Screening for Highly Active Plasmid Promoters via Fusion to β-Galactosidase Gene

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A plasmid containing promoter-deleted inactive β-galactosidase gene [1] was used to select promoters of the pEP121 plasmid [2]. Colonies of cells harboring reactivated β-galactosidase gene were identified by their red color on McConkey plates. The quantitative amounts of β-galactosidase produced in each clone were estimated by assaying enzyme activity and by measuring the specific β-galactosidase protein following fractionation of total cells' proteins on polyacrylamide gel. A wide range of enzyme activities was observed. The most active promoter isolated was shown to promote β-galactosidase production more efficiently, compared with the original β-galactosidase promoter, amounting to 20% of all cell proteins. Such highly active promoters may be utilized in the future, to promote expression of cloned genes in bacteria.

Introduction

It was previously shown that bacterial promoters can promote expression of foreign genes in bacteria [3, 4]. The amount of protein produced depends mainly on the activity of the promoter itself. The isolation and molecular manipulation of bacterial promoters became, therefore, of prime interest in utilizing *E. coli* for the production of high yields of biological compounds coded by foreign genes.

Casadaban *et al.*, [1] have recently developed a plasmid vector specially constructed for the cloning and identification of translational initiation signals *via* fusion to a promoter-deleted inactive β-galactosidase gene deleted in the coding region of the first 8 amino acids. Its subsequent reactivation was obtained by insertion of translation initiation signals in a proper phase in front of this gene. We have applied this vector, in the present work, to screen for highly active promoters containing translation initiation signals originating from the pEP121 plasmid, which carries the *trp* operon as well as an ampicillin resistant gene [2].

Materials and Methods

Plasmid and bacterial strains: The plasmid pEP121 which carries part of the *trp* operon [2] was a gift from Dr. P. H. Pouwels. The plasmid pMC1403 which carries a promoter deleted β-galactosidase gene and *E. coli* strain MC1061, restriction minus, deleted in the lactoase operon [1] were received from Dr. M. J. Casadaban and given to us by Dr. M. Mevorach.

Preparation of plasmid: Extraction of plasmid DNA was performed according to Betlach *et al.* [5].

Transformation of cells with plasmid DNA was carried out as described by Cohen *et al.* [6].

DNA digestion and gel electrophoresis: Restriction endonuclease enzymes and T_4 DNA ligase were purchased from New England BioLabs. Digestions were carried out in the assay buffer recommended by the supplier. DNAs wer electrophoresed through horizontal 1.4% agarose slab gel or 7.5% polyacrylamide verticle gel.

Blotting of DNA onto nitrocellulose filter paper was carried out essentially as described by Southern [7].

Hybridization probe: Labelling of DNA by nick-translation was carried out by the procedure of Maniatis *et al.* [8].

Hybridization of the labelled probe to the DNA on the filter was performed as in Mory *et al.* [9].

β-Galactosidase assay was carried out as described elsewhere [10].

Protein gels: Proteins were fractionated in 10% polyacrylamide gels and stained according to Laemmli [11].

Results and Discussion

In a search for strong promoters the pEP121 plasmid [2] was cleaved by restriction endonuclease

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Table I. Cells of several of the isolated red colonies were grown in 1 ml of M9 medium to a mid-log phase and assayed for β-galactosidase activity as in Miller [10]. A white colony was also picked from the McConkey plate to serve as a β-galactosidase negative control. HB 101 *E. coli* cells (containing the original active chromosomal β-galactosidase gene) were grown under the same conditions in the presence of 10 mM IPTG (induced) or without IPTG (non-induced) and the assay was carried out as above.

Colony No.	Red colonies							White colony	HB 101 cells	
	01	02	03	04	05	06	07		non-induced	induced
β-galactosidase activity (enzyme units)	2440	2360	3680	226	5350	450	45	19	10	150

and the resulting fragments were fused to promoter deleted β-galactosidase gene, which was thus reactivated. The plasmid pEP121 was cleaved with *Hha* I restriction endonuclease, followed by S1 nuclease treatment to create blunt ends. The DNA fragments were ligated to a single *Sma* I cut located in front of the promoter deleted β-galactosidase gene of the pMC1403 plasmid [1]. Lactose negative cells (MC1061) were transformed with the ligated mixture and transformants were selected on McConkey plates containing ampicillin. Most of the colonies grown were white (containing the parental pMC1403 plasmid). A few red colonies, containing an active β-galactosidase gene were also observed and subsequently purified.

