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ORIGINAL PAPER

Construction and characterization of a novel maltose inducible expression vector in *Bacillus subtilis*

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Abstract A maltose-inducible expression vector in *Bacillus subtilis* has been developed and characterized. The vector permitted β-galactosidase expression at a high level (maximum activity, 8.16 U/ml) when induced and its expression was markedly repressed by glucose. Using this vector, we successfully expressed the other two genes, *bioA* and *vgb*. This thus provided a potential expression system for cloned genes in *B. subtilis.*

Keywords *Bacillus subtilis* · Expression vector · *glv* promoter · Thermostable β-galactosidase

Introduction

Bacillus subtilis is an important organism both from the basic and applied research perspectives (Bron 1990). Because it is amongst the best-characterized prokaryotic organism at the genetic, biochemical and physiological level, *B.subtilis* has become the second most frequently used model, next to *Escherichia coli*, for research on bacilli (Bolhuis et al. 1996). *B. subtilis* is of interest for producing growth factors, antigens and proteins in the fermentation industry because it is secretive and non-pathogenic, and because it does not produce an endotoxin (Bron et al. 1998; Tjalsma et al. 2000). It has received considerable attention from those wishing to develop genetic tools for efficient gene expression. Several promoters and regulatory genes have been used to develop regulated expression systems for *B. subtilis* (Hartl et al. 2001; Kim et al. 1996). However, to avoid genetic instability of the plasmid-based expression vector in *B. subtilis* (Eijnenburg et al. 1987; Eima et al. 1996), these systems are often integrated into chromosomal DNA, which limits high-level expression. In addition, application of these systems to industry is relatively limited because of the cost of inducers.

The maltose utilization operon (*glv* operon) in *B.subtilis* was well-characterized, and the promoter of *glv* operon (P_{glv}) is positively regulated through maltose and the regulator GlvR encoded by *glvR* of *glv* operon (Thompson et al. 1998; Yamamoto et al. 2001).

We have recently developed and characterized a plasmid-based maltose-inducible expression

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vector in *B. subtilis* that takes advantage of P_{glv} promoter, and have demonstrated the effectiveness of the system through the over-production of homologous and heterologous proteins in *B.subtilis*.

Materials and methods

Bacterial strains, plasmids and culture conditions

Bacillus subtilis 1A747 (prototroph), plasmids pGDV1 and pDL were generously provided by Bacillus Genetic Stock Centre of the Ohio State University (Columbus, OH, USA).The plasmid pUC8:16 was kindly supplied by Dr. Webster (Wei et al. 2000). The primers used in this study are listed in Table 1. Bacteria were cultured in Luria–Bertani (LB) medium at 37°C. When specified, maltose and glucose were supplemented. The antibiotics ampicillin (100 μg/ml) and chloramphenicol (5 μg/ml) were added as required.

Transformation of *E. coli* and *B. subtilis*

Transformation of *E. coli* cells was performed as described by Sambrook et al. (1989). *B. subtilis* cells were transformed by electroporation (Bron 1990).

Construction of the P_{glv}-inframe promoter

The P_{glv} promoter and the first 57 nucleotides of the *glvA* gene were amplified as a single product from *B. subtilis* 1A747 chromosomal DNA, using the primers P_{glv}-up and P_{glv}-down. After digestion with *Apa*I and *Bam*HI, the 360 bp PCR-amplified fragment was cloned into the corresponding site of pBluskm, resulting in pGJ213. To generate a ribosome binding site sequence, a pair of synthetic oligonucleotides (inSD-1 and inSD-2) was annealed, yielding a 40 bp INSD fragment. The fragment overhanged the cohesive end of *Bgl*II at the 5′ end and *Sac*I at the 3′ end, the location of the additional stop codons and an *Eco*RI close to the *Sac*I site. The INSD was ligated into the pGJ213 digested with *Bam*HI and *Sac*I, resulting in pGJ240.

Construction of the expression vector

A 2500 bp DNA fragment was amplified using pGDV1 (Bron et al. 1990) as a template, with pGDV1-2-up and pGDV1-2-down as primers. The fragment was digested with *Bam*HI and *Sac*I, and ligated with a 700 bp *Bgl*II–*Sac*I fragment excised from pE3 that cloned the CoEI replicon between *Apa*I and *Kpn*I, and resulted in the *E. coli–B. subtilis* shuttle vector pGJ103.

