Biotecnología Aplicada, Vol. 7, No. 3, 313-319 (1990)

Bacteriophage T7 DNA Polymerase and *Escherichia coli* K-12 Thioredoxin: Cloning and High-Level Expression

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Received in August 1990
Approved in September 1990

SUMMARY

The bacteriophage T7 gene 5 and the trxA gene of *Escherichia coli* K-12 have been cloned into multicopy plasmids using the polymerase chain reaction (PCR). When placed under the control of the *trp* promoter, the phage T7 gene 5 and the trxA gene can be stably maintained in *Escherichia coli* trxA$^-$ and yield high levels of the recombinant proteins upon induction. On the basis of a previously published report (Brinkmann *et al.*, 1989) and of these and other results from our laboratory, the relationship between the content of rare Arg codons and the expression of recombinant proteins in *Escherichia coli* is discussed.

RESUMEN

El gen 5 del bacteriofago T7 y el gen trxA de *Escherichia coli* K-12 fueron clonados en plasmidios multicopias empleando la reacción en cadena de la polimerasa (PCR). Cuando se situan bajo el control del promotor *trp*, los genes 5 del fago T7 y trxA se mantienen establemente en *Escherichia coli* trxA$^-$ y se expresan con altos niveles después de la inducción. En el trabajo también se discute la relación que existe entre el contenido de codones raros de Arg y la expresion de proteinas recombinantes en *Escherichia coli* sobre la base de un reporte anterior (Brinkmann *et al.*, 1989) y algunos de los resultados de nuestro laboratorio.

INTRODUCTION

Upon infection of *E. coli*, bacteriophage T7 induces its own DNA polymerase (Grippo and Richardson, 1971). Purification of the polymerase activity reveals a complex consisting of two polypeptides in a one-to-one stoichiometry: the 80 kDa gene 5 protein encoded by the phage and the 12 kDa thioredoxin specified by the trxA gene of *E. coli* (Modrich and Richardson, 1975; Mark and Richardson, 1976). The gene 5 protein alone is a DNA polymerase with low processivity (Tabor *et al.*, 1987). Thioredoxin increases the stability of the gene 5 protein-primer-template complex, conferring a high processivity to the polymerization reaction (Huber *et al.*, 1987). This fact has made the T7 DNA polymerase-thioredoxin complex ideal for DNA sequencing (Tabor and Richardson, 1987).

Both genes have been cloned and expressed with high yield in *E. coli* (Tabor *et al.*, 1987; Lim *et al.*, 1985; Reutimann *et al.*, 1985), but never under the control of the very strong and regulable *trp* promoter.

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Here, we report on the cloning of the phage T7 gene 5 and the *E. coli* trxA gene by PCR method and its high-level expression in *E. coli* trxA$^-$ using the *trp* promoter (Platt, 1980) and a pBR322 derived plasmid.

The system described allows the obtainment of high yields of T7 DNA polymerase and thioredoxin. On the basis of a previously published report (Brinkmann *et al.*, 1989) and of these and other results from our laboratory, in the paper we also discussed the relationship between the content of rare Arg codons and the expression of recombinant proteins in *E. coli*.

MATERIALS AND METHODS

Bacterial strains, phages, plasmids, growth conditions and enzymes

E. coli strain MC1066 *hsd*R *pyr*F74:Tn5 (Km^R)*leu*B6 *ara*$^+$*trp*C9830 (*lacIPOZYA*)X74, *gal*U, *gal*K, *str*A^R was used as a source for the cloning of the trxA gene and *E. coli* B strain BL24 [BL21 (Studier and Moffat, 1986; Grodberg and Dunn,1988) trxA (am)] for analysis of phage T7 gene 5 and trxA gene expression. Phage T7 (kindly provided by Drs. Hans Weber, Hubert Hug and Rudolf Hausmann) was used as a source for the cloning of the gene 5.

The plasmid pT5 (Platt, 1980) has been described elsewhere. For plasmid analysis and expression experiments, *E. coli* cells bearing plasmid were grown overnight at 37°C in L broth supplemented with 50-100 μg Ampicillin/ml and 100 μg tryptophan/ml. The promoter was induced essentially according to Halleweld and Emtage, 1980. Briefly, from the overnight culture the cells were grown in 50 ml of M9 medium (2% hydrolyzed casein, 0.4% glucose, 0.1 mM $CaCl_2$ and 100 mM $MgSO_4$) with an initial O.D. of 0.05 for 12 h at 37°C.

