Versatile Low-Copy-Number Plasmids for Temperature-Inducible Overexpression of Bacterial Genes in *Escherichia coli*


Today, many plasmids for expression studies in strains of *Escherichia coli* are available. Most of them are based on high-copy-number vectors like pBR322 or the pUC-series plasmids. Transcription of the gene of interest is commonly initiated from powerful promoters whose activity is controlled by an inactivatable repressor. The gene encoding the repressor is either located in the host chromosome, on a compatible co-plasmid or directly on the expression vector, which increases the number of suitable host-vector combinations.

One of the most popular promoter systems for use in expression studies is the *lac*-UV5 promoter, a catabolite-insensitive mutant of the *E. coli lac* operon promoter $p_{lac}$ (10). Initiation of transcription from *lac*-UV5 and $p_{lac}$ is controlled by the *lac*I repressor, which is inactivated by addition of isopropyl β-D-thiogalactopyranoside (IPTG). However, induction of promoter activity with IPTG is expensive and therefore sometimes not suitable when using bacterial expression systems in a large-scale bioreactor process. