High level production and rapid purification of the *E. coli* *trp* repressor

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ABSTRACT

Two small, multicopy, expression plasmids were constructed that permit convenient insertion of *trpR*, the structural gene for the *trp* repressor of *Escherichia coli*, with its natural ribosome binding site or adjacent to the ribosome binding site for the *trp* leader peptide. In these plasmids *trpR* is positioned between the strong regulated *tac* promoter and the *rpoC* transcription terminator. IPTG induction of *lacI^q* strains bearing these plasmids results in the production of 25-50% of the soluble cell protein as *trp* repressor. Mutant and wild type repressors overproduced in this manner have been purified by simple procedures.

INTRODUCTION

The recent determination of the three-dimensional structure of the *trp* repressor of *Escherichia coli* (1) and the isolation and characterization of many classes of *trp* repressor mutants (2) have increased the need for a general expression vector that may be used to produce large amounts of mutant and wild type repressors. High level expression of *trpR* from its own expression signals is not feasible since the *trpR* promoter is relatively weak and translation initiation at the *trpR* ribosome binding site is inefficient (3). In addition, overproduced active *trp* repressor can be toxic to growing cells, so regulated production seems essential (4). The *trp* repressor expression vector previously employed, pRLK18, contained tandem *trp* promoter-operators rather than the *trpR* promoter (5). Expression directed by this vector was induced somewhat by addition of the tryptophan analog indole-3-acrylic acid to growing cultures (5). Using this plasmid and optimal growth and expression conditions, cell extracts were obtained that contained 2 to 5% of their protein as *trp* repressor (5). To improve the yield of wild type and mutant *trp* repressors, we have developed expression plasmids that incorporate the following general features: (a) high plasmid copy number, (b) the strong, regulated *tac* promoter directing transcription (6,7), (c) appropriate cloning sites allowing insertion of a 430 bp fragment that

contains the trp repressor coding region, (d) the strong rpoC transcription termination region (8) immediately following the cloning sites, and (e) sequences that permit the replacement of the trpR ribosome binding site region by synthetic DNA containing the ribosome binding site for the trp leader peptide. Using these plasmids in appropriate lacI^Q strains and delayed IPTG induction, cells can produce 25-50% of their soluble protein as mutant or wild type repressor. Uninduced cultures contain about 0.5% repressor. The high repressor content of crude extracts permits rapid purification by simple procedures: a heat step, ammonium sulfate fractionation, and a single chromatographic run on a phosphocellulose column. Mutant repressor peaks are readily identifiable in the phosphocellulose column eluate. The development of these plasmids should facilitate studies with wild type and mutant trp repressors.

MATERIALS AND METHODS

Strains

Three trpR lacI^Q strains were constructed and tested for basal level control of the tac promoter/operator of pJPR1 and pJPR2. Strain JM101 SRT4 is a trpR derivative of the JM101 F' lacI^Q strain of Messing (9) into which the serB-trpR-thr segment of the E. coli chromosome was introduced by cotransduction with an adjacent Tn10. Strain CY15070, a derivative of E. coli W3110, is tnaA2 trpR2 lacI^Q (lacI^Q was introduced by cotransduction with Tn10 from strain DS1210 (6)). Strain CY15071 is a derivative of CY15070 in which the trpR thr region has been deleted. The three lacI^Q strains mentioned behaved similarly in expression studies.

Construction of the trpR insert of pJPR2

A 403 bp BamHI fragment was constructed that contained the E. coli trpR coding region fused to a synthetic trp leader ribosome binding site. This fragment was inserted into an expression vector designated ptacterm (Figure 1 and Results) to construct the trpR expression plasmid pJPR2 (Figure 2). To construct the 403 bp BamHI fragment two overlapping oligonucleotides, the 22-mer 5'GGCCATTGTTATCTCTAATTG3' and the 23-mer 5'GATCCAATTAGAGAATAACAATG3', were designed so that when annealed they would form a synthetic trp leader ribosome binding site fragment with BamHI and Sau96I ends. These oligonucleotides were kindly provided by Karl Pope and Gerard Zurawski of the DNAX Research Institute. Annealing of the oligonucleotides was performed at 85°C in 20 µl of buffer containing 25 mM Tris-HCl, pH 7.5, 10 mM MgCl₂ and 5 µg of each oligonucleotide. The mixture was slowly cooled to room temperature

