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BIOTECHNOLOGY METHODS

A method for simultaneous gene overexpression and inactivation in the *Corynebacterium glutamicum* genome

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Abstract The gene integration method is an important tool to stably express desirable genes in bacteria. To avoid heavy workload and cost, we constructed a rapid and efficient method for genome modification. This method depended on a mobilizable plasmid, which contains a P_{tac} promoter, an introduced multiple cloning site (iMCS), and *rrnBT1T2* terminator. Briefly, the mobilizable plasmid pK18-MBPMT with the P_{tac}-iMCS-*rrnBT1T2* cartridge derived from pK18*mobsacB* was prepared to directly integrate hetero-/homologous DNA into the *Corynebacterium glutamicum* genome. Like our previous method, this method was based on insertional inactivation and double-crossover homologous recombination, which simultaneously achieved gene overexpression and inactivation in the genome without the use of genetic markers. Compared to the previous method, this protocol omitted the construction of a recombinant expression plasmid and clone of the target gene(s) cassette, which significantly decreased the workload, cost, and operational time. Using this method, the heterologous gene *amy* and the homologous gene $lysC^{T311I}$ were successfully integrated into the *C. glutamicum* genome at *alaT* and *avtA* loci, respectively. Moreover, the operation time of this method was shorter than that of the previous method, especially for repeated integration. This method, which is based on the mobilizable plasmid pK18-MBPMT, thus represents a potentially attractive protocol for the integration of genes in the course of genetic modification of *C. glutamicum*.

Keywords *Corynebacterium glutamicum* · Mobilizable plasmid · Homologous double crossover · Integration · Marker free

Abbreviations

AK^{Fr}	Allosterically feedback-resistant aspartokinase
AlaT	Aspartate aminotransferase
AvtA	PLP-dependent aminotransferase
iMCS	Introduced multiple cloning sites
Km	Kanamycin
DCW	Dry cell weight
μ_{max}	Maximum specific growth rate
LB	Luria-Bertani
LBG	LB + 5 g L^{-1} glucose
LBS	LB + 100 g L^{-1} sucrose
LBK	LB + 50 μg mL^{-1} Km
LBHIS	LB + brain heart infusion + sorbitol

Introduction

Regulation of gene expression in *Corynebacterium glutamicum* is one of the hot research areas, because this non-pathogenic and Gram-positive bacterium has shown to be a versatile microorganism. *C. glutamicum* has been widely used in large-scale fermentative production of many biochemicals, such as amino acids, nucleic acids, and organic

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acids [1, 2]. Metabolic engineering via genetic modification in *C. glutamicum* is based on intensifying or shifting carbon flux towards the biosynthetic pathway of the aimed product or on enhancing the degradation of some special substrates [3]. Main strategies used in remodeling the cell metabolic balance involved introducing heterologous genes, boosting the activity of key genes, relieving the regulation of enzymes, and reducing gene expression of by-product biosynthesis [4–7]. However, genetic modification of *C. glutamicum* is mainly dependent on the application of antibiotic resistance genes that are used as positive markers for screening target-recombinant strains, especially for the introduction of heterologous genes and overexpression of key genes [8].

Plasmid-mediated hetero-/homologous gene overexpression in amino acid producers has some shortcomings, such as high cost, changes in *Corynebacteria*'s physiological function, problems in food safety, etc [7, 8]. Although Hu et al. [9] have constructed a novel expression system for gene overexpression using a plasmid system, the antibiotic resistance gene was also introduced into the cell. To bypass the application of the expression plasmid, many researchers have turned to developing integrative plasmids that carry a homologous chromosomal DNA fragment and an antibiotic resistance marker gene [10, 11]. However, the integration involved homologous single-crossover between the plasmid and the recipient genome [8], and the selectable resistance marker was introduced into the cell [10, 11]. In addition, Suzuki et al. [12] have reported a new Cre/mutant *lox* system for integrating heterologous genes into *C. glutamicum* genome. However, the host cell must contain a mutant *lox* gene, and the integrated foreign gene must contain a selective marker, such as antibiotic resistance genes [12]. Therefore, a method that can realize the integration of diverse genes that avoid introducing antibiotic resistance genes is needed.

Previously, we reported on a method to genetically modify the *C. glutamicum* genome, which involved the integration of diverse genes at a specific gene locus [7]. The method is composed of two recombinant plasmids: the expression plasmid derived from pDXW-8 [13] for cloning the gene cassette, and the mobilizable plasmid derived from pK18*mobsacB* [14] for targeted gene integration. The recombinant *C. glutamicum* strains exhibited unmarked gene overexpression and fragment deletion in the genome. However, the recombinant expression plasmid must be constructed every time during targeted gene integration, which was time- and cost consuming. To overcome these problems of heavy workload and high cost, we developed a rapid and efficient method based on a mobilizable plasmid, pK18-MBPMT. The mobilizable plasmid pK18-MBPMT contains RP4 *mob*, kanamycin (Km) resistance gene *kan*, genetically modified *sacB*, promoter $P_{tac,}$ and terminator *rrnBT1T2* cartridge with introduced multiple cloning sites (iMCS), derived from pK18*mobsacB* [14]. This method avoided the construction of expression plasmids, and required only two steps to construct the target integrative plasmids. Therefore, the operation time of this protocol was shorter than the previous method, especially for repeated integration.

As an application example, we demonstrate the locus-specific integration of the heterologous gene *amy* (encoding α-amylase) and the homologous gene $lysC^{T311I}$ (encoding allosterically feedback-resistant aspartokinase, AK^{Fr}) into the *C. glutamicum* ATCC13032 genome at *alaT* (encoding aminotransferase T, AlaT) and *avtA* (encoding alanine-valine aminotransferase, AvtA) loci. The results demonstrated that this method could be used to efficiently modify the genome of *C. glutamicum*.

Materials and methods

Strains, growth media, and culturing conditions

Strains and plasmids used in this study are listed in Table 1. Luria-Bertani (LB) was used as the standard medium for *Escherichia coli*. LBG (LB supplemented with 5 g L^{-1} glucose) was used for *C. glutamicum* [2]. Epo medium was used for preparing electroporation-competent cells. LB, brain heart infusion, sorbitol (LBHIS) medium was used for obtaining recombinant strains [15]. LBS (LB supplemented with 100 g L^{-1} of sucrose) and LBK (LB supplemented with 50 μg mL^{-1} of Km) media were used for screening the recombinant strains [7]. CgXII medium was prepared according to Keilhauer et al. [16]. *E. coli* and *C. glutamicum* were grown at 37 and 30 °C, respectively. Where appropriate, strains were cultured with kanamycin (Km 50 μg mL^{-1}), and a reduced concentration of Km (25 μg mL^{-1}) was used to obtain recombinant *C. glutamicum*.

