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Research Article

Development of A Novel Gene Expression System for Secretory Production of Heterologous Proteins via the General Secretory (Sec) Pathway in *Corynebacterium glutamicum*

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Background: *Corynebacterium glutamicum* (*C. glutamicum*) is a potential host for the secretory production of the heterologous proteins. However, to this date few secretion-type gene expression systems in *C. glutamicum* have been developed, which limit applications of *C. glutamicum* in a secretory production of the heterologous proteins.
Objectives: In this study, a novel and efficient general secretory (Sec) pathway-dependent type gene expression system for the production of heterologous proteins was developed in *C. glutamicum*.
Materials and Methods: The synthesized cloning/expression cassette C was assembled into the basic *E. coli-C. glutamicum* shuttle vector pAU2, generating the Sec-dependent type gene expression vector pAU5. Subsequently, the applicability of the *C. glutamicum*/pAU5 system was tested using the α-amylase AmyE from *Bacillus subtilis* as a reporter protein.
Results: The vector pAU5 was successfully constructed. The SDS-PAGE experiment showed the AmyE protein band could be observed in the original culture supernatant of the 14067/pAU5-*amyE*. The Western blotting experiment showed that the AmyE polypeptide could be detected in the culture supernatant of the 14067/pAU5-*amyE*, not in the cell lysate of 14067/pAU5-*amyE*. The α-amylase specific activity of the culture supernatant of 14067/pAU5-*amyE* was 103.24±7.14 $U.mg^{-1}$ protein, while no α-amylase activity was detected in the cell homogenate supernatant of 14067/pAU5-*amyE*. These results demonstrate that the recombinant AmyE was efficiently expressed and completely secreted into the extracellular environmentin an active form in *C. glutamicum*/pAU5 system.
Conclusions: A novel efficient Sec-dependent type gene expression vector pAU5 was constructed in the *C. glutamicum*. The vector pAU5 employs the strong promoter *tac-M* for controlling a constitutive transcription of the target gene, the consensus ribosome binding site (RBS) sequence of *C. glutamicum* to ensure protein translation, and the efficient Sec-type cgR_2070 signal sequence to mediate protein secretion in the *C. glutamicum*. The *C. glutamicum*/pAU5 system is an efficient expression system for the secretory production of the heterologous proteins.

Keywords: *Corynebacterium glutamicum*; Gene expression system; General secretory (Sec) pathway; Protein secretion

1. Background

The secretory production of the recombinant proteins can greatly simplify separation and purification processes and significantly decrease their production costs. *Bacillus subtilis* is the most widely used host in the industry for the secretory production of the recombinant proteins. However, *B. subtilis* is mainly applied in the production of its host-derived proteins; in contrast, there exist multiple extracellular and cell wall-associated proteases that degrade heterologous proteins, so attempts to use *B. subtilis* for secretory production of heterologous proteins often has failed (1). *Corynebacterium glutamicum* has been widely used for the production of amino acids and other low-molecular metabolites (2). Due to its many advantages, such as the GRAS (generally regarded as safe) status (3), the ability to secrete proteins into culture media (4), little extracellular proteolytic activity (5), and the excellent cultural characteristics (6), *C. glutamicum* has attracted attention as a host for secretory production of the heterologous proteins.

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C. glutamicum possesses two major protein secretory pathways: the general secretory (Sec) pathway that translocates unfolded proteins to the extracellular spacer, and the twin-arginine translocation (Tat) pathway that catalyzes the export of the folded proteins (7). A signal peptide is a short peptide in the N-terminal region of the secretory protein precursor, and it plays a key role in determining secretory pathway and efficiency of the secreted protein. A number of *C. glutamicum* vectors have been developed, some of which were used for the secretory production of heterologous proteins (8-10). Due to the lack of signal sequences in the developed vectors, the two experimental steps were performed to construct secretion-type recombinant vectors. The first step was to fuse the target gene with the signal sequence by an *in vitro* PCR overlap, followed by the cloning the fused gene into the vector; alternatively, the first step was to clone the signal sequence into the vector, followed by cloning of the target gene downstream of the signal sequence in the vector. The use of secretion-type gene expression vector harboring an efficient signal sequence can simplify the construction process of the recombinant vector and improve the yield of the secreted protein. However, to this date, few secretion-type gene expression vectors in *C. glutamicum* have been developed (11, 12). The lack of secretion-type gene expression vectors limits applications of *C. glutamicum* in the secretory production of the heterologous proteins. Therefore, it is valuable to develop new secretion-type gene expression vectors harboring efficient signal sequences in *C. glutamicum*. A series of signal peptides have been identified in *C. glutamicum* (13-19), which lays the foundation for the development of the new efficient secretion-type gene expression vectors.

