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A novel expression vector for high-level synthesis and secretion of foreign proteins in *Escherichia coli*: overproduction of bovine pancreatic phospholipase A_2

(Recombinant DNA; T7 promoter; OmpA signal peptide; phospholipase A_2; prophospholipase A_2)

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SUMMARY

A novel expression plasmid (pTO-N) has been constructed that allows for the production of large quantities of foreign proteins (or fragments thereof) in an unfused state. The vector has a strong and tightly regulated T7 gene *10* promoter and the *ompA* Shine–Dalgarno (SD) sequence, followed by the *ompA* sequence and a cloning linker region. The mRNAs produced by the vector are protected by secondary structures at both ends of the mRNAs. The OmpA signal peptide directed the synthesized proteins into the periplasmic space of *Escherichia coli*. Phospholipase A_2 and prophospholipase A_2 from bovine pancreas have been produced to a high level by using this expression vector. One additional feature, which is essential for the stable maintenance of the plasmid in the *E. coli* expression host, BL21(DE3)[pLysS], is the shortened distance between the 5′ secondary structure sequence (immediately following the gene *10* promoter) and the SD sequence. This vector could be particularly useful for synthesis of toxins in *E. coli*.

INTRODUCTION

Although PLA2 from porcine pancreas has been produced in *E. coli* (De Geus et al., 1987) and that from porcine and bovine pancreas in yeast (Van den Bergh et al., 1987; Tanaka et al., 1988), the yield has been quite low. We have reported (Noel and Tsai, 1989) the design and synthesis of a gene coding for bovine pancreatic proPLA2 (fused to the *ompA* gene; Movva et al., 1980) and its high-level expression in *E. coli*. However, the expression system (pJPNPLA2) was not tightly regulated and leaky expression occurred even before induction by IPTG. Since the synthesized fusion product is lethal to the cells, high-level expression cannot be achieved in large-scale culture. The

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Abbreviations: Ap, ampicillin; bp, base pair(s); Cm, chloramphenicol; dATPαS, 2′-deoxyadenosine 5′-*O*-(1-thiotriphosphate); *ϕ10*, phage T7 promoter preceding gene *10* in T7 DNA; IPTG, isopropyl-β-D-thiogalactopyranoside; kb, kilobase(s) or 1000 bp; LCA, Luria–Bertani medium (pH 7.5) consisting of 1% tryptone/0.5% yeast extract/0.5% NaCl/0.4% glucose/15 μg Cm per ml/25 μg Ap per ml; nt, nucleotide(s); *ori*, origin of DNA replication; PAGE, polyacrylamide-gel electrophoresis; PLA2 (or pla2 in Figs.), phospholipase A_2; PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; RF, replicative form; SD, Shine–Dalgarno; SDS, sodium dodecyl sulfate; ss, single strand(ed); *Tϕ*, transcription terminator that follows gene *10*; [ ], denotes plasmid-carrier state.

aim of this study was to construct an expression vector which allows high-level synthesis and secretion of bovine pancreatic proPLA2 and PLA2 in *E. coli*.

RESULTS AND DISCUSSION

(a) General methods

Standard methods were used for DNA manipulation (Maniatis et al., 1982). DNA fragments were electrophoresed and isolated by a Geneclean kit (BIO 101) (for fragments > 350 bp) or by electroelution (for fragments < 350 bp). Dideoxy sequencing (Sanger et al., 1977) was performed on ss M13 templates using the sequenase system (U.S. Biochemical) and [^{35}S]dATPαS (Amersham). Site-specific mutagenesis was performed according to Nakamaye and Eckstein (1986) using the Amersham kit.

