Construction of a Shuttle Vector for Inducible Gene Expression in *Escherichia coli* and *Bacillus subtilis*

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The construction of a shuttle vector for inducible gene expression allowing fast and easy cloning in *Escherichia coli* and subsequent transformation of *Bacillus subtilis* is presented. The expression is based on the regulation of the *tac* promoter by the Lac repressor which was assayed with the *xylE* gene from *Pseudomonas putida* as a marker gene. The *lacI^a* gene, transcribed by the strong *spo* promoter, allowed full repression of the weak *tac* promoter.

INTRODUCTION

A number of proteins produced by genetic engineering techniques are detrimental to cell growth. These problems can be overcome by the use of either chemically inducible or thermoinducible expression systems. Yansura & Henner (1983) adapted the *lac* operator–repressor system to achieve IPTG-inducible expression of interferon in *Bacillus subtilis*. Another approach is to use a promoter–repressor system from phage $\phi 105$. The repressor itself may be temperature-sensitive (Osburne *et al.*, 1985) or its gene can be placed on a plasmid temperature-sensitive for replication (Dhaese *et al.*, 1984); in other words, the repression can be relieved by incubation at a non-permissive temperature. In these thermoinducible systems the expression of cloned genes can be repressed only about 30-fold while a chemically inducible system is repressed no more than 100-fold.

Utilizing the well-characterized *lac* repressor–operator system, which operates in *B. subtilis* (Yansura & Henner, 1983), we have developed an expression system regulated at the level of transcription. The use of the weak *tac* promoter (Moran *et al.*, 1982; Peschke *et al.*, 1985) should allow a high repression and, therefore, avoid interference with cell growth.

METHODS

Bacteria. *Bacillus subtilis* strain PSL1 (Ostroff & Pène, 1983) and *Escherichia coli* strain HB101 (Boyer & Roulland-Dussoix, 1969) were used.

Ty broth for *B. subtilis* (Rottländer & Trautner, 1970) and L broth for *E. coli* (Luria & Burrous, 1957) were used throughout both as liquid and solid medium. The media were supplemented either with ampicillin (50 $\mu\text{g ml}^{-1}$), neomycin (5 $\mu\text{g ml}^{-1}$) or phleomycin (0.1 $\mu\text{g ml}^{-1}$).

Plasmids. pKK223-3 (Brosius & Holy, 1984), *placI^a* (Bagdasarian *et al.*, 1983), pTG402 (Zukowski *et al.*, 1983) and pUB110 (Lacey & Chopra, 1974) were used. Genes encoded by pUB110 are *kan* and *ble* (conferring Nm^R and Pm^R , respectively) and *repU*, the replication initiator protein (unpublished results).

Recombinant DNA procedures. Restriction endonucleases and DNA modification enzymes were purchased from Boehringer and used as described by the manufacturer.

Transformation. *E. coli* cells were transformed as described by Cohen *et al.* (1972). Transformation of competent *B. subtilis* cells was done as described previously (Rottländer & Trautner, 1970).

Catechol 2,3-oxygenase assay. This was done essentially as described by Zukowski *et al.* (1983). Cell cultures were grown to an OD_{560} of 0.2 units; if required, IPTG was added and cells were further incubated for 120 min. All values reported are the mean of four independent experiments with SE values within 15%.

Abbreviation: IPTG, isopropyl β -D-thiogalactopyranoside.

Dot blot analysis. RNA was isolated from *E. coli* and from *B. subtilis* by the methods of Brosius *et al.* (1982) and Sarachu *et al.* (1980) respectively. Dot blotting was done essentially as described by Thomas (1980); a nylon membrane (GeneScreen) was used instead of nitrocellulose. For nick-translation the *Pst*I–*Bam*HI fragment from pEB113 containing the *xylE* gene was chosen.

RESULTS AND DISCUSSION

To construct a shuttle vector which is able to replicate and express a selectable marker in *E. coli* and *B. subtilis*, parts of pKK223-3 and pUB110 were fused. The pBR322-based plasmid, pKK223-2, was digested completely with *Pvu*II and partially with *Bam*HI to delete the truncated *tet* gene. From pUB110 the large *Eco*RI–*Pvu*II fragment was used. Cohesive ends were filled in and ligation of both fragments resulted in plasmid pEB111, which confers Ap^R, Nm^R and Pm^R in *E. coli* and Nm^R and Pm^R in *B. subtilis* (Fig. 1). This vector comprises the entire expression device of pKK223-3, i.e. the *tac* promoter followed by the M13mp8-derived multicloning site and two tandemly ordered transcriptional terminators that work both in *E. coli* and in *B. subtilis*. Truncation of the pBR322 *rom* gene (see Tomizawa & Som, 1984) in pEB111 causes an increased copy number in *E. coli* as compared to the wild-type (data not shown). The 1.1 kb *Eco*RI fragment carrying the *lacI^s* gene (Bagdasarian *et al.*, 1983) was placed downstream of a strong promoter derived from the *B. subtilis* bacteriophage SPO2 (*spo*) (Williams *et al.*, 1981), and this construct was subsequently cloned into the unique *Pvu*II site of pEB111. The resulting plasmid was named pEB112 (Fig. 1).

To show that the shuttle vector provides an appropriate tool for an inducible gene expression we chose the *xylE* gene from *Pseudomonas putida* as a marker gene. The *xylE* gene was recovered on a 1.9 kb fragment by digesting pTG402 completely with *Bam*HI and partially with *Sal*I. This fragment was cloned into the unique *Bam*HI and *Sal*I restriction sites of the multicloning site in pEB111 or pEB112. Plasmid derivatives of pEB111 and pEB112 expressing the *xylE* gene were named pEB113 and pEB114 respectively.

