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Short Communication

Construction of pOGOduet – An inducible, bicistronic vector for synthesis of recombinant proteins in *Corynebacterium glutamicum*

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ABSTRACT

The Gram-positive *Corynebacterium glutamicum* is widely known for its application in the industrial production of amino acids and as a non-pathogenic model organism for cell wall biosynthesis in the group of CMN bacteria. For biotechnological and physiological studies often co-expression of recombinant genes is required, however for *C. glutamicum* no vector for the independent co-expression of two genes was described. We here created the novel expression vector pOGOduet for *C. glutamicum*, which carries the ColE1 replicon of *E. coli* and the pBL1 replicon of *C. glutamicum* and two independently inducible promoters Ptac and Ptet each followed by unique multiple cloning sites. Functionality of pOGOduet is tested by coexpression of genes for the fluorescent proteins eCFP and mVenus; fluorescence of the reporters varies in dependence of the inducer concentrations present in the culture broth. These experiments demonstrate that the vector pOGOduet fulfills the task for individually inducible expression of two genes of interest in *C. glutamicum*.

1. Introduction

Vector based expression of genes is a powerful tool in microbiology for the investigation of various questions in the fields of physiology, biotechnology and biochemistry (Tolia and Joshua-Tor, 2006). Often co-expression of two or more genes is required e.g. to investigate protein-protein interactions (Karimova et al., 1998), to support correct folding of proteins (Lobstein et al., 2012; Shriver-Lake et al., 2017), to accomplish protein modifications (Nguyen et al., 2011), to produce protein complexes (Kerrigan et al., 2011) or to simultaneously express genes for several metabolic pathways (Matano et al., 2014). For these purposes co-expression is often conducted by the use of two or more plasmids, each carrying one gene of interest, a different selection marker, and different compatible replicons (Akiba and Tsumoto, 2016; Rosano and Ceccarelli, 2014; Sorensen and Mortensen, 2005). Beside the use of multiple vectors for co-expression also several genes can be inserted into the same vector (Sorensen and Mortensen, 2005). In these vectors genes can be transcribed either from a single promoter as long polycistronic mRNA or from individual promoters, which leads to several-fold higher expression compared to polycistronic transcription (Rosano and Ceccarelli, 2014). Transcription from individual promoters can be obtained by the use of dual-expression vectors like pETDuet-1 and pACYCDuet-1 for *Escherichia coli* or pDUO for *Pseudomonas fluorescens* (Nakata, 2017; Tolia and Joshua-Tor, 2006).

For the Gram-positive *Corynebacterium glutamicum*, which is used for industrial-scale amino acid production (Becker et al., 2016; Eggeling and Bott, 2015) and serves as model organism for related pathogens like *Mycobacterium tuberculosis* (Schubert et al., 2017; Varela et al., 2012), hitherto a system for dual expression of genes has not been available. So far, vector based expression in *C. glutamicum* was accomplished by the use of several expression plasmids with single insertion sites under the control of constitutive promoters (e.g. in pBB1 (Krause et al., 2010)), isopropylβ-D-thiogalactopyranoside (IPTG) inducible promoters (e.g. in pEKEx2 and pVWEx1 (Eikmanns et al., 1991)), or anhydrotetracycline (aTc) inducible promoters (e.g. in pCLTON1 (Lausberg et al., 2012)). Co-expression of genes was achieved in *C. glutamicum* hitherto by the use of compatible plasmids such as pEKEx3 and pVWEx1 (Matano et al., 2014) or pZ8-1 and Corynebrick vectors (Kang et al., 2014), polycistronic operons (Henke et al., 2016), and combinations thereof (Jo et al., 2017; Perez-Garcia et al., 2017).

In this work we created a novel expression vector for *C. glutamicum* consisting of the two independently inducible promoters Ptac and Ptet each followed by unique multiple cloning sites, which enable the specific introduction of target genes. The creation of such a vector bypasses the two vector solutions used hitherto that often came along with disadvantages due to the need of two resistance markers and different copy numbers of the plasmids (Witthoff et al., 2015). Our solution to these problems is the new vector pOGOduet, which carries induction

Abbreviations: CMN, Corynebacterium-Mycobacterium-Nocardia; WT, wild-type; IPTG, Isopropyl β-D-1-thiogalactopyranoside; aTC, Anhydrotetracycline; MCS, Multiple Cloning Site

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Table 1
Strains and plasmids used in this study.

Strains and plasmids	Relevant properties and applications	Sources and references
Strains		
E. coli DH5a	F⁻ *thi-1 endA1 hsdR17* (r ⁻ m ⁻) *sup*E44 Δ*lac*U169 (φ80*lacZ*ΔM15) *recA1 gyr*A96 *rel*A1 F⁻ λ⁻ *ilvG rfb*-50 *rph*-1	(Hanahan, 1983)
C. glutamicum ATCC13032	Wild type	American Type Culture Collection
Plasmids		
pEKEx2	Ptac, *lacI*^{q}, Km^r	(Eikmanns et al., 1991)
pCLTON1	Ptet, *tetR*, Km^r	(Lausberg et al., 2012)
pOGOstart	pCLTON1 carrying the pEKEx2 derived fragment with the *ptac* promoter and *lacI*^{q}	This work
pEKEx2_mVenus	pEKEx2 carrying mVenus	This work
pOGOduet	*C. glutamicum* expression vector with the pCLTON1 derived *ptet* promoter under the control of TetR and the pEKEx2 derived *ptac* promoter under the control of LacI^q	This work
pOGOduet_mVenus	pOGOduet carrying the genes for the red fluorescent protein mVenus	This work
pOGOduet_*mCherry*	pOGOduet carrying the gene for the red fluorescent protein mCherry	This work
pOGOduet_*mVenus_eCFP*	pOGOduet carrying the genes for the red fluorescent protein mVenus and the cyan fluorescent protein eCFP	This work
pRSETb_AT1.03	template for the amplification of mVenus and eCFP via PCR	(Imamura et al., 2009)
pK19msB-Div-mCherry	template for the amplification of mCherry via PCR	(Donovan et al., 2012)

Table 2
Oligonucleotides used in this study.