All enzymes were obtained from ENZIBIOT (CIGB, Cuba) and used according to the manufacturers recommendations.

DNA: analysis and cloning

Plasmid DNA was prepared according to Birnboim and Doly, 1979. Agarose-gel electrophoresis was performed for analysis of DNA as described by Maniatis *et al.*(1975) and nucleotide sequencing was carried out according to Chen and Seeburg, 1985. PCR followed essentially the procedure of Saiki *et al.*(1988) with the modifications pointed out in Results and Discussion section.

Preparation of crude extracts and T7 DNA polymerase assay

For T7 DNA partial purification, 10 ml of *E. coli* BL24 [pTT7] induced cultures were centrifuged at 7000 xg and 0°C. The cell pellet was resuspended in 0.5 ml of 50 mM Tris-HCl pH 7.4, 10% sacarose and 5 mM EDTA. Then, 20 μl of lysozyme (10 mg/ml) and 20 μl of 5 M NaCl were added and the cells were lysed by incubating at 0°C for 45 min, 37°C for 3 min and freeze-thawing two times. Finally, 50 μl of 5 M NaCl were added and cell debris was removed by centrifugation.

The cell extracts containing thioredoxin were prepared from induced BL24 [pTx6] cultures. After centrifugation the cell pellets were resuspended in 0.5 culture volume of 20 mM Tris-HCl pH 7.5, 0.1 mM EDTA, 0.1 mM DTT, 0.1 mM glycerol and 100 mM NaCl. The cells were then lysed by sonication. Cell extracts were heated to 75-80°C and then centrifuged to remove the cell debris.

The DNA polymerase assay using calf thymus single stranded DNA (ssDNA) (Tabor *et al.*, 1989; Table II) was carried out in 200 μl reaction volume (40 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 0.5 mM calf thymus ssDNA, 0.3 mM dATP, dCTP, dTTP and 8 μCi [^{3}H]dGTP (16 Ci/mmol) with the addition of 2 μl of BL24 [pTT7] cell extract and 20 μl of BL24 [pTx6] cell extract. Incubation was at 37°C for 15 min.

The reaction was stopped by the addition of 10 μl HCl 10 N and 5 μl Na_2HPO_4 1 M at 0°C, and the acid-insoluble radioactivity was measured.

RESULTS AND DISCUSSION

Cloning and expression of the trxA gene in *E. coli*

Two oligodeoxyribonucleotides (oligos; 30- and 31-mer primers, Figure 1) were synthesized complementary to the 5' and 3' ends of the sense and antisense strand of the trxA gene, respectively. The 3' oligo contained a Bgl II site. This allowed us to clone the amplified fragment into pT5 in an oriented way (Figure 1).

