SHORT COMMUNICATION

Activity of Two Strong Promoters Cloned into *Bacillus subtilis*

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Two DNA fragments, one encoding the *Escherichia coli trc* promoter and the other encoding a sequence from the early region of *Bacillus subtilis* phage SPO1, were cloned into the *B. subtilis* promoter-probe vector pPL603. Both fragments effected strong *in vivo* promoter activity in vegetative *B. subtilis* cells.

INTRODUCTION

Among the requirements for effective expression of eukaryotic genes in *Bacillus subtilis* is an efficient promoter sequence. To avoid potential problems caused by protein degradation in late exponential and early stationary phase cultures, we chose to examine promoter sequences that are active during vegetative growth. Using the promoter-probe vector pPL603 (Williams *et al.*, 1981), we cloned two strong promoter sequences into *B. subtilis*. One sequence encodes the *Escherichia coli trc* promoter (Brosius *et al.*, 1985), which has the –10 region of the *E. coli lac* promoter and the –35 region of the *E. coli trp* promoter: TTGACA 17 bp TATAAT. The other sequence is derived from phage SPO1 *EcoRI** fragment 15, which encodes a strong early phage promoter (Lee & Pero, 1981).

METHODS

Organism. *Bacillus subtilis* strain BR151 (Shapiro *et al.*, 1974) was used.

Plasmids. Plasmids pUBI10 (Keggins *et al.*, 1978) and pPL603 (Williams *et al.*, 1981) have been described.

Growth conditions. Cells were grown aerobically at 37 °C in LB broth (Sonenshein *et al.*, 1974), unless otherwise indicated. Culture turbidity was measured with a Klett–Summerson colorimeter (green filter).

Transformation. Transformation of competent *B. subtilis* was done by the method of Sonenshein *et al.* (1974).

Chloramphenicol acetyltransferase (CAT) assays. Cell pellets were disrupted by sonication (for a total of 2 min in 10 s pulses). Cell extracts were assayed for CAT activity by the method of Shaw (1975). Protein was determined by the Lowry method.

Recombinant DNA procedures. Digestion of DNA with restriction endonucleases, ligation of DNA, small-scale and large-scale plasmid preparations, agarose gel electrophoresis, and Southern blotting experiments were done by standard procedures described by Maniatis *et al.* (1982).

DNA sequencing. Sequencing of the 290 bp *EcoRI* fragment from the early region of phage SPO1 was achieved by labelling the 5' ends of the fragment with [³²P]ATP, separating the strands, and subjecting them to Maxam–Gilbert sequencing reactions (as described by Maxam & Gilbert, 1980).

RESULTS AND DISCUSSION

To clone the *trc* promoter fragment, a 200 bp *EcoRI*–*PstI* fragment (obtained from J. Brosius) containing the *trc* promoter and the *lac* operator sequence was ligated into plasmid pPL603 which had been cut to completion with *EcoRI* and *PstI*. Plasmid pPL603, with its relevant

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Abbreviation: CAT, chloramphenicol acetyltransferase.

Fig. 1. Diagrams of promoter-probe plasmid pPL603 (a) and its promoter-containing derivatives p52-4, containing the *E. coli trc* promoter (b) and p3-8, containing a promoter from the early region of phage SPO1 (c).

Table 1. *CAT* activities of strains bearing plasmids with various promoter-containing fragments

Cells were grown in LB broth containing 50 µg chloramphenicol ml⁻¹ (except for cells bearing plasmid pPL603, which were grown in LB broth containing 10 µg neomycin ml⁻¹), harvested in mid-exponential phase, and assayed as described in Methods. All plasmids were derived from plasmid pPL603 and contain a promoter inserted in front of the *cat-86* gene. Assays were repeated a minimum of five times for each strain, and the results were highly reproducible.

Plasmid	Source of promoter	CAT activity [ΔA ₄₁₂ min ⁻¹ (mg protein) ⁻¹]
pPL603	None	Undetectable
p3-8	Derivative of SPO1 <i>Eco</i> RI* fragment 15	90.0
p52-4	<i>E. coli trc</i> promoter + <i>lac</i> operator	47.5
pPL608	SPO2 (Williams <i>et al.</i> , 1981)	33.1
p1	φ105 <i>Eco</i> RI fragment F	5.0
p603-900	φ105, subclone of <i>Eco</i> RI fragment F (Osburne <i>et al.</i> , 1985)	2.8

restriction sites, is shown in Fig. 1(a). This plasmid carries the *cat-86* gene from *B. pumilis*. Strains containing pPL603 are normally sensitive to chloramphenicol (Cm^S) during growth. Insertion of a promoter in front of *cat-86* renders the growing cell chloramphenicol resistant (Cm^R). To clone the early promoter of phage SPO1, *E. coli* plasmid pMB9 (Bolivar & Backman, 1979), with *Eco*RI* fragment 15 of SPO1 ligated into the *Eco*RI site, was digested to completion with *Eco*RI. SPO1 fragment 15 was purified by electroelution from an 0.8% agarose gel and ligated into the *Eco*RI site of plasmid pPL603. For both fragments, the ligation mixes were transformed into competent cells of *B. subtilis* BR151(pUB110), and Cm^R colonies were selected. Our transformation procedure made use of the resident plasmid recombination technique described by Gryczan *et al.* (1980), with pUB110 as the resident plasmid.