Using *Apa*I and *Eco*RI, the 400 bp P_{glv}-inframe fragment was excised from pGJ240 and ligated into pGJ103 digested with the same enzymes,

Table 1 Primers and oligonucleotides used in this study

Primer	Sequence (5′ → 3′)[a]	Restriction site
pGDV1-2-up	ggGGATCCTAGCACTTTGCCACTC	*Bam*HI
pGDV1-2-down	ttGAGCTCGCATGCAAGCTCTAGC	*Sac*I
P*glv*-up	ttGGGCCCGGCATGTATCCGAATC	*Apa*I
P*glv*-down	gcGGATCCTGGAGTGAAAGTGCTCCC	*Bam*HI
inSD-1	**GATCT**TAAATAAATAAATTTATCAA*GGAGG*TGAATTC***GAGCT**	*Eco*RI
inSD-2	**C**GAATTCACCTCCTTGATAAATTTATTTATTTA**A**	
bga-up	ggCGAATTCATGAATGTGTTATCCTC	*Eco*RI
bga-down	gcGGATCCAGGAGCTCTTAGC	*Sac*I
bioA-up	ggcGAATTCATGACTCATGATTTGATAG	*Eco*RI
bioA-down	ggcGAGCTCAGGAATCAATCTTCAAGG	*Sac*I
vgb-up	gcgGAATTCATGTTAGACCAGC	*Eco*RI
vgb-down	gccGAGCTCTTTATTCAACCGCTTG	*Sac*I

[a] The additional sequence (lower case) and restriction site (underlined) are indicated. The INSD-1 and INSD-2 annealed and overhanged the *Bgl*II and *Sac*I adhesive end (bold)

yielding the expression vector pGJ113. The *bgaB* gene, 2.0 kb, coding for thermostable β-galactosidase was produced with *bga*-up and *bga*-down; the *vgb* gene coding for VHB, 450 bp, was produced with *vgb*-up and *vgb*-down; and the *bioA* gene, 1.1 kb, coding for diaminopelargonic acid aminotransferase was produced with *bioA*-up and *bioA*-down. These were generated by PCR using plasmid pDL (Yuan and Wong 1995), pUC8:16 (Wei et al. 2000), and *B. subtilis* 1A747 chromosomal DNA as templates, respectively. These fragments were digested with *Eco*RI and *Sac*I, and cloned into pGJ113 digested with the same enzymes, resulting in pLJ-7, pGJ203, and pDCA, respectively.

SDS-PAGE

SDS-PAGE was performed as described elsewhere (Sambrook et al. 1989) using the Bio-Rad Mini ProteinIII, with 5% stacking and 15% resolving gels.

β-Galactosidase activity assay

The method to measure β-galactosidase activity has been described previously (Hirata et al. 1986; Schrogel and Allmansberger 1997; Shimotsu and Henner 1986). One unit of β-galactosidase activity was defined as the amount of enzyme necessary to release 1 μmol 2-nitrophenol from *o*-nitrophenyl-galactopyranoside. β-Galactosidase activity is expressed as units per ml sample.

Results and discussion

Construction of expression vector

Based on the versatile *E. coli–B. subtilis* shuttle vector pGJ103, we constructed the expression vector pLJ (Fig. 1A), using P_{glv} as a control element. The P_{glv} was fused with a synthetic ribosome binding site fragment, which resulted in the P_{glv}-inframe, and the tandem stop codons(TAAATAAATAAA) were introduced (Fig. 1B) to efficiently avoid read-through.

The presence of unique restriction sites downstream of the maltose-inducible promoter allows appropriate ligation of coding regions for target protein overproduction (Fig. 1A).

Expression of β-galactosidase in B. subtilis

To verify the efficiency of gene expression driven by this system, we constructed pLJ-7 in which the heat-stable β-galactosidase from *B. stearothermophilus* was used as reporter. Figure 2A demonstrates the over-production of β-galactosidase from pLJ-7 induced by 5% maltose in *B. subtilis*.

To further characterize the expression vector, *B. subtilis* 1A747 harbouring pLJ-7 was cultured in liquid LB and in LB supplied with maltose at various concentrations. Figure 2B shows that the β-galactosidase activity was maximal (8 U/ml) after 24 h with both 5% and 10% maltose. This activities was about 16 times higher than under non-induction conditions.

