A Controllable Expression-secretion Vector Constructed from the Multiple trp Promoter-operator, the Signal Peptide Region of the ompF Gene and the trpR Gene in Escherichia coli

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We constructed a hybrid plasmid to allow controlled expression of a gene and the subsequent secretion into the culture medium of the gene product in Escherichia coli. This was achieved by the use of five trp promoter-operator regions in tandem followed by the DNA fragment coding for the signal peptide and the N-terminus of the OmpF protein, and the trpR gene coding for the Trp repressor. Multiplication of the trp promoter-operator appreciably enhanced expression of the gene that followed. A single copy of the trpR gene on the chromosome was insufficient for controlling the enhanced expression. The expression was, however, completely controlled when the trpR gene was cloned onto the same plasmid. When the multiple trp promoter-operator was followed by the DNA fragment coding for the signal peptide and the N-terminus of the OmpF protein that was further followed by the gene for human β-endorphin, a β-endorphin-containing polypeptide was synthesized under the complete control of the trp promoter-operator, and secreted to the culture medium across both the cytoplasmic membrane and the outer membrane. Controlled expression of a foreign gene and subsequent secretion into the medium of the product were thus achieved.

The ompF gene codes for major outer membrane protein OmpF of Escherichia coli. The gene has been cloned,1) sequenced2) and characterized intensively.3,4) OmpF is one of the most abundant proteins in E. coli cells, indicating that the gene is expressed very actively in the cells. In addition, OmpF is a secretory protein in that it has to be exported across the cytoplasmic membrane to reach the outer membrane. On the basis of these facts, the use of the ompF gene as an expression-secretion vector has been investigated.

Recently Nagahari et al.5) constructed a hybrid plasmid in which the gene for human β-endorphin is preceded by the upstream region of the ompF gene consisting of the promoter region and the coding region for the signal peptide and a few N-terminal amino acid residues of OmpF. Interestingly, the β-endorphin-containing peptide was secreted into the culture medium across both the cytoplasmic and outer membranes. The passage through the cytoplasmic membrane was found to be a signal peptide-dependent process, whereas the mechanism of the outer membrane penetration remains unclear. Although these results indicate the usefulness of the ompF gene for the construction of an expression-secretion vector, the gene has a disadvantage in terms of regulation. Expres-
sion of the ompF gene can be controlled by the osmolarity of the growth medium\(^6,7\): low expression in a medium containing a high concentration of sucrose or NaCl, but derepressed when the culture medium is deprived of such supplements. The regulation is often incomplete, however, and derepression by removal of such supplements from the medium is not practicable.

In the present work, we constructed hybrid plasmids to allow strictly inducible expression of the ompF gene by insertion of the trp promoter-operator (trpPO) as a transcriptional control switch. We further inserted trpR, the gene coding for the repressor of the tryptophan operon (Trp repressor), into the plasmid. In the presence of both trpPO and trpR on a plasmid, high expression of the ompF gene was perfectly controlled. Finally a gene coding for human \(\beta\)-endorphin was inserted downstream of the DNA fragment coding for the OmpF signal peptide. trpPO-controlled synthesis and subsequent secretion into the culture medium of a \(\beta\)-endorphin-containing peptide were achieved.

**MATERIALS AND METHODS**

**Bacteria and plasmids.** *E. coli* K-12 strains

<table>
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<tr>
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<tr>
<td>HO201</td>
<td>ompF14 envZ derivative of HO201;</td>
<td>(9)</td>
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<tr>
<td>YO180</td>
<td>spontaneous Tula-resistant mutant</td>
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<tr>
<td>YO181</td>
<td>mal(^-) envZ(^+) transductant of YO180; donor, JF568</td>
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</tr>
<tr>
<td>N99</td>
<td>weaker protease activity</td>
<td>(5, 15)</td>
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**Plasmids**

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<td>P. L. Biochemicals</td>
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<td>pNM100</td>
<td>Ap(^+); cloned gene: ompF-(\beta)E</td>
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Ap, Ampicillin; Tc, tetracycline; \(\beta\)E, the gene coding for human \(\beta\)-endorphin.

**DNA manipulations.** Procedures used for DNA mani-
Controllable Expression-secretion Vector

Fig. 1. Structures of trpPO-ompF Chimeric Genes.

A: The promoter structure of the ompF gene is shown. The mRNA start site is numbered +1. AUG starting at +111 is the initiation codon for OmpF translation. A and B represent the promoter region, the untranslated leader region and the translated region of the ompF gene, respectively. The −35 region (−35), Pribnow box (PB), and the Shine-Dalgarno sequence (SD) are also indicated.

B: Upstream ends of the trpPO-ompF chimeric genes. The trpPO-carrying DNA fragment was inserted into the promoter-deficient ompF gene with various lengths of the untranslated leader region. A represents the trpPO-carrying fragment. In pIAT140, five trpPO-carrying fragments were arranged in tandem in the same orientation. Other symbols are the same as those for A.

