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Construction of a novel twin-arginine translocation (Tat)-dependent type expression vector for secretory production of heterologous proteins in *Corynebacterium glutamicum*

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ABSTRACT

Corynebacterium glutamicum is recognized as a favorable host for the secretory production of heterologous proteins. However, there are few secretion-type expression vectors available for protein production in *C. glutamicum*. In this study, we constructed a shuttle expression vector pAU3, which harbors the strong promoter *tac-M* for constitutive gene transcription, the consensus RBS sequence for protein translation, and the strong *cgR_0949* signal sequence for protein secretion via the Tat pathway in *C. glutamicum*. The applicability of pAU3 was confirmed by the highly efficient expression and secretion of the CAT protein in *C. glutamicum*. The vector pAU3 is highly useful for secretory production of heterologous proteins in *C. glutamicum*.

1. Introduction

Corynebacterium glutamicum has been widely used for the production of various small metabolites, including amino acids, vitamins, ethanol and certain organic acids, such as lactate and succinate (Becker and Wittmann, 2012). Recently, considerable research has been conducted on the application of *C. glutamicum* as a host for the secretory production of heterologous recombinant proteins (Yim et al., 2014). *C. glutamicum* is recognized as a favorable host for the secretory production of heterologous proteins because of the following important features. Firstly, *C. glutamicum* is a Gram-positive nonpathogenic organism and is generally regarded as safe (GRAS status). Secondly, *C. glutamicum* possesses the ability to secrete proteins into the medium (Suzuki et al., 2009), which would be convenient for protein purification. Thirdly, extracellular proteolytic activity is not detected (Yukawa et al., 2007), which may keep heterologous secretory proteins from being degraded. In addition, for *C. glutamicum*, the fermentation conditions for mass production methods are well-established (Yin et al., 2012), which serves to improve the productivity of target heterologous proteins.

Another key factor for achieving highly efficient secretory production of heterologous proteins is to employ a suitable gene expression vector harboring a strong promoter, RBS sequence and signal peptide sequence. In *C. glutamicum*, no strong natural promoters have been reported (Pátek and Nešvera, 2013). Briefly, the developed expression vectors in *C. glutamicum* use the *lac*, *tac* or *trp* promoters from *Escherichia coli*. These promoters are relatively weak in *C. glutamicum* (Srivastava and Deb, 2005; Xu et al., 2010a), which limits the expression level of recombinant proteins. By comparing different RBS sequences, sequence 5′-GAAAGGA-3′ is regarded as a consensus RBS sequence in *C. glutamicum* (Morbach et al., 2000). The general secretory (Sec) pathway and the twin-arginine translocation (Tat) pathway are two major protein secretory pathways of *C. glutamicum*. Generally, the Sec pathway catalyzes the export of unfolded proteins that are folded after secretion from the cytoplasm across the cytoplasmic membrane, whereas the Tat pathway serves the role of transporting folded proteins (Lee et al., 2006). A signal peptide in the N-terminal regions of preproteins, which is removed from the secreted protein by signal peptidases on the plasma membrane, is necessary to initiate export (Lee et al., 2006). A number of Sec-type and Tat-type signal peptide sequences have been identified in *C. glutamicum* (Suzuki et al., 2009; Watanabe et al., 2009). Using a transgenic approach, *C. glutamicum* has successfully been made to display amylase and lysine decarboxylase on its cell surface (Tateno et al., 2007a, 2007b, 2009a,b; Yao et al., 2009) and secrete amylase, protease, nuclease, subtilisin-like serine protease, transglutaminase, epidermal growth factor, antibody fragments, green fluorescence protein, isomaltodextranase, and sorbitol-xylitol oxidase into culture medium (Tateno et al., 2007a, 2007b; Date et al., 2003,

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Table 1
Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Sources
Strains		
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
C. glutamicum ATCC14067	Wild-type *C. glutamicum*	ATCC
Plasmids		
pBR322	A cloning vector in *E. coli*, Ap^{r}, Tc^{r}	Pharmacia
pET-28a	A expression vector in *E. coli*, Km^{r}	Novagen
pC2	A shuttle vector between *E. coli* and *C. glutamicum*, Km^{r}	Goyal et al. (1996)
pDXW-10-*cat*	A recombinant plasmid harboring the *cat* gene from Tn9	Xu et al. (2010b)
pAU1	pBR322 *rep* (pMB1) (900 bp) plus pET28a *aphI* (1051 bp), a mini vector in *E. coli*, Km^{r}	This work
pAU2	pAU1 (*Sac*I/*Apa*I) Ω *rep* (pBL1) (*Sac*I/*Apa*I; 2686 bp, pC2), a basic shuttle vector between *E. coli* and *C. glutamicum*, Km^{r}	This work
pAU3	pAU2 (*Nco*I/*Aat*II) Ω cassette A (*Nco*I/*Aat*II; 280 bp, synthesized fragment), a shuttle expression vector between *E. coli* and *C. glutamicum*, Km^{r}	This work
pAU4	pAU2 (*Nco*I/*Aat*II) Ω cassette B (*Nco*I/*Aat*II; 184 bp, synthesized fragment), a shuttle expression vector between *E. coli* and *C. glutamicum*, Km^{r}	This work
pAU3-*cat*	pAU3 (*Not*I/*Hin*dIII) Ω *cat* (*Not*I/*Hin*dIII; 660 bp, pDXW-10-*cat*), a recombinant pAU3 plasmid harboring the *cat* gene	This work
pAU4-*cat*	pAU4 (*Not*I/*Hin*dIII) Ω *cat* (*Not*I/*Hin*dIII; 660 bp, pDXW-10-*cat*), a recombinant pAU4 plasmid harboring the *cat* gene	This work

2004, 2006; Billman-Jacobe et al., 1995; Liebl et al., 1992; Meissner et al., 2007; Yim et al., 2014; Teramoto et al., 2011; Kikuchi et al., 2006; Scheele et al., 2012). In the above protein secretory production systems of *C. glutamicum*, target genes were fused with signal peptide sequences and promoters based on cloning vectors or ordinary expression vectors, which was neither convenient nor efficient. To the best of our knowledge, the only reported secretion-type expression vector pASJ100 in *C. glutamicum* was constructed by An et al. (2013), which employs the PorB promoter and the PorB signal peptide to express and secrete recombinant proteins, respectively. The PorB signal peptide mediates the Sec-dependent secretion pathway in the *C. glutamicum*/pASJ100 system. The post-translocational folding in the cell wall environment sometimes occurs only inefficiently or not at all, leading to low or no activity of proteins secreted via the Sec pathway in *C. glutamicum*. Certain proteins can be heterologously secreted in active forms via the Tat pathway but not via the Sec pathway (Meissner et al., 2007; Kikuchi et al., 2003). Thus, it is obvious that the Tat pathway is of importance for production of some heterologous proteins; therefore, it is valuable to construct a novel Tat-dependent secretion-type expression vector in *C. glutamicum*.

Previously, by rational mutagenesis to the extended −10 region of the *tac* promoter, we obtained a strong promoter *tac-M* used for highly efficient gene transcription in *C. glutamicum* (Xu et al., 2010b). The putative consensus RBS sequence (GAAAGGA) was demonstrated to be strong in our previous work (Xu et al., 2011). Among the identified Tat-type signal peptides from *C. glutamicum*, CgR_0949 is the strongest signal sequence that mediates protein secretion (Suzuki et al., 2009; Watanabe et al., 2009). In this study, we developed a novel secretion-type gene expression vector pAU3 for the efficient production of heterologous proteins in *C. glutamicum*. pAU3 harbors the promoter *tac-M* for gene transcription, the consensus RBS sequence (GAAAGGA) for protein translation, and the *cgR_0949* signal sequence for protein export via the Tat pathway. The applicability of pAU3 was confirmed by the highly efficient expression and secretion of the chloramphenicol acetyltransferase (CAT) in *C. glutamicum*. pAU3 is an attractive vector for expression and secretion of heterologous proteins in *C. glutamicum*.

2. Materials and methods

2.1. Strains, plasmids, and growth conditions

All bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* was grown in LB media (Sambrook et al., 1989) at 200 rpm and 37 °C, and *C. glutamicum* was grown in LBHI media (Xu et al., 2010a) at 200 rpm and 30 °C. The concentration of kanamycin used was 50 μg/mL for *E. coli* and 30 μg/mL for *C. glutamicum*.

2.2. DNA preparation and PCR techniques

Restriction enzymes and T4 DNA ligase were purchased from Sangon (Shanghai, China). Plasmid DNA was prepared using the Plasmid Minipreps Purification System B (BioDev-Tech, Beijing, China). The protein molecular weight marker was purchased from TaKaRa (Dalian, China). DNA was separated on agarose gels and purified with the EZ-10 Spin Column DNA Gel Extraction Kit (Bio Basic Inc., Markham, Canada). The DNA sequencing was performed by Sangon (Shanghai, China).

The PCR experiments were performed by using a Mastercycler (Eppendorf, Hamburg, Germany). The amplification of DNA was conducted using PrimeSTAR™ HS DNA Polymerase according to the manufacturer's protocol (TaKaRa, Dalian, China). The primers were synthesized by Sangon, and their sequences are listed in Table 2. Generally, The PCR reaction mixture was 50 μl, which contained 10 μl 5 × PrimerSTAR buffer (Mg2 + plus), 4 μl dNTP mixture (2.5 mM each), 1 μl plasmid template (100 ng/μl), 1 μl forward primer (20 μM), 1 μl reverse primer (20 μM), and 0.5 μl PrimeSTAR™ HS DNA Polymerase. Each reaction mixture was first

Table 2
Primers used for PCR experiments in this study. The restriction sites are underlined.

Names	Sequences	Restriction site
oriE-F	ATATGGTACCATTCAACACTTAAGTCACTCAAAG GCGGTAATACGGTTA	*Kpn*I
oriE-R	AGCTGAGCTCGCCTCACTGATT AAGCATTGGTAA	*Sac*I
kan-F	AATCGAGCTCAACTTTCTGGGCCCCTTTGATCTTTT CTACGGGGTCTGACG	*Sac*I, *Apa*I
kan-R	AGCAGGTACCCAAAAAGGCCATCCGTCAGGATGG CCTTTCAGGTGGCACTTTTCGGGGAA	*Kpn*I
oriC-F	AGTAGAGCTCCCATGGTAATCCTAAAGACGTCCATT GTCAACAACAAGACCCATCA	*Sac*I, *Nco*I, *Aat*II
oriC-R	ATGAGGGCCCGTCGACAATTCTTACACTGCAGGTCT ACGTCTGATGCTTTGAATCG	*Apa*I
cat-F	ATGAGCGGCCGCGAGAAAAAAATCACTGGATATACCACC	*Not*I
cat-R	ATCTAAGCTTCGCCCCGCCCTGCCACTC	*Hin*dIII