*Gene*, 151 (1994) 131–135



GENE 08327

# Use of the tetracycline promoter for the tightly regulated production of a murine antibody fragment in *Escherichia coli*

(Expression vector; *tet* repressor; anhydrotetracycline; induction; immunoglobulin; secretion; *Strep tag*)

Arne Skerra

*Max-Planck-Institut für Biophysik, Frankfurt am Main, Germany*

Received by H.M. Krisch: 26 March 1994; Revised/Accepted: 16 June/28 June 1994; Received at publishers: 4 August 1994

SUMMARY

A generic vector, pASK75, was developed for the synthesis of foreign proteins in *Escherichia coli* under transcriptional control of the *tetA* promoter/operator. Tight regulation was achieved by placing the structural gene for the *tet* repressor, as a transcriptional fusion, downstream from the β-lactamase-encoding gene (*bla*) on the same plasmid. Strong expression of the foreign gene was conveniently induced by adding anhydrotetracycline at a low concentration. Using the production of a recombinant murine immunoglobulin $F_{ab}$ fragment as an example, the system was shown to function independently of the host-strain background and to be extremely well repressed in the absence of the inducer. Thus, it represents an economic and independent alternative to IPTG-inducible promoter constructs. Additional features of pASK75 include a signal sequence and a multiple cloning site followed by a region encoding the *Strep tag* affinity peptide to facilitate purification of a bacterially produced protein.

## INTRODUCTION

Inducible promoter systems have proven generally useful for the production of foreign proteins in *Escherichia coli*, because the synthesized recombinant gene product often has a deleterious effect on bacterial cell growth and viability. Particularly, for proteins secreted into the periplasm it is advisable to ensure tight repression of the promoter not only during vector construction steps, but also in order to achieve high cell densities prior to protein production. The bacterial secretion of antibody fragments is a typical example in this respect, since toxicity and lysis resulting from heterologous gene expression can be readily observed (Plückthun and Skerra, 1989).

Among common promoter systems, the IPTG-inducible *lac* promoter and its derivatives (like the *lac*UV5 mutant or the *tac* fusion promoter) are most prominent (Reznikoff and Gold, 1986) and have frequently been used for the bacterial production of immunoglobulin (Ig) fragments (reviewed in Skerra, 1993). However, the level of transcription from the *lac* promoter depends on the genotype and metabolic state of the host cell because of the endogenous concentration of *lac* repressor molecules on one hand, and the catabolite repression effect on the other. Therefore, significant variations of the level of expression from a given vector may be observed depending on the host strain used. This can be either due to reduced inducibility – especially when the *lac* repressor

*Correspondence to:* Dr. A. Skerra, Max-Planck-Institut für Biophysik, Abteilung Molekulare Membranbiologie, Heinrich-Hoffmann-Straße 7, D-60528 Frankfurt am Main, Germany. Tel. (49-69) 96769-389; Fax (49-69) 96769-423.

Abbreviations: *A*, absorbance (1 cm); Ap, ampicillin; aTc, anhydrotetracycline; *bla*, gene encoding β-lactamase; bp, base pair(s); Ig, immunoglobulin; IMAC, immobilized metal affinity chromatography; IPTG, isopropyl-β-D-thiogalactopyranoside; *lac*, lactose operon; MCS, multiple cloning site; nt, nucleotide(s); OmpA, outer membrane protein A; PAGE, polyacrylamide-gel electrophoresis; PCR, polymerase chain reaction; $^R$, resistance; RBS, ribosome-binding site; SDS, sodium dodecyl sulfate; *Strep tag*, affinity tail of 10 aa (available from Biometre; see Fig. 1b); Tc, tetracycline; *tet*, $Tc^R$ determinant; *tetA*, gene encoding $Tc^R$ function; *tetR*, gene encoding *tet* repressor.