In order to quantitatively evaluate the activity of the acquired promoters in each of the isolated red colonies, an assay of β-galactosidase enzyme was carried out as described by Miller [10]. The results are given in Table I. Although the red colour of the colonies indicated the presence of an active β-galactosidase enzyme the quantitative measurements (Table I) revealed large differences in the degree of activity among the various clones isolated (marked 01–07). The lowest activity was observed in clone 07 which was almost as low as the activity found in cells of a white (β-galactosidase negative) colony, used as a negative control, or in non-induced HB101 cells (containing the original intact lactose operon in the chromosome). Clones 04 and 06 showed a relatively mild activity of β-galactosidase, while the activity in clone 05 was shown to be higher in comparison with IPTG induced HB101 cells.

The relative amount of β-galactosidase protein made in clone 05 was measured by fractionating total

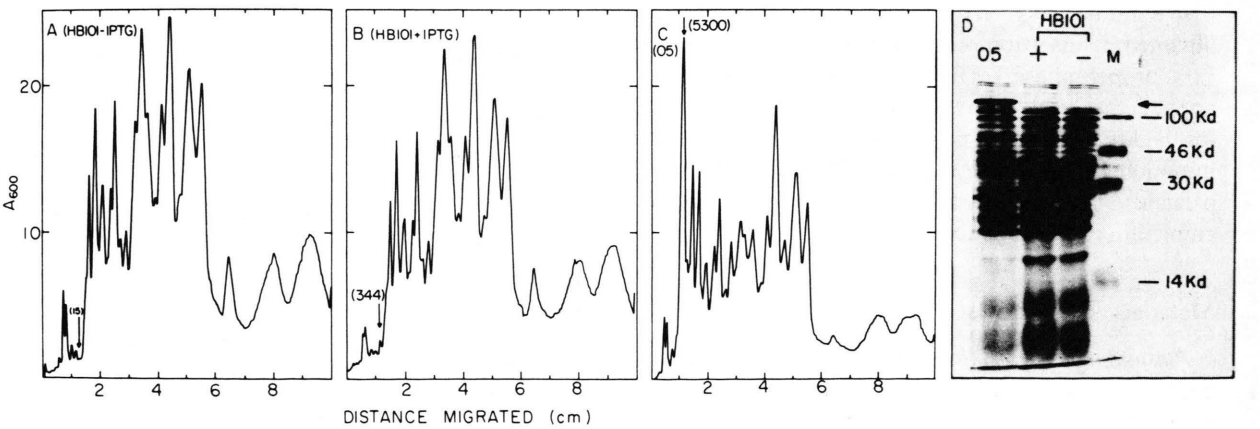

Fig. 1. β-galactosidase protein produced in clone 05 compared with HB101 cells. *E. coli* HB101 cells were grown in 1 ml M9 minimal medium with (+) or without (–) 10 mM IPTG to a mid-log phase, and 0.1 ml samples of each culture were taken for analysis of total proteins by gel electrophoresis (D) and for β-galactosidase assay which was carried out as described by Miller [10]. Cells were grown without IPTG under the same conditions and β-galactosidase was measured as above (D). The stained patterns in D were scanned (A, B, and C, respectively). The arrows mark the position of the β-galactosidase band in each scan and β-galactosidase activity values are given in brackets.

cell proteins on polyacrylamide gel in parallel with induced and non-induced HB101 cells (Fig. 1D). Scans of the stained patterns are given in Fig. 1A, B and C. While in the non-induced HB101 cells the amounts of β-galactosidase protein made were below detection level (Fig. 1A and D), and only a weak band is seen in fully induced HB101 cells (Fig. 1B and D), in clone 05, β-galactosidase protein was the major protein produced consists up to 20% of all cell proteins as calculated from the area under the β-galactosidase peak in Fig. 1C and D (marked with an arrow).

