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Construction of a novel expression system for use in *Corynebacterium glutamicum*

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ABSTRACT

Corynebacterium glutamicum is an important microorganism for production of amino acids in industrial fermentation. Suitable vectors are needed for metabolic engineering in *C. glutamicum*. Most available vectors used in *C. glutamicum* carry antibiotic resistant genes as a genetic labeling for rapid identification of recombinant strains, and antibiotics have to be added to maintain the vector when growing the cells. These vectors, though excellent for laboratory use, are not preferable choices for industry-scale fermentation. In this work, we developed a novel expression system for use in *C. glutamicum*, which do not require antibiotics when used for industrial fermentation. This system includes two vectors: the shuttle vector pJYW-4 for expression of genes and the vector pJYW-6 for deletion of the essential gene *alr* in *C. glutamicum*. The vector pJYW-4 contains a large multiple cloning site for cloning multiple genes and two selective markers: one is the kanamycin-resistant gene *kan* and the other is an essential gene *alr*. The selective marker *kan* facilitates molecular manipulation or fermentations in the laboratory, and the selection marker *alr* is good for use in industry-scale fermentation, allowing *in vivo* maintenance of the expression vector through auxotrophic complementation; therefore, the two selection markers in pJYW-4 make it useful for both laboratory research and industrial fermentation, and convenient to transfer valuable laboratory-developed strains into industrial production. This newly-constructed expression system was successfully used to increase L-valine production in *C. glutamicum* ATCC 14067, indicating its potential on developing amino acid-producing *C. glutamicum* strains.

1. Introduction

Corynebacteria are gram-positive bacteria with moderately high G + C content, and have been widely used in the industrial production of amino acids (Hermann, 2003) and vitamins (Huser et al., 2005). *Corynebacterium glutamicum*, one of the most used Corynebacteria in industrial fermentation, has been used for production of L-valine (Miyajima and Shiio, 1972), L-glutamate (Yamada et al., 1972), L-threonine (Ishida et al., 1993) and L-lysine (Tada et al., 2000). The production strains initially obtained by classical mutagenesis (Nakayama, 1981) and protoplast fusion (Furukawa et al., 1988) now can be developed by metabolic engineering, based on the sequenced genomes of several *C. glutamicum* strains (Lv et al., 2012; Kalinowski et al., 2003). Metabolic engineering could draw metabolic flux towards a specific amino acid in *C. glutamicum*; and overexpression of genes encoding the rate-limiting enzymes in biosynthetic pathways is one of the most efficient strategies (Dong et al., 2010).

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Various vectors have been built as tools for metabolic engineering in *C. glutamicum*, and most of them are *Escherichia coli-C. glutamicum* shuttle vectors which contain an antibiotic resistant gene for rapid identification of the recombinant strains (Eggeling and Bott, 2005; Burkovski, 2008; Xu et al., 2010a, 2010b, 2011). Antibiotic resistance is the most preferable and reliable selection marker in laboratory study, however, in industrial fermentation, the inevitable antibiotic supplement to the media for *in vivo* maintenance of the vectors would raise the cost and contaminate the final product (Baneyx, 1999; Balbas, 2001). Moreover, constitutively expressed antibiotic resistance genes may impose a metabolic burden on the host cells, resulting in the reduced growth rate and cell density (Bentley et al., 1990; Smith and Bidochka, 1998). Based on a strain auxotrophic for an essential metabolite which can be complemented with the plasmid-borne selection gene, auxotrophic complementation system is an alternative selection marker which does not require antibiotics. This system is efficient in numerous bacterial species (Nakayama et al., 1988; Morona et al., 1991; Ryan et al., 2000; Glenting et al., 2002; Tauch et al., 2002a; Borsuk et al., 2007).

In this work, a novel *C. glutamicum* auxotrophic complementation expression system has been developed. This system includes two vectors: pJYW-4 for expression and pJYW-6 for deletion of the essential gene *alr* in *C. glutamicum*. The gene *alr* encodes alanine racemase which catalyzes the pyridoxal 5′-phosphate-dependent reversible racemization between L-alanine and D-alanine. D-alanine is an essential component of the peptidoglycan in bacteria (Hayashi et al., 1990). Therefore, *alr* is a good target for constructing the auxotrophic complementation system in *C. glutamicum* (Tauch et al., 2002a). The plasmid pJYW-4 harbors two selective markers, one is the kanamycin-resistant gene *kan* and the other is *alr*. The selective marker *kan* facilitates molecular manipulation or fermentations in the laboratory, and the selection marker *alr* is good for use in industry-scale fermentation, allowing *in vivo* maintenance of the expression vector. Therefore, the plasmid pJYW-4 is useful for both laboratory research and industry fermentation, and is convenient to transfer valuable laboratory-developed strains into industrial production. This newly-constructed expression system was successfully used to increase L-valine production in *C. glutamicum* ATCC 14067.