(ca. 30 minutes) and kept on ice for an additional 10 minutes. The *E. coli* trpR gene has a unique Sau96I site which overlaps its translation initiation codon (Figure 2). A 380 bp trpR-containing Sau96I-BamHI fragment was isolated from pRLK18 (5) on a 5% acrylamide/TBE gel. The general method for linker ligation described by Seth (10) was then followed with little variation. Under standard ligation conditions in a volume of 80 μ l, 670 pmoles of the non-phosphorylated synthetic trp leader ribosome binding site fragment and 3.8 pmoles of the 380 bp trpR fragment with Sau96I and BamHI ends were ligated at 15°C overnight. This mixture was then heated at 90°C for 1 minute and quick-cooled in ice/water. Sodium acetate was added and the DNA precipitated with 3 volumes of ethanol. The dried pellet was dissolved in buffer and the DNA was redissolved and phosphorylated with ATP and polynucleotide kinase. The mixture containing the 380 bp Sau96I-BamHI trpR fragment with a single oligonucleotide (23-mer) attached by its 3' end to the Sau96I site of the trpR fragment and a single oligonucleotide (22-mer) attached similarly at the BamHI end was then run on a 5% acrylamide/TBE gel. Two major fragments ca. 390 bp and 420 bp were observed and isolated separately. The two fragments were independently self-ligated to circularize. Upon recutting with BamHI, a 403 bp linear fragment was obtained in both instances that contained the synthetic trp leader ribosome binding site in proper position before the *E. coli* trpR gene. This fragment was inserted into the BamHI site of pJPR2 to form pJPR2 (Fig. 2). No difference was found between the plasmids obtained using the two original slightly different sized fragments.

trp aporepressor production, purification and assay

A single colony was picked from an LB-Amp plate and inoculated into 10 ml of minimal medium (11) containing 0.2% glucose, 0.1% acid casein hydrolysate, 0.1% yeast extract and 200 μ g/ml ampicillin. After overnight growth at 37°C the culture was added to 1 liter of fresh medium (identical to above except that it contained 0.4% glucose). The culture was grown to a density of about 4×10^8 cells/ml and a filter-sterilized solution of 0.1 M IPTG was added to a final concentration of 1 mM. The culture was then shaken overnight at 37°C and the cells harvested. The final yield was 3-5 grams of cells/liter.

To purify the trp repressor we followed the procedure described in Joachimiak et al. (5) through the ammonium sulfate precipitation step. The 45-70% ammonium sulfate precipitate was dissolved in 10 mM NaPO_4 , pH 7.6 containing 0.1 mM EDTA and 0.1 M NaCl (P11 buffer), and dialyzed overnight against the same buffer. This sample (about 100 mg. protein) was applied to a P11 column (1.4 x 20 cm.) equilibrated with the same buffer. The column was

washed with about 20 ml of P11 buffer and then eluted with a gradient containing 80 ml of 10 mM NaPO₄, pH 7.6, 0.1 mM EDTA, 0.15 M NaCl in the mixing flask and 160 ml of this buffer with 0.75 M NaCl in the second flask. The cross-sectional area of the second flask was twice that of the mixing flask. Repressor eluted in a sharp peak in the middle of the gradient. The overall yield obtained in the peak fractions was 25-50 mg pure repressor per liter of cells.

Repressor was assayed in a restriction site protection assay (5). We define 5 units of repressor activity as the amount required to give 50% protection of the RsaI site of the trp operator in 0.68 µg of pRK9 DNA (5). Pure repressor has a specific activity of approximately 6-8 x 10⁵ units/mg protein.

Plasmid copy number estimations

Cultures of CY15070 containing ptacterm, pJPR1 or pJPR2 were grown as described above to a density of 4 x 10⁸ cells/ml in the absence of inducer and plasmid DNA was isolated (12). DNA samples were digested to completion with EcoRI endonuclease and various dilutions were run separately and mixed with known amounts of a digested reference plasmid DNA. (There are single EcoRI sites in each of the above plasmids.) Plasmid DNA was visualized by ethidium bromide staining and the amount of test DNA that was equivalent to the digested internal standard was determined by visual inspection. We estimated that there were approximately 200, 100 and 60 plasmid copies/cell in strains containing ptacterm, pJPR1 and pJPR2, respectively.

trpR mRNA measurements

Cultures were grown with shaking at 37°C in single strength minimal medium (13) containing 0.2% acid hydrolyzed casein, 0.2% glucose and 200 µg/ml ampicillin. When each culture reached a density of 4 x 10⁸ cells/ml, 10 ml samples were removed and pulse labeled for 30 sec. with 0.2 ml of [³H]-uridine (25 Ci/mmol). IPTG was added to a separate sample of each culture to a final concentration of 1 mM and 10 ml samples were removed after 10 min and 120 min for pulse labeling as performed with uninduced cultures. RNA was isolated and hybridized to an excess (2 µg) of M13 DNAs immobilized on nitrocellulose filters (13). The DNAs employed were those of m19, and mp19 containing a 430 nucleotide insert with the entire trpR coding sequence.