2. Objective

In this study, a novel efficient general secretory (Sec) pathway-dependent type gene expression system for the production of heterologous proteins was developed in *C. glutamicum*.

3. Materials and Methods

3.1. Strains, Plasmids, and Growth Conditions

All bacterial strains and plasmids used in this study are listed in **Table 1**. *E. coli* was grown in the LB medium at 37 □ (20), and *C. glutamicum* was grown in LBHI medium at 30 □ (21). If necessary, kanamycin used was at a concentration of 50 μg.mL^{-1} in the LB medium and 30 μg.mL^{-1} in the LBHI medium.

3.2. DNA Preparation and PCR Amplification

Preparation, separation, and purification of the DNA were carried out using the corresponding kits according to the instructions. The synthesis of the primers and the cloning/expression cassette C and the DNA sequencing were carried out by Sangon (Shanghai, China). The signal-less *amyE* gene (1878 bp) was amplified using *B. subtilis* 168 genomic DNA as the template and the forward primer (ATATAGATCTGAAACGGCGAACAAATCGAAT, the underlined bases indicate *Bgl*II site) and the reverse primer (ATAT CTCGAGATGGGGAAGAGAACCGCTTAA, the

Table 1. Bacterial strains and plasmids used in this study.

Strain	Description	Reference or Sources
E. coli JM109	*el4-(McrA-) recA*1 *endA*1 *gyrA*96 *thi*-1 *hsdR*17 *supE*44 *relA*1 Δ（*lac-proAB*）[F′ *traD*36 *proAB*$^+$*lacI*^{q}*ZΔM15*]	Stratagene
B. subtilis 168	*trpC2*	(20)
*C. glutamicum*ATCC14067	Wild-type *C. glutamicum*	ATCC
Plasmid		
pAU2	A shuttle vector between *E. coli* and *C. glutamicum*, Km^r	(21)
pAU5	pAU2 (*Nco*I/*Aat*II) Ω cassette C (*Nco*I/*Aat*II; 295 bp, synthesized fragment), A shuttle expression vector between *E. coli* and *C. glutamicum*, Km^r	This work
pAU5-*amyE*	pAU5 (*Bgl*II/*Xho*I) Ω *amyE* (*Bgl*II/*Xho*I; 1878 bp, *B. subtilis* 168 chromosomal DNA), a recombinant pAU5 plasmid harboring the *amyE* gene	This work

underlined bases indicate *Xho*I site) were used for amplification. Annealing temperature and time are 60 □ and 15 s, and the PCR reaction was carried out using the high fidelity PrimeSTAR™ HS DNA polymerase (TaKaRa, Dalian, China) according to the instruction using a Mastercycler (Eppendorf, Hamburg, Germany) PCR machine.

3.3. Preparation of the Competent Cells and Transformation

Preparation of the competent *E. coli* and *C. glutamicum* cells as well as DNA transformation was carried out using the methods described by Sambrook *et al*. and in our previous work (20, 21).

3.4. Construction of the Plasmids pAU5 and pAU5-amyE

The DNA sequence of the artificially synthesized cloning/expression cassette C (295 bp, flanked by *Nco*I and *Aat*II sites) contains the *tac-M* promoter, ribosome binding site (RBS) sequence, *cgR_2070* signal sequence, multiple cloning sites (MCS) (*Eco*RI, *Not*I, *Bgl*II, *Nhe*I, *Xho*I, and *Hin*dIII), and the His-tag and *rrnB*T2 terminator (**Fig. 1**). The cassette C was digested with *Nco*I and *Aat*II and then ligated into the *E. coli-C. glutamicum* shuttle vector pAU2 constructed in our previous study (22), which was also digested with *Nco*I and *Aat*II, generating the target plasmid pAU5 (4993 bp) (Fig. 1). The cassette C in the plasmid pAU5 was sequenced toconfirm its correct insertion. The PCR product of *amyE* gene was digested with *Bgl*II and *Xho*I, and cloned into pAU5, which was also digested with *Bgl*II and *Xho*I, generating the recombinant plasmid pAU5-*amyE* (6865 bp).

