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Short Communication

A tetracycline inducible expression vector for *Corynebacterium glutamicum* allowing tightly regulable gene expression

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ABSTRACT

Here we report on the construction of a tetracycline inducible expression vector that allows a tightly regulable gene expression in *Corynebacterium glutamicum* which is used in industry for production of small molecules such as amino acids. Using the green fluorescent protein (GFP) as a reporter protein we show that this vector, named pCLTON1, is characterized by tight repression under non-induced conditions as compared to a conventional IPTG inducible expression vector, and that it allows gradual GFP synthesis upon gradual increase of anhydrotetracycline addition.

1. Introduction

Corynebacterium glutamicum is a Gram-positive soil bacterium that has been used for industrial amino acid production for decades (Yamada et al., 1972; Aida et al., 1986; Leuchtenberger, 1996; Ikeda, 2003). Besides that it has become a platform organism for the production of different chemicals and fuels (Becker and Wittmann, 2011; see also references therein), as well as for the secretory production of heterologous proteins (Liebl et al., 1992; Kikuchi et al., 2006; Meissner et al., 2007). In addition, it serves as a model organism for the investigation of cell envelope biogenesis and composition in *Actinobacteria* (Hoffmann et al., 2008; Bansal-Mutalik and Nikaido, 2011; Marchand et al., 2011).

Both for basic and applied research it is essential to have genetic tools which allow controlled expression of homologous and heterologous genes. Accordingly, a variety of such vectors have been developed for *C. glutamicum*. Most of these are IPTG inducible (Eikmanns et al., 1991; Jakoby et al., 1999; Kirchner and Tauch, 2003), but also heat inducible (Tsuchiya and Morinaga, 1988; Park et al., 2008) and carbon source inducible (Okibe et al., 2010) expression vectors have been constructed. A great disadvantage of the so far available regulable expression vectors is the leaky expression of the target gene in the absence of an inducer (Pátek et al., 2003 and our own unpublished observations).

An expression system which is known to allow tightly regulated gene expression is the tetracycline inducible Tet repressor (TetR) based expression system derived from the *Escherichia coli* Transposon Tn*10* (Hillen and Berens; 1994). This system has been employed successfully in a variety of Gram-positive bacteria (Geissendörfer and Hillen, 1990; Bateman et al., 2001; Ehrt et al., 2005; Fagan and Fairweather, 2011). In fact, a previous report has shown that a TetR based expression system allows controlled expression of a chromosomally encoded gene in *C. glutamicum* (Radmacher et al., 2005).

To also allow the use of the TetR-dependent, tetracycline inducible expression system for regulated expression of plasmid encoded genes, we have constructed a tetracycline inducible expression vector for *C. glutamicum*. The vector is based on the ColE1 replicon of *E. coli*, the pBL1 replicon of *C.*

Abbreviations: IPTG, isopropyl-β-D-thiogalactopyranoside; ATc, anhydrotetracycline.

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glutamicum, it carries the *tetR* gene under the control of a strong constitutive *C. glutamicum* promoter (Radmacher et al., 2005) and a TetR controllable promoter derived from *Bacillus subtilis* (Kamionka et al., 2005). It could be shown that this newly constructed expression vector allows a gradual induction of a reporter protein and shows a tightly repressed basal expression under non-induced conditions.

2. Material and methods

2.1. Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* was grown at 37 °C in Luria Bertani medium (Miller, 1972). *C. glutamicum* was grown at 30 °C in BHI medium (Difco). If required, isopropyl-β-D-thiogalactopyranoside (IPTG) was used at a concentration of 1 mM, anhydrotetracycline (ATc) was used at concentrations ranging from 10 to 250 ng/ml. Antibiotic supplements were at the following concentrations: kanamycin, 50 mg/l (*E. coli*); 25 mg/l (*C. glutamicum*). *E. coli* was transformed with plasmid DNA by the $CaCl_2$ method (Cohen et al., 1972). *C. glutamicum* was transformed with plasmid DNA by electroporation as described previously (Reyes and Eggeling, 2005).

