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A method for gene amplification and simultaneous deletion in *Corynebacterium glutamicum* genome without any genetic markers

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ABSTRACT

A method for the simultaneous replacement of a given gene by a target gene, leaving no genetic markers, has been developed. The method is based on insertional inactivation and double-crossover homologous recombination. With this method, the *lysC*^{T311I}, *fbp* and *ddh* genes were inserted into *Corynebacterium glutamicum* genome, and the *pck*, *alaT* and *avtA* genes were deleted. Mobilizable plasmids with *lysC*^{T311I}, *fbp* and *ddh* cassettes and two homologous arms on the ends of *pck*, *alaT* and *avtA* were constructed, and then transformed into *C. glutamicum*. The target-expression cassettes were inserted in the genome via the first homologous recombination, and the genetic markers were removed via the second recombination. The target-transformants were sequentially screened from kanamycin-resistance and sucrose-resistance plates. The enzyme activities of transformants were stably maintained for 30 generations under non-selective culture conditions, suggesting that the integrated cassettes in host were successfully expressed and maintained as stable chromosomal insertions in *C. glutamicum*. The target-transformants were used to optimize the L-lysine production, showing that the productions were strongly increased because the selected genes were closely linked to L-lysine production. In short, this method can be used to construct amino acid high-producing strains with unmarked gene amplification and simultaneous deletion in genome.

1. Introduction

Corynebacterium glutamicum are non-pathogenic soil *Corynebacteria* that have significant economic importance. They are widely used for the industrial production of various amino acids, such as L-glutamate (Georgi et al., 2005), L-lysine (Becker et al., 2011; van Ooyen et al., 2012; Xu et al., 2014), L-valine (Hou et al., 2012) and other metabolites (Correia et al., 1996). Fortunately, the development of genetic engineering of this microorganism has enabled us to construct the L-lysine high-producing strains. Several reports of successful metabolic engineering of L-lysine producers have been reported (Becker et al., 2011; van Ooyen et al., 2012). At present, however, the industrial L-lysine producing strains are almost auxotroph, regulatory and combined mutants, which have been obtained by

Abbreviations: AK, aspartokinase; FBPase, fructose-1,6-bisphosphatase; DDH, diaminopimelate dehydrogenase; PEPCk, phosphoenolpyruvate carboxykinase; AlaT, aminotransferase T; AvtA, aminotransferase A; DCW, dry cell weight; LB, Luria–Bertani; LBG, LB + 0.5% glucose; LBS, LB + 10% sucrose; LBK_{25}, LB + 25 μg ml^{-1} kanamycin; $LBSK_{25}$, LBS + 25 μg ml^{-1} kanamycin; LBHIS, LB + brain heart infusion + sorbitol.

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repeated physical and/or chemical mutagenesis (Xu et al., 2013). One of the reasons why the genetically modified bacteria were not widely used in industry is that a certain amount of antibiotics must be supplemented in media to achieve a stable maintenance of plasmids (Tauch et al., 2003).

Plasmid-mediated structural gene amplification is a common strategy in genetic engineering (Ikeda and Katsumata, 1998). Plasmids generally carry genetic markers, such as antibiotic resistance marker. However, there are several disadvantages to the application of antibiotic resistance marker in genetic modification of amino acid-producing strains: (i) high price and instability (Zhou et al., 2009); (ii) effects on physiological function of *Corynebacteria*; (iii) raising fears of the contribution of antibiotic resistance gene (Tauch et al., 2002). And plasmids are easily lost in the absence of selective pressures. In addition, overexpression of a gene via plasmid is not beneficial to the cell growth because of metabolic imbalance and accumulation of intermediates (Ikeda and Katsumata, 1998). In the past decades, fortunately, constructions of integration plasmids which carry a homologous chromosomal DNA fragment and an antibiotic resistance marker have made it possible for inserting a gene into the genome. The plasmid sequence driving homologous recombination is a dispensable element for cell growth, such as *IS13869* (Correia et al., 1996) and rRNA gene (Amador et al., 2000). The transformants are yielded by single crossover between plasmid and the recipient genome occurring at the region homology (Ikeda and Katsumata, 1998). However, the antibiotic resistance gene is also introduced into the transformants genome. In addition, single-crossover integration of a plasmid after transformation generally occurs at a low frequency in the *Corynebacteria* when the plasmid carries chromosomal sequences (Ikeda and Katsumata, 1998). Consequently, there is a necessity to develop method which can be used for efficiently inserting a gene into the genome rather than recombinant plasmids and avoiding the inclusion of any exogenous genetic markers in genome.

The mobilizable plasmid pK18*mobsacB* is widely used in gene disruption and allelic exchange by homologous recombination (Schäfer et al., 1994). This plasmid carries kanamycin-resistance gene and genetically modified *sacB* gene which confers sucrose-sensitively to Gram$^-$ and some Gram$^+$ species (Schäfer et al., 1994). The target-transformants are obtained by double crossover events. Only cell in which the plasmid is deleted by the two homologous recombinations can grow in the medium with 10% sucrose (Ohnishi et al., 2002). The efficiency and regulation of strong *Escherichia coli* promoters, such as *Ptac*, *Ptrp*, *PlacUV5*, $P\lambda P_R P_L$ and *ParaBAD* are found to exhibit similar properties in *C. glutamicum* (Srivastava and Deb, 2005). In addition, Hou et al. (2012) has reported that DNA segment *Ptac-cat-rrnBT1T2* integrated into *Brevibacterium flavum* genome can be effectively expressed in *B. flavum*. Based on the above observations, we developed a method to construct a *C. glutamicum* strain which contains simultaneously critical enzyme gene over-expression and fragment deletion in genome. This method is based on the theories of insertional inactivation and double-crossover homologous recombination. The recombinational *C. glutamicum* strains with marker-free insertion of target gene and simultaneous fragment deletion in genome were obtained via this method.

As proof or principle, the generation of amplification of *lysC*^{T311I} gene (encoding allosterically feedback-resistant aspartokinase, AK), *fbp* (encoding fructose-1,6-bisphosphatase, FBPase) and *ddh* (encoding diaminopimelate dehydrogenase, DDH), and simultaneous deletion of *pck* gene (encoding phosphoenolpyruvate carboxykinase, PEPCk), *alaT* gene (encoding aminotransferase T, AlaT) and *avtA* gene (encoding aminotransferase A, AvtA) in the genome of *C. glutamicum* are represented in this manuscript (The related information is presented in Fig. 1.). The AK and DDH are the key enzymes in the L-lysine biosynthesis pathway, and the FBPase is crucial enzyme for increasing the availability of L-lysine cofactor NADPH. However, the PEPCk, AlaT and AvtA will decrease the availability of L-lysine precursor pyruvate or OAA. In the end, these target-transformants are used to optimize the L-lysine production.

2. Materials and methods

2.1. Strains, growth media and culturing conditions

Strains and plasmids used in this study are listed in Table 1, and oligonucleotides used in this study are listed in Table S1. Luria–Bertani (LB) was used as the standard medium for *E. coli* BL21. LBG (LB supplemented with 5 g l^{-1} glucose) was used for *C. glutamicum* (Hou et al., 2012). The Epo medium used for growing electroporation-competent cells and the LBHIS (LB, brain heart infusion, sorbitol) plates used for obtaining transformants of *C. glutamicum* were prepared according to the descriptions of van der Restá et al. (1999). *E. coli* and *C. glutamicum* were respectively grown at 37 and 30 °C. When appropriate, *E. coli* and *C. glutamicum* strains were cultured with kanamycin (50 μg ml^{-1}) and 120 rpm, and a reduced concentration of kanamycin (25 μg ml^{-1}) was used to obtain recombinant strains of *C. glutamicum* (Georgi et al., 2005).

2.2. Construction of mobilizable plasmid pK18mobsacB-ΔA::B

The construction process of plasmid pK18*mobsacB*-Δ*A::B* (representative pK18*mobsacB*-Δ*pck::lysC*^{T311I}, pK18*mobsacB*-Δ*alaT::fbp* and pK18*mobsacB*-Δ*avtA::ddh*) was illustrated in Fig. 2A. DNA was extracted from *C. glutamicum* using the TaKaRa MiniBEST Universal Genomic DNA Extraction Kit according the protocol supplied by the manufacturer (TAKARA, Dalian, China). The left and right arm of *A* gene (representative *pck*, *alaT* and *avtA*) were amplified with high-fidelity *pyrobest* DNA polymerase (TAKARA, Dalian, China) from the chromosomal DNA of *C. glutamicum* by corresponding primers, respectively (Table S1), and designated as *A*-L/*A*-R (representative *pck*-R/*pck*-L, *alaT*-L/*alaT*-R and *avtA*-L/*avtA*-R). The fragments of *A*-L/*A*-R were purified and digested by suitable restriction enzyme, respectively (Table S1), and then were sequentially ligated into pK18*mobsacB* (Schäfer et al., 1994) which was similarly digested. The resulting plasmid was designated as pK18*mobsacB*-Δ*A* (representative