3.5. Flask Cultivation and Sample Preparation of C. glutamicum

Plasmids pAU5 and pAU5-*amyE* were used to transform *C. glutamicum* ATCC14067, resulting in ATCC14067/pAU-5 and ATCC14067/pAU5-*amyE*, respectively. The seed culture of the *C. glutamicum* strain was prepared by overnight cultivation and was then transferred to 500 mL flasks containing 100 mL of the medium for shake-culturing to produce AmyE protein. The cell pellets and culture supernatants of the ATCC14067/pAU-5 and ATCC14067/pAU5-*amyE* cultures were separated by centrifugation at 13680 ×g for 5 min, respectively. The samples of the culture's supernatants were directly used to test the production of AmyE by SDS-PAGE, Western blotting, and amylase activity analysis. The cell pellets were washed and re-suspended in the phosphate buffer (pH 6.0) in accordance to 1:20 (v:v) ratio of the buffer:

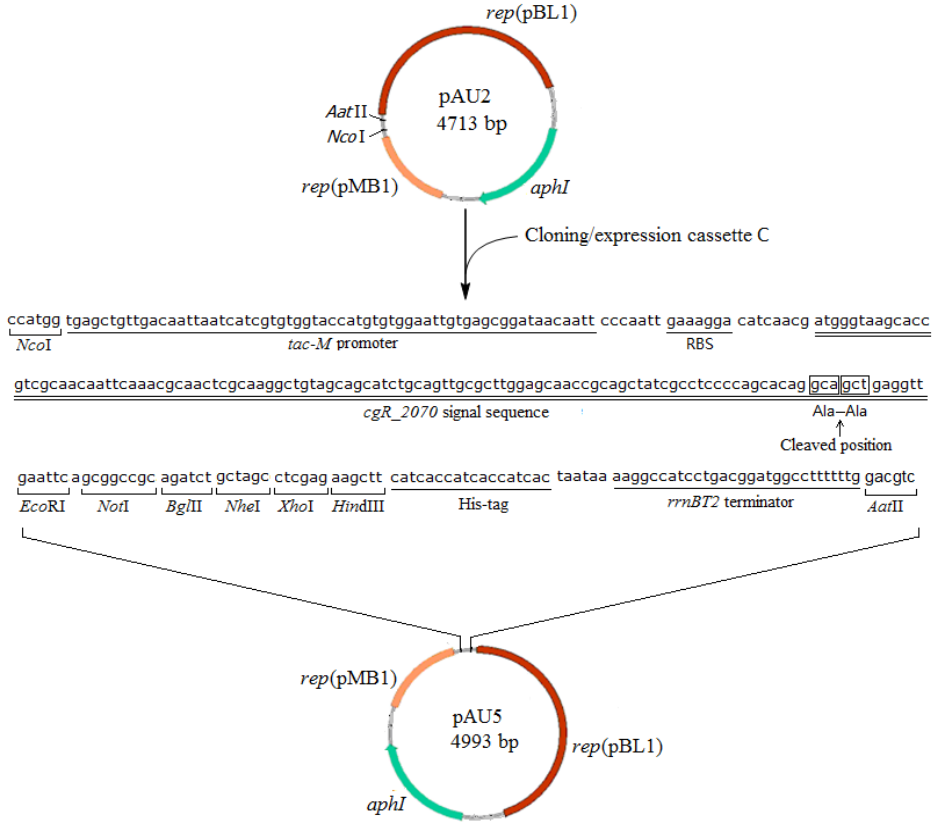

Figure 1. The construction map of the gene expression vector pAU5*rep* (pMB1), the origin of *E. coli* plasmid pBR322; *rep* (pBL1), the origin of *C. glutamicum* plasmid pC2; *aphI*, kanamycin resistance gene. Nucleotides encoding Ala are boxed, and the peptide bond between the two alanine residues is cleaved position of the signal peptide of CgR2070 by the signal peptidase.

culture medium. The cell suspension was mixed with a quarter volume of 5X SDS-PAGE loading buffer, boiled for 10 min, and centrifuged in order to remove debris, generating a sample of cell lysate for SDS-PAGE and Western blotting. The re-suspended cells were also disrupted by ultrasonication and centrifuged, generating the sample of cell homogenate supernatant for the amylase activity assay.