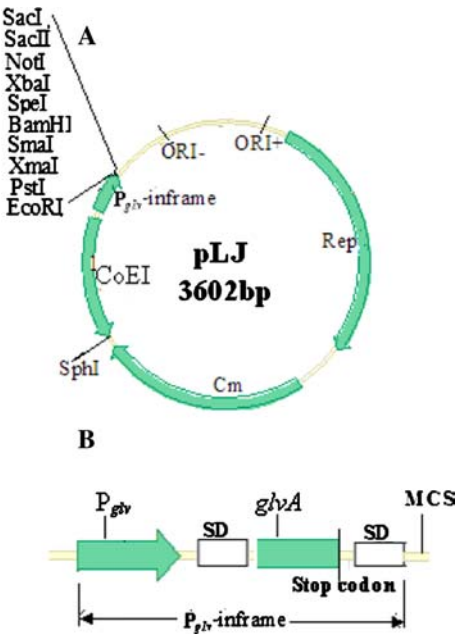

Fig 1 (**A**) Map of the expression vector pLJ. ORI+, ORI– and Rep represent the single-strand replication origin, the double strand origin and replication protein in *B. subtilis*, respectively. CoEI and Cm represent *E. coli* CoEI replicon and chloramphenicol-resistance marker, respectively. P_{glv}-inframe refers to the P_{glv}-inframe promoter. The unique restriction sites are marked on the outside of the map. (**B**) Close-up of the key elements of the P_{glv}-inframe promoter. The P_{glv} promoter, ribosome binding site (SD), the 5′ partial of *glvA* followed by the stop codon in frame, and the restriction sites (MCS) are shown

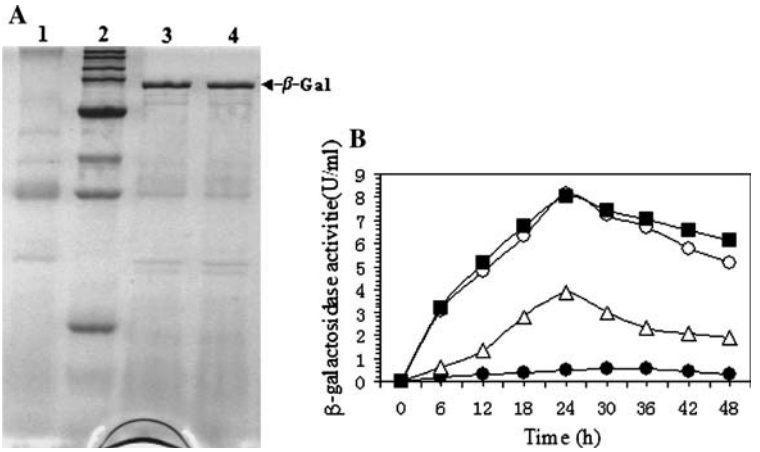

Fig 2 Assay of the β-galactosidase production from *B. subtilis* 1A747 harbouring pLJ-7. (**A**) SDS-PAGE analysis of β-galactosidase. After 24 h of induction, the cell extract was prepared and SDS-PAGE was performed. Lane 1, crude extract of *B. subtilis* 1A747 harbouring pLJ as a negative control; lane 2, molecular mass markers (top to bottom: 225, 150, 100, 75, 50, 35, 25 and 10 kDa); lanes 3 and 4, crude extract of *B. subtilis* 1A747 harbouring pLJ-7. (**B**) β-Galactosidase activity (U/ml) in *B. subtilis* 1A747 harbouring pLJ-7 under non-induction conditions (●), and under induction by 2.5% (Δ), 5%(○), or 10%(■) maltose

To compare this maltose-inducible expression vector with that of the commonly used strong promoter, we replaced the P_{glv}-inframe promoter with the P43 promoter (Wang and Doi 1984). The β-galactosidase maximum activity, driven by the P43 promoter in *B. subtilis*, reached 2.7 U/ml (at 48 h), which was about 33% of the maximum activity from pLJ-7, suggesting that the pLJ is a high-level expression system.