Construction of mobilizable plasmid pK18-MBPMT

The construction process of plasmid pK18-MBPMT is illustrated in Fig. 1. The expression plasmid pDXW-8 was used as DNA template for amplifying the promoter P_{tac} with iMCS (i.e., *Sca*I, *Not*I, *Xma*III, *Xho*I, *Nde*I, and *Mlu*I) and the terminator *rrnBT1T2* with the same iMCS by polymerase chain reaction (PCR) using high-fidelity DNA polymerase (Finnzyme, Espoo, Finland) and corresponding primers, respectively (Table 2). It should be pointed out that iMCS was introduced via 5′-extensions of the primers. The PCR fragments were purified by DNA Purification Kit and designated as P_{tac}-iMCS and *rrnBT1T2*-iMCS, respectively.

Table 1 Strains and plasmids used in this study

	Relevant characteristic(s)	Reference
Strains		
E. coli JM109	*recA*1 *endA*1 *gyrA*96 *thi*-1 *hsdR*17 *e*14^{-}(*mcrA*$^{-}$) *supE*44 *relA*1 Δ(*lac-proAB*)/F' [*traD*36 *proB*$^{+}$ *lacI*^{q} lacZΔM15]	Stratagene
B. amyloliquefaciens Y-2	Isolated from Chinese fermented bean (designated as *douchi*); China Center for Type Culture Collection (CCTCC) M2013493	Our lab
C. glutamicum ATCC13032	Wild-type strain	[26]
C. glutamicum lysC^{T311I}	Exchange T311I in the *lysC* gene	[7]
C. glutamicum/*amy*	*C. glutamicum* ATCC13032 harboring pDXW-8-*amy*	This work
C. glutamicum IA-1	The *amy* amplification and *alaT* deletion in *C. glutamicum* ATCC13032 genome	This work
C. glutamicum IA-2	The *lysC*^{T311I} amplification and *avtA* deletion in *C. glutamicum* ATCC13032 genome	This work
C. glutamicum IAA	The *lysC*^{T311I} amplification and *avtA* deletion in *C. glutamicum* IA-1 genome	This work
Plasmids		
pDXW-8	*E. coli–C. glutamicum* shuttle vector, P_{tac} promoter, *rrnBT1T2* terminator, Km^{r} and Ampicillin-resistant	[13]
pDXW-8-*amy*	pDXW-8 carrying *amy* gene	This work
pK18*mobsacB*	Integration vector, Km^{r}	[14]
pK18-MBPMT	pK18*mobsacB* carrying promoter P_{tac} and terminator *rrnBT1T2* cartridge with iMCS	This work
pK18-MBPMT/Δ*alaT*	pK18-MBPMT carrying *alaT*-L and *alaT*-R segments	This work
pK18-MBPMT/Δ*alaT::amy*	pK18-MBPMT carrying *alaT*-L, *alaT*-R segments and *amy* gene	This work
pK18-MBPMT/Δ*avtA*	pK18-MBPMT carrying *avtA*-L and *avtA*-R segments	This work
pK18-MBPMT/Δ*avtA::lysC*^{T311I}	pK18-MBPMT carrying *avtA*-L, *avtA*-R segments and *lysC*^{T311I} gene	This work

Next, the plasmid pK18*mobsacB* was digested with BamHI and XbaI and purified by the Gel Extraction Kit. The digested fragments of pK18*mobsacB*, P_{tac}-iMCS, and *rrnBT1T2*-iMCS were mixed and supplemented with *pEASY*®-Uni Seamless Cloning and Assembly Kit (TRANSGEN, Beijing, China) to assemble these fragments (Fig. 1). After incubation at 50 °C for 15 min, the mixture was electroporated into *E. coli* JM109 competent cells, and spread onto LBK. The plasmid was extracted from Km-resistant colonies and designated as pK18-MBPMT. The plasmid map is presented in Fig. 2a.

Construction of target integrative plasmid pK18-MBPMT/Δ*A::B*

DNA was extracted from *Bacillus amyloliquefaciens* or *C. glutamicum* using the DNA Extraction Kit according to the protocol supplied by the manufacturer (Sangon, Shanghai, China). The construction process of plasmid pK18-MBPMT/ΔA::B is shown in Fig. 3. The left and right arms of *A* gene (representative *alaT* and *avtA*) were amplified with high-fidelity DNA polymerase (Finnzyme, Espoo, Finland) from the DNA of *C. glutamicum* (Table 2) and designated as *A*-L/*A*-R (representative *alaT*-L/*alaT*-R or *avtA*-L/*avtA*-R). The fragments of *A*-L/*A*-R were purified, digested with suitable restriction enzymes, respectively (Table 2), and ligated into pK18-MBPMT, which was similarly digested. The resulting plasmid was designated as pK18-MBPMT/ΔA (representative pK18-MBPMT/ΔalaT or pK18-MBPMT/ΔavtA).

Next, DNA fragment of *B* gene (representative *amy* or *lysC*^{T311I} genes) was amplified with high-fidelity DNA polymerase (Finnzyme, Espoo, Finland) from the relevant host cell (such as *amy* from *B. amyloliquefaciens* CCTCC M2013493 and *lysC*^{T311I} from *C. glutamicum*) by corresponding primers (upstream primer contains an SD sequence [17]; Table 2). The fragment was purified, digested with suitable restriction enzymes (Table 2), and ligated into mobilizable plasmid pK18-MBPMT/Δ*A*, which was similarly digested. The resulting plasmid was designated as pK18-MBPMT/Δ*A::B* (representative pK18-MBPMT/Δ*alaT::amy* or pK18-MBPMT/Δ*avtA::lysC*^{T311I}). Plasmids were extracted from *E. coli* using the Mini Plasmid Kit according to the protocol supplied by the manufacturer (Sangon, Shanghai, China). The plasmid maps are listed in Fig. 2b and c.