Oligonucleotid	Sequence (3′–′5)	Restriction sites[a]
GIBS_lacI-ptac-MCS-fw	AACCTGTCGTGCCAGAGTCGAAAGACTGGGCCTTTTCAAGCCTTCGTCACTGGTC	–
GIBS_ptac-MCS_rev	CCGATTCATTAATGCAGTTATAA**CCATGGATTTAAATACCTGGTGCGGCCGC**CATGCAAGCTTGGCGTAATC	*Nhe*I, *Nco*I, *Swa*I, *Sex*AI, *Not*I
F1-term_fw	GG**CCATGG**CGGGTACC**GCTAGC**AGGAGTATGG	*Nco*I, *Nhe*I
F1-term_rev	GG**TTATAA**CAAAAGAGTTTGTAGAAACGC	*Psi*I
mCherry_fw	**ACCTGGT**ATGGTGAGCAAGGGCGAGG	*Sex*AI
mCherry_ rev	**GCGGCCGC**CTTGTACAGCTCGTCC	*Not*I
eCFP_fw	GG**GTCGAC**AGGAGACTAGATGGTGAGCAAGGGCGAG	*Sal*I
eCFP_rev	GG**GAGCTC**CTTGTACAGCTCGTCCATG	*Sac*I
F1_fw	**GGTACCGCTAGC**AGGAGTATGGTGAGCAAGGGCG	*Kpn*I, *Nhe*I,
F1_rev	C**GAATTC**GCTAGCTCACTTGTACAGCTCGTCC	*Eco*RI

[a] Restriction sites are indicated in bold.

systems based on aTc and IPTG. The vector holds the ColE1 replicon of *E. coli* and the pBL1 replicon of *C. glutamicum* and fulfills the need for individually inducible expression of two genes of interest in *C. glutamicum*.

2. Material and methods

2.1. Bacterial strains, plasmids, and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* and all pre-cultures of *C. glutamicum* were grown aerobically in baffled Erlenmeyer flasks containing 50 ml 2 × TY complex medium (Sambrook and Russell, 2001) on a rotary shaker at 130 rpm at 37 °C and 30 °C, respectively. For the main cultures of *C. glutamicum*, cells of an overnight pre-culture were washed twice with 0.9% (w/v) NaCl and then inoculated into Brain Heart Infusion medium (37 g/L). When appropriate, kanamycin (50 µg/ml), IPTG (0 to 1 mM) and/or aTc (0–500 ng ml^{-1}) were added to the media. Growth of *E. coli* and of *C. glutamicum* was followed by measuring the optical density at 600 nm in a Ultrospec 2100 pro spectrophotometer (GE Healthcare life sciences GmbH, Freiburg, Germany).

2.2. DNA manipulation and plasmid construction

Standard procedures were employed for plasmid isolation, cloning and transformation of *E. coli* DH5α, as well as for electrophoresis (Sambrook and Russell, 2001). Transformation of *C. glutamicum* was performed by electroporation using the methods of (Tauch et al., 2002). The recombinant strains were selected on 2 × TY-agar plates containing kanamycin (50 µg/ml). All molecular biology enzymes used were purchased from Thermo Scientific (Waltham MA, USA) and used according to the instructions of the manufacturer. PCR experiments were performed in a C100 thermocycler (Bio-Rad Laboratories, Munich, Germany). Deoxynucleotide triphosphates were obtained from Bio-Budget (Krefeld, Germany) and oligonucleotides (primers) from biomers (Ulm, Germany), oligonucleotide sequences are provided in Table 2. Conditions of the PCR were chosen according to primer composition and fragment length. PCR products were separated in agarose gels and purified using the Nucleo-Spin DNA extraction kit from Macherey & Nagel (Düren, Germany).

2.3. Fluorescence spectroscopy

Fluorescence measurements were performed in a TECAN infinite M200 plate reader (Männedorf, Switzerland) using black 96 well plates (Sarstedt, Nümbrecht, Germany). Cells were harvested by centrifugation (4000 rpm, 15 min, 4 °C) and suspended afterwards in PBS (137 mM NaCl, 10 mM Na_2HPO_4, 1.8 mM NaH_2PO_4). Fluorescence emission was recorded at 480 nm for eCFP, 530 nm for mVenus and 610 nm for mCherry. Excitation was performed at 430 nm for eCFP, 480 nm for mVenus and 570 nm for mCherry, respectively.

3. Results

3.1. Construction of pOGOduet

To date there is no duet-expression vector available for *C. glutamicum*, while several of these vectors exist for *E. coli*. For the *C. glutamicum* single vector duet-expression system we wanted to combine the strong expression and LacI dependent-control brought about by the Ptac