We further analyzed plasmids from two of the red colonies isolated (clones 02 and 05): To determine the size of the insert (containing the presumed promoter) DNAs of both clone 02 and 05 were double-digested with *Bam*HI-*Eco*RI which cut closely on both sides of the *Sma*I inserted fragment [1], and fractionated on a 7.5% polyacrylamide gel (Fig. 2, lanes 1 and 2, respectively), together with a known size marker of pBR322/*Hinf* digest (lane 4, and ref. [12]) and a *Hha*I digest of the pEP121

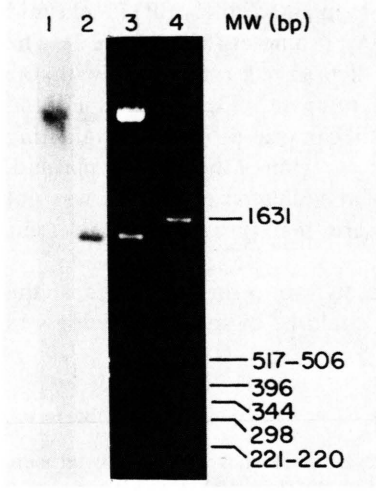

Fig. 3. Localizing the origin of the isolated promoters on the pEP121 plasmid. The pEP121 plasmid was double-cleaved with *Bgl*II-*Xho*I (to cut out a fragment containing the *trp* promoter) and the digest was fractionated on a 1% agarose gel (lane 3) together with a size marker (lane 4). The DNA fragments were blotted onto a nitrocellulose paper and hybridized to the purified ^{32}P-labelled inserts of either the 02 (lane 1) or the 05 (lane 2) clones prepared as described in the text.

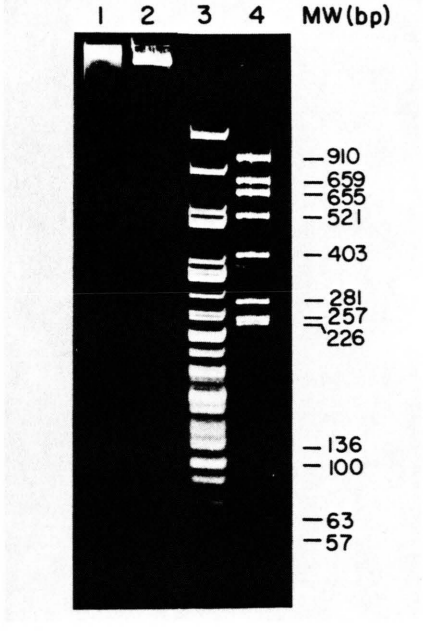

Fig. 2. Size analysis of the cloned promoters. Plasmid DNA (1 μg) of clones 02 and 05 was double-cleaved with *Bam*HI-*Eco*RI and the excised insert was separated by electrophoresis on 7.5% polyacrylamide gel (lanes 1 and 2, respectively) in parallel with plasmids pEP121/*Hha*I (lane 3) and pBR322/*Alu*I digests used as a size marker (lane 4) as in Sutcliffe [12].

plasmid (lane 3). The size of the 02 insert was calculated to be about 500 base pairs, while that of the 05, was 350 base pairs; both longer than, for instance, the 139 base pairs *Hha*I fragment containing the *E. coli trp* promoter [13], and seems, therefore, to be sufficiently large to contain the information required for promoter functions. The fractionated inserts were eluted from the gel and thus purified to be used later for hybridization studies.

The pEP121 plasmid carries the *trp* operon, among other genes which could serve as a source for the isolated promoters. The *trp* promoter region was defined by Brown *et al.* [14]. Since this region did not contain any *Hha*I site, its integrity was not likely to be broken by cleaving the plasmid with this enzyme. To distinguish between promoters originating from the *trp* operon region and those located on other parts of the pEP121 plasmid, the purified inserts of clones 02 and 05 were labelled by nick-translation and used for hybridization with a blot of the pEP121 plasmid which was double-cleaved with *Bgl*II-*Xho*I to cut out the region containing the *trp* promoter and separated it from the rest of the plasmid (2, and Fig. 3, lane 3). Only the insert of clone 05 positively hybridized with the

small *Bgl*II-*Xho*I fragment of the pEP121 plasmid which contains the *trp* promoter (Fig. 3, lane 2). The insert of clone 02 hybridized exclusively with the large *Bgl*II-*Xho*I fragment (Fig. 3, lane 1) and seems, therefore, to carry a promoter originating outside the *trp* operon region of the pEP121 plasmid. The exact origin of the isolated promoters was not followed as we were mainly interested in their relative activity.

Insertion of promoters into the *Sma*I site of the PMC1403 plasmid could be of special usefulness as they can be then "lifted-up" by cleaving on both sides with *Bam*HI-*Eco*RI restriction endonucleases, thus creating a convenient "mobile-promoter" which can be linked to any other gene.

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