Construction of recombinant strains

The plasmid pK18-MBPMT/Δ*A::B* was electroporated into *C. glutamicum*-competent cells, and the recombinant strains were obtained by screening colonies from Km-resistance

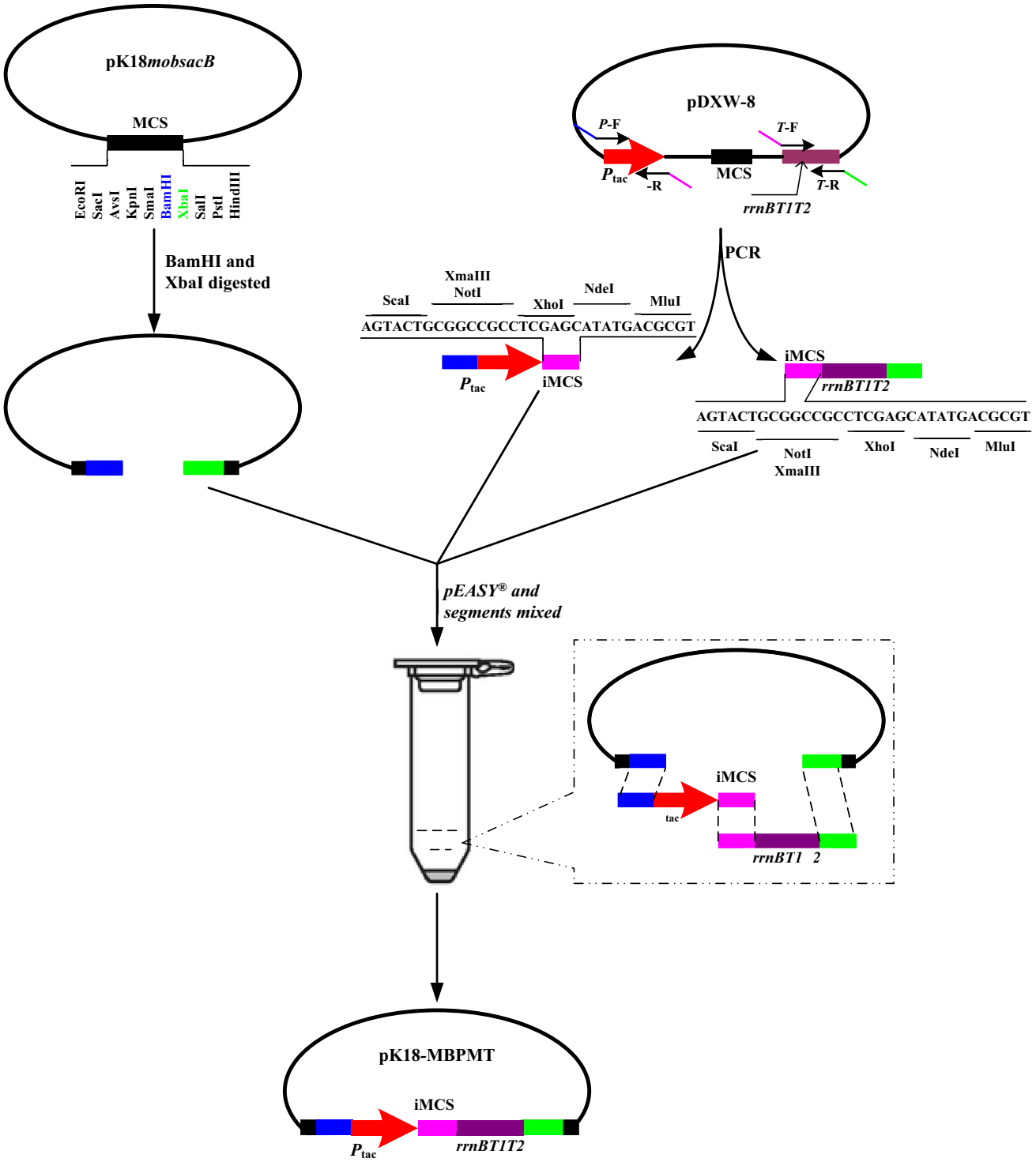

Fig. 1 Strategy used for the construction of the integrative plasmid pK18-MBPMT. P_{tac} *tac* promoter, *rrnBT1T2* terminator, *MCS* multiple cloning sites, *iMCS* introduced multiple cloning sites, *P*-F/*P*-R primers for amplifying the promoter P_{tac} fragment, and *T*-F/*T*-R primers for amplifying the terminator *rrnBT1T2* fragment. The *lines* indicated in same *color* represent homologous sequences (color figure online)

and sucrose-sensitivity plates. To clarify the role of this method, we modified *amy*, *lysC*^{T311I}, *alaT,* and *avtA* in *C. glutamicum* ATCC 13032. *C. glutamicum* ATCC 13032 was transformed with plasmid pK18-MBPMT/Δ*alaT::amy*, and the resulted strain was *C. glutamicum* IA-1. *C. glutamicum* ATCC 13032 or *C. glutamicum* IA-1 was transformed with

Table 2 Oligonucleotides used in this study

Oligonucleotide	5′ → 3′ sequence[a]	Cloning sites	Purposes
P-F	*ATTACGAATTCGAGCTCGGTACCCGGG*GATCCGGAGCT-TATCGAC	–	Amplifying promoter P_{tac} segment
P-R	***ACGCGTCATATGCTCGAGGCGGCCGCAGTACT***TTCTGTTC-CTGTGAAATTG	–	
T-F	***AGTACTGCGGCCGCCTCGAGCATATGACGCGT***CAGAT-TAAATCAGAACGC	–	Amplifying terminator *rrnBT1T2* segment
T-R	*GCTTGCATGCCTGCAGGTCGACTCTAG*AATAAAACGAAA-GGCCCAG	–	
lysC-F	GATGCGGCCGC**GAAAGGAGATATACC**GTGGCCCTG-GTCGTACAG	*Not*I	Amplifying $lysC^{T311I}$ gene
lysC-R	CCGCTCGAGTTAGCGTCCGGTGCCTGCAT	*Xho*I	
amy-F	GATGCGGCCGC**GAAAGGAGATATACC**ATGTGCACGCTGT-TATTTG	*Not*I	Amplifying *amy* gene
amy-R	CCGCTCGAGTTATTTCTGAACATAAATG	*Xho*I	
alaT-L-F	CGGAATTCTGTAATAGGAGAAGGAAATG	*Eco*RI	Amplifying the *alaT* left arm, *alaT*-L
alaT-L-R	CGGGATCCGGGAAGTTGGAAGGTTGGCG	*Bam*HI	
alaT-R-F	GCTCTAGAGACTCCTGGAACAGCGCAAC	*Xba*I	Amplifying the *alaT* right arm, *alaT*-R
alaT-R-R	CACGCGTCCGAGTTTGCGCCATAGGGATATAT	*Sal*I	
avtA-L-F	CGGAATTCGGCTGCCTGAAGATGGCGTC	*Eco*RI	Amplifying the *avtA* left arm, *avtA*-L
avtA-L-R	CGGGATCCGAATCCACCTGAACAACCGGTG	*Bam*HI	
avtA-R-F	ACGCGTCGACCTTGATGCCCACGTTGAAGC	*Sal*I	Amplifying the *avtA* right arm, *avtA*-R
avtA-R-R	CACAAGCTTATGAGGTCAAGCATGTCTTG	*Hin*dIII	