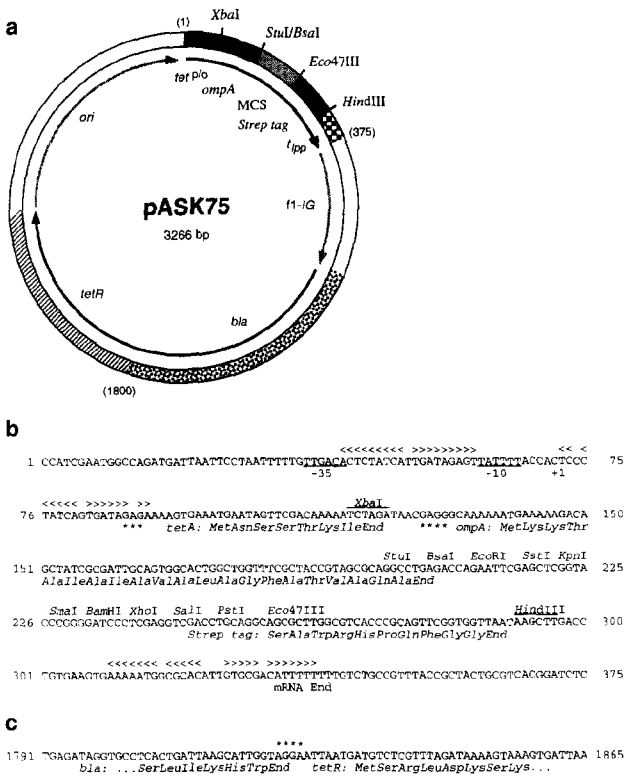


Fig. 1. Features of the pASK75 vector for the expression of foreign genes in *E. coli* under control of the *tetA* promoter/operator. (**a**) pASK75. $tet^{p/o}$, Tn*10* $Tc^R$ promoter/operator region; $t_{lpp}$, lipoprotein transcription terminator; *ori*, origin of DNA replication (ColE1); f1-*IG*, intergenic region of filamentous phage f1. The unique restriction sites flanking the expression cassette, *Xba*I and *Hin*dIII, as well as useful restriction sites for the precise insertion of foreign genes between the *ompA* signal sequence and the *Strep tag* affinity peptide are indicated. (**b**) The nt sequence of the *tet* regulatory region on pASK75, beginning with the *tetA* promoter (−35 and −10 consensus sequences, as well as the transcription start point (+1) are indicated) and ending with the transcription terminator. Palindromic patterns corresponding to the two *tet* repressor-binding sites in the promoter region and to the terminator structure are labeled by arrowed brackets. Putative RBS elements are marked by asterisks. Unique restriction sites in the MCS are shown. Due to conservation of the unique *Xba*I and *Hin*dIII sites (underlined), compatibility with earlier and more specialized constructs, e.g., for the expression of Ig fragments (Essen and Skerra, 1993; Skerra, 1994; Schmidt and Skerra, 1994b) or lipocalin proteins (Müller and Skerra, 1993; Schmidt and Skerra, 1994a), was maintained. (**c**) Artificial intercistronic region between the *bla* and *tetR* genes. The putative RBS sequence of *tetR* is indicated by asterisks. **Constructions:** pASK75 was constructed from pASK60-Strep (Schmidt and Skerra, 1993) in several steps using PCR and site-directed mutagenesis according to standard methods (Sambrook et al., 1989). All critical regions were confirmed by dideoxy sequencing. The segment on pASK60-Strep comprising the *lac* repressor gene and the *lac* promoter/operator was replaced by a short fragment that was amplified from pWH1012 (Sizemore et al., 1990), and contained the *tet* promoter/operator region (see Fig. 1b). Efficient translational initiation was ensured (cf., Flache et al., 1992) by keeping the start region of *tetA*, which was followed by a stop codon, the *Xba*I restriction site, and the segment encoding the OmpA signal peptide endowed with its own RBS. In order to avoid interference of transcription and translation from the two *tetR* promoters with the plasmid *ori*, the double Met codon at the 5′ end of the original *tetR* reading frame was removed, together with a *Xba*I restriction site following behind. The *tetR* structural gene itself, which was amplified from pWH520 (Berens et al., 1992), was inserted directly downstream from a modified translational stop for the *bla* gene. The *tetR* RBS was retained, but the *Xba*I site at the beginning of the reading frame was eliminated by mutagenesis of the codon for $Arg^3$ (see panel **c**). The translational initiation region of *tetR* was found to be rather sensitive to sequence variation, since other mutational variants tested during construction of pASK75 led to significantly poorer repression of the *tetA* promoter. Downstream of the *tetR* reading frame, a unique *Spe*I site was introduced, and the internal *Eco*47III site (as well as *Ase*I in the *bla* gene) was removed.

is both chromosomally and plasmid encoded, or to cell death prior to induction because of insufficient repression.