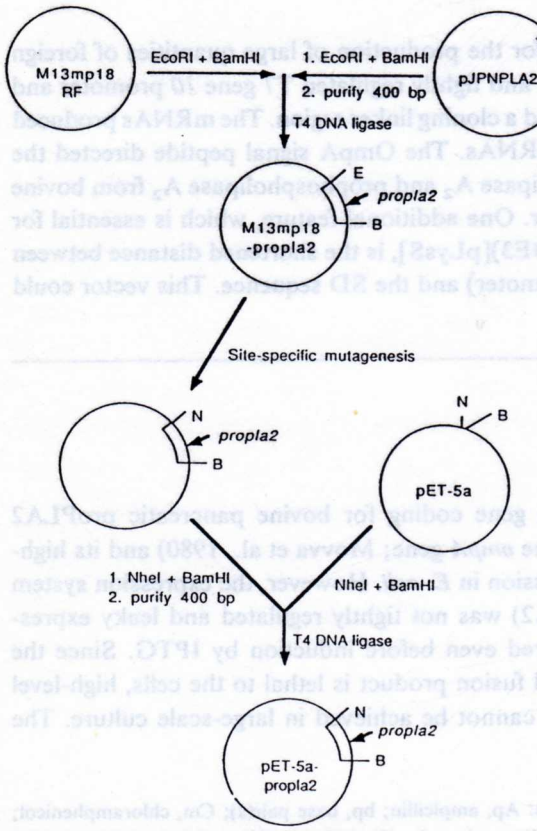

Fig. 1. Construction of pET-5a-propla2. The *Eco*RI-*Bam*HI fragment (containing the coding sequence for proPLA2) from pJPNPLA2 was cloned into M13mp18. The ss DNA was isolated and used for site-specific mutagenesis (using primer 5′–CTCAAAGCTAGCAATTCG) to create a *Nhe*I site immediately upstream from the *Eco*RI site (*Eco*RI site was destroyed by the mutation). The *Nhe*I-*Bam*HI fragment, which would allow in-frame fusion of the proPLA2-encoding gene with the reading frame of pET-5a, was then cloned into pET-5a to generate pET-5a-propla2. Sites: B, *Bam*HI; E, *Eco*RI; N, *Nhe*I.

(b) Initial steps to improve the system

Attempting to overcome the problem of regulation, we inserted the *ompA-proPLA2* gene into some other, more tightly regulated, expression systems such as λ-based expression vectors (Rosenberg et al., 1983). However, leaky expression still occurred significantly enough to destabilize the system (data not shown).

One of the factors for lethality could be the presence of the OmpA signal peptide, which is not cleaved in some of the expressed proteins. The signal peptide (in the fused protein) may insert into the inner membrane of bacteria and affect the normal function of the membrane. To test this idea, we inserted *proPLA2* gene without the *ompA* sequence (from pJPNPLA2) into pKK-233-2 (Amann and Brosius, 1985). However, the level of expression was very poor due to two possible reasons: the promoter (*trc*) of pKK-233-2 is weak, or the mRNA produced by pKK-233-2-propla2 is degraded because of the lack of protective secondary structure(s).

Recently, Studier and coworkers developed a set of expression vectors (the pET series) carrying the strong pro-

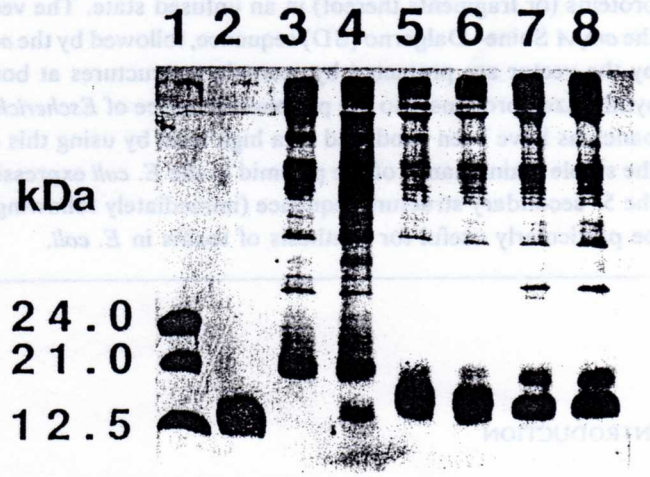

Fig. 2. 0.1% SDS–20% PAGE analysis. Lanes: **1**, M_r standards; **2**, mature PLA2; the following lanes are whole-cell lysates containing different plasmids: **3**, pTO-A2M, no induction; **4**, pET-5a-propla2, 5 h induction; **5**, pTO-propla2, 3 h induction; **6**, pTO-propla2, 5 h induction; **7**, pTO-A2M, 3 h induction; **8**, pTO-A2M, 5 h induction. Note that the proPLA2 bands in lanes **5** and **6** run slightly higher than the PLA2 bands due to slightly higher M_r, and that the weaker band (17 kDa) next to the proPLA2 or PLA2 band in lanes **5–8** arises from the corresponding pre-enzyme carrying the signal peptide. **Procedure**: a single colony of the *E. coli* host strain BL21(DE3)[pLysS] transformed with different expression plasmids was used to inoculate 250 ml of LCA medium which was then grown overnight in a 37°C shaker. The culture was used to inoculate 8–12 liters of LCA, which was again grown at 37°C. When the absorbance of the culture at 550 nm reached 0.8, IPTG was added to a final concentration of 0.25 mM. The culture was then grown for additional 3–5 h. 0.4-ml aliquots of bacteria were pelleted, resuspended in 0.25 ml sample buffer (Laemmli, 1970), boiled for 5 min, and used for SDS–PAGE on a Phast system (Pharmacia). Proteins were detected by $AgNO_3$ staining.

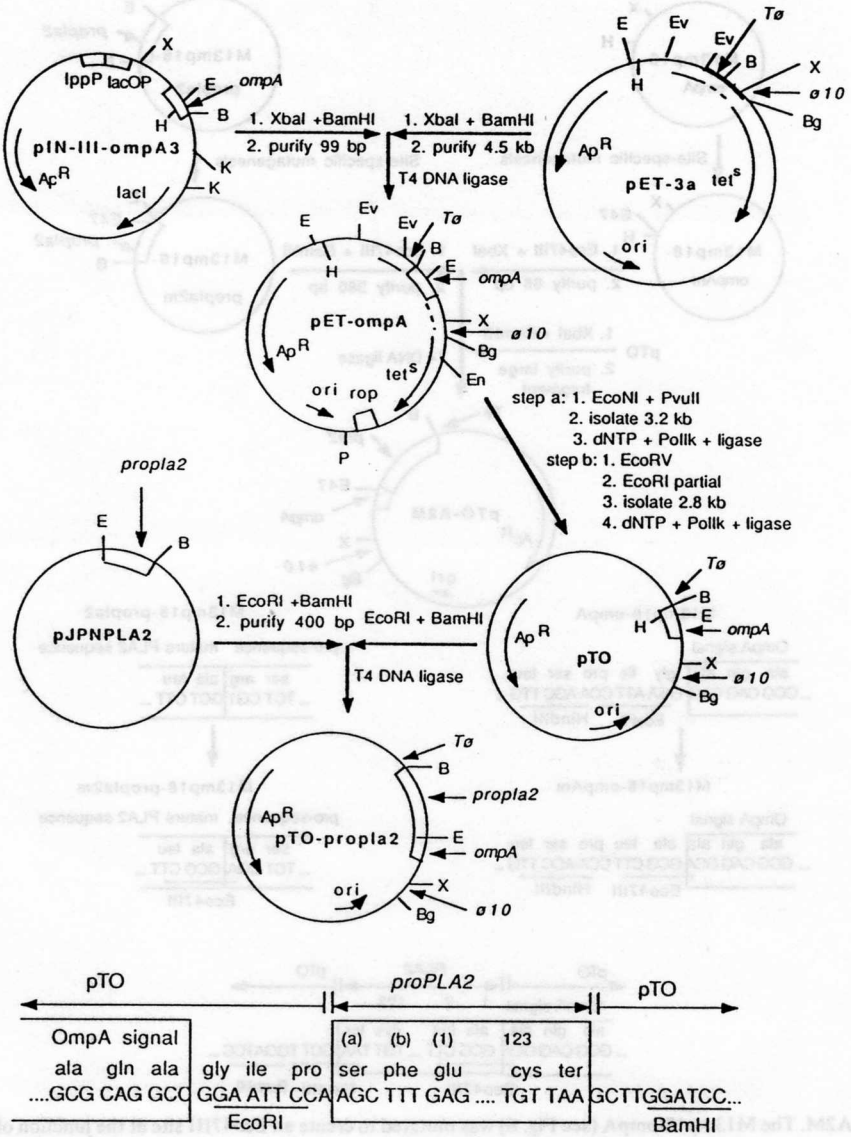

Fig. 3. Construction of pTO and pTO-propla2. The 99-bp *Xba*I-*Bam*HI fragment (containing the SD and the *ompA* sequences) from pIN-III-ompA3 (Ghrayeb et al., 1984) was used to replace the small *Xba*I-*Bam*HI fragment in pET-3a. The new plasmid pET-ompA was further modified by two steps. (a) The sequence between *Eco*NI and *Pvu*II sites was deleted to remove the *rop* gene. (b) The sequence between the *Eco*RV site at the end of *Tϕ* and the *Eco*RI site located immediately upstream from the *bla* promoter (not the *Eco*RI in the cloning linker region) was deleted to remove both the *Eco*RI and the *Hin*dIII sites outside of the cloning linker region. Sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; En, *Eco*NI; Ev, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; P, *Pvu*II; X, *Xba*I.

moter from the gene *10* of T7 phage (Rosenberg et al., 1987). In addition, the mRNAs produced by these vectors are protected by stem-and-loop secondary structures at both ends. To test the proPLA2 production in this system, the plasmid pET-5a was used to construct a bovine proPLA2 expression vector pET-5a-propla2 as outlined in Fig. 1. As shown in Fig. 2 (lane 4), pET-5a-propla2 directed the synthesis of proPLA2 in BL21(DE3)[pLysS] (Studier et al., 1990) following IPTG induction, but the level of xpression was still low.

(c) Construction of new plasmids pTO, pTO-propla2, pTO-A2M, and pTO-N

Since pET-5a-propla2 can be stably maintained in BL21(DE3)[pLysS], and since the mRNAs should be protected by secondary structures at both 5′ and 3′ ends, the low level expression is unlikely to be due to a low level mRNA for the bovine proPLA2. One possible reason is proteolytic degradation of the expressed proteins. It is therefore desirable to secrete the protein into the periplasmic space where the protease activity is low. Furthermore,

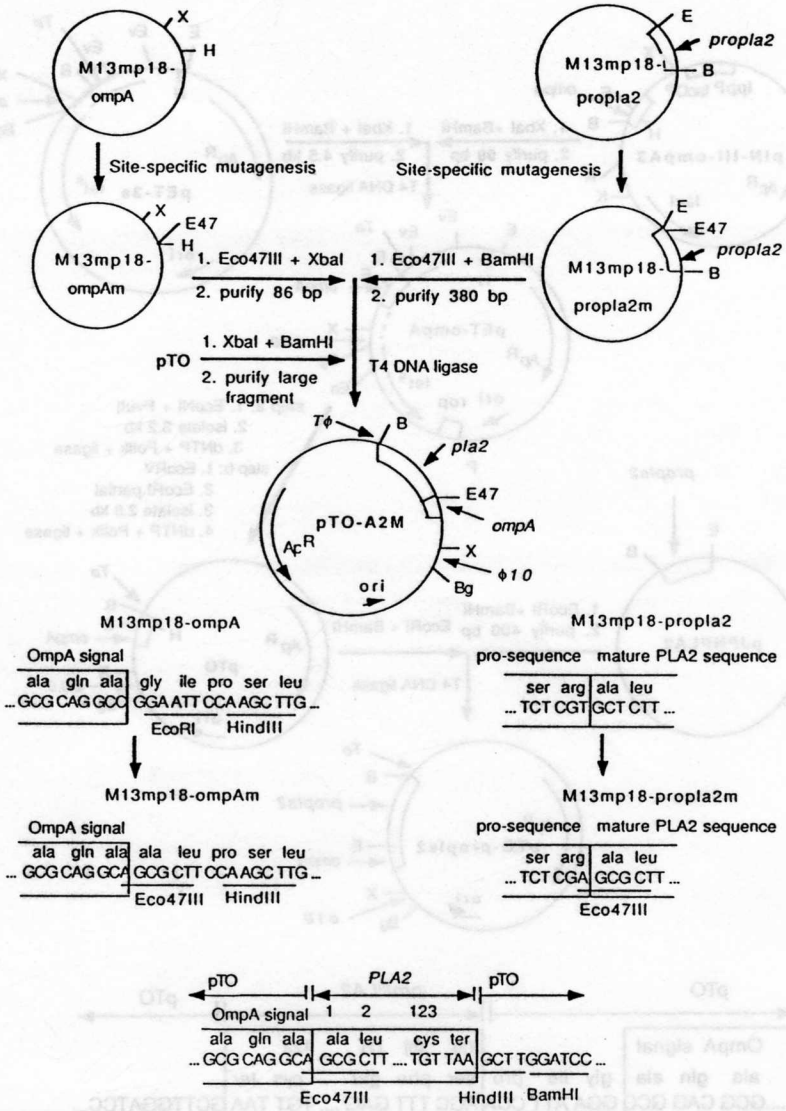

Fig. 4. Construction of pTO-A2M. The M13mp18-ompA (see Fig. 6) was mutated to create an *Eco*47III site at the junction of the OmpA-coding sequence and the *Eco*RI site in the cloning linker region. The M13mp18-propla2 (from Fig. 1) was mutated to create an *Eco*47III site at the junction of the coding sequence for the pro-sequence and the sequence for the mature PLA2. The 380-bp *Eco*47III-*Bam*HI fragment from M13mp18-propla2m and the 86-bp *Eco*47III-*Xba*I fragment from M13mp18-ompAm were cloned into the *Xba*I and *Bam*HI sites of pTO to generate pTO-A2M in NM522 competent cells. Positive clones were obtained by restriction analyses. The lower part of the figure shows the relevant sequences and changes. Sites: E47, *Eco*47III; others are as defined in Fig. 3.

the signal peptide appears to be necessary for high level expression of bovine proPLA2 even when the protein was not secreted (Noel and Tsai, 1989). Thus, we constructed a novel vector, designated as pTO, by combining useful features from both pET and pIN-III-ompA3 (Ghrayeb et al., 1984) vectors. The procedures are outlined in Fig. 3. Since the *ompA* gene is highly expressed in *E. coli* (Movva et al., 1980), use of the SD sequence and the sequence surrounding the AUG start codon of the *ompA* gene should allow efficient expression and secretion of proteins fused downstream from its signal peptide. Removal of the *rop* gene has been shown to increase the copy number of the derived plasmid (Tomizawa and Som, 1984), which in turn increases the synthesis of chicken lysozyme (Miki et al., 1987) but not in other cases (e.g., Hsiung et al., 1989). We found that removal of the *rop* gene indeed improved proPLA2 production to some extent (data not shown).

Cloning of the *proPLA2* gene from pJPNPLA2 into pTO between the *Eco*RI and the *Bam*HI sites in the cloning linker region gave pTO-propla2 as also outlined in Fig. 3. Although proPLA2 is useful for some studies, it is desirable to synthesize mature PLA2 and avoid the low-yield tryptic digestion step in purification. We therefore constructed a new plasmid derived from pTO, designated as pTO-A2M, which contains the coding sequence for mature PLA2 instead of proPLA2. The procedures are outlined and explained in Fig. 4.

To make pTO useful for expressing other eukaryotic

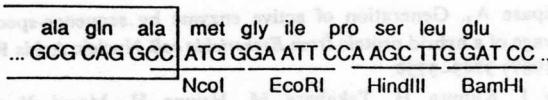

Fig. 5. The nt sequence of the cloning linker region of pTO-N. The complete sequence of pTO-N can be obtained from GenBank (Accession number M34008).

proteins, we created an *Nco*I site in the cloning linker region since this site commonly occurs at the eukaryotic translation initiation site (Kozak, 1983). The new plasmid is designated as pTO-N. The nt sequence of the cloning linker region of pTO-N is shown in Fig. 5. Digestion of *Nco*I site should produce a 4-nt 5′ overhang containing the ATG start codon, and allow direct ligation and expression of a foreign protein as an unfused product after the OmpA signal peptide is cleaved. The cloning linker region can be easily modified to meet a specific cloning requirement when

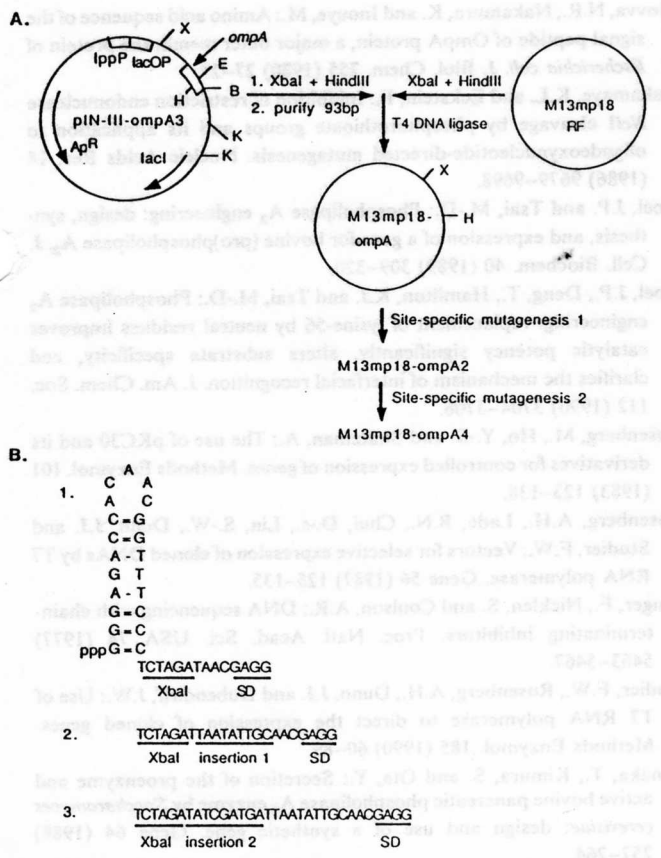

Fig. 6. Construction of expression vectors with different distances between the stem-and-loop sequence and the SD sequence. In panel A, the small *Xba*I-*Hin*dIII fragment (containing the SD and the *ompA* sequences) was cloned into M13mp18 and used for site-specific mutagenesis. The first mutation was to insert a 9-bp sequence between the *Xba*I site and the SD sequence (panel B.2) and the second mutation was to insert additional 9-bp sequence (panel B.3). In panel B.1, the potential stem-and-loop structure of pTO is shown. The *Xba*I-*Hin*dIII fragments from M13mp18-ompA2 and M13mp18-ompA4 were then used to replace the corresponding region in pTO to generate pTD and pTD2, respectively (not shown). Sites are as defined in Fig. 3.

synthesizing a protein or a portion of a protein of which the terminal amino acid residue is not methionine.

(d) Expression of pTO-propla2 and pTO-A2M

Colonies containing either pTO-propla2 or pTO-A2M from LCA plates were used for expression in BL21-(DE3)[pLysS] (Rosenberg et al., 1987). After 1 h induction with IPTG, significant synthesis of proPLA2 or PLA2 could be observed. The expression reached a maximal level in about 5 h. Continued culture of the induced bacteria increased the synthesis of other proteins but not proPLA2 or PLA2. Based on SDS–PAGE, the synthesized proPLA2 or PLA2 was estimated to be more than 25% of total cellular proteins (Fig. 2) under optimal conditions: rich media, good aeration, and use of 15 μg Cm/ml in both plates and media. Most of the expressed proteins were exported to the periplasmic space, and both vectors appear to be stable in the expression host BL21(DE3)[pLysS]. Kinetic properties of purified PLA2 and its mutants obtained from pTO-propla2 have been reported elsewhere (Noel et al., 1990).

(e) Effects of the distance between the 5′ stem-and-loop and the SD sequences

The main significance of the above result is that it has overcome the problem of instability of pJPNPLA2 (Noel and Tsai, 1989) and a λ expression plasmid (T.D., unpublished results) containing the coding sequence for the fusion protein OmpA-proPLA2. Since λ expression system is fairly tightly regulated, it is interesting to know why pTO-propla2 (and pTO-A2M) is stable in our expression system. A possible explanation is that the T7 promoter itself is regulated tightly enough in the expression host for the stable maintenance of pTO-propla2. Another possible reason is that one of the modifications introduced in the construction of pTO, the reduction of the distance between the 5′ secondary structure sequence and the SD sequence (from 28 bp in pET-3a to 10 bp in pTO), could stabilize pTO-propla2 in BL21(DE3)[pLysS]. To test this possibility, we increased the distance between the 5′ secondary structure sequence and the SD sequence in pTO-propla2, first by 9 bp and then by another 9 bp (Fig. 6). The altered region was then used to replace the corresponding region in pTO-propla2 to generate pTD-propla2 and pTD2-propla2, respectively. Both were found to be stable in a non-expressing host such as NM522. When pTD-propla2 was transformed into BL21(DE3)[pLysS], colonies formed on LCA plates. However, when the colonies were used for expression in liquid media, leaky expression occurred and caused lethality. When pTD2-propla2 was transformed into BL21(DE3)[pLysS], no colony formed on LCA plates (using the same competent cells which worked for pTO-propla2 and pTD-propla2). Thus it is clear that the distance

between the 5′ secondary structure sequence and the SD sequence of target mRNA can affect the stability and expression of an expression plasmid in expression hosts.

A possible explanation for the above observation is that the decreased distance reduces the efficiency of recognition of mRNAs by ribosomes possibly by steric hindrance, because the decreased distance brought the 5′ secondary structure sequence closer to the SD sequence, which might reduce the efficiency of the recognition of the SD sequence by ribosomes. Thus, the level of leaky expression of the fusion protein might be reduced to a level which cells could tolerate although the level of leaky transcription of the fusion gene might be significant. When the expression system was induced by IPTG, the level of transcription was so high that the reduced efficiency of translation initiation could still make significant amount of the fusion protein.

pTD-propla2 and pTD2-propla2 can be stably maintained in BL21(DE3)[pLysE]. However, the expression levels were always considerably lower than that achieved by pTO-propla2 in BL21(DE3)[pLysS] even when bacteria containing either pTD-propla2 or pTD2-propla2 were induced by high concentrations (1 mM) of IPTG. Low-level expression is usually the case for other genes in BL21(DE3)[pLysE] (Studier et al., 1990).

Whatever mechanism could be responsible for the stable maintenance and efficient expression of pTO-propla2 in BL21(DE3)[pLysS], the system may be useful for expression of those genes which cannot be stably maintained in BL21(DE3)[pLysS] when placed under the control of T7 promoter (Studier et al., 1990).

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REFERENCES

Amann E. and Brosius, J.: 'ATG vector' for regulated high-level expression of cloned genes in *Escherichia coli*. Gene 40 (1985) 183–190.