2.2. DNA manipulation and plasmid construction

All DNA manipulations followed standard procedures (Sambrook et al., 1989). The following oligonucleotides were used as PCR primers (restriction enzyme recognition sites are underlined):

Primer1 (AACTGCAGAAGGAGATATAGATATGAGTAAAG GAGAAGAACTTTTCACTG),
Primer 2 (CGGAATTCTTATTTGTAGAGCTCATCCATGCC),
Primer 3 (AGAGAGCGTACGCGTTTGTTGAACTAATGG GTGCTTTAG),
Primer 4 (CTCTCTCTGCAGGTGTATCAACAAGCTGGGGA TCTTAAG).

Plasmid pEKEx2-GFP was constructed by amplifying the *gfp* gene via PCR using primers 1 and 2 and vector pCGTorA-GFP (Meissner et al., 2007) as the template. The PCR product was digested with *Pst*I and *Eco*RI and ligated into the *Pst*I/*Eco*RI digested pEKEx2 vector. pTc105-GFP was constructed by replacing the $laqI^q$ and *ptac* regions from pEKEx2-GFP by a TetR controllable promoter from pWH105 (Kamionka et al., 2005). The promoter was amplified via PCR using primers 3 and 4 and pWH105 as the template, digested with *Bsi*WI and *Pst*I and ligated with the 6.9 kb fragment of the *Bsi*WI/*Pst*I digested pEKEx2-GFP. Plasmid pCLTON1-GFP was constructed by incorporating the *tetR* gene under control of the strong *C. glutamicum* promoter of the glyceraldehyde-3-phosphate dehydrogenase (*pgap*) from the vector pJC1-*pgap-tetR* (Radmacher et al., 2005) into pTc105-GFP vector. Therefore, the 1.4 kb *Bam*HI/*Dra*I fragment from pJC1-*pgap-tetR* was filled in using the Klenow fragment of DNA polymerase and ligated into the pTc105-GFP vector which had been digested with *Bsi*WI and was also filled in using the Klenow fragment of DNA polymerase. After ligation and transformation of *E. coli*, different clones where checked by an asymmetric digestion with *Xba*I. *Xba*I cuts once at the beginning of the *tetR* gene and once elsewhere in the vector. Thus a clone could be selected in which the *pgap* promoter was oriented in opposite direction to the pWH105 promoter. The pCLTON1 empty vector was constructed by replacing the 3.3 kb *Pst*I/*Pvu*I fragment encompassing the *gfp* gene with the 2.6 kb *Pst*I/*Pvu*I fragment of pEKEx2, thus restoring the pEKEx2 multiple cloning site.

2.3. Preparation of whole cell extracts

For protein analysis, *C. glutamicum* was grown over night in BHI medium. The overnight culture was washed

Table 1
Bacterial strains and plasmids.

Bacterial strains and plasmids	Relevant properties[a]	Source
Bacterial strains		
E. coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB* $lacI^q$ *ZΔM15* Tn*10* (Tet^r)]	Stratagene (Heidelberg)
C. glutamicum ATCC13032	Wild-type	Abe et al. (1967)
Plasmids		
pEKEx2	*ptac*, $lacI^q$, Km^r	Eikmanns et al. (1991)
pWH105	pWH102 derivative with second *tetO* cloned in *Bam*HI site; Ap^r, Cm^r	Kamionka et al. (2005)
pJC1-*pgap-tetR*	pJC1 containing the *tetR* gene under control of *pgap* promoter from *C. glutamicum*; Km^r	Radmacher et al. (2005)
pCGTorA-GFP	pEKEx2 containing a *torA-gfp* hybrid gene	Meissner et al. (2007)
pEKEx2-GFP	pEKEx2 containing the *gfp* gene	This study
pTc105-GFP	*ptac* and $lacI^q$ deficient variant of pEKEx2-GFP with the *B. subtilis* derived *ptet* promoter from pWH105	This study
pCLTON1-GFP	pTc105-GFP with *pgap-tetR* from pJC1-*pgap-tetR*	This study
pCLTON1	*C. gluatmicum* expression vector with the *B. subtilis* derived *ptet* promoter from pWH105 and the *tetR* gene under control of *C. glutamicum pgap* promoter from pJC1-*pgap-tetR*; Km^r	This study

[a] Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Cm^r, chloramphenicol resistance; Tet^r, tetracycline resistance.