3.6. SDS-PAGE and Western Blotting Analysis

The protein samples (10 μL each) were loaded in the wells of a 12% (w/v) SDS-polyacrylamide gel. After electrophoresis, the gel was stained using Coomassie Brilliant Blue dye. According to the experimental design, the recombinant AmyE was fused with 6×His-tag at its C-terminus. In order to further confirm production yield and localization, Western blotting was carried out according to the procedure described by An *et al*. (11). A mouse anti-6×His tag antibody (BioDev-Tech, Beijing, China) was used as the first antibody. The second antibody was a goat alkaline phosphatase-labeled anti-His-antibody (BioDev-Tech, Beijing, China), and the polyvinyl difluoride membrane was finally incubated with the color-substrate solution (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, 0.01 mM NBT, 0.01 mM BCIP, pH 9.5) to observe color reaction.

3.7. Analysis of Amylase Activity

The α-amylase activity in the cell homogenate supernatant and the culture supernatant of ATCC14067/pAU5-*amyE* were quantitatively analyzed using the standard blue value method (23). Briefly, 20 mL 2.0% (w/v) of the soluble starch and 5 mL of phosphate buffer (pH 6.0) were mixed and heated at 60 □ for 5 min. One mL of the cell homogenate supernatant or culture supernatant was then added and incubated for 5 min. One mL of the reaction mixture was added to 5 mL I_2/KI solution (I_2 0.088 $g.L^{-1}$, KI 40 $g.L^{-1}$). The absorbance of the reactant was measured at 660 nm. One Unit (U) of the enzyme activity was defined as the amount of amylase required to liquefy 1 g soluble starch completely for 1 h at 60 □ and pH 6.0. The determination of the total protein concentration in the both supernatants was carried out using the Protein dotMETRIC™ Kit (Sangon, Shanghai, China).

4. Results

4.1. Construction of a Novel Gene Expression Vector pAU5 in C. glutamicum

The synthesized cloning/expression cassette C was assembled into our previously constructed basic *E. coli*-*C. glutamicum* shuttle vector pAU2 (22), and DNA sequencing further confirmed its correct assembly, resulting in the target plasmid pAU5 (Fig. 1). The *rep* (pMB1) of the plasmid pBR322 and the *rep* (pBL1) of the plasmid pC2 were used as replication origins of the vector pAU5 in *E. coli* and *C. glutamicum*, respectively. The kanamycin resistance gene *aphI* was used as the selection marker for both *E. coli* and *C. glutamicum* transformants. The cloning/expression cassette of pAU5 employs the MCS (*Eco*RI, *Not*I, *Bgl*II, *Nhe*I, *Xho*I, and *Hin*dIII) to ensure insertion of the target gene, the *tac-M* promoter and *rrnBT2* terminator to control gene transcription, the consensus RBS sequence GAAAGGA of *C. glutamicum*to initiate protein translation, the Sec-type *cgR_2070* signal sequence to mediate protein secretion, and the His-tag to simplify purification procedure of the recombinant proteins and provide convenience for their Western blotting analysis in *C. glutamicum*.

The *tac-M* promoter was obtained by the site-directed mutagenesis of the *tac* promoter in our previous work and has been proved to be strong (22, 24, 25). The *tac-M* promoter performs induced transcription in *E. coli*; in contrast, due to the lack of *lacI* gene encoding repressor protein on both the vector pAU5 and *C. glutamicum* chromosome, *tac-M* performs the constitutive transcription in *C. glutamicum*. The consensus RBS sequence GAAAGGA ensures an efficient initiation of the protein translation in *C. glutamicum* (26). The efficient Sec-type cgR_2070 signal sequence was identified by investigating the secretome of the *C. glutamicum* using two-dimensional gel electrophoresis (19). Here, applications of the *tac-M* promoter, the consensus RBS sequence of *C. glutamicum*, and the efficient Sec-type cgR_2070 signal sequence ensure efficient expression and secretion of the recombinant proteins in the *C. glutamicum*/pAU5 system. Using the *C. glutamicum*/pAU5 system, the high-efficient secretory production of a recombinant protein was achieved as shown below (**Fig. 2**).