Repression of expression by glucose

The P_{glv} promoter is negatively regulated through catabolite repression, (Yamamoto et al. 2001). To examine the effect of glucose on this expression system, we added glucose at 2% or 4% into LB medium and induction medium (LB with 5% maltose), respectively. Table 2 shows that glucose supplementation (at 2% and 4%) severely repressed the expression of β-galactosidase. This repression disappeared gradually after 36 h cultivation probably due to. We speculate that this may have resulted from exhaustion of glucose from the culture medium.

Expression of the bioA and vgb gene in *B. subtilis*

To further demonstrate the effectiveness of the expression vector for cloned genes, the genes *bioA*, which is involved in biotin biosynthesis of *B. subtilis* (Streit and Entcheva 2003), and *vgb*, coding for *Vitreoscilla* haemoglobin (VHB), were cloned down-stream of P_{glv}-inframe in pLJ. The resultant pDCA and pGJ203 were transformed into *B. subtilis* 1A747. The expression experiments showed that the two genes were expressed successfully (Fig. 3).

An inducible and efficient expression system, which results from a strong regulation element and versatile vector backbone, is critical in genetic engineering. The P_{spac} promoter (Hartl et al. 2001), P_{xylA} promoter (Kim et al. 1996) and

Table 2 Effect of glucose concentration on β-galactosidase expression

Supplement[a]	β-Galactosidase activity (U/ml)			
	12 h	24 h	36 h	48 h
Control[b]	0.31	0.52	0.55	0.32
2% (w/v) glucose	0.06	0.13	0.23	0.28
4% (w/v) glucose	0.04	0.08	0.19	0.22
5% (w/v) maltose	4.86	8.01	6.45	5.17
5% (w/v) maltose and 2% (w/v) glucose	0.53	0.51	4.77	4.05
5% (w/v) maltose and 4% (w/v) glucose	0.42	0.47	3.87	4.22

[a] The supplement was based on LB medium. [b] The control was LB medium

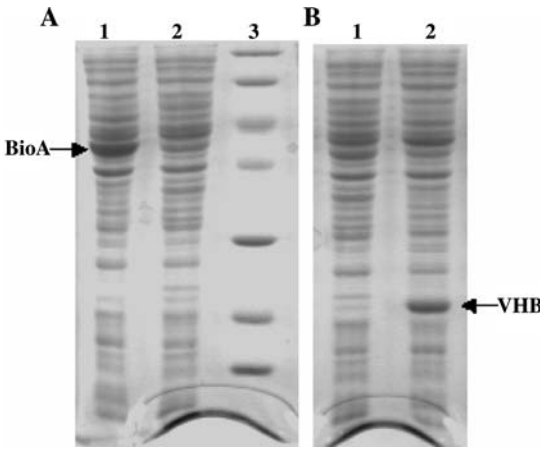

Fig 3 Analysis of the BioA from *B. subtilis* 1A747 harbouring pDCA and VHB from *B. subtilis* 1A747 harbouring pGJ203. (A) SDS-PAGE analysis of BioA. Lane 1, crude extract of *B. subtilis* 1A747 harbouring pDCA; lane 2, crude extract of *B. subtilis* 1A747 harbouring pLJ as a negative control; lane 3, molecular mass markers (top to bottom: 116, 66, 45, 35, 25, 18 and 14 kDa). (**B**) SDS-PAGE analysis of the VHB. Lane 1, crude extract of *B. subtilis* 1A747 harbouring pLJ; crude extract of *B. subtilis* 1A747 harbouring pGJ203

constitutive promoters such as P43 promoter (Xiao-Zhou Zhang et al. 2005) are used frequently as strong promoters in the construction of expression systems in *B. subtilis*. Compared to these systems, the maltose inducible expression system constructed in this study has several advantages. For example, the expression strength of the maltose inducible expression system is much higher than that of P43 system; and the inducer, maltose, is used as a nutrient and is much cheaper and more convenient than IPTG or xylose that are used as inducers of P_{spac} and P_{xylA} systems, respectively. The vector backbone used in this study, which confers single-strand replication origin, double strand origin in *B.subtilis* and sufficient unique restriction sites, is smaller and more versatile than the derivatives of pUB110.

In conclusion, we have constructed a novel and efficient inducible expression vector in *B. subtilis.* The expression vector has particular advantages including the strength of the promoter and nature of the inducer. Three proteins were overproduced to demonstrate the effectiveness of the vector. Therefore, this system provides a useful expression system for cloned genes in *B. subtilis*.

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