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A plasmid vector for isolation of strong promoters in *Escherichia coli*

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Abstract

In order to isolate very strong promoters from bacteria and bacteriophage a plasmid named **pProm** was constructed. It possesses an origin (ORI) for replication in Gram-negative bacteria, an ORI for replication in Gram-positive bacteria, a promoterless ampicillin resistance gene with a multiple cloning site (MCS) in the position formerly occupied by the ampicillin promoter, a tetracycline resistance gene for selection in Gram-negative bacteria and a chloramphenicol resistance gene for selection in Gram-positive bacteria. Insertion in the MCS of DNA fragments of *Staphylococcus aureus* bacteriophages resulted in isolation of several clones very resistant to ampicillin. The DNA fragments inserted in these recombinant plasmids were sequenced and all of them contained putative promoter motifs. Direct measurement of the penicillinase activity indicated that one of the isolated promoters could be included within a group of the stronger known prokaryotic promoters. According to these results **pProm** is a powerful tool to perform studies on promoter strength and for industrial applications.

Keywords: Bacterial promoter; Bacteriophage promoter; Strong promoter; Promoter isolation; Vector

1. Introduction

DNA dependent RNA polymerase (RNAP) initiates the transcription process in bacteria after binding to a specific DNA sequence named the promoter. The efficiency of this process strongly depends on the structure of the promoter. In *Escherichia coli* several studies indicate that canonical hexanucleotides at the −35 (TTGACA) and −10 (TATAAT) regions are important for σ^{70}-RNAP recognition [1]. Recently, a very A+T rich region between positions −40 and −60 (named the UP element) was also recognized in some promoters as an important determinant of promoter efficiency [2]. On the other hand, a relationship between promoter efficiency and the presence of intrinsically curved DNA upstream of the promoter has also been demonstrated [3]. Thus, promoter efficiency seems to be a complex matter related not only to the kind of nucleotides directly involved in the contact with the RNAP but also to the three-dimensional structure of the whole region.

In order to further investigate the bases for promoter efficiency we thought that it would be useful

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to isolate a large collection of very efficient (strong) promoters from different sources.

A possible strategy for promoter isolation is to construct a vector having a gene marker devoid of its own promoter (a silent gene). The insertion of a DNA fragment containing a promoter in place of the original promoter would activate the silent gene. Using this strategy several promoter-probe plasmids have been constructed using as reporters antibiotic resistance genes [4–7], the β-galactosidase gene [8–10], the galactokinase gene [11], the gene for a fluorescent protein [12], the β-1,4-endogluconase gene [13], the *xylE* gene [14], the chloramphenicol acetyltransferase gene [15–18], and the β-glucuronidase gene [19]. The main problem with this strategy is that if a very strong promoter is present in the inserted DNA fragment, the reporter gene product will accumulate in very large amounts inside the cell, resulting in reduced cell viability.

To avoid this problem, we have constructed a vector based on the ampicillin resistance gene of the pBR322 plasmid whose product is secreted into the periplasmic space of *E. coli*. Cell viability is probably not affected as seen in high copy number vectors such as pUC [20,21]. Three strong promoters and one very strong promoter were isolated from bacteriophages, which confirmed the correctness of this approach. To our knowledge, this is the first vector which allows the trapping of very strong bacterial or bacteriophage promoters.

2. Materials and methods

2.1. Strains and growth conditions

E. coli DH5αF′Iq *ϕ80dlacZΔM15*, Δ(*lacYZA-argF*)*U169*, *recA1, endA1, hsdR17* (r_{k-}, m_{k+}), *supE44* λ−, *thi-1*, *gyrA*, *relA1*/*F′proAB*$^+$, *lacI*^{q}*ZΔM15zzf::Tn5*(*kn*^{r}) was used in cloning experiments. Bacteria were usually grown in Luria-Bertani broth (LB) liquid or solid media at 37°C. *Staphylococcus aureus* CDC phages φ 83, φ 85, φ 95 and φ 3E/C were used as a source of DNA to isolate promoters.

2.2. Cloning and sequencing

Plasmid DNA was purified according to the method described by Birnboim and Doly [22] and was digested and ligated using standard protocols [23]. Transformations were performed according to the method described by Hanahan [21]. The screening of the inserts was performed by digestion of plasmid DNA with restriction enzymes followed by analysis of the products by agarose gel electrophoresis [23]. DNA was sequenced on both strands using the fmol DNA Sequencing kit (Promega) [24] after purification with the Wizard Clean Up system (Promega).