When the *trc* promoter was inserted in pPL603, hundreds of Cm^R transformants bearing plasmids which contained the *trc* fragment were isolated. One such recombinant plasmid, p52-4, is shown in Fig. 1(b). Digestion of this plasmid with *Eco*RI yielded the parent plasmid pPL603

TCCCACTGGCCTTGGTTAAGTTAAGATGTGGACGGAATGGGTAAGTGTAGTAAAGTACAATTAATCGGGAGCTTAGATGTCCTTC
 AACATCTTATATAGAAGGGAAGGTTGGCAATGGAAATTGAAAGAATTAACGAGCATACAGTAAAATTTATATGTCTTACGGAGATATT
 GAAGATCGCGGTTTGACAGAGAAGAAATTTGGTATAACCGTGAGCGCAGTGAAGAAGTCTTCTGGGAAGTCATGGATGAAGTTCATGAA
 GAAGAGGGGA

Fig. 2. DNA sequence of the SPO1 promoter fragment in plasmid p3-8. The direction of transcription is left to right, and the underlined regions represent the putative -35 and -10 promoter sequences on this fragment.

and the 200 bp *trc* fragment (data not shown). When the SPO1 promoter was inserted into pPL603 several hundred Cm^R colonies were also isolated. The plasmids carried by 43 of these colonies were examined by agarose gel electrophoresis after *Eco*RI digestion. Each of the 43 recombinant plasmids had sustained a promoter fragment deletion. Compared with the original size of approximately 500 bp, the inserts were all approximately 290 bp. However, we do not know whether the deleted regions of the promoter fragments were identical. We chose to work with one of these recombinant plasmids, p3-8 (Fig. 1c). The CAT activities of strains containing p52-4 or p3-8 are presented in Table 1. For comparison, the activities of a number of other promoter sequences cloned in front of the *cat-86* gene are also presented. Cells bearing either p52-4 or p3-8 had higher CAT activity than cells bearing plasmid pPL608. Previously, the promoter in plasmid pPL608, derived from phage SPO2 (Williams *et al.*, 1981), was the strongest known vegetative promoter for *B. subtilis*, as determined by the CAT activity encoded by pPL608. Thus, the two promoter-containing fragments had very strong promoter activities. It should be noted that, despite the large differences in CAT activities, strains bearing any of the plasmids listed in Table 1, except for the original probe plasmid pPL603, were able to grow with normal doubling times in LB medium containing up to 50 µg chloramphenicol ml⁻¹.

It is evident from Table 1 that the DNA fragment bearing the *trc* promoter works well in *B. subtilis*. This promoter displays perfect homology to the *E. coli* consensus promoter sequence TTGACA 17 or 18 bp TATAAT, which is thought to be very efficient in vegetative *B. subtilis* cells (Moran *et al.*, 1982). The *trc* promoter also behaves as a strong promoter in *E. coli* (Brosius *et al.*, 1985). The DNA fragment containing the *trc* promoter in plasmid p52-4 also contains the *lac* operator sequence. It has been shown that the *lacI* (repressor) gene can be transcribed in *B. subtilis* from a *Bacillus* promoter, and that the resulting *lacI* repressor protein is functional (Yansura & Henner, 1984). Yansura & Henner (1984) also showed that this repression can be lifted by the addition of isopropyl β-D-thiogalactoside (IPTG), as in *E. coli*. Therefore, regulation of the *trc* promoter by the *lacI* gene is theoretically feasible in *B. subtilis*.

The sequence of the SPO1 promoter fragment in p3-8 is presented in its correct orientation in Fig. 2. No part of this sequence is homologous to the 90 bp promoter region of the original fragment 15 as described by Lee & Pero (1981). However, our 280 bp fragment hybridized to the original fragment 15 (data not shown). We therefore surmise that the original early promoter in SPO1 fragment 15 had been deleted from our 280 bp fragment, and that a second strong promoter was thereby created or unmasked. A possible putative promoter sequence is underlined in Fig. 2.

That we were unable to clone the intact early SPO1 promoter onto a small multicopy plasmid in *B. subtilis* is consistent with the fact that, to our knowledge, no other research group has been able to clone this promoter into *B. subtilis*. Nevertheless, the promoter that we have cloned is a strong one; its activity is nearly twice that of the strong *trc* promoter in *B. subtilis*. Both of these strong promoter sequences can potentially be used for efficient expression of foreign genes in *B. subtilis*. The *trc* promoter, followed by the *lac* operator, confers an additional advantage for gene expression, namely that promoter activity may be regulated by IPTG.

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