A New Hybrid Promoter and Its Expression Vector in *Escherichia coli*

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A new hybrid promoter, "pac", comprising the ‘−35’ region of the bacteriophage T5 P25 gene promoter and the ‘−10’ and the operator regions of the lacUV5 promoter, was chemically synthesized and used to construct a new expression vector. The activity of the hybrid promoter was compared with that of the tac (trp: lac fusion) promoter, which is widely used as a strong and controllable promoter. The activity of the pac promoter was found to be stronger by about 3-fold than that of tac when assayed with the chloramphenicol acetyltransferase (CAT) system. The pac promoter, however, was not repressed as efficiently as the tac promoter.

With the development of techniques in gene engineering, useful proteins and peptides which had been prepared in small amounts can be produced easily and in large amounts in bacterial cells. In the production of a foreign gene product in bacteria, however, the elements which are necessary for its production must be replaced with those effective in bacterial cells. One of those most important elements is the promoter.

Sequences of a large number of promoters in *E. coli* and coliphages are known and many of these have been used for many purposes.\(^1\) Promoters are divided into two types with regard to the mechanism of expression, i.e. constitutive expression and controllable expression. Constitutive promoters include *lpp*,\(^2\) *bla*,\(^5\) and phage T5 P25 promoters,\(^6,7\) which are relatively strong, and controllable promoters include *lac*,\(^8,9\) *lacUV5*,\(^10\) *trp*,\(^11\) and lambda *P*,\(^12\) promoters, which can be induced under certain conditions.

At present, it is difficult to correlate the strength of a promoter with its primary structure despite the known sequences of more than 50 promoters.\(^13\) Comparison of these sequences, however, has revealed two consensus regions; 5′TATAAT3′ at the ‘−10’ region\(^14,15\) and 5′TTGACA3′ at the ‘−35’ region.\(^16,17\)

Strong and controllable promoters are useful in genetic engineering. A good example of a strong and controllable promoter is *tac*, which was constructed as a fusion promoter of the *trp* (the ‘−35’ region) and the *lacUV5* (the ‘−10’ region).\(^18,19\) The ‘−35’ region of the *trp* promoter and the ‘−10’ region of the *lacUV5* promoter have consensus sequence of *E. coli* promoters. The *tac* promoter can be under a control of the *lacI* gene\(^20\) and induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG),\(^21\) because the *tac* promoter has a *lac* operator region. On the basis of these facts, *tac* is commonly used as a strong and controllable promoter for the expression of many foreign genes. On the other hand, promoters in bacteriophage T5 early genes P25 and P26 are known to have a strong affinity for RNA polymerase.\(^22,23\)

Since these T5 promoters are very strong and expressed constitutively, a transcriptional terminator should be set downstream for a stable cloning.\(^24\)

In this study, we chemically synthesized the hybrid promoter *pac*, consisting of the ‘−35’ region of the strong T5 P25 gene promoter and the ‘−10’ and the operator regions of the *lacUV5* promoter, examined its regulation by
the lacI gene, and compared its promoter activity with that of tac.

**MATERIALS AND METHODS**

*Escherichia coli* strains. HB101<sup>25,26</sup> (hsdS20, recA13, ara-14, proA22, lacY1, galK2, rpsL20 (Sm), xyl-5, mtl-1, supE44) was used in the construction of pac promoter and for chloramphenicol acetyltransferase assay. JM109<sup>26</sup> (recA1, {\it flac pro, endA1, gryA97, thi-1, hsdR17, supE44, relA1, F<sup>+</sup>; trdD36, proAB, lacI<sup>8</sup>, Z M15) was used in the constructions of plasmids pMTI2 (pac) and pUSI2 (tac).

*Media*. M9S medium containing M9 salts mix (5.8 g of Na<sub>2</sub>HPO<sub>4</sub>, 3 g of KH<sub>2</sub>PO<sub>4</sub>, 5 g of NaCl, and 1 g of NH<sub>4</sub>Cl per liter), 0.4% casaminoacid, 0.4% glycerol, 1 mm MgCl<sub>2</sub>, 1 mm CaCl<sub>2</sub>, and 20 mg of ampicillin per liter was used to grown cells for the expression of chloramphenicol acetyltransferase, and for the isolation of plasmid DNAs. L plates (10 g of Bactotryptone, 5 g of Bactoyeast extract, and 10 g of NaCl per liter) containing ampicillin 20 mg/l were used for the selection of the transformants.

*Enzymes.* All the enzymes used in this study were purchased from Toyobo Co., Ltd., and used under the conditions recommended by the supplier.

*DNA sequencing.* DNA sequencing was done by the dideoxy methods.<sup>25,27</sup> The sequencing primers (16 mer) were purchased from Takara Shuzo.

*Plasmids.* pMC9,<sup>28</sup> carrying a 1.7-Kbp EcoRI DNA fragment which codes for the lacI gene, was obtained from Promega Co., Ltd. pCM4,<sup>29</sup> carrying a 750-bp BamHI DNA fragment of chloramphenicol acetyltransferase (CAT) cartridge, was purchased from Pharmacia Co., Ltd.

*Transformation.* The bacteria were transformed by the CaCl<sub>2</sub> method.<sup>25</sup>

*Culture conditions.* *E. coli* cells harboring the appropriate plasmid were cultured overnight at 30°C with shaking in M9S medium. One ml of the culture was inoculated into 50 ml of fresh medium, which was incubated at 30°C with shaking. After two hours, IPTG was added to a final concentration of 2 mm to induce the promoter function, and the incubation was continued for four hours. Then, the cells were harvested by centrifugation at 6500 rpm for 10 min. The products in the cell pellets were analyzed.

*SDS polyacrylamide gel electrophoresis.* A portion of the collected cells was dissolved in Laemmlis sample buffer,<sup>30</sup> and analyzed by sodium dodecyl sulfate (SDS) 12.5% polyacrylamid gel electrophoresis (PAGE).<sup>30</sup>

*Densitometric analysis of the gel.* After SDS-PAGE, the gel was stained with Coomassie Brilliant Blue. Densitometric scanning of the stained gel was done by a densitometer (Dual-Wavelength TLC Scanner, CS-930, Shimadzu).

*Chloramphenicol acetyltransferase assay.* Chloramphenicol acetyltransferase (CAT) was assayed by the method of Close et al.<sup>29,31</sup> One unit of the enzyme activity was defined as the amount of enzyme required to acetylate one nanomole of chloramphenicol per minute.

*The N-terminal amino acid sequence analysis.* After SDS-PAGE, the gel was stained with Coomassie Brilliant Blue. The stained protein band was cut out of the gel and the protein was purified by electro-elution by the method of Hunkapiller et al.<sup>32</sup> Automated N-terminal amino acid sequence analysis of the purified protein was done with a gas-phase microsequenator (Applied Biosystems, Model 470A). The phenylthiohydantoins were analyzed by reversed-phase HPLC.

**RESULTS**

*Construction of the pac promoter.*

The pac promoter was designed as shown in Fig. 1, and constructed from four single-stranded DNA fragments which were synthesized with an Applied Biosystems Model 380A DNA synthesizer, phosphorylated with T4 polynucleotide kinase, annealed, and cloned between an EcoRI and a HindIII site of plasmid pBR322. As the tetracyclin resistant gene of pBR322 has its own promoter between the EcoRI and the HindIII sites, the resistance would be regenerated when a DNA fragment having the promoter activity was inserted at this position. One tetracyclin resistant clone was selected, and a plasmid was isolated from it and named pBRpac. The DNA sequence of the inserted EcoRI–HindIII fragment of plasmid pBRpac was confirmed as described under MATERIALS AND METHODS.