2.3. Penicillinase activity

Penicillinase assays were performed using a colorimetric method [25]. Briefly, 50 μl of ampicillin resistant *E. coli* cells (OD_{600} 0.7) were incubated for 5 min at 30°C in 0.1 M phosphate buffer pH 7,0. 6 μmol of penicillin G was added at different times ($t = 0$–170 min). The reactions were ended by adding 1.8 ml of a 10% iodine solution (0.32 M I_2, 1.2 M potassium iodine, in 0.5 M buffer sodium acetate pH 4.0) and the resulting color was spectrophotometrically measured at 540 nm. Activity was defined as the difference between the initial OD_{540} and the OD_{540} at a given time ($\Delta OD_{540(t)}$), divided by the time in minutes, as a percentage of the initial OD_{540} ($OD_{540(to)}$): $\text{Act.} = [(\Delta OD_{540(t)}/t_{(min)})/OD_{540(to)}] \times 100$.

3. Results and discussion

3.1. Vector construction

In order to isolate very strong promoters, a plasmid named **pProm** was constructed (Fig. 1). First, a small fragment containing the promoter of the ampicillin gene of the pBR322 was eliminated using the endonucleases *Ssp*I and *Eco*RI. The plasmid was reconstructed by inserting a *Hinc*II-*Eco*RI fragment recovered from the M13mp19 multiple cloning site (MCS). A 1700-bp *Cla*I DNA fragment from pRIT5 [26] containing a chloramphenicol resistance gene and ORI for Gram-positive bacteria of the *S. aureus* plasmid pC194 [27] was inserted into the *Acc*I site of M13mp7. It was recovered from this vector by digestion with *Eco*RI and inserted into the *Eco*RI site of the modified pBR322. The resulting vector named