RESULTS AND DISCUSSION

Construction and features of the expression plasmids

A small, high copy number plasmid derived from colE1, pDEPO (Dorothy

Figure 1 - Construction of ptacterm. See Materials and Methods and Results and Discussion for details not included in figure. H = HindIII, P = PstI, S = SalI, B = BamHI, RI = EcoRI, Sa = Sau3AI, S96 = Sau96I, p/o = promoter/operator, rpoCt = rpoC transcription terminator. Filled-in segments of the circular representations of the plasmids are from the polycloning region.

Figure 2 - Construction of pJPR1 and pJPR2. See Materials and Methods and Results and Discussion for details not included in figure. H = HindIII, P = PstI, S = SalI, B = BamHI, RI = EcoRI, Sa = Sau3AI, S96 = Sau96I, p/o = promoter/operator, rpoCt = rpoC transcription terminator, RBS = ribosome binding site. Filled-in segments of the circular representations of the plasmids are from the polycloning region. The trpR start codon is marked by brackets on the bottom line of the figure.

ligated fragment was such that the BamHI site was restored. A 330 bp BamHI fragment containing the rpoC terminator (8) was isolated from an M13mp8 derivative, pSVS24 (Valley Stewart and Charles Yanofsky, unpublished). The ends of this fragment were filled-in and the fragment inserted into a SalI-digested, filled-in, pJptac vector to construct the plasmid designated ptacterm (Figure 1). This 2.2 kb vector contains unique BamHI and SalI sites flanked by the tac promoter/operator and the rpoC terminator and should be generally useful for the regulated overproduction of proteins in *E. coli*. The rpoC terminator is functional in either orientation (8); the natural orientation, in ptacterm, provides more efficient termination.

Expression vector pJPR1, a 2.6 kb plasmid, contains the *E. coli* trpR gene

Table 1
trp repressor content of extracts of strains
 containing trpR plasmids

Strain	Induced ⁺	Repressor sp. act. x 10 ³
JM101*/pJPR1	-	0.84
	+	220
JM101*/pJPR2	-	0.93
	+	411
CY15070/pJPR2	-	7.5
	+	323
CY15071/pJPR2	+	159

⁺ addition of IPTG to a final concentration
 of 1 mM.

* trpR derivative SRT4

with its natural ribosome binding site (Figure 2). This vector was constructed by isolating a 430 bp trpR containing BamHI fragment from pRLK18 (5) and ligating this fragment in the correct orientation into the unique BamHI site of ptacterm.

To improve the translation initiation efficiency of trpR mRNA we constructed a second expression plasmid, pJPR2 (Figure 2). In this plasmid

Table 2
trpR mRNA production by hyper-producers

Strain	Induced ⁺	Time of pulse labeling	% of labeled RNA as <u>trpR</u> mRNA
CY15070	-	0 min.	0.015
	+	10 min.	0.01
CY15070/pJPR1	-	0 min.	0.17
	+	10 min.	9.4
	+	120 min.	12.1
CY15070/pJPR2	-	0 min.	0.19
	+	10 min.	5.4
	+	120 min.	2.1

⁺ addition of IPTG to a final concentration of
 1 mM at 0 min.

the trpR ribosome binding site was replaced by a synthetic ribosome binding site identical to that for the trp leader peptide of E. coli (14). The ribosome binding site of trpR was removed by cleavage at a Sau96I site that overlaps the sequence encoding the trpR translation initiation codon. This site is unique to trpR as well as to pRLK18, the plasmid from which a Sau96I-BamHI fragment containing trpR was obtained. Short oligonucleotides of 22 and 23 nucleotides were synthesized, annealed and attached at the Sau96I end of the 380 bp Sau96I-BamHI trpR fragment, placing the E. coli trp leader peptide ribosome binding site in proper position before the trpR start codon (see Materials and Methods and Figure 2). The resulting fragment was then ligated in the correct orientation into the unique BamHI site of ptacterm, to give plasmid pJPR2. This strategy is also applicable to mutant E. coli trpR-containing fragments.