[a] Sequence in italic: homologous recombination sequences with plasmid pK18*mobsacB*; nucleotides in bold and italic: introduced homologous recombination sequences and cleavage sites; underlined nucleotides: cloning site; nucleotides in bold: SD sequences

plasmid pK18-MBPMT/Δ*avtA::lysC*^{T311I}, and the resulted strain was *C. glutamicum* IA-2 or *C. glutamicum* IAA, respectively. The recombinant strains were screened and confirmed according to Ohnishi et al. [18].

Analytical methods

The cell growth was monitored according to the method as described previously [7]. The glucose and L-lysine concentrations were determined by an SBA-40E immobilized enzyme biosensor (Shandong, China) according to the suggestion of Xu et al. [7]. The cell-free extracts were prepared according to the method as described previously [6]. The cell-free extracts were immediately used for the protein expression analysis by SDS-PAGE [19], and enzyme activities were determined. Protein concentration was determined using the Bradford Protein Quantification Kit according to the protocol supplied by the manufacturer (Sangon, Shanghai, China) using bovine serum albumin as standard. Enzyme activities and protein concentrations were analyzed in biological triplicates. Enzymatic activities of AK [7], AlaT, and AvtA [20] were determined according to the previous reports. The activity of α-amylase was determined by a starch plate test described previously [21] or by Amylase Assay Kit (Biovision, San Francisco, USA) using ethylidene-pNP-G7 as the substrate.

Results

Construction of a mobilizable plasmid pK18-MBPMT

The mobilizable plasmid pK18*mobsacB* was widely used for gene disruption and exchange by homologous double crossover because of its high performance and absence of genetic markers [14]. Given the composition structure of this plasmid, it cannot be used for gene overexpression. The original pK18*mobsacB* is composed of MCS, *lacZα* fragment, *kan*, genetically modified *sacB*, etc., but is devoid of promoter and terminator [14]. The constructed plasmid pK18-MBPMT contained not only the elements of pK18*mobsacB*, but also a P_{tac}-iMCS-*rrnBT1T2* cartridge (Fig. 2a). The general scheme for constructing pK18-MBPMT based on pK18*mobsacB* was shown in Fig. 1. A 319-bp long fragment contained with homologous sequences upstream of the BamHI site in pK18*mobsacB*, promoter P_{tac} and iMCS, a 270-bp long fragment contained with iMCS, terminator *rrnBT1T2* and homologous sequences downstream of the XbaI site in pK18*mobsacB*, and the plasmid pK18*mobsacB* digested with BamHI and XbaI were ligated using the *pEASY*®-Uni Seamless Cloning and Assembly Kit. Successful constructions were selected by plating transformed *E. coli* JM109 on LBK. The target plasmid was extracted from