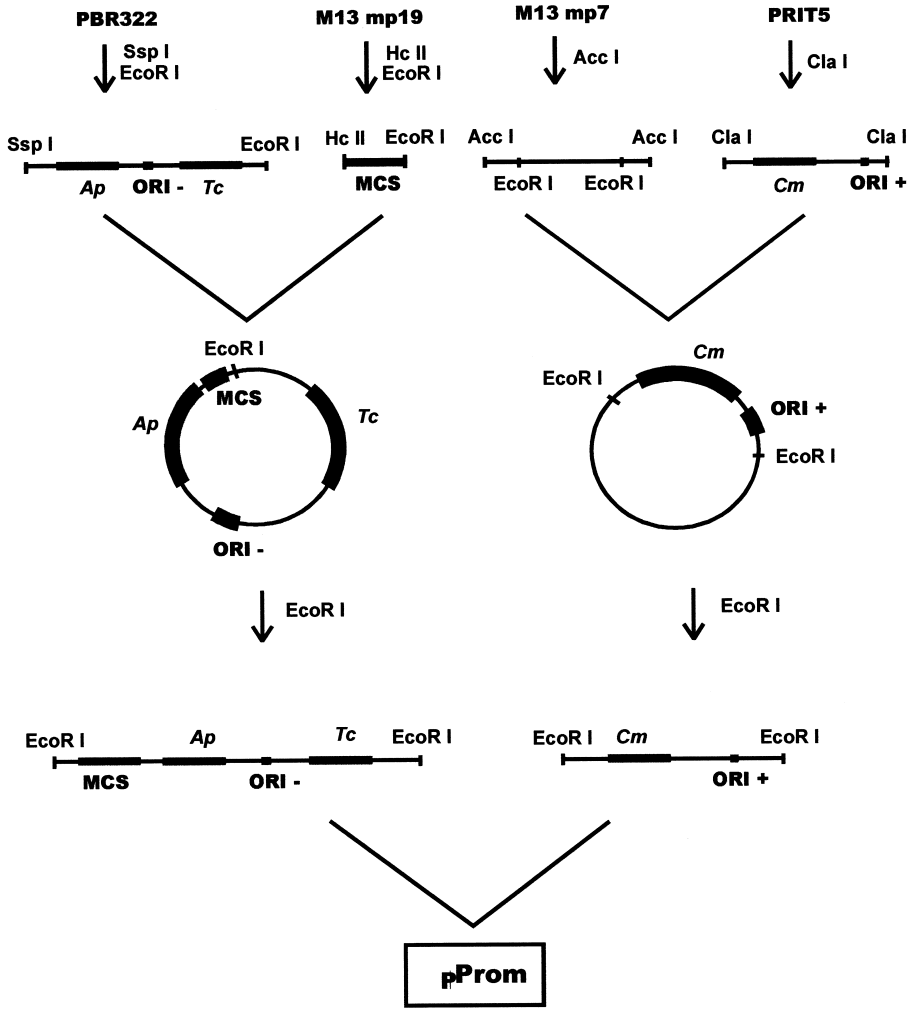

Fig. 1. Construction of the **pProm** plasmid for strong promoter isolation. *Ap*: ampicillin resistance gene; *Tc*: tetracycline resistance gene; *Cm*: chloramphenicol resistance gene; ORI+: replication origin for Gram-positive bacteria; ORI−: replication origin for Gram-negative bacteria; MCS: multiple cloning site.

pProm (Fig. 2) possesses the following features: an ORI(−) to replicate in Gram-negative bacteria, an ORI(+) to replicate in Gram-positive-bacteria, a promoterless ampicillin gene with an MCS including a unique *Sma*I site which replaces the promoter of the ampicillin resistance gene, a tetracycline resistance gene for selection in Gram-negative bacteria and a chloramphenicol resistance gene for selection in Gram-positive bacteria.

Insertion into the MCS of DNA fragments containing promoters should activate the ampicillin resistance gene allowing the selection of bacteria transformed with the plasmid in ampicillin-containing agar plates.

The ORI for replication in Gram-positive bacteria was introduced into **pProm** in order to allow transformation into *Bacillus subtilis* cells where the product of the ampicillin resistance gene would be secreted. However, preliminary experiments showed that upon transformation in these bacteria **pProm** suffers a number of structural alterations, which limits its usefulness (data not shown).

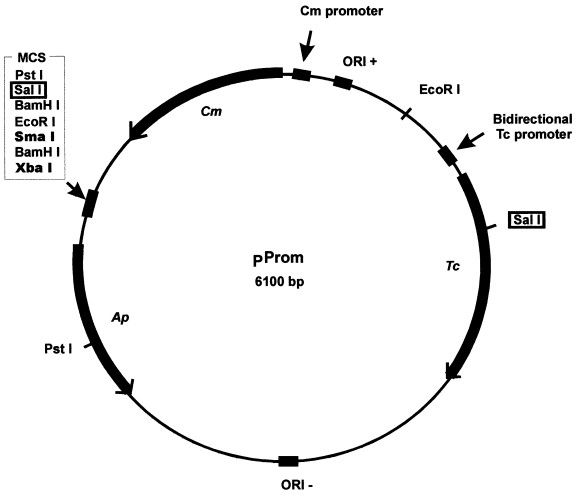

Fig. 2. **pProm** vector map. Endonucleases of the MCS shown in bold letters have unique restriction sites.

3.2. Isolation of promoters

Using a mix of restricted DNA of the *S. aureus* bacteriophages CDC φ 83, φ 85, φ 95 and φ 3E/C, the capability of **pProm** to isolate strong promoters was assayed. These phages were chosen in order to have a reasonable chance that strong, foreign promoters were isolated. Phage DNAs were purified and digested with the restriction enzymes *Alu*I and *Hae*III and inserted into the *Sma*I site of **pProm**. Recombinant plasmids were introduced into *E. coli* cells by transformation and bacterial clones selected on LB agar plates with a high concentration of ampicillin (1 mg ml^{-1}) in order to: (a) isolate only strong promoters and (b) avoid a background of colonies of low resistance to ampicillin because of the chloramphenicol promoter or because a divergent promoter expressing tetracycline resistance (Fig. 3).

Four of the selected bacterial colonies (X1, X2, X3 and X8) were chosen for characterization. An assay performed using agar plates containing different amounts of ampicillin (Table 1) demonstrated that the selected clones were able to grow at very high concentrations of this antibiotic. Bacteria transformed with **pProm** activated by insertion of well-known strong promoters (P_{Tac} and lambda P_L) were used as controls. The selected clones were able to grow at concentrations over 1 mg ml^{-1} of ampicillin (Table 1). The most resistant clone, X8, was able to grow on media containing up to 7 mg ml^{-1} of ampicillin. This level of resistance was not reached by the positive controls.

Subsequently, the DNA fragments inserted into **pProm** X1, X2, X3 and X8 plasmids were sequenced and putative promoter areas localized (Fig. 3). DNAs fragments X1, X2 and X3 possess hexanucleotides with different degrees of homology to the canonical −10 and −35 elements of the σ^{70}-RNAP of *E. coli* promoters. In addition X1 has, in the −44 region and downstream of the −10 element, sequences coincident with motifs pointed out by Ozoline et al. [28,29] as frequently present in these *E. coli* promoters. The X8 fragment is particularly interesting because within it lies a putative promoter, which

Table 1
Ap resistance of *E. coli* DH5αF′Iq transformed with derivatives of **pProm** as estimated by agar plate assay

Ap	Transforming plasmid							
	pProm-X1	**pProm**-X2	**pProm**-X3	**pProm**-X8	**pProm**-Tac	**pProm**-P_L	**pProm**	*E. coli*
0.5 mg ml^{-1}	+++	+++	+++	+++	+++	+++	+	−
1 mg ml^{-1}	++	++	++	+++	+++	++	−	−
2 mg ml^{-1}	+	+	++	+++	+++	+	−	−
3 mg ml^{-1}	+	+	++	+++	++	−	−	−
4 mg ml^{-1}	+	−	+	+++	+	−	−	−
5 mg ml^{-1}	−	−	−	+++	−	−	−	−
7 mg ml^{-1}	−	−	−	++	−	−	−	−

The number of + indicates if colonies produced after incubation of plates seeded with equal amounts of cells were abundant (+++), regular (++), scarce (+), or absent (−).
1.5% agar-LB medium plates with different amount of Ap were inoculated with 10 μl of a cell suspension containing about 300 viable bacteria and were evenly distributed on the agar surface using small glass spheres. Incubation was carried out for 15 h at 37°C.

X1

```
TAATGCATCC GTTACATGAT ATTTTTGCGT ATGATTTCGC CATTTTCGTA TCTCTTGCTT  60
CCTCCGTTTT TTACGTTTTA TATTGACTGA ATTTTTTCTT AAGTGTTTTG TTTGATACTG 120
TTACTTGTTT TGGCGCAGTA GCAGTTTTTT TATTGTTCAT AAAAGTATTC TTTATAAAAT 180
                  -44        -35                          -10
ATGAATGTTG CGATACTTGC GAATCCCGCA ATTGACCACG CTGTAGTGAA GTATAGAAAC 240
down-10
GGCATGAGTA CAATCGCTAA GACTGTGAAG CATAATACTG CTAATAGATA            290
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X2