4.2. Secretory Production of the AmyE in C. glutamicum

The exocellular α-amylase AmyE from *Bacillus subtilis* is natively secreted via Sec pathway (27). To test the applicability of pAU5, the signal peptide of AmyE was analyzed using online signal peptide prediction software SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP), and the signal-less *amyE* was used as the reporter gene and cloned into pAU5, generating the recombinant plasmid pAU5-*amyE*. The growth curves showed that growth rate and biomass of ATCC14067/pAU5-*amyE* were significantly lower than those of ATCC14067/pAU-

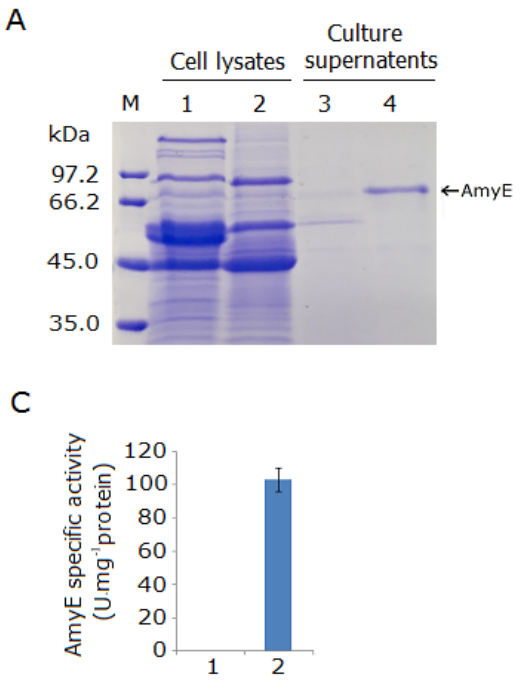

Figure 2. Applicability of pAU5 in *C. glutamicum*. (**A**) SDS-PAGE analysis of the culture medium of the different *C. glutamicum* strains. M, the protein molecular weight marker; 1 and 3, 14067/pAU5; 2 and 4, 14067/pAU5-*amyE*. (**B**) Western blotting of the AmyE localization in the culture of 14067/pAU5-*amyE*. M, prestained protein molecular weight marker; 1, cell lysate; 2, culture supernatant. (**C**) The specific activity of AmyE in the culture of 14067/pAU5-*amyE*. 1, cell homogenate supernatant; 2, culture supernatant. Data expressed as the mean ± SEM from the three independent experiments.

5 (**Fig. 3**), suggesting that the secretory production of the AmyE protein has negatively affected the cell growth of *C. glutamicum*.

To determine whether the AmyE sequence was expressed and the protein secreted into the *C. glutamicum* culture medium, the 28-hour-old cultures of 14067/pAU5-*amyE* and 14067/pAU5 were firstly analyzed by SDS-PAGE. The SDS-PAGE results showed that no specific protein band was observed in the cell lysate of the 14067/pAU5-*amyE*, while the specific protein band corresponding to the expected molecular mass of AmyE (69 kDa) was observed in the original culture supernatant of 14067/pAU5-*amyE* (Fig. 2A). The concentration of the total protein in the culture supernatant of ATCC14067/pAU5-*amyE* was 0.49 $mg.mL^{-1}$. SDS-PAGE showed that AmyE accounted for the vast majority of the total protein (Fig. 2A, lane 4). Therefore, the concentration of AmyE should be very close to that of the total protein, and it was a good yield for the secretory production of protein. The above results have suggested that the AmyE protein was efficiently expressed and secreted in 14067/pAU5-*amyE*.

To detect whether the expressed AmyE protein was partially retained in *C. glutamicum* cells, localization of the AmyE polypeptide in the cells and culture supernatant of the 28-hour-old culture of 14067/pAU5-*amyE* was analyzed by Western blotting using anti-His antibody. As shown in Fig. 2B, the AmyE polypeptide was detected in the original culture supernatant of the 14067/pAU5-*amyE*, not in the cell lysate of the 14067/pAU5-*amyE*, indicating that the expressed AmyE polypeptide did not accumulate in the *C. glutamicum* cells, and was completely secreted into the culture medium.