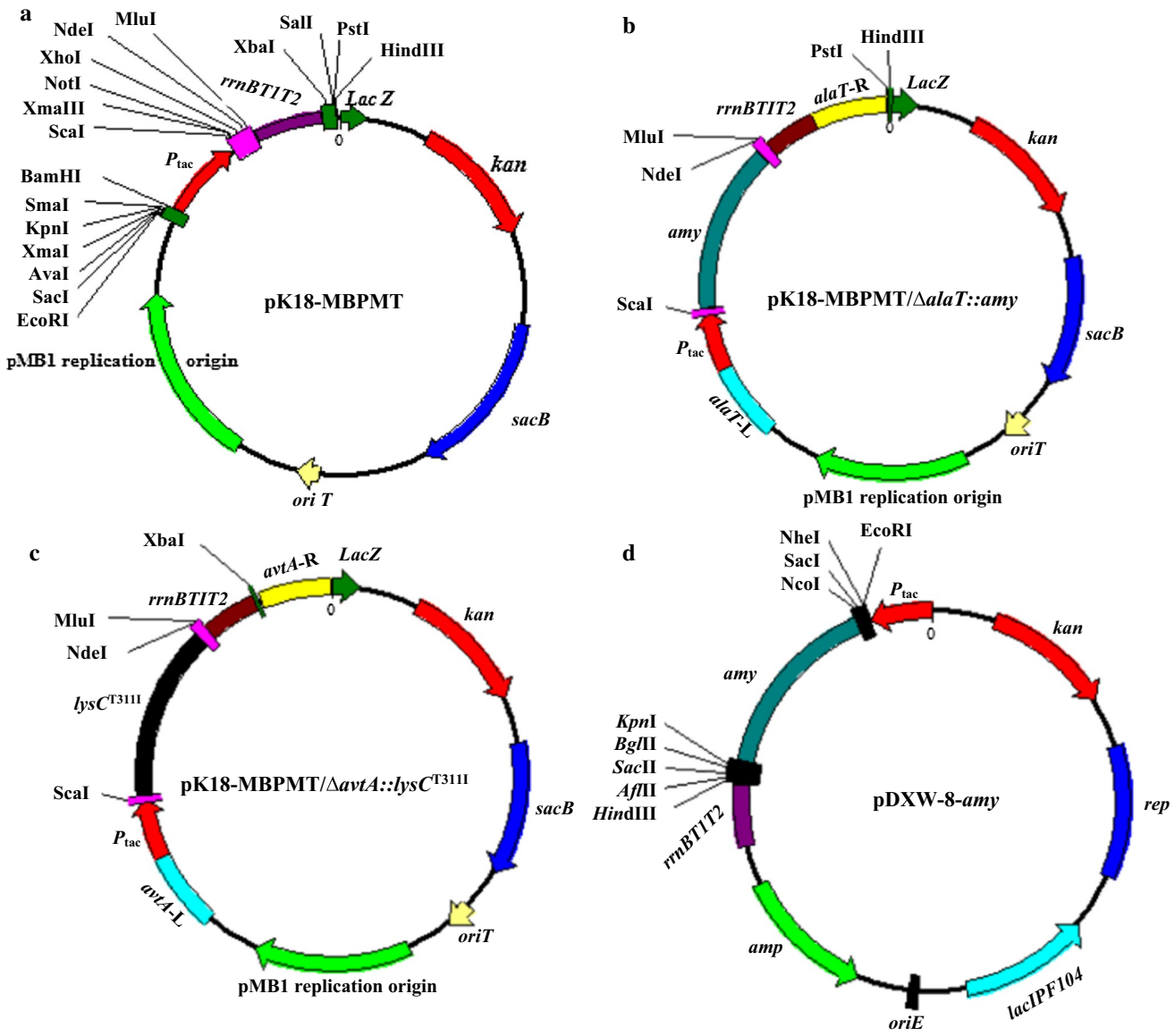

Fig. 2 Plasmid maps of **a** pK18-MBPMT, **b** pK18-MBPMT/ΔalaT::amy, **c** pK18-MBPMT/ΔavtA::lysC^{T311I}, and **d** pDXW-8-*amy*

E. coli and examined by the restriction endonuclease analysis (data not shown). The target mobilizable plasmid was selected and designated as pK18-MBPMT.

Construction of a target integrative plasmid pK18-MBPMT/Δ*A::B*

In contrast to our previous report [7], there were two steps to construct pK18-MBPMT/ΔA::B (representative pK18-MBPMT/ΔalaT::amy or pK18-MBPMT/ΔavtA::lysC^{T311I}) (Fig. 3): (1) construction of pK18-MBPMT/ΔA carrying the left and right arms of *A* gene, which provided homology for recombination and (2) insertion of *B* gene into pK18-MBPMT/ΔA, the resulting plasmid designated as pK18-MBPMT/ΔA::B. Since the plasmid pK18-MBPMT contained the P_{tac} and *rrnBT1T2* (Fig. 2a), the construction of pDXW-8-*B* was not required to obtain the cassette P_{tac}-*B*-*rrnBT1T2*. Moreover, there was an iMCS between P_{tac} and *rrnBT1T2* (Fig. 2a), which was beneficial to allow direct insertion of *B* gene into plasmid pK18-MBPMT/Δ*A*. All procedures were performed within 48 h, whereas our previous method required at least 72 h [7].

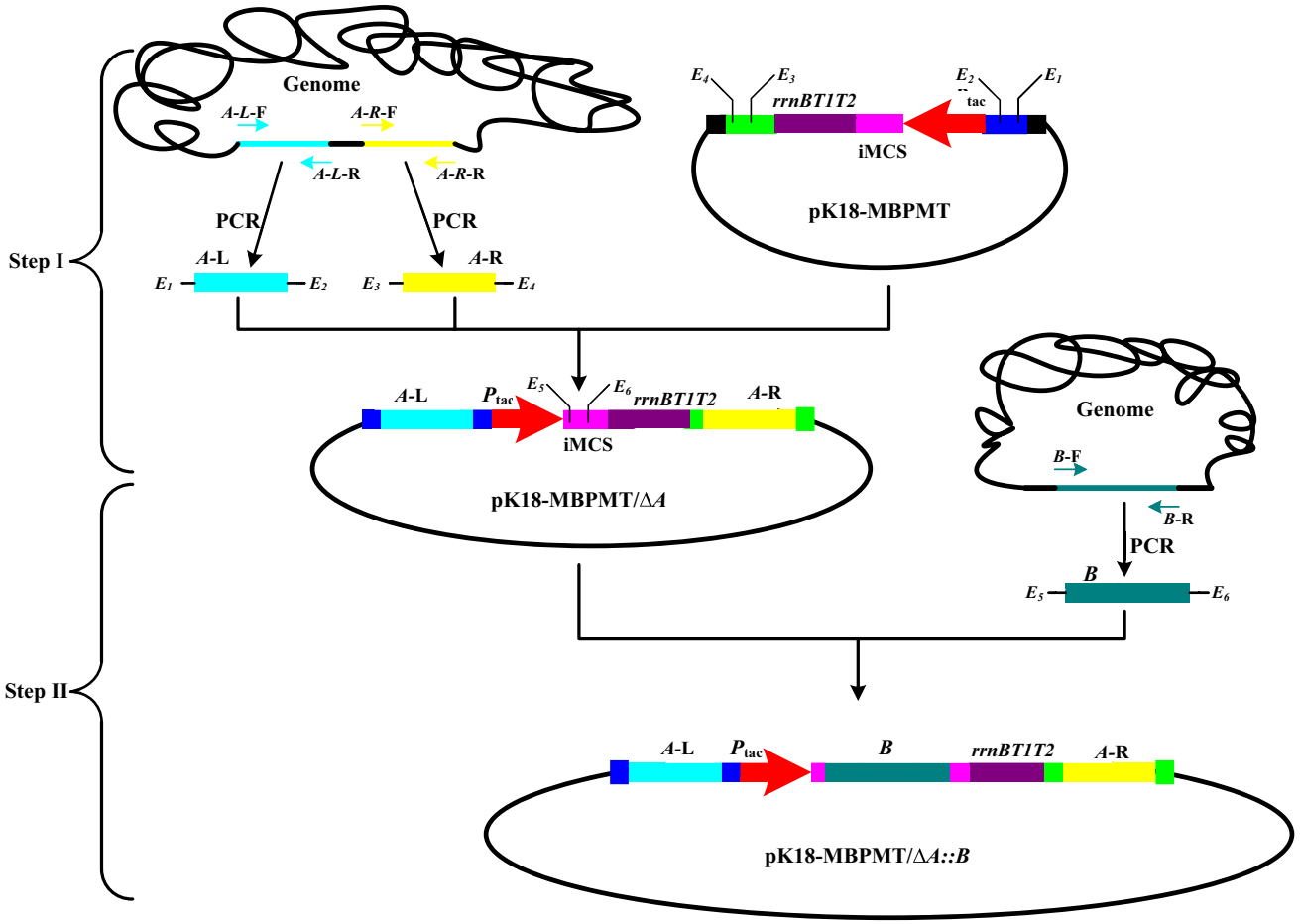

Fig. 3 Strategy used for the construction of integrative plasmid pK18-MBPMT/ΔA::B. *A* represents knock-out gene (e.g., *alaT* and *avtA*); *B* represents over-expressed gene (e.g., *amy* and $lysC^{T311I}$); *A*-L *left* arm of *A* gene, and *A*-R *right* arm of *A* gene, P_{tac} *tac* promoter; *rrnBT1T2* terminator, *iMCS* introduced multiple cloning sites, *A*-L-F/*A*-L-R primers for amplifying the *left* arm of *A* gene, and *A*-R-F/*A*-R-R primers for amplifying the *right* arm of *A* gene, *B*-F/*B*-R primers for amplifying the *B* gene