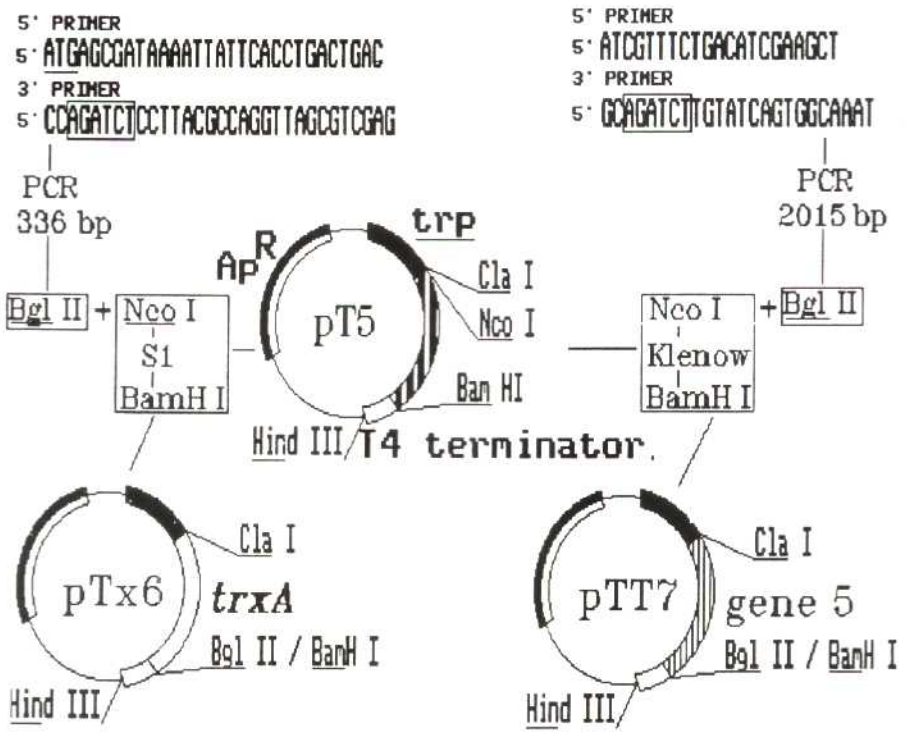

FIG. 1. Cloning of the phage T7 gene 5 and the *E. coli* trxA gene. Two oligos were synthesized complementary to the 5' and 3' ends of the sense and antisense strand of the genes, respectively. The 3' oligo contained a Bgl II site. This allowed us to clone the amplified fragment into pT5 in an oriented way. The PCR was carried out as described in Results and Discussion section. The oligos used for PCR are indicated as 5' and 3' primers and the cloning sites introduced are boxed. The thioredoxin ATG codon is underlined. The structure of the expression plasmid pT5 and of the final plasmids pTx6 and pTT7 are shown. Klenow, (large) fragment of *E. coli* DNA polymerase I.

The PCR was carried out with 1 μg of *E. coli* K-12 (strain MC1066) DNA and 100 pmoles of each oligo primer. The DNA was denatured in 100 μl of the reaction buffer (50 mM KCl, 100 mM Tris-HCl pH 8.8, 25 mM $MgCl_2$) by heating to 100°C in boiling water for 5 min. Then, 2.5 units of *Thermus thermophilus* DNA polymerase and dNTPs to a final concentration of 200 μM each were added, and the samples were subjected to 30 cycles of PCR, each consisting of 1 min of denaturation at 93°C, 30 sec at 55°C, and 1 min of polymerization at 70°C. After amplification, the DNA was concentrated by ethanol precipitation. The fragment corresponding in size (336 bp) to the trxA gene was extracted from a low melting point (LMP) agarose gel, and cloned into the expression vector, pT5, to generate the final plasmid pTx6 (Figure 1).

The plasmid pTx6 was transformed into the *E. coli* trxA$^-$ BL24 strain and as shown in Fig.2A, the thioredoxin produced is about 5% of total cellular protein as estimated by densitometer scans of Coomassie-blue- stained acrylamide gels.

The thioredoxin activity was assayed by the ability of the BL24 [pTx6] strain

([ ] denotes plasmid-carrier state) to support the growth of the phage T7 (Table I) and by measuring its ability to complement partially purified gene 5 protein to form an active DNA polymerase (Tabor *et al.*, 1989; Table 2).

Table 1
GROWTH OF BACTERIOPHAGE T7 IN *E. COLI* BL24 [PTX6] STRAIN

E. coli strain	Multiplicity of infection (pfu/ml)
BL24	6.7×10^{11}
BL24 [pTx6]	2.7×10^{13}

Overnight cultures of *E. coli* grown in L broth were diluted to a concentration of 2.5×10^8 cells/ml into 5 ml of minimal medium (0.5% glucose, 0.2 μg/ml thiamine and 0.2% casaminoacids) and incubated at 30°C for 3 min with vigorous shaking. Then, the cultures were infected with T7 phage at a multiplicity of 20, incubated at 30°C for another 30 min with shaking, and then at 37°C overnight. The cells were treated with chloroform (500 μl per 5 ml culture) and pelleted by centrifugation. The phage titer in the supernatant was assayed in *E. coli* B.

Table 2
T7 DNA POLYMERASE ASSAY

Cell extracts	Activity (cpm)
BL24	2.6×10^5
BL24 [pTT7]	5.4×10^5
BL24 [pTx6]	2.0×10^3
BL24 + BL24 [pTx6]	3.5×10^5
BL24 [pTT7] + BL24 [pTx6]	3.0×10^6

The preparation of *E. coli* BL24 [pTT7] and BL24 [pTx6] crude extracts and the T7 DNA polymerase assay were carried out as described in Materials and Methods section. In the T7 DNA polymerase assay, 2 μl of BL24 [pTT7] and/or 20 μl of BL24 [pTx6] or BL24 cell extracts were used. After the reaction was stopped, the acid-insoluble radioactivity was measured. (+) denotes crude extract addition to the same reaction.