```
TCGTCTGTAG TCTCTACTGC ATTTTTGTTG TATGTCGCTT TGATACGATC CATCAACATT  60
GGTATGTGCA TAAAAGCAAC CGTATGCCCT TTAGCTGAAC AATCAAAATG GACACGCCAA 120
AAGGCTTTTA AAGAATATAT GTTAAAGAAT GTAGCTGACT GCATACTTAA ACCACCCATA 180
                                      -54
CTAGTTGCTG GGTGGTTTTT ATGTTATAAT ATAAATGTGA ATGGTCATTC TTGAAATGAC 240
   -35                 extended -10
TCGGTCTCTA CTGGCACATC GTTTAAAGTG TCACCACAAC ATGAACTGAG AATTCATATA 300
CGTTGCTACG AGC                                                    313
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X3

```
TATAAATGGG ATACCTTAGC AGGCAGTAGT GATATTGAGG ATTGCCTCAA GACGGTATCA  60
TTTCGGTAGA TGATAAAAAT AAATTAGATA ATTTAAAAAT AGGCGAAGCA GGAATTATTC 120
      -35                          -10
AAAATAGCAT TGTACAGAAA TCCCCAAACG GTAAATTGTG GAAAATAACA GTTGACGATA 180
GTGGGAAACT TGGTACAGTG CTATTTTATT AGAAAGGAAG GTGCATTATG GAAAATTTGT 240
ATTTAATAAA GGATTTGGGA                                             260
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X8

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TGAGAATATG TCATAACCTG CATCCGTCTT ATGATTTCGT TCGGCGATTC TAGCATTTTT  60
TGATAATATG TCATAATAGT TTTACTTGTA ATGTGTTAGT CATTAAGTTC AATACCTTCT 120
TTAACTGAAT GTTGAATAGC ATTTGTCATC CAAGATTTCA CAACAGCTTG ACGACTTTCT 180
TATACACGTC CTCAATCTCT TTTAGCAACC CCTCTGTGTC ATTACCGTTA TACGCACTAG 240
CACTAATAAC GGACTGTTCG ATTTTTTTCGC GATTATTCAT TTGTGTCATC CTCCATAAAA 300
                -54          -44          -35  homopyrimidines
ATTTTATTGT TTAATTCCAT TCCGAATTTA ACGCTTTCAT CATCGTTACC GAATTCAGCT 360
       extended -10
CGGTAA                                                            365
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Fig. 3. Sequence of DNA fragments inserted into **pProm** plasmid, which activate the promoterless *Ap*. The EMBL accession number for X8 is Y12633.

possesses most of the motifs described by Ozoline et al. [28] as follows:

1. A −10 region between nucleotides 310 and 315, TTTAAT, which shows only one nucleotide difference to the canonical TATAAT −10 region of *E. coli* σ^{70}-RNAP promoters. It also bears the TG dinucleotide upstream which increases the contact with the σ^{70}-RNAP [30,31].
2. A −35 region between nucleotides 283 and 288, GTGTCA, shows only two mismatches with the canonical TTGACA −35 region of the *E. coli* σ^{70}-RNAP promoters.
3. A homopyrimidine box TCCTCC downstream of the −35 region, which has been previously reported for promoters with longer than usual spacer length [32].
4. A −44 region between nucleotides 273 and 278, TTATTC, which shows only one nucleotide difference with TTTTTC of the −44 region previously reported as typical of the *E. coli* σ^{70}-RNAP promoters [29,33].
5. A −54 region between nucleotides 262 and 267, TTTTTT, which is identical to one of the −54 regions previously reported as typical of the *E. coli* σ^{70}-RNAP promoters [29,33].

These homologies, together with the fact that major RNA polymerases of Gram-positive bacteria have consensus nucleotide sequences very similar to those of *E. coli* promoters [34–39], strongly suggest that fragment X8 possesses a real promoter.

However, this may not be the case for X1, X2 and X3 which might be random sequences that act as good promoters. Further studies are necessary to evaluate this possibility.

Using hybridization techniques, fragment X8 was demonstrated to belong to the *S. aureus* bacteriophage φ 85 genome (not shown). According to its exceptional strength, the promoter of this DNA fragment may direct the synthesis of a highly expressed phage component. However, sequence data to corroborate this are not yet available in databases.

Although putative promoters were recognized in the X1, X2, X3 and X8 DNA fragments, it is possible that the inserted DNA sequences may be acting as enhancer elements of the basal transcriptional activity of the chloramphenicol promoter or the tetracycline divergent promoter in **pProm** (see above). In order to rule out this possibility, we removed these promoters in **pProm**-X1, X2, X3 and X8 by deletion. For this, plasmids were first digested with the *Sal*I restriction enzyme that releases two fragments, one of them containing both promoters, the chloramphenicol and tetracycline resistance genes, and the ORI for Gram-positive bacteria and the other containing the activated ampicillin gene and the ORI for Gram-negative bacteria (Fig. 2). Recircularization of this last fragment resulted in plasmids with only one resistance marker (ampicillin). The ampicillin resistance levels were about the same in cells transformed with deleted or undeleted plasmids (not shown), a fact that supports the idea that the inserted DNA fragments are acting as promoters.

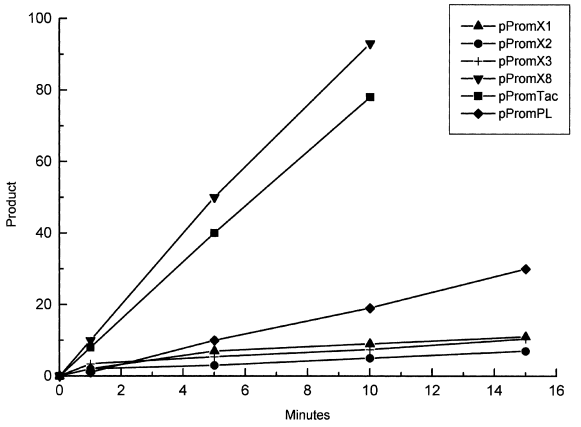

Fig. 4. Penicillinase assay of *E. coli* clones transformed with **pProm** derivatives containing DNA inserts which activated the promoterless ampicillin gene.

3.3. Promoter strength evaluation

The penicillinase activity of cell suspensions was measured in order to assess the relative efficiencies of the isolated promoter sequences (Fig. 4). According to this, the promoter present in the X8 DNA fragment is stronger than the well-known P_{Tac} and Lambda P_L promoters. This result confirms our initial expectation that very strong promoters could be isolated using the **pProm** vector and opens the possibility of collecting many such promoters.

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