Other promoters were also utilized, but most of them suffer from disadvantages, particularly when moderate secretion of the gene product or expression at lower temperature is desired. Thus, there is still demand for a system that is independent of individual host properties and that is reversibly inducible by means of a commercially available chemical compound. Here the promoter/operator of the Tn*10* $Tc^R$ gene (reviewed in Hillen and Berens, 1994) has been successfully employed for the tightly regulated bacterial secretion of a recombinant Ig $F_{ab}$ fragment and a corresponding generic vector for the cloning and expression of foreign genes is described.

## EXPERIMENTAL AND DISCUSSION

### (a) Vector construction

Plasmid pASK75 (Fig. 1a), which carries the *tet* promoter region and the *tet*-repressor-encoding gene (*tetR*), was derived from the *lac* promoter plasmid pASK60-Strep (Schmidt and Skerra, 1993), which was previously developed for the bacterial secretion of proteins carrying the *Strep tag* affinity peptide at their C terminus (Schmidt and Skerra, 1994b). The expression cassette on pASK75 (i.e., the sequence flanked by the *Xba*I and *Hin*dIII sites; see Fig. 1b) is identical to that on pASK60-Strep, and consists of a gene fragment encoding the OmpA leader peptide with its translational initiation region, an MCS and the sequence coding for the *Strep tag*.

On pASK75, the expression cassette was put under transcriptional control of the *tetA* promoter. The translational initiation site and the first seven codons of the *tetA* structural gene were kept, followed by a stop codon and the nt sequence from pASK60-Strep (Fig. 1b). Thus, a tandem RBS for efficient initiation of translation was created. A foreign gene can be inserted in several different ways (see Schmidt and Skerra, 1993) to achieve either protein production in the cytoplasm or its secretion to the periplasm via precise fusion with the signal sequence.

On transposon Tn*10*, the three promoters of the *tet* control region act bidirectionally (Hillen and Berens, 1994): the *tetA* promoter transcribes the structural gene for the $Tc^R$ protein in one direction, and two overlapping weaker promoters direct transcription of the *tetR* gene, encoding the repressor, in the opposite direction. In this arrangement the repressor regulates both its own synthesis and transcription of *tetA*, so that a basal level of promoter activity is retained even in the absence of tetracycline (Tc), the natural inducer.

In order to establish tight repression of the *tetA* promoter, the *tetR* gene was introduced into pASK75 uncoupled from the *tet* control region. For this purpose the *tetR* gene, including its RBS, was placed downstream from the constitutively expressed *bla* gene (Fig. 1a), resulting in a transcriptional fusion (Fig. 1c).

**(b) Recombinant gene expression**

The characteristics of the pASK75 vector were exemplified using a recently described system for the functional secretion of a murine $F_{ab}$ fragment-$His_6$ fusion in *E. coli* (Skerra, 1994). The expression plasmid pASK85-D1.3 was constructed from pASK75 by inserting via *Xba*I and *Hin*dIII the corresponding cassette from the published vector pASK84-D1.3 (Skerra, 1994), which carries the structural genes for the two chains of the $F_{ab}$ fragment. Thus, the heavy chain and the light chain-encoding sequences, both preceded by a bacterial signal peptide, were placed under transcriptional control of the *tetA* promoter as part of an artificial operon.

The time-course of induction was investigated by a Western blot of total *E. coli* cell protein using commercial antisera directed against mouse Ig (Fig. 2a). The cells were grown to a mid-log phase, and the promoter was then induced by adding 200 µg aTc/l. 1 h thereafter both chains of the $F_{ab}$ fragment could be clearly detected. Their amount increased progressively during 4 h and became even larger after overnight incubation. Comparison with the purified recombinant $F_{ab}$ fragment revealed that both chain precursors were quantitatively processed, and it was estimated that 3–4 h post induction, roughly 20 mg Ig protein/l had been synthesized.