Bunulations were described previously. Restriction endonucleases, and T4 DNA polymerase and T4 DNA ligase were purchased from Takara Shuzo Co., and Toyobo Biochemicals, respectively. The conditions for restriction endonuclease digestions were those recommended by the commercial suppliers. Bal31 was obtained from Bethesda Research Laboratories.

**Assaying of β-endorphin.** A radioimmunoassay (RIA) kit from New England Nuclear was used for assaying of β-endorphin-containing peptides.

**RESULTS**

**trpPO-controlled expression of OmpF**

For expression of the intact ompF gene, the domain consisting of about 110 nucleotides upstream of the mRNA start site and about 17 nucleotides downstream of the start site is indispensable (see Fig. 1). The role of the region between this domain and the translation start site is unclear. trpPO-ompF hybrid genes in which the ompF promoter is replaced by trpPO were constructed previously. Some of them, shown in Fig. 1, were tested as to repression by tryptophan and derepression by indoleacrylic acid, together with pIAT500 that was constructed in the present work (Fig. 2).

For all the hybrid genes, the OmpF synthesis was stimulated by indoleacrylic acid and suppressed by tryptophan, indicating that the expression was under the control of trpPO. OmpF synthesis, at maximum in the presence of indoleacrylic acid and the absence of tryptophan, was suppressed in the absence of indoleacrylic acid and the presence of tryptophan.

**Effect of the copy number of trpPO**

In the course of the construction of the hybrid plasmids, pIAT140 carrying multiple trpPO in tandem was obtained. In this plasmid, the ompF gene was preceded by five successive trpPO that were arranged in the same orientation (Figs. 1 and 3). The number and the orientation of trpPO were determined by restriction endonuclease analyses with BglII and PvuI.

Introduction of the multiple trpPO appreciably enhanced the OmpF synthesis (Fig. 4). The synthesis was, however, only partially suppressed even in the presence of tryptophan and the absence of indoleacrylic acid, probably due to a shortage of the Trp repres-
Fig. 2. Expression of trpPO-ompF Chimeric Genes. YO181 (ompF) harboring the indicated plasmids was grown in the presence or absence of tryptophan (Trp) (100 µg/ml) and 3-β-indoleacrylic acid (IAA) (20 µg/ml) as indicated. Outer membrane proteins were prepared and analyzed on polyacrylamide gel. The positions of the OmpC, OmpF and OmpA proteins are indicated by △, ▲ and ○, respectively.

Construction of pIAT141 carrying the multi-copy trpPO and the trpR gene

We, therefore, introduced into pIAT140 the trpR gene coding for Trp repressor (Fig. 3). Plasmid pIAT141, thus constructed, expressed a huge amount of OmpF in the presence of indoleacrylic acid (Fig. 4). It accounted for more than 60% of the total envelope proteins. It should be noted that such high level expression was almost completely suppressed when indoleacrylic acid was removed from the culture medium and replaced by tryptophan.

The switching-on of gene expression by the removal of a substance (tryptophan in this case) is not convenient. We sought, therefore, conditions under which switching from complete suppression to high expression can be achieved simply by the addition of indoleacrylic acid. Figure 5 shows that the OmpF synthesis, that was completely suppressed in the presence of 40 µg/ml of tryptophan, was derepressed significantly by the addition of indoleacrylic acid (40 ~ 80 µg/ml). The amount of OmpF thus expressed was nearly as high as that reached in the absence of tryptophan (see Fig. 4). This high level expression of OmpF did not affect the bacterial growth rate. Higher concentrations of indoleacrylic acid resulted, however, in slower bacterial growth (data not shown).

Construction of a hybrid plasmid, and expression and secretion of β-endorphin under the control of trpPO and the OmpF signal sequence

We previously demonstrated the secretion into the culture medium of a human β-endorphin-containing peptide by the use of a hybrid gene consisting of the ompF gene and the gene for β-endorphin.5) The expression was ompF promoter-dependent, and the secretion was OmpF signal peptide-dependent. In the present work, we constructed a hybrid plasmid, pIAT141-βE, in which the β-endorphin gene was linked in frame to the preceding trpPO-ompF fragment, as shown in Fig. 6. The OmpF-β-endorphin fused peptide, which was expected to be synthesized, is also shown in Fig. 6. The plasmid thus constructed efficiently directed the indoleacrylic acid-induced synthesis of the β-endorphin-containing peptide in the E. coli N99 cells (Fig. 7), which indicates that the expression was controlled by trpPO. The peptide predominantly accumulated in the culture medium; accumulation in the periplasmic space and the cytosol being negligible.

DISCUSSION

We constructed a hybrid plasmid to allow in
Fig. 3. Construction of pIAT141 Carrying the Multicopy trpPO and the trpR Gene.

The BamHI small fragment from ptrpR3 was treated with S1 exonuclease and inserted into the StuI site of pIAT140 by blunt-end ligation. □ and ▨ represent the regions of the ompF gene coding for the signal peptide and the OmpF protein, respectively. ▨ represents the trpR gene. ▫ represents trpPO and ▫ represents the tandem repeat of five trpPO. Restriction endonuclease cleavage sites are shown by the following abbreviations: Ba, BamHI; S, StuI; Bg, BglII; E, EcoRI.