To evaluate the activity of the *tac* promoter in the shuttle vector plasmids the *tac* promoter in pEB114 was deleted (pEB104) or replaced by the constitutively expressed *spo* promoter (pEB4). The first step was performed by replacing the *Eco*RI–*Bgl*II fragment from pEB114 containing the *tac* promoter by the *Eco*RI–*Bgl*II fragment from pUB110 to create plasmid pEB104 which is identical to pEB114 except for the *tac* promoter. Plasmid pEB4 was derived from pEB114 by inversion of the *Eco*RI fragment comprising the pUB110 moiety and concomitant deletion of the smallest *Eco*RI fragment containing the *lacI^s* gene so that the *xylE* gene is transcribed by the *spo* promoter.

The catechol 2,3-oxygenase activities obtained with these plasmids in *E. coli* and *B. subtilis* are listed in Table 1. Comparing the *xylE* expression of plasmids pEB113 and pEB4 we infer that in *E. coli* the *tac* promoter is slightly stronger than the *spo* promoter. The plasmid pEB114 bearing the *lacI^s* repressor gene gave rise to a more than 500-fold lower level of catechol 2,3-oxygenase activity compared with an homologous plasmid (pEB113) lacking the repressor gene. The addition of IPTG to a final concentration of 1 mM increased the expression of the *xylE* gene 330-fold. Higher concentrations of IPTG did not show any further increase (data not shown). In *E. coli* the control plasmid lacking the *tac* promoter, pEB104, shows an 8-fold higher level of enzyme activity than plasmid pEB114 carrying the *tac* operator region. This increase was attributed to upstream promoters. In other words, the operator not only hinders the entrance of RNA polymerase but also may block transcriptional readthrough. This second mode of action, the termination of transcription at or near the operator site by the repressor, was recently reported by Deuschle *et al.* (1986).

The enzyme activities obtained with these vectors in *B. subtilis* were, in general, lower than in *E. coli*. Even for the strong *B. subtilis* *spo* promoter the values obtained were about two orders of magnitude lower. This lower expression of the *xylE* gene in *B. subtilis* allows a better distinction of different states of regulation. The regulation of gene expression, however, works as well as in *E. coli*, as shown by the at least 180-fold increase in the specific enzyme activity in cells containing pEB114 after the addition of IPTG (1 mM).

Fig. 1. Physical map of plasmids constructed in this work. Only restriction sites used for the respective cloning step are shown. The origin of the fragments can be distinguished by their different representation: —, pUB110; □, pKG402; ■, *lacP* gene from *E. coli* fused to the *spo* promoter. Arrows denote open reading frames (see text). The direction of transcription is indicated (▶). Abbreviations: A, *AccI*; B, *Bam*HI; E, *Eco*RI; P, *Pvu*II; S, *Sal*I; MCS, multicloning site (*Eco*RI, *Sma*I, *Bam*HI, *Sal*I, *Pst*I, *Hind*III; see text).

Fig. 2. Determination of specific *xylE* transcripts in *E. coli* and *B. subtilis*. Both *E. coli* and *B. subtilis* total RNA (10 µg per sample) were blotted onto a nylon membrane and hybridized to 0.1 µg of nick-translated DNA with a total activity of 5×10^6 Cerenkov counts min^{-1} . RNAs were isolated from *B. subtilis* PSL1 except for samples A1, A2 and A3 which were isolated from *E. coli* HB101. Cells in B3 and B4 were induced with 1 mM-IPTG. B3/B4 and B5/B6 represent identical samples from independent experiments. B1 represents a plasmid-free control.

Table 1. Catechol 2,3-oxygenase activities

Plasmid	Promoter	Repressor	IPTG (mM)	Specific enzyme activity (milliunits mg^{-1})	
				<i>E. coli</i> HB101	<i>B. subtilis</i> PSL1
pEB113	<i>tac</i>	—	—*	14000	2.5
pEB114	<i>tac</i>	+	0	24	0.005
pEB114	<i>tac</i>	+	0.25	6700	0.6
pEB114	<i>tac</i>	+	0.5	7250	1.06
pEB114	<i>tac</i>	+	1	8000	1.07
pEB4	<i>spo</i>	—	—*	11000	110
pEB104	—	+	—*	200	0.005

* Identical results were obtained after addition of 1 mM-IPTG.

In *B. subtilis* the *tac* activity measured under repressed conditions (pEB114 without addition of IPTG) was not distinguishable from the background level of enzyme activity found in plasmid free strains.

To verify that regulation takes place at the level of transcription and to investigate the reasons for the low expression of the *xylE* gene in *B. subtilis* we determined the relative amount of specific *xylE* mRNA by dot blot analysis. The comparison between *E. coli* and *B. subtilis* in Fig. 2 shows that the amount of specific mRNA produced with the *spo* promoter is of the same order of magnitude in both species, suggesting that the host difference in the catechol assay is post-

transcriptional. In *B. subtilis* we failed to detect specific *xylE* mRNA transcribed from the *tac* promoter under repressed conditions (Fig. 2B).

Derepression of these cultures with IPTG (1 mM) however, gave rise to detectable specific transcripts (about 8% of what was found for the *spo* promoter). This is in good agreement with results reported by Peschke *et al.* (1985).

The high repressibility should make this shuttle vector an appropriate tool for expressing genes which are detrimental to cell growth. Replacing the weak *tac* promoter by the related *trc* promoter, which is reported to belong to the strong *B. subtilis* promoters (Osburne & Craig, 1986), should adapt this vector for purposes which require higher expression.

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