The cell homogenate supernatant and the culture supernatant of the 14067/pAU5-*amyE* were used to measure α-amylase activity, respectively. The α-amylase specific activity of the culture supernatant

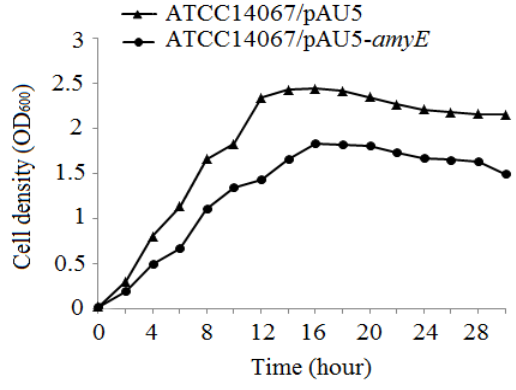

Figure 3. The growth curves of the ATCC14067/pAU5 and ATCC14067/pAU5-*amyE* strains. According to the same cell concentration at the initial stage of cultivation, the seed cultures of ATCC14067/pAU5 and ATCC14067/pAU5-*amyE* were inoculated into 100 mL LBHI media in 500-mL flasks, respectively. The cultivation was performed at 200 rpm and at 30 □, and cell growth was monitored by measuring OD_{600} of the culture.

was 103.24±7.14 U.mg^{-1} protein, while no α-amylase activity was detected in the cell homogenate supernatant (Fig. 2C) further confirming the efficient expression and secretion of the active-form of AmyE in the 14067/pAU5-*amyE*. These experimental results demonstrated high-level of secretory production of the AmyE *in C. glutamicum/*pAU5 system.

5. Discussion

Under the condition of flask cultivation, the reporter protein AmyE in the original culture supernatant can be detected by SDS-PAGE analysis, confirming that the *C. glutamicum*/pAU5 system is very efficient. In bacteria, the vast majority of the native extracytosolic proteins are translocated by the Sec pathway (28). Of the identified signal sequences in *C. glutamicum*, over 90% are Sec type (18). The high-level expressed AmyE did not accumulate in the cytosol, further confirming the high efficiency of the Sec machinery in *C. glutamicum*. In comparison to other available vectors, pAU5 possesses the following overall advantages: (i) the vector employs the strong promoter: *tac-M*, and the consensus RBS sequence of *C. glutamicum*, leading to an efficient expression of the inserted gene; (ii) the vector employs the efficient Sec-type cgR_2070 signal sequence, leading to an efficient secretion of the expressed protein via Sec pathway in *C. glutamicum*; and (iii) the promoter in pAU5 performs a constitutive expression of the target gene in *C. glutamicum*, which is more suitable for the large-scale industrial production of the recombinant proteins. These advantages make pAU5 an excellent Sec-dependent type gene expression vector in *C. glutamicum*.

For secretion of heterologous proteins in *C. glutamicum*, some proteins can be secreted in active forms through either Sec or Tat pathway only (10, 29), while the others can be actively secreted through both Sec and Tat pathways (18, 19). Generally, for a certain heterologous protein, there should be a more suitable secretion pathway between Sec and Tat pathway in *C. glutamicum*. Therefore, the presence of a different and efficient secretion-type gene expression vectors is valuable in *C. glutamicum*. Previously, we constructed an efficient Tat-dependent type expression vector pAU3 in *C. glutamicum* (22). In this study, the efficient Sec-dependent type expression vector pAU5 was constructed. The only difference between pAU5 and pAU3 is the signal sequence. pAU5 and pAU3 constitute a pair of attractive secretion-type sister vectors and would be highly useful for the secretory production of the heterologous proteins in the *C. glutamicum*.

6. Conclusion

In this study, a novel and efficient Sec-dependent type gene expression system *C. glutamicum*/pAU5 was developed. The vector pAU5 employs the strong promoter *tac-M* for controlling a constitutive transcription of the target gene, the consensus ribosome binding site (RBS) sequence of *C. glutamicum* to ensure protein translation, and the efficient Sec-type cgR_2070 signal sequence to mediate protein secretion in *C. glutamicum*. The secretion experiment showed that the reporter protein AmyE was efficiently expressed and secreted using *C. glutamicum*/pAU5 system. The *C. glutamicum/*pAU5 system is an efficient expression system for the secretory production of the heterologous proteins.

Acknowledgments

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