Plasmid 67 (2012) 44–52

Contents lists available at SciVerse ScienceDirect

Plasmid

journal homepage: www.elsevier.com/locate/yplas

Construction of a novel *sacB*-based system for marker-free gene deletion in *Corynebacterium glutamicum*

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ARTICLE INFO

Article history:
Received 15 August 2011
Accepted 2 November 2011
Available online 12 November 2011
Communicated by C. Jeffery Smith

Keywords:
Marker-free vector
sacB
Gene deletion
pDXW-3
Corynebacterium glutamicum

ABSTRACT

Bacillus subtilis sacB gene with its 463 bp upstream region including its native promoter has been used for marker-free gene deletion in *Corynebacterium glutamicum*, but the role of this upstream region is not clear. In this study, it was demonstrated that the upstream region of *sacB* failed to efficiently promote its expression in *C. glutamicum*, and the native promoter of *sacB* is weak in *C. glutamicum*. The expression level of *sacB* under its native promoter in *C. glutamicum* is not high enough for cells to confer sucrose sensitivity. Therefore, a new promoter P*lacM* and a novel vector pDXW-3 were constructed. P*lacM* is 18 times stronger than the native promoter of *sacB* in *C. glutamicum*. The pDXW-3 contains *B. subtilis sacB* with the P*lacM* fused at the 5′-end, a general *Escherichia coli* replicon *oriE* for easy cloning, a kanamycin resistance marker for selection, and a multiple unique restriction sites for *Xho*I, *Not*I, *Eag*I, *Sal*I, *Sac*I, *Bam*HI, and *Nhe*I, respectively. By using pDXW-3, the *aceE* gene in the chromosome of *C. glutamicum* was deleted. This *sacB*-based system should facilitate gene disruption and allelic exchange by homologous recombination in many bacteria.

1. Introduction

After *Corynebacterium glutamicum* was first isolated to produce L-glutamate, it has been widely developed for production of different amino acids (Kinoshita et al., 2004). In recent years, information on the physiology, biochemistry and genetics of *C. glutamicum* has accumulated, and metabolic engineering in *C. glutamicum* has been initiated (Burkovski, 2008; Eggeling and Bott, 2005). Several expression vectors have been developed for gene expression in *C. glutamicum* (Xu et al., 2010a,b). The chromosomal gene deletion in *C. glutamicum*, however, is still difficult due to lack of proper methods.

The classical method for chromosomal gene deletion in bacteria is to replace the gene with an antibiotic resistance marker by homogenous recombination, but it is not efficient in *C. glutamicum* (Sanchez et al., 2007; Schwarzer and Puhler, 1991; Vertes et al., 1993). This classical method needs a number of selectable markers when it is required to introduce several independent mutations into a single strain. In addition, the antibiotic resistance marker inserted in the chromosome could pose a biohazard, considering most products produced by *C. glutamicum* are used for food or feed.

To solve this problem, the vector containing *Bacillus subtilis sacB* coupled with an antibiotic resistance marker has been developed (Schafer et al., 1994). The *sacB* gene encodes levansucrase, a 50-kDa enzyme which can catalyze sucrose hydrolysis followed by levan synthesis. When the *sacB* gene was expressed in Gram-negative bacteria, such as *Escherichia coli*, *Agrobacterium tumefaciens*, and *Rhizobium meliloti*, the production of levansucrase is lethal in the presence of sucrose (Gay et al., 1985), probably because

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doi:10.1016/j.plasmid.2011.11.001

the periplasm is encumbered by the accumulation of levans. Gram-positive bacteria lack the periplasm, and therefore levansucrase usually is not lethal to them. However, the expression of *sacB* in Gram-positive *C. glutamicum*, the most used production strain for amino acids, confers sucrose sensitivity (Holatko et al., 2009; Jager et al., 1992; Mishra et al., 2008; Schafer et al., 1994); this might be due to the particular organization of cell walls in these bacteria, where mycolic acids form an outer membrane-like structure and therefore a periplasm-like space (Jager et al., 1992).

The *sacB*-based vectors currently used for gene deletions in corynebacteria were derived from the mobilizable pK18*mobsacB* (Burkovski, 2008; Eggeling and Bott, 2005; Holatko et al., 2009). This vector contains a kanamycin resistant marker, a *lacZ* fragment, a genetically modified *sacB* gene with its 463 bp upstream region including its native promoter, and the RP4 *mob* DNA region for sufficient mobilization (van der Geize et al., 2008). When the pK18*mobsacB* system was used for the *hom-thrB* gene deletion in *C. glutamicum*, only 55% of the colonies grown on sucrose were kanamycin sensitive (Schafer et al., 1994), suggesting this *sacB*-based vector needs to be modified.