In order to confirm the formation of correctly folded Ig protein, preparative expression was performed on the 1-litre scale as described before (Skerra, 1994), with the exception that aTc was used for induction. Employing *E. coli* K-12 JM83 (Yanisch-Perron et al., 1985) as expression host, the yield obtained using the *tetA* promoter on pASK85-D1.3 was indistinguishable from that of the *lac*UV5 promoter on pASK84-D1.3, when the functional $F_{ab}$ fragment was purified from the periplasmic cell fraction via IMAC (not shown). Furthermore, the time-course of induction and the total amount of Ig protein synthesized were essentially identical between both sys-

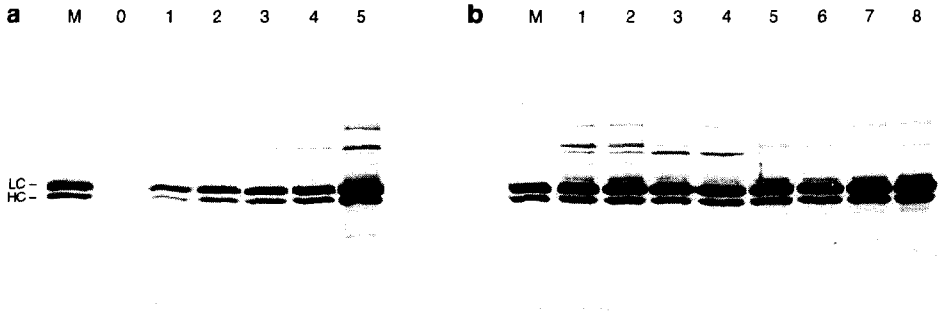


Fig. 2. Bacterial synthesis of an Ig $F_{ab}$ fragment from the *tet* promoter plasmid pASK85-D1.3, a derivative of pASK75. (**a**) Time-course of induction. Detection of recombinant $F_{ab}$ fragment by Western blot of total *E. coli* cell protein using the K-12 strain JM83 (Yanisch-Perron et al., 1985). Lanes: 0, sample taken immediately before induction; 1 to 4, samples from 1–4 h after induction; 5, sample after overnight incubation; M, 1 µg purified $F_{ab}$ fragment. LC and HC denote the light and heavy chain of the Ig fragment, respectively. (**b**) Comparison of host strains. Detection of recombinant $F_{ab}$ fragment by Western blot of total *E. coli* cell protein 3 h after induction. Lanes: 1, JM83 (Yanisch-Perron et al., 1985); 2, WK6 (Zell and Fritz, 1987); 3, *E. coli* B (ATCC 11303); 4, BL21 (Studier and Moffatt, 1986); 5, MG1655 (Jensen, 1993); 6, W3110 (Jensen, 1993); 7, W3110 in glucose minimal medium; 8, XL1-Blue (Bullock et al., 1987); M, 1 µg purified $F_{ab}$ fragment. **Methods:** Cells were grown in Luria-Bertani medium (or M9 glucose minimal medium; Sambrook et al., 1989) in the presence of 100 µg Ap/ml. For expression at the analytical scale, as shown here, 100 ml medium in a 250-ml shaking flask was inoculated with 2 ml (4 ml in the case of minimal medium) of an overnight culture and incubated at 22°C (30°C for XL1-Blue; 25°C for W3110 grown in minimal medium) until an $A_{550}$=0.5 was reached. Expression was then induced by the addition of aTc (Janssen Chimica, Neuss, Germany), dissolved in dimethylformamide, to a final concentration of 0.2 µg/ml. At the time indicated, cells from 1 ml culture were spun down and resuspended in 80 µl 100 mM Tris·HCl pH 8.0/5 mM $MgSO_4$ in the presence of 1 unit benzonase (Merck, Darmstadt, Germany). After adding 20 µl 5 × SDS gel loading buffer, the samples were kept on ice (1 h) to break down the chromosomal DNA. 5 µl of the solution (equivalent to 0.05 ml of the culture) were then heated to 95°C and subjected to 0.1% SDS-12% PAGE in the presence of 6 M urea (Skerra, 1994). Transfer onto nitrocellulose membrane (Schleicher&Schüll, Dassel, Germany), immunodetection with rabbit-anti-mouse Ig and swine-anti-rabbit Ig-alkaline phosphatase conjugate (Dako, Hamburg, Germany) and staining were performed as described (Schmidt and Skerra, 1993).

tems. However, trace amounts of the $F_{ab}$ fragment could be clearly detected in the absence of IPTG in the case of the *lac*UV5 plasmid.

Because of the toxicity of secreted Ig fragments in *E. coli* the degree of repression in a promoter system can be assessed qualitatively based on the viability and growth behavior of the transformed bacteria. For this purpose a variety of different host strains were transformed with pASK85-D1.3. In contrast to the *lac*UV5 promoter plasmid, no indication of toxicity, as the appearance of satellite colonies on Ap-containing plates or lysis of overnight cultures, was observed when strains different from JM83 were used. In addition, cell densities of overnight cultures (grown at 37°C) were usually higher and plasmid preparations gave consistently good yields, as expected for a high-copy-number vector. The routine preparation of rather large amounts of single-stranded phagemid DNA was also possible from the *tet* promoter vectors.

When small-scale expression experiments were performed with pASK85-D1.3, almost identical amounts of the $F_{ab}$ fragment were detected in the different *E. coli* host strains, irrespective whether belonging to class K-12 or B (Fig. 2b). No influence was observed when an episomal copy of the Tn*10* $Tc^R$ gene was present, as in the case of XL1-Blue (Bullock et al., 1987), or when cell growth and induction were performed in glucose minimal medium.

The inducer aTc, utilized here for induction of expression, is commercially available. Since it is active at an extremely low concentration, its use is much more economical as compared to IPTG. Furthermore, the anhydro-derivative binds more strongly to the *tet* repressor (Degenkolb et al., 1991) and is less antibiotic (Oliva et al., 1992) than the natural inducer Tc.

**(c) Conclusions**

The *tetA* promoter was shown to be useful for the tightly regulated high-level synthesis of a foreign gene product in a variety of *E. coli* K-12 and B strains. Its strength was comparable to that of *lac*UV5, but its repression on pASK75 and derivatives thereof is significantly better. Cell lysis effects, which were otherwise frequently observed in the work with recombinant antibody fragments, were thus prevented.

In contrast to practical experience with *lac*-based regulons the *tet* promoter system was found to be largely independent of the *E. coli* strain used. Therefore, the host strain can be mainly chosen, for example, according to optimized growth characteristics in the high cell density fermentation. The *tetA* promoter is inducible even when cells are grown in a minimal medium; thus, the system appears to be well suited for the large-scale production of recombinant proteins.

Although the potential advantages of the *tet* promoter for the expression of foreign genes in *E. coli* were recognized, in principle, 10 years ago (De la Torre et al., 1984) the development of a convenient expression system has not been described to date. The vector pASK75 has the *tetA* promoter/operator, as well as its cognate repressor gene, implemented in a way that makes it useful under a variety of circumstances. In conjunction with the recently established *Strep tag* purification technology (Schmidt and Skerra, 1994b) it should significantly facilitate the expression and isolation of heterologous gene products in *E. coli*.

ACKNOWLEDGEMENTS

The author wishes to thank Christina Wardenberg for expert technical assistance and Prof. Wolfgang Hillen for an inspiring discussion and providing the plasmids pWH520 and pWH1012. This work was supported by the Deutsche Forschungsgemeinschaft.

REFERENCES

Berens, C., Altschmied, L. and Hillen, W.: The role of the N terminus in Tet repressor for *tet* operator binding determined by a mutational analysis. J. Biol. Chem. 267 (1992) 1945–1952.

Bullock, W.O., Fernandez, J.M. and Short, J.M.: XL1-Blue: a high efficiency plasmid transforming *recA Escherichia coli* strain with beta-galactosidase selection. BioTechniques 5 (1987) 376–379.