De Geus, P., Van den Bergh, C.J., Kuipers, O., Verheij, H.M., Hoekstra, W.P.M. and De Haas, G.H.: Expression of porcine pancreatic phospholipase A_2. Generation of active enzyme by sequence-specific cleavage of a hybrid protein from *Escherichia coli*. Nucleic Acids Res. 15 (1987) 3743–3759.

Ghrayeb, J., Kimura, H., Takahara, M., Hsiung, H., Masui, Y. and Inouye, M.: Secretion vectors in *Escherichia coli*. EMBO J. 3 (1984) 2437–2442.

Hsiung, H.M., Cantrell, A., Luirink, J., Oudega, B., Veros, A.J. and Becker, G.W.: Use of bacteriocin release protein in *Escherichia coli* for excretion of human growth hormone into the culture medium. Biotechnology 7 (1989) 267–271.

Kozak, M.: Comparison of initiation of protein synthesis in prokaryotes, eukaryotes and organelles. Microbiol. Rev. 47 (1983) 1–45.

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227 (1970) 680–685.

Maniatis, T., Fritsch, E.F. and Sambrook, J.: Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.

Miki, T., Yasukochi, T., Nagatani, H., Furuno, M., Orita, T., Yamada, H., Imoto, T. and Horiuchi, T.: Construction of a plasmid vector for the regulatable high level expression of eukaryotic genes in *Escherichia coli*: an application to overproduction of chicken lysozyme. Prot. Eng. 1 (1987) 327–332.

Movva, N.R., Nakamura, K. and Inouye, M.: Amino acid sequence of the signal peptide of OmpA protein, a major outer membrane protein of *Escherichia coli*. J. Biol. Chem. 255 (1980) 27–29.

Nakamaye, K.L. and Eckstein, F.: Inhibition of restriction endonuclease *Nci*I cleavage by phosphorothioate groups and its application to oligodeoxynucleotide-directed mutagenesis. Nucleic Acids Res. 14 (1986) 9679–9698.

Noel, J.P. and Tsai, M.-D.: Phospholipase A_2 engineering: design, synthesis, and expression of a gene for bovine (pro)phospholipase A_2. J. Cell. Biochem. 40 (1989) 309–320.

Noel, J.P., Deng, T., Hamilton, K.J. and Tsai, M.-D.: Phospholipase A_2 engineering: replacement of lysine-56 by neutral residues improves catalytic potency significantly, alters substrate specificity, and clarifies the mechanism of interfacial recognition. J. Am. Chem. Soc. 112 (1990) 3704–3706.

Rosenberg, M., Ho, Y.-S. and Shatzman, A.: The use of pKC30 and its derivatives for controlled expression of genes. Methods Enzymol. 101 (1983) 123–138.

Rosenberg, A.H., Lade, B.N., Chui, D.-s., Lin, S.-W., Dunn, J.J. and Studier, F.W.: Vectors for selective expression of cloned DNAs by T7 RNA polymerase. Gene 56 (1987) 125–135.

Sanger, F., Nicklen, S. and Coulson, A.R.: DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74 (1977) 5463–5467.

Studier, F.W., Rosenberg, A.H., Dunn, J.J. and Dubendirff, J.W.: Use of T7 RNA polymerase to direct the expression of cloned genes. Methods Enzymol. 185 (1990) 60–89.

Tanaka, T., Kimura, S. and Ota, Y.: Secretion of the proenzyme and active bovine pancreatic phospholipase A_2 enzyme by *Saccharomyces cerevisiae*: design and use of a synthetic gene. Gene 64 (1988) 257–264.

Tomizawa, J. and Som, T.: Control of ColE1 plasmid replication: enhancement of binding of RNA I to the primer transcript by the Rom protein. Cell 38 (1984) 191–201.

Van den Bergh, C.J., Bekkers, A.C.A.P.A., De Geus, P., Verheij, H.M. and De Haas, G.H.: Secretion of biologically active porcine prophospholipase A_2 by *Saccharomyces cerevisiae*. Eur. J. Biochem. 170 (1987) 241–246.