*Construction of an expression vector using the pac promoter.*

pBRpac was digested with EcoRI and BamHI, and a 73-bp DNA fragment was isolated. The EcoRI–BamHI fragment was then ligated to a plasmid pUS2 (tac) (T. Shibui, manuscript in preparation) previously linearized with EcoRI and BamHI to construct pMT2 (pac). To regulate the activity of the pac promoter in any *E. coli* strain, a 1.7-Kbp
**A New Hybrid Promoter Expression Vector**

**FIG. 1.** A): The Promoter Sequences of T5 P25, pac, lacUV5, tac, and trp. The ‘-35’ and the ‘-10’ regions are underlined. Arrows indicate the junction points of the components in the hybrid promoters. --- indicate the lac repressor binding site (lac operator).

B): The DNA Sequence of the Chemically Synthesized pac Promoter.

The left half (37-bp) is mainly from the T5 P25 gene promoter and the right half (43-bp) is mainly from the lacUV5 promoter.


*Abbreviations:* lpp3', *E. coli* lipoprotein gene 3'-region including transcriptional terminator; T4 DNA pol, T4 DNA polymerase; B, BamHI; Bg, BglII; E, EcoRI; H, HindIII.
EcoRI DNA fragment containing the lacI gene from pMC9 was inserted into the EcoRI site of pMT2 (pac) to construct pMT2 (pac). The same construction was done in pUS2 (tac) to construct pUS2 (tac) (Fig. 2B).

Construction of plasmids pMTCAT (pac) and pUSCAT (tac)

Figure 3 shows the construction of the CAT expression vectors. pMC4 was digested with BamHI and a 750-bp BamHI fragment containing the CAT cartridge was isolated by 5% PAGE by the methods of Maniatis et al.\(^{29}\) This CAT cartridge was inserted into the BamHI sites of pMT2 (pac) and pUS2 (tac) to construct plasmids pMTCAT (pac) and pUSCAT (tac).

Comparison of the promoter activities

_E. coli_ HB101 cells harboring pMTCAT (pac) and pUSCAT (tac) were cultured and treated as described in MATERIALS AND METHODS. Figure 4 shows the Coomassie Brilliant Blue stained SDS–12.5% polyacrylamide gel electrophoresis patterns of _E. coli_ HB101 cells harboring the independently isolated plasmids pMTCAT (pac) [pMTCAT (pac) 1 and 2] and pUSCAT (tac) [pUSCAT (tac) 1 and 2]. The CAT gene product (25 Kd), which was identified by N-terminal amino acid sequencing analysis (data not shown), only appears in lanes of the cells induced with IPTG. From the densitometric analysis of this Coomassie Brilliant Blue stained gel, the CAT gene product was estimated to be 6–7%.

**TABLE I. CAT ACTIVITIES IN E. coli STRAIN HB101 CARRYING VARIOUS PLASMIDS**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Induction</th>
<th>Activity (U/mg prot.) (^{a})</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMTCAT (pac)</td>
<td>– IPTG</td>
<td>758</td>
</tr>
<tr>
<td></td>
<td>+ IPTG</td>
<td>13,515</td>
</tr>
<tr>
<td>pUSCAT (tac)</td>
<td>– IPTG</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>+ IPTG</td>
<td>5,017</td>
</tr>
<tr>
<td>pCM4</td>
<td></td>
<td>1,028</td>
</tr>
</tbody>
</table>

\(^{a}\) These values were average of independently isolated clones (see Fig. 4. legend). The assays were done 3 times each.
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[pUSCAT (tac)] and 10–12% [pMTCAT (pac)] of the total cellular protein. To compare the activities of the pac and the tac promoters more precisely, we assayed CAT activity in the E. coli transformants harboring each plasmid (Table I). With induction by the addition of IPTG, CAT activity expressed in E. coli harboring pMTCAT (pac) was 2.7-fold higher than that expressed in E. coli harboring pUSCAT (tac). Without induction (in the repressed state), however, CAT activity detected in E. coli harboring pMTCAT (pac) was 14-fold higher than that in E. coli harboring pUSCAT (tac). Ratios of CAT activities in induced cells and non-induced cells were 92:1 in pUSCAT (tac) and 18:1 in pMTCAT (pac).