In this study, a novel vector pDXW-3 for marker-free gene deletion in *C. glutamicum* has been constructed. It contains a general *E. coli* replicon for easy cloning, a set of restriction sites, an antibiotic marker, and a *sacB* gene expressed from a new promoter P*lacM*. The pDXW-3 was used for deleting the *aceE* gene in the chromosome of *C. glutamicum*. Compared to pK18mobsacB, the vector pDXW-3 is smaller in size and contains a new promoter to replace the native 463 bp upstream region of *sacB* which failed to efficiently promote expression in *C. glutamicum*. Most importantly, when the pDXW-3 system used for the *aceE* gene deletion in *C. glutamicum*, 100% of the colonies grown on sucrose were kanamycin sensitive, suggesting this *sacB*-based vector is more efficient than pK18*mobsacB*. The vector pDXW-3 should facilitate gene disruption and allelic exchange by homologous recombination in many bacteria.

Table 1
Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Source
Strains		
E. coli JM109	Wild type *E. coli*	Stratagene
C. glutamicum ATCC13032	Wild type *C. glutamicum*	ATCC
B. subtilis subsp. *subtilis* 168	Wild type *B. subtilis*	ATCC
JM109/pDXW-2	*E. coli* JM109 harboring pDXW-2	This work
JM109/pDXW-2-Ups-sacB	*E. coli* JM109 harboring pDXW-2-Ups-sacB	This work
ATCC13032/pDXW-2	ATCC13032 harboring pDXW-2	This work
ATCC13032/pDXW-2-Ups-sacB	ATCC13032 harboring pDXW-2-Ups-sacB	This work
ATCC13032/pDXW-2-PF104-*sacB*	ATCC13032 harboring pDXW-2-PF104-*sacB*	This work
ATCC13032/pDXW-2-Pneo-*sacB*	ATCC13032 harboring pDXW-2-Pneo-*sacB*	This work
ATCC13032/pDXW-2-P*lacM-sacB*	ATCC13032 harboring pDXW-2-P*lacM-sacB*	This work
YTW-1	ATCC13032, *ΔaceE*	
Plasmids		
pGEM-T	Cloning vector in *E. coli*, Amp^r	Promega
pDXW-1	Derived from pET-28a by deleting *lacI* and T7 promoter, Km^r	Xu et al. (2011)
pDXW-2	Derived from pDXW-1 by inserting a replication origin from pC2, Km^r	(Xu et al. (2011)
pDXW-2-Ups-sacB	pDXW-2 harboring the *sacB* gene with its native promoter, Km^r	This work
pDXW-2-PF104-*sacB*	pDXW-2 harboring the *sacB* gene with a PF104 promoter, Km^r	This work
pDXW-2-Pneo-*sacB*	pDXW-2 harboring the *sacB* gene with a Pneo promoter, Km^r	This work
pDXW-2-P*lacM-sacB*	pDXW-2 harboring pDXW-2 harboring the *sacB* gene with a P*lacM*, Km^r	This work
pDXW-3	pDXW-1 harboring the *sacB* gene with a P*lacM*, Km^r	This work
pDXW-3-LR*aceE*	pDXW-3 harboring the *aceE* gene flanking regions, Km^r	This work

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. Both *E. coli* and *B. subtilis* were grown in LB media at 37 °C. *C. glutamicum* was grown at 30 °C in LBG media (LB media supplemented with 5 g/L glucose) or the modified CGXII minimal media (Keilhauer et al., 1993) in which 0.03 mg/L of protocatechuic acid was replaced by 1 mg/L citrate. Modified Epo media (van der Rest et al., 1999) are used for the preparation of competent cells of *C. glutamicum*, it contains 10 g/L trypton, 5 g/L yeast extract, 10 g/L NaCl, 30 g/L glycine and 0.1% Tween 80. LBHIS media (van der Rest et al., 1999) was used for the transformation of *C. glutamicum*, it contains 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. When necessary, 30 μg/mL kanamycin was supplemented in media.

2.2. DNA preparation and PCR techniques

Restriction enzymes, calf intestine alkaline phosphatase (CIAP), T4 DNA ligase, and 1 kb DNA Ladder were purchased from Sangon (Shanghai, China). PrimeSTAR™ HS DNA Polymerase was purchased from TaKaRa (Dalian, China). The pGEM-T Easy Vector System was purchased from Promega (Madison, USA). Plasmid Minipreps Purification System B used for isolating the plasmid DNA from *E. coli* or *C. glutamicum* was from BioDev-Tech (Beijing, China). TIAnamp Bacteria DNA Kit used for isolating the genomic DNA from *B. subtilis* or *C. glutamicum* 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