Fig. 4. Effect of the Multiplicity of trpPO and the Cloned trpR Gene on the Expression of the OmpF Protein.

YO180 (ompF) harboring pIAT100(A), pIAT140(B) and pIAT141(C) was grown in the presence or absence of tryptophan (Trp) (100 μg/ml) and 3-β-indoleacrylic acid (IAA) (20 μg/ml) as indicated. Analyses of outer membrane proteins on polyacrylamide gel were carried out as described in the legend to Fig. 2. The positions of the OmpC, OmpF and OmpA proteins are indicated.

Fig. 5. Switching off and on of OmpF Expression with Tryptophan and 3-β-Indoleacrylic Acid in pIAT141-Harboring YO180.

YO180 (ompF) harboring pIAT141 was grown in media containing the indicated concentrations (μg/ml) of tryptophan (Trp) in the absence of 3-β-indoleacrylic acid (A) or in media containing tryptophan (40 μg/ml) and the indicated concentrations of 3-β-indoleacrylic acid (IAA) (B). Outer membrane proteins were analysed as described in the legend to Fig. 2. The positions of the OmpC, OmpF and OmpA proteins are indicated.
Fig. 6. Construction of a Hybrid Plasmid Expressing $\beta$-Endorphin under the Control of trpPO and the OmpF Signal Sequence.

A: A series of treatments with enzymes was carried out in one test tube without fraction of the DNA fragments. BAP, bacterial alkaline phosphatase; and $\square$ and $\Box$ represent the DNA regions coding for the ompF signal peptide and the OmpF protein, respectively. $\square$ represents the linker DNA and the presequence of the $\beta$-endorphin gene, and $\Box$ the $\beta$-endorphin gene (for details see ref. 9). $\Rightarrow$ represents the tandem repeat of five trpPO and $\Rightarrow$ the ompF promoter region. $\Box$ represents the ompR gene. The region responsible for ampicillin resistance (bla) is also indicated. Restriction endonuclease cleavage sites are shown by the following abbreviations: Ps, PstI; B, BglII; Pv, PvuII.

B: Structure of the OmpF-$\beta$-endorphin fused peptide (precursor form) deduced from the DNA sequence. The peptide from Met$^{-22}$ to Ala$^{1}$ is the signal peptide of the OmpF protein. The first amino acid of the mature OmpF protein is numbered +1. Human $\beta$-endorphin is from Tyr$^{41}$ to Glu$^{71}$. 
Controllable Expression-secretion Vector

**Fig. 7.** *trp*PO-Controlled Expression and Secretion of a β-Endorphin-containing Peptide.

E. coli N99**†** harboring pIAT141-βE was precultured in L-broth containing 40 μg/ml of tryptophan at 37°C. A one-twentieth volume of the preculture was transferred to medium of the same composition. After about 2 hr-cultivation, indoleacrylic acid was added to the final concentration of 80 μg/ml (time 0; also indicated by an arrow). Then a portion of the culture was withdrawn periodically, and the amounts of β-endorphin in the culture media (○), the periplasm (▲) and the cytosol (△) were determined. Cell growth was followed densitometrically and expressed in Klett units (---□---).

**E. coli** the controlled high-expression of the *ompF* gene and of the gene for human β-endorphin, and the subsequent secretion of the gene products. This was achieved by incorporation into a plasmid of DNA fragments carrying, respectively, 1) five copies of *trp*PO arranged in tandem in the same orientation, 2) the signal peptide region of the OmpF protein, and 3) the *trpR* gene coding for the Trp repressor.

*trp*PO has been widely used as a transcriptional control switch. The use of the multiple *trp*PO for efficient gene expression has also been studied.¹⁶ When the multiple *trp*PO was used, however, effective control of gene expression was difficult, probably due to a shortage of the Trp repressor (see Fig. 4). To achieve high-expression of a gene with effective control, we constructed a hybrid plasmid carrying both the multiple *trp*PO and the *trpR* gene. In pIAT141 thus constructed, the expression of the *ompF* gene was very high and completely controlled by tryptophan and indoleacrylic acid, a co-repressor and derepressor, respectively. Furthermore, effective control could be achieved only by the addition of indoleacrylic acid, when an appropriate concentration of tryptophan was chosen. Controllable expression plasmid vectors have been also constructed using the lac promoter-operator¹⁷ and the lambda Pₗ promoter¹⁵ in combination with their repressor genes.

The *ompF* gene has been successfully used for the construction of an expression-secretion vector.⁵ Human β-endorphin was specifically secreted into the culture medium across both the cytoplasmic membrane and the outer membrane of the host *E. coli* cells, when the gene coding for β-endorphin was inserted downstream of the DNA fragment coding for the OmpF signal peptide. In the present work, the gene coding for β-endorphin was inserted into pIAT141 downstream of the DNA fragment coding for the OmpF signal peptide. The controlled accumulation in the culture medium of β-endorphin was observed. Thus the controlled expression of a foreign gene and the subsequent secretion into the medium of its product was achieved. The use of a high copy-numbered plasmid for the construction of such a vector has so far been unsuccessful.

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**REFERENCES**