Cloning and expression of the phage T7 gene 5 in *E. coli*

The PCR reaction and the cloning of the phage T7 gene 5 into the expression vector pT5, to generate the final plasmid pTT7 (Figure 1), were carried out as described for the trxA gene with the following modifications: the oligo primers used were of 21 and 24 mer (Figure 1); in the PCR reaction, 1 μg of phage T7 DNA was used and the cycles were of 1 min of denaturation at 93°C, 30 sec at 50°C, and 2.5 min of polymerization at 70°C.

The level of enzyme activity achieved in our system is around 100 times that obtained from T7-infected cells (Modrich *et al.*, 1975a; Nordström, 1981). As shown in Fig.2B, the T7 polymerase produced is about 10% of total cellular protein as estimated by densitometer scans of Coomassie-blue- stained acrylamide gels.

The polymerase assay using calf thymus DNA was carried out according to Tabor *et al.*(1989) (Table 2).

High-level expression of recombinant genes in *E. coli* is affected by the content of AGA/AGG codons

High-level expression of several recombinant proteins in *E. coli* has not been achieved despite optimizing transcriptional and translational signals. One of the possible reasons discussed has been the unfavorable codon usage (Robinson *et al.*,1984; Bonekamp and Yensen, 1988; Spanjaard and Van Duin, 1988).

Recently it was published by Brinkmann *et al.*(1989) that the high-level expression of recombinant genes in *E. coli* is dependent on the availability of the dnaY gene product, which codes for the minor $tRNA^{Arg}_{AGA/AGG}$.

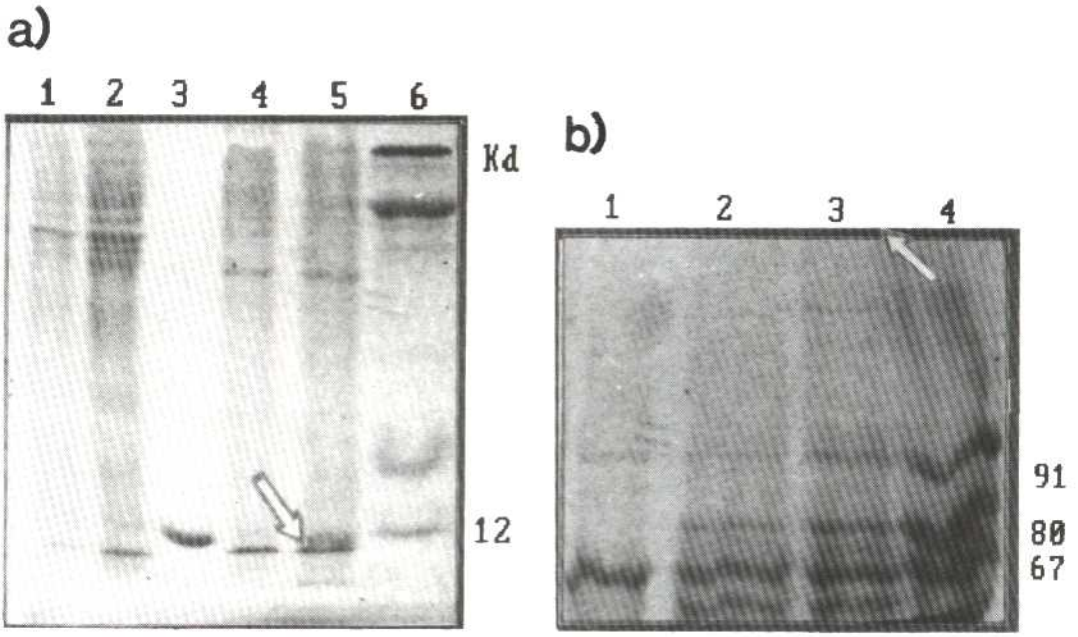

FIG. 2. Analysis of polypeptides after induction of the BL24 [pTx6] and BL24 [pTT7] strains. The polypeptides were separated on an 0.1% SDS-20% polyacrylamide (A, thioredoxin) or 0.1% SDS-7.05% polyacrylamide (B, T7 polymerase) gels and stained with Coomassie blue. [A] Lane 1) induced BL-24 (4h); Lane 2) induced BL-24 (16h); Lane 3) cytochrome C (12 kDa); Lane 4) induced BL-24/ptx6 (4h); Lane 5) induced BL-24/ptx6 (16h); Lane 6) molecular weight standards. [B] Lane 1) induced BL-24; Lane 2) induced BL-24/pTT7-1; Lane 3) induced BL-24/pTT7-2; Lane 4) molecular weight standards.

In the light of these recent results we have looked at the amount of AGA and AGG codons in the phage T7 gene 5, the trxA gene, and in other genes expressed in our laboratory (human growth hormone, hGH), (Estrada *et al.*, 1988); human tissue-type plasminogen activator (ht-PA), R.Lleonart unpublished results). As it was shown previously (Brinkmann *et al.*, 1989), there is an inverse relationship between the abundance of rare Arg codons and expression yield (Figure 3). Nevertheless, the content of AGA/AGG codons is not the only fact that accounts for the differences in the expression yield of recombinant proteins in *E. coli* since the phage T7 gene 5 and the *E. coli* trxA gene have no AGG and AGA codons but differ in 2.5 times in the expression yield (Figure 3).

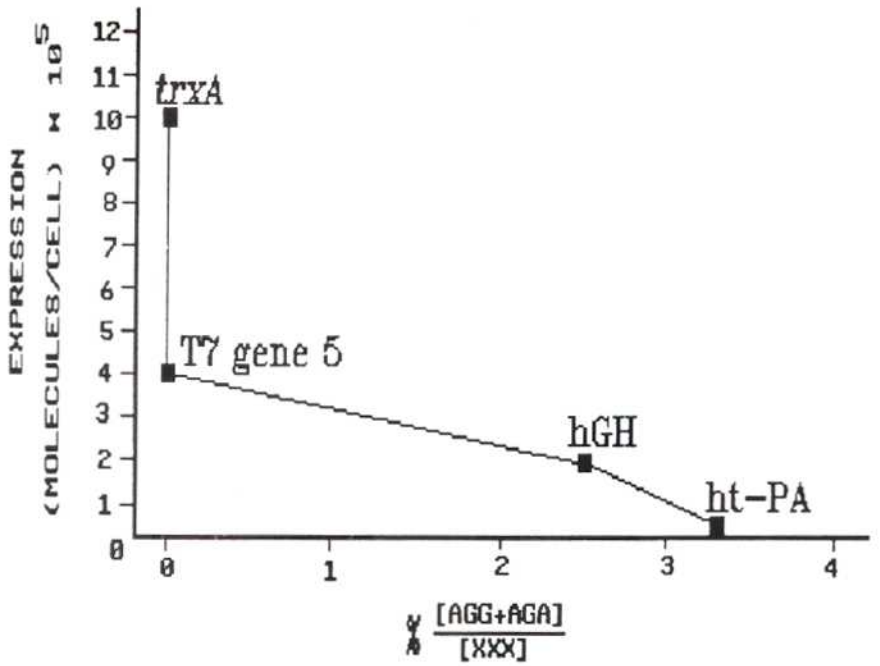

FIG. 3. Synthesis of recombinant proteins in relation to the amount of AGG and AGA codons. (■), Recombinant proteins expression yield (as the number of protein molecules per cell). The % is expressed as the number of rare Arg codons [AGG+AGA] over the total number of codons [XXX] per translated sequence. Total cellular protein was extracted from cultures 12 h after trp induction. Cells corresponding to 30 μg protein were boiled for 30 min in sample buffer (Estrada *et al.*, 1988) and separated as indicated in Fig. 2 legend. The expression yield was determined by densitometrical scanning of Coomassie-blue- stained gels. Expression plasmids used for this analysis were: pTx6 and pTT7 (thioredoxin and T7 DNA polymerase respectively, this paper), pTHH43 (hGH), (Estrada *et al.*, 1988), pT8 (ht-PA, R.Lleonart unpublished results).

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Don Oliver for the *E. coli* B strain BL24 and Drs. Hans Weber, Hubert Hug and Rudolf Hausmann for phage T7.

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