Degenkolb, J., Takahashi, M., Ellestad, G.A. and Hillen, W.: Structural requirements of tetracycline-Tet repressor interaction: determination of equilibrium binding constants for tetracycline analogs with the Tet repressor. Antimicrob. Agents Chemother. 35 (1991) 1591–1595.

De la Torre, J.C., Ortin, J., Domingo, E., Delamarter, J., Allet, B., Davies, J., Bertrand, K.P., Wray Jr., L.V. and Reznikoff, W.S.: Plasmid vector based on Tn*10* DNA: gene expression regulated by tetracycline. Plasmid 12 (1984) 103–110.

Essen, L.-O. and Skerra, A.: Single-step purification of a bacterially expressed antibody Fv fragment by metal chelate affinity chromatography in the presence of betaine. J. Chromatogr. A 657 (1993) 55–61.

Flache, P., Baumeister, R. and Hillen, W.: The Tn*10*-encoded tetracycline resistance mRNA contains a translational silencer in the 5′ nontranslated region. J. Bacteriol. 174 (1992) 2478–2484.

Hillen, W. and Berens, C.: Mechanisms underlying expression of Tn*10* encoded tetracycline resistance. Annu. Rev. Microbiol. 48 (1994) 345–369.

Jensen, K.F.: The *Escherichia coli* K-12 "wild types" W3110 and MG1655 have an *rph* frameshift mutation that leads to pyrimidine starvation due to low *pyrE* expression levels. J. Bacteriol. 175 (1993) 3401–3407.

Müller, H.N. and Skerra, A.: Functional expression of the uncomplexed serum retinol-binding protein in *Escherichia coli*: ligand binding and reversible unfolding characteristics. J. Mol. Biol. 230 (1993) 725–732.

Oliva, B., Gordon, G., McNicholas, P., Ellestad, G. and Chopra, I.: Evidence that tetracycline analogs whose primary target is not the

bacterial ribosome cause lysis of *Escherichia coli*. Antimicrob. Agents Chemother. 36 (1992) 913–919.

Plückthun, A. and Skerra, A.: Expression of functional antibody Fv and Fab fragments in *Escherichia coli*. Methods Enzymol. 178 (1989) 497–515.

Reznikoff, W. and Gold, L.: Maximizing Gene Expression. Butterworth, Stoneham, MA, 1986.

Sambrook, J., Fritsch, E.F. and Maniatis, T.: Molecular Cloning. A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

Schmidt, T.G.M. and Skerra, A.: The random peptide library-assisted engineering of a C-terminal affinity peptide, useful for the detection and purification of a functional Ig Fv fragment. Protein Eng. 6 (1993) 109–122.

Schmidt, F.S. and Skerra, A.: The bilin-binding protein of *Pieris brassicae* cDNA sequence and regulation of expression reveal distinct features of this insect pigment protein. Eur. J. Biochem. 219 (1994a) 855–863.

Schmidt, T.G.M. and Skerra, A.: One-step affinity purification of bacterially produced proteins by means of the "Strep tag" and immobilized recombinant core streptavidin. J. Chromatogr. A 676 (1994b) 337–345.

Sizemore, C., Wissmann, A., Gülland, U. and Hillen, W.: Quantitative analysis of Tn*10* Tet repressor binding to a complete set of *tet* operator mutants. Nucleic Acids Res. 18 (1990) 2875–2880.

Skerra, A.: Bacterial expression of immunoglobulin fragments. Curr. Opin. Immunol. 5 (1993) 256–262.

Skerra, A.: A general vector, pASK84, for cloning, bacterial production and single-step purification of antibody $F_{ab}$ fragments. Gene 141 (1994) 79–84.

Studier, F.W. and Moffatt, B.A.: Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. 189 (1986) 113–130.

Yanisch-Perron, C., Vieira, J. and Messing, J.: Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33 (1985) 103–119.

Zell, R. and Fritz, H.-J.: DNA mismatch-repair in *Escherichia coli* counteracting the hydrolytic deamination of 5-methyl-cytosine residues. EMBO J. 6 (1987) 1809–1815.