Integration of heterologous α-amylase gene in *C. glutamicum* genome

A heterologous α-amylase-encoding gene, *amy* from *B. amyloliquefaciens* was used to confirm the potential of mobilizable plasmid pK18-MBPMT-mediated DNA integration approach in *C. glutamicum*. To this end, the plasmid pK18-MBPMT/ΔalaT::amy (Fig. 2b) was introduced into *C. glutamicum* ATCC 13032 by electroporation (see "Materials and methods"). The target-recombinant strains were obtained according to Xu et al. [7]. The colonies were randomly selected from LBS plates and confirmed by individual bacterial colonies PCR, indicating that 92 % of the resulting colonies contained the *amy* gene (data not shown). We randomly selected one colony and sequenced it via Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China) to further reduce the false positive, and the resulted strain was designated as *C. glutamicum* IA-1. To confirm whether the integration happened at *alaT* locus, PCR was performed using the *alaT*-L-F/*alaT*-R-R primer pair, and approximately 3.8- and 2.5-kbp DNA fragments were amplified from *C. glutamicum* IA-1 and the wild-type strain *C. glutamicum* ATCC13032, respectively (Fig. 4b). Moreover, to confirm the normal expression of integrated *amy* in *C. glutamicum* genome, α-amylase activity was determined and compared to the expression plasmid pDXW-8-*amy* (Fig. 2d) mediating *amy* gene expression. As shown in Fig. 4a, *C. glutamicum* IA-1 possessed the capability of hydrolyzing starch at about the same level as the strain harboring pDXW-8-*amy*. These results were consistent with results obtained from the Amylase Assay Kit (Table 3). The α-amylase activity of *C. glutamicum* IA-1 was 670 ± 2.4 mU (mg protein)$^{-1}$, while *C. glutamicum*/*amy* showed 840 ± 9.1 mU mg^{-1}. In addition, cell-free extracts prepared from the above-mentioned strains were analyzed using SDS-PAGE (Fig. 4c). The thick protein band observed in the cell-free extracts from *C. glutamicum* IA-1 and *C. glutamicum*/*amy* but not in *C.*

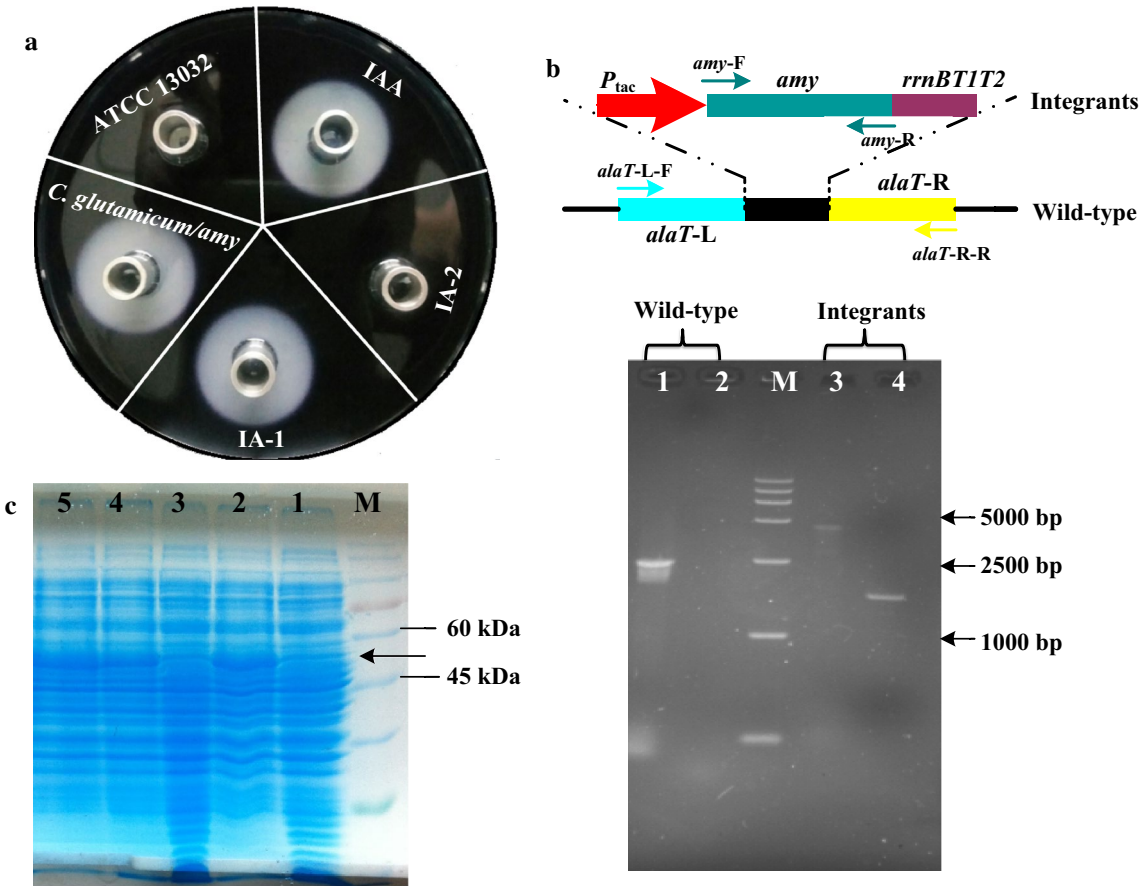

Fig. 4 Site-directed integration of *amy* in *C. glutamicum* ATCC13032. **a** Iodine staining of 1 % starch-containing agar plates after inoculation with wild-type strain and recombinant strains. *Clear zones* indicate hydrolysis of starch around recombinant colonies. **b** PCR amplification of wild-type strain and strain IA-1 with *alaT*-L-F/*alaT*-R-R (*Lanes 1* and *3*) or *amy*-F/*amy*-R (*Lanes 2* and *4*) as primer pairs. *M* is the DNA marker (DL15000). **c** SDS-PAGE analysis of α-amylase expression in *C. glutamicum* ATCC13032. *Lane 1 C. glutamicum* ATCC13032; *Lane 2 C. glutamicum/amy*; *Lane 3 C. glutamicum* ATCC13032 harboring pDXW-8; *Lane 4C. glutamicum* IA-1; *Lane 5C. glutamicum* IAA; *M* is the protein marker. *Arrow* indicates bands corresponding to α-amylase protein

