately amplified and then linked by overlapping PCR. The resulting fragment was ligated into pK18*mobsacB* and pK18*mobrpsL* that were linearized by BamHI, generating pK18*mobsacB-gfp* and pK18*mobrpsL-gfp* respectively. For construction of the *aroG*^{fbr}-*pheA*^{fbr} integration plasmid, the *aroG*^{S180F} mutant and C-terminal truncated *pheA* were obtained from genome of *E. coli* MG1655 and inserted into pXTuf and pXSod, generating pXTuf-*aroG*^{fbr} and pXSod-*pheA*^{fbr} respectively. The P*sod-pheA* fragment was amplified from pXSod-*pheA*^{fbr} and ligated with PstI-linearized pXTuf-*aroG*^{fbr}, generating pXTuf-*aroG*^{fbr}P*sod-pheA*^{fbr}. The upstream and downstream arms of *cgl1675* and Ptuf-aroG-Psod-pheA fragment were amplified, linked and then ligated into pK18*mobrpsL* to generate pK18*mobrpsL-aroG*^{fbr}*pheA*^{fbr}.

Streptomycin-resistant strains *rpsL*^{K43T}, *rpsL*^{K43R} and *rpsL*^{K43N} were obtained via electroporation of DNA fragments containing corresponding mutations, followed by selection with streptomycin pressure. Primers containing mutations were designed, which were introduced into these fragments using overlapping PCR. The single-cross-over strains *rpsL*^{K43T}-pk18*mobrpsL-gfp*, *rpsL*^{K43R}-pk18*mobrpsL-gfp* and *rpsL*^{K43N}-pk18*mobrpsL-gfp* were obtained by kanamycin selection of competent cells of each starting strain electroporated with the

Table 1. Strains and plasmids used in this study

Name	Genotype or characteristic	Source
Strains		
E. coli DH5α	F^-, △(lacZYA-*argF*) U169, *hsdR17* (rk^-mk^+), *recA1*, *endA1*, *relA1*	Laboratory stock
MG1655	F^-, λ^-	Laboratory stock
C. glutamicum ATCC 13032	Wild-type (WT) strain	Laboratory stock
rpsL^{K43T}	WT with *rpsL*^{A128C}	This study
rpsL^{K43R}	WT with *rpsL*^{A128G}	This study
rpsL^{K43N}	WT with *rpsL*^{G129T}	This study
WT-pk18*mobsacB-gfp*	WT integrated with pk18*mobsacB-gfp* at *iolD* (*cgl0162*) locus via single cross-over; kan^R, $sucrose^S$	This study
rpsL^{K43T}-pk18*mobrpsL-gfp*	*rpsL*^{K43T} integrated with pk18*mobrpsL-gfp* at *iolD* locus; kan^R, $strep^S$	This study
rpsL^{K43R}-pk18*mobrpsL-gfp*	*rpsL*^{K43R} integrated with pk18*mobrpsL-gfp* at *iolD* locus; kan^R, $strep^S$	This study
rpsL^{K43N}-pk18*mobrpsL-gfp*	*rpsL*^{K43N} integrated with pk18*mobrpsL-gfp* at *iolD* locus; kan^R, $strep^S$	This study
WT-*gfp*	WT integrated with *gfp* at *iolD* locus via double cross-over; kan^S, $sucrose^R$	This study
rpsL^{K43N}-*gfp*	*rpsL*^{K43N} integrated with *gfp* at *iolD* locus via double cross-over; kan^S, $strep^R$	This study
AP	*rpsL*^{K43N} integrated with P*tuf-aroG*^{fbr}P*sod-pheA*^{fbr} at *cgl1675* locus via double cross-over	This study
Plasmids		
pK18*mobsacB*	The suicide vector containing the *B. subtilis sacB* gene; kan^R	Tauch *et al*. (2002)
pXTuf	Derived from *E. coli*/*C. glutamicum* shuttle cloning vector pXMJ19, P*tac* replaced by P*tuf*; cm^R	Lab stock
pXSod	Derived from *E. coli*/*C. glutamicum* shuttle cloning vector pXMJ19, P*tac* replaced by P*sod*; cm^R	Lab stock
pEGFP-N1	*gfp* template vector; kan^R	Lab stock
pK18*mob*	pK18*mobsacB* removing *sacB* gene	This study
pXTuf-*rpsL*	pXTuf containing *C. glutamicuam rpsL* gene	This study
pK18*mobrpsL*	pK18*mobsacB* with *sacB substituted by Ptuf-rpsL*	This study
pXTuf-*gfp*	pXTuf containing *gfp* gene	This study
pK18*mobsacB-gfp*	pK18*mobsacB* containing P*tuf-gfp* for integration into *iolD* (*cgl0162*) locus	This study
pK18*mobrpsL-gfp*	pK18*mobrpsL* containing P*tuf-gfp* for integration into *iolD* (*cgl0162*) locus	This study
pXTuf-*aroG*^{fbr}	pXTuf containing *aroG*^{fbr} gene	This study
pXSod-*pheA*^{fbr}	pXSod containing *pheA*^{fbr} gene	This study
pXTuf-*aroG*^{fbr}P*sod-pheA*^{fbr}	pXTuf-*aroG*^{fbr} containing P*sod-pheA*^{fbr} cassette	This study
pK18*mobrpsL-aroG*^{fbr}*pheA*^{fbr}	pK18*mobrpsL* containing P*tuf-aroG*^{fbr}P*sod-pheA*^{fbr} for integration at *cgl1675* locus	This study

corresponding plasmids. The strain WT-pk18*mobsacB-gfp* was constructed for investigating the efficiencies of sucrose selection of the second-cross-over strains. After *sacB*- and *rpsL*-mediated counter selection, the second-cross-over strains WT-*gfp* and *rpsL*^{K43N}-*gfp* were obtained. As proof of concept, the strain AP was constructed using the method developed in this study.

Shake-flask fermentation for L*-phenylalanine and* L*-tyrosine production*

Shake-flask fermentation of *C. glutamicum* AP harbouring *aroG*^{fbr}*pheA*^{fbr} expression cassettes in its chromosome was performed, using the *rpsL*^{K43N} strain as a control. Agar slants were cultivated for 24 h at 32°C, after which the bacterial cells were transferred into 250 ml baffle flasks containing 30 ml CGXII medium for seed culture. After 12-h cultivation at 200 rpm at 32°C, the seed cultures were inoculated at 10% (v/v) into fermentation flasks containing CGXII medium. Fermentation was conducted under the same conditions used for the seed culture, with samples taken at 0, 12 and 24 h for determination of L-phenylalanine and L-tyrosine concentrations. The L-phenylalanine and L-tyrosine in culture were quantified by HPLC using precolumn derivatization as previously described (Zhang *et al*., 2018). All experiments were conducted in triplicate.

Statistical analysis

All experiments were performed at least in triplicate, and statistical significance was determined by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Differences with $P < 0.05$ were considered statistically significant.

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Conflict of interest

None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Primers used in this study.