2. Materials and methods

2.1. Growth and transformation of E. coli and C. glutamicum

All bacterial strains used in this study are listed in Table 1. When necessary, 30 mg/L kanamycin was supplemented in the media.

E. coli JM109 was grown at 37 °C in LB media (10 g/L trypton, 5 g/L yeast extract, 10 g/L NaCl). Transformation of *E. coli* was performed according to the published protocol (Sambrook and Russell, 2001). Briefly, overnight *E. coli* culture was inoculated into 50 mL LB media to an initial OD_{600} of 0.02, grown at 200 rpm and 37 °C until OD_{600} reached 0.5. The cells were cooled on ice for 30 min, centrifuged and washed 3 times with ice-cold 0.1 M $CaCl_2$, and stored at −70 °C in 1.5 mL aliquots. For transformation, an aliquot of competent cells was thawed on ice, and DNA was added. The mixture was incubated on ice for 30 min, and put in a water-bath at 42 °C for 1.5 min. The mixture was then cooled on ice for 3 min, and 1 mL of LB media was added. The mixture was incubated at 37 °C and 100 rpm for 1 h, and plated on LB agar containing proper antibiotics for selection.

C. glutamicum was usually grown at 30 °C in LBG media (LB supplemented with 5 g/L glucose) or minimum media (20 g/L glucose, 7 g/L $(NH_4)_2SO_4$, 3 g/L urea, 3 g/L K_2HPO_4, 1 g/L KH_2PO_4, 0.4 g/L $MgSO_4{\cdot}7H_2O$, 2 mg/L $FeSO_4{\cdot}7H_2O$, 2 mg/L $MnSO_4{\cdot}H_2O$, 50 mg/L NaCl, 10 mg/L $CaCl_2{\cdot}2H_2O$, 1 mg/L biotin, and 10 mg/L thiamine). 2 mL of the special solution (88 mg/L $Na_2B_4O_7{\cdot}7H_2O$, 40 mg/L $(NH_4)_6Mo_7O_{27}{\cdot}4H_2O$, 10 mg/L $ZnSO_4$, 270 mg/L $CuSO{\cdot}5H_2O$, 7.2 mg/L $MnCl_2{\cdot}H_2O$, and 870 mg/L $FeCl_3$) was added to 1 L of minimal media when it was used for growing *C. glutamicum* (Follettie and Sinskey, 1986).

Transformation of *C. glutamicum* ATCC14067 was performed according to the previous publication (Xu et al., 2010a). Briefly, overnight *C. glutamicum* culture was inoculated into 30 mL of the modified Epo media (10 g/L trypton, 5 g/L yeast extract, 10 g/L NaCl, 25 g/L glycine, 0.1% Tween-80, and 4 g/L isonicotinic acid hydrazide) to an initial OD_{600} of 0.2. The culture was grown at 200 rpm and 30 °C until OD_{600} reached 0.9. The cells were cooled on ice for 30 min, centrifuged and washed 4 times with ice-cold 10% glycerol, and stored at −70 °C in 1.5 mL aliquots. For electro-transformation, an aliquot of the competent cells was thawed on ice, and DNA was added. The mixture was transferred to a cold electroporation cuvette (0.1 cm), and electroporated twice at 1.8 kV with 5 ms pulse. Immediately after the electroporation, 1 mL LBHIS media (5 g/L trypton, 5 g/L NaCl, 2.5 g/L yeast extract, 18.5 g/L brain heart infusion powder and 91 g/L sorbitol) was added to the cuvette. The mixture was transferred to a 1.5 mL Eppendorf tube, incubated at 30 °C and 100 rpm for 1 h, and plated on LBHIS agar containing proper antibiotics for selection.

2.2. DNA manipulations

Restrictions enzymes, T4 DNA ligase, and 1 kb DNA ladder were purchased from Sangon (Shanghai, China). PrimeSTAR™ HS DNA polymerase was purchased from TaKaRa (Dalian, China). Plasmid minipreps purification system B used for isolating plasmids was from BioDev-Tech (Beijing, China). TIAnamp bacteria DNA kit used for isolating the genomic DNA was from Tiangen (Beijing, China). EZ-10 spin column DNA gel extraction kit used for purifying DNA from agarose gels was purchased from Bio Basic Inc (Markham, Canada). DNA synthesis and sequencing were performed by Sangon (Shanghai, China).

PCR experiments were performed by using Mastercycler from Eppendorf (Hamburg, Germany). The sequences of all primers used in this study are listed in Table 2. Generally, 50 μL PCR reaction mixture includes 10 μL 5× PrimerSTAR buffer (Mg^{2+} plus), 4 μL dNTP mixture