Table 3 In vitro specific activities of α-amylase, AK, AlaT, and AvtA in genetically modified *C. glutamicum* strains and wild-type strain *C. glutamicum* ATCC 13032

Strains	Specific activity (mU [mg protein]$^{-1}$)			
	α-Amylase	AK	AlaT	AvtA
C. glutamicum ATCC 13032	ND	63 ± 2.5	89 ± 4.3	51 ± 1.7
C. glutamicum/amy	840 ± 9.1	61 ± 4.6	87 ± 6.1	51 ± 3.0
C. glutamicum IA-1	670 ± 2.4	64 ± 3.3	ND	62 ± 4.5
C. glutamicum IA-2	ND	131 ± 9.7	96 ± 2.4	ND
C. glutamicum IAA	650 ± 10.6	139 ± 5.2	ND	ND

Each value represents mean with standard error of three biological replicative experiments; the corresponding deviations are given

ND not detectable

glutamicum ATCC13032 was consistent with the expected molecular mass of α-amylase (~56 kDa) (Fig. 4c). These results indicated that the expression of α-amylase by the *C. glutamicum* strains was either via integration into genome or plasmid-mediated expression.

Integration of homologous AK^{Fr} gene in *C. glutamicum* genome

To further demonstrate the feasibility of this strategy for integrating homologous gene into *C. glutamicum* genome, AK^{Fr}-encoding gene *lysC*^{T311I} from *C. glutamicum* was integrated into *C. glutamicum* genome. To achieve this, the pK18-MBPMT/ΔavtA::lysC^{T311I} (Fig. 2c) was introduced into *C. glutamicum* ATCC 13032 or *C. glutamicum* IA-1 by electroporation. Colonies were randomly selected from LBS plates and confirmed by colony PCR using *avtA*-L-F/*avtA*-R-R as primer pair, indicating that about 90 % of the resulting colonies contained the desired mutations (Fig. 5a). The target-recombinant strains were designated as *C. glutamicum* IA-2 or *C. glutamicum* IAA, respectively. The expected chromosomal localization of integrated

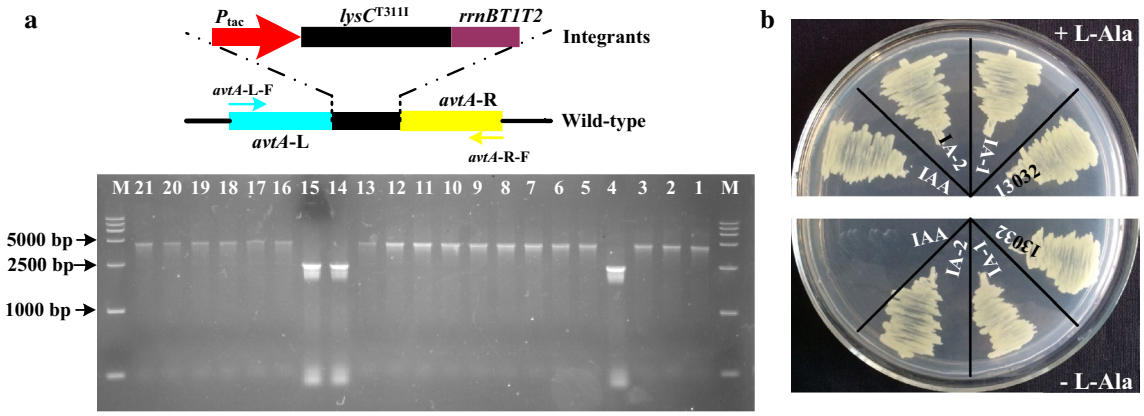

Fig. 5 Site-directed integration of $lysC^{T311I}$ in *C. glutamicum* ATCC13032 and *C. glutamicum* IA-1. **a** PCR amplification of wild-type strain and recombinant strains with *avtA*-L-F/*avtA*-R-R as primer pairs. *Lane 1–10* indicates site-directed integration of $lysC^{T311I}$ in *C. glutamicum* ATCC13032; *Lane 11–21* indicates site-directed integration of $lysC^{T311I}$ in *C. glutamicum* IA-1. *M* is the DNA marker (DL15000). **b** Growth of wild-type strain and recombinant strains on CgXII medium containing 40 g L^{-1} glucose (−) or supplemented with 1 g L^{-1} L-alanine (+); *13032* indicates *C. glutamicum* ATCC13032

Table 4 Kinetic and stoichiometrics characteristics of the recombinant *C. glutamicum* strains and wild-type strain *C. glutamicum* ATCC13032

C. glutamicum	DCW (g L^{-1})	Growth rate (μ_{max}, h^{-1})	L-Lysine (mmol L^{-1})	$Y_{P/X}$ (mmol [g DCW]$^{-1}$)	$Y_{P/S}$ (mmol [mol Glc]$^{-1}$)
ATCC13032	24.6 ± 2.12	0.43 ± 0.020	ND	–	–
IA-1	24.1 ± 1.08	0.44 ± 0.033	ND	–	–
IA-2	23.5 ± 1.32	0.42 ± 0.012	17.1 ± 0.07	0.73 ± 0.018	78.0 ± 1.76
IAA	22.7 ± 1.01	0.40 ± 0.009	22.8 ± 0.51	1.01 ± 0.064	102.6 ± 3.15

Each value represents mean with the standard error of three replicative experiments; the corresponding deviations are given

$Y_{P/X}$ biomass-specific L-lysine production, $Y_{P/S}$ substrate-specific L-lysine production, *Glc* Glucose, *ND* not detectable, – no computed data

pK18-MBPMT/ΔavtA::$lysC^{T311I}$ (Fig. 5a) was confirmed by amplifying a specific 3.7-kbp DNA fragment from recombinant strains by PCR using primer pair *avtA*-L-F/*avtA*-R-R, whereas the fragment from strain ATCC13032 and IA-1 was about 2.4 kbp (Fig. 5a). In addition, AK activity of *C. glutamicum* IA-2 and *C. glutamicum* IAA was higher than that of the wild-type strain ATCC13032 and strain IA-1 (Table 3). Moreover, AvtA activity of strain IA-2 and IAA was not detected (Table 3). As described previously, the inactivation of *alaT* and *avtA* led to the recombinant strain unable to grow in CgXII medium containing glucose unless supplemented with L-alanine (Fig. 5b). These results indicated that $lysC^{T311I}$ was inserted into *C. glutamicum* genome at the *avtA* locus and expressed successfully.