Overproduction of the trp repressor

Plasmids pJPR1 and pJPR2 were introduced into a trpR derivative of the F'lacI^q strain JM101 SRT4 (9) by transformation. Plasmid pJPR2 also was introduced into two strains with lacI^q in their chromosome. These strains, CY15070 and CY15071, contain a trpR point mutation and a trpR-thr deletion, respectively. The various strains were grown in a moderately rich medium and were induced by IPTG addition when they reached a density of 4×10^8 cells per ml. They were then grown overnight to final densities of $4-5 \times 10^9$ cells per ml. The specific activities of the various crude extracts of cultures grown with and without induction are summarized in Table 1. Repressor specific activities ranging from 1.5 to 4×10^5 units/mg protein were routinely obtained. The repressor content of non-induced cultures was about one percent that of induced cultures. Pure trp repressor has a specific activity of $6-8 \times 10^5$ units/mg. An SDS polyacrylamide gel displaying the protein bands in various crude extracts as well as purified wild type aporepressor is shown in Figure 3. It is evident that the trp aporepressor is the predominant band in extracts of induced strains containing pJPR1 or pJPR2. The data in Table 1, and the results of many experiments with the JM101 strains, indicate that the yield of wild type repressor is about double in strains containing pJPR2 vs pJPR1.

We examined plasmid stability in strains with pJPR1 or pJPR2 under inducing and non-inducing conditions. Under non-inducing conditions strains with either plasmid were stable. Following induction, however, strains with either plasmid were moderately unstable. Most of the cells in harvested induced cultures of strains with pJPR2 were non-viable.

To gain greater insight into relative trpR expression in strains containing the two plasmids, trpR mRNA was measured both before and after induction. In both plasmid-containing strains an appreciable fraction of the mRNA synthesized was trpR mRNA (Table 2); the level present is particularly impressive given that trpR mRNA is only about 400 nucleotides in length. Notice that the fraction of newly synthesized RNA that was trpR mRNA remained fairly constant in the pJPR1-containing strain, while it dropped with time in the strain with pJPR2. Despite this drop, pJPR2 cultures generally produced more trp repressor (Table 1). This result is consistent with the expectation that the synthetic ribosome binding site introduced in pJPR2 would be more efficient than the natural trpR ribosome binding site (3) in pJPR1. We also estimated plasmid copy number in cultures of strains with pJPR1 and pJPR2 at the time of addition of IPTG. We found that there were approximately 100 and

Figure 3 - Repressor content of induced and uninduced cultures. Electrophoresis was performed in 15% polyacrylamide/0.1% NaDodSO₄ gels (15). Lane 1, standards, in order of decreasing molecular weight: bovine serum albumin (67 kd), ovalbumin (45 kd), cytochrome C (12 kd). Lane 2, 16 μ g of pure trp aporepressor. Lane 3, 8 μ g of pure trp aporepressor. Lane 4, 50 μ g pRLK18/CY15070 crude extract (+ indole acrylic acid). Lane 5, 50 μ g pJPR1/CY15070 crude extract (-IPTG). Lane 6, 50 μ g pJPR1/CY15070 crude extract (+IPTG). Lane 7, 50 μ g pJPR2/CY15070 crude extract (-IPTG). Lane 8, 50 μ g pJPR2/CY15070 crude extract (+IPTG). Lane 9, 50 μ g pJPR2/JM101 SRT4 crude extract (+IPTG). Lane 10, 50 μ g pJPR2/CY15071 crude extract (+IPTG). Protein bands were stained with Coomassie Brilliant Blue dye.

60 copies/cell in cultures of CY15070/pJPR1 and CY15070/pJPR2, respectively. Thus despite the lower copy number and trpR mRNA content of strains with pJPR2, it is apparent that pJPR2 is the more efficient expression plasmid.

We also performed experiments in which indole acrylic acid was added along with IPTG in an attempt to inactivate the repressor. We expected indole acrylate to minimize the toxic effects of the repressor and thereby increase plasmid stability and the yield of repressor. The results of these tests were inconclusive.

We have prepared essentially pure mutant and wild type trp aporepressors (Figure 3) using the procedure outlined in Materials and Methods.

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