**DISCUSSION**

Promoters of the bacteriophage T5 early genes P25 and P26 have been shown to far exceed other promoters in the rate of complex formation with RNA polymerase.22 Neither of these T5 promoters contains the ‘−35’ region fully matched to the consensus sequence (5’TGGACA3’). The sequence corresponding to these ‘−35’ region is 5’TTGCTT3’ and this sequence is probably important for the high affinity with RNA polymerase. Based on this speculation, we chose the sequence of the ‘−35’ region from the T5 P25 gene promoter to construct a strong promoter. For other parts of the promoter, i.e. the ‘−10’ and the control regions, we chose the lacUV5 promoter and operator regions. As this ‘−10’ region is a consensus sequence of E. coli promoters and the operator region is the binding site of the lac repressor, we were able to construct a strong and controllable promoter by combining these three parts of the promoter elements.

Using a DNA synthesizer, we chemically synthesized these three promoter elements and cloned them between an EcoRI and a HindIII site of PBR322 to construct pBRpac. In the cloning of promoter-active DNA fragments, using these sites is very effective, because if promoter-less DNA fragments were inserted between an EcoRI and a HindIII site of pBR322, E. coli cells harboring these plasmids are not tetracyclin resistant (<1 mg/l) (data not shown). E. coli cells harboring the pBRpac acquired a resistance to tetracyclin (≥20 mg/l), which was conferred by the pac promoter. Using the pac promoter cloned in this way, we constructed a new expression vector pMTI2 (pac).

In construction of pMTI2 (pac) and pUSI2 (tac), we first constructed pMT2 (pac) and pUS2 (tac), which lacked the repressor gene (lacI) for promoters. As the replication origin of these plasmids was derived from pBR322 and the rop (repressor of primer) region33 of pBR322 was removed in the construction of these plasmids, the copy number of these plasmids is relatively high (it was estimated at >40 copies/cell34). In wild type strains, the copy number of lac repressor is about 10–20 per cell.21 As the copy number of plasmids is higher than that of the repressor, pMT2 (pac) and pUS2 (tac) were less stable in wild type strains than in lacI1 strains, which synthesize the lac repressor at 100–200 copies per cell.35 We could construct pMT2 (pac) and pUS2 (tac) only in a lacI strain (i.e. JM109).

To use any other strains than lacI strains, we inserted the lacI gene into pMT2 (pac) and pUS2 (tac) to construct pMTI2 (pac) and pUSI2 (tac). These plasmids can regulate the pac or tac promoter by themselves and we could assay their promoter activity by using the CAT system in HB101 (not lacI strain).

In addition, the cloning sites of these expression vectors have the following character. As the 3’-end of the initiation methionine (Met) codon in the cloning sites can be converted to a blunt end by digestion with KpnI followed by T4 DNA polymerase digestion29 and any 5’blunt ended DNA fragment can be ligated to it, the encoded polypeptide or protein can be expressed directly with only one additional Met to its N-terminus (Fig. 2B).

In comparison of promoter activities using the CAT system, the pac promoter was estimated to be about 3-fold higher than tac in promoter activity, although the repression of
the pac promoter was less strict than tac. It is likely that the affinity of the pac promoter for RNA polymerase may be so strong that the lac repressor could not bind to its operator site as effectively as tac.

To repress the pac promoter activity more completely, we tried to produce more repressors in the cells. We changed the lac gene promoter of pMT12 (pac) to a more effective one, the tetracyclin resistance gene promoter of pBR322. But the repression efficiency did not improve (data not shown). The higher background of the pac promoter activity in the repressed state seems to be not easy to improve, as far as we examined. Inserting another operator into an appropriate locus near the pac promoter might ensure its repression.

As we reported here, the pac promoter and its expression vector are very effective in over-producing a gene product, although its higher background in the repressed state remains to be improved.

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