L-Lysine production of genetically defined *C. glutamicum* strains

The results above indicated that the integrated $lysC^{T311I}$ could be successfully expressed in *C. glutamicum*, and the activities of AlaT and AvtA were successfully inactivated (Table 3). However, $lysC^{T311I}$, *alaT*, and *avtA* have important roles in regulating L-lysine production by *C. glutamicum*. To investigate whether integration or inactivation of these genes via this method was beneficial to the improvement of L-lysine production, all strains were grown in CgXII minimal medium containing 40 g L^{-1} glucose and/or 1 g L^{-1} L-alanine, and growth and L-lysine accumulation were monitored over the course of the experiment. The recombinant strains IA-1, IA-2, and IAA showed slightly lower cell growth than wild-type strain ATCC13032 (Table 4). However, L-lysine production in the recombinant strains IA-2 and IAA was significantly increased: 78.0 ± 1.76 mmol mol^{-1} (i.e., C-mmol L-lysine/C-mol glucose) and 102.6 ± 3.15 mmol mol^{-1}, respectively, whereas L-lysine was not detected in strains ATCC13032 and IA-1 (Table 4). In addition, the biomass-specific L-lysine yield ($Y_{P/X}$) of strains IA-2 and IAA was also significantly increased (Table 4).

Discussion

Plasmid-mediated gene overexpression in *Corynebacterium* is a common strategy in genetic engineering [7]. However,

gene overexpression via plasmid is not beneficial to cell growth because of metabolic imbalance and accumulation of intermediates [8]. In addition, antibiotics must be added to the broth to maintain plasmid stability, which will increase production cost for companies and causes worry among the public [7, 22]. Therefore, many researchers dedicate into the research of DNA integration techniques, because it is the best genetic tool to stably express desirable genes in bacteria [12]. *C. glutamicum* shows complicated restriction systems (e.g., a specific DNA methylation pattern) that can severely affect the integration of foreign gene into its genome [12, 23]. To date, despite the development of many methods used to integrate foreign genes into the *C. glutamicum* genome, most of them will also introduce antibiotic resistance genes into the host cell [9–12]. In addition, homologous single-crossover between integrated plasmid and genome is the main strategy in these methods, which has low integration efficiency [8]. To increase the efficiency of integration, Vertès et al. [24] developed a new method, which depended on homologous double crossover. Although the Km-resistance gene was introduced into the host cell, the integration frequency of target insertion reached 2.4×10^2 integrants $(\mu g\ DNA)^{-1}$ [24]. Therefore, methods based on homologous double crossover might be useful to increase the integration efficiency.

The mobilizable plasmid pK18*mobsacB* is widely used for gene disruption and allelic exchange by homologous double crossover without genetic markers and showed high performance [14]. This plasmid is mainly used in gene disruption and exchange, but it is seldom used to gene overexpression [14, 18, 25]. Previously, we confirmed that it could work well in modifying the *C. glutamicum* genome to implement gene overexpression [7]. To overcome the heavy workload and high-cost of the previous method [7], a method based on mobilizable plasmid pK18-MBPMT has been developed. The mobilizable plasmid pK18-MBPMT contains promoter P_{tac}, terminator *rrnBT1T2*, and iMCS except for the components of pK18*mobsacB*. This method avoids the construction of expression plasmids, and only needs two steps to construct the target integrative plasmids. Using this method, the operation time of this protocol was shorter than the previous method (2 vs. 3 days), especially for repeated integration.

We showed the feasibility of the present strategy by integrating pK18-MBPMT/ΔalaT::amy harboring the *amy* gene from *B. amyloliquefaciens*, which provided *C. glutamicum* ATCC13032 with functional α-amylase activity. Normally, *C. glutamicum* does not possess functional α-amylase activity in media [24], but recombinant strains integrated with the *B. amyloliquefaciens amy* can break down soluble starch, indicating that the heterologous *amy* gene can be successfully expressed in *C. glutamicum* (Fig. 4a). To further demonstrate the potential of this strategy for integrating a homologous gene into the *C. glutamicum* genome, the $lysC^{T311I}$ gene from *C. glutamicum* was integrated into the genome at the *avtA* locus. The $lysC^{T311I}$ gene, encoding AK^{Fr}, is a key enzyme in L-lysine biosynthetic pathway [18]. The *alaT* and *avtA*, encoding aminotransferase T and A, respectively, are necessary for L-alanine biosynthesis [2]. Overexpression of $lysC^{T311I}$ proved to beneficially improve L-lysine production [4], whereas the inactivation of *alaT* or *avtA* showed a decrease in L-alanine accumulation [2, 4]. Therefore, the increased L-lysine production indicated that $lysC^{T311I}$ has been successfully integrated into the *C. glutamicum* genome and expressed via plasmid pK18-MBPMT/ΔavtA::$lysC^{T311I}$. In addition, integrating *amy* and $lysC^{T311I}$ at *alaT* and *avtA* loci, respectively, in strain *C. glutamicum* IAA resulted in its inability to grow in CgXII medium containing glucose unless supplemented with L-alanine (Fig. 5b). This indicated that *avtA* and *alaT* were successfully inactivated. These results are in accordance with the previous results [2, 4], indicating that the gene unmarked site-specific integration and fragment deletion in *C. glutamicum* genome using mobilizable plasmid pK18-MBPMT was successfully applied in improving L-lysine production.

In conclusion, genetically modifying the genome is an important strategy to stably express desirable genes in bacteria. The mobilizable plasmid pK18-MBPMT-mediated gene integration is useful for the improvement of the *C. glutamicum* genome. It could even create a *C. glutamicum* minimum genome factory (MGF), because this method can achieve gene overexpression and inactivation at the same time, and the genetically modified strains do not contain any genetic markers. In addition, the operation time of this protocol is shorter than the previous method, especially for repeated integration. Therefore, we believe the method described in this study has promising applications in genetically modifying industrial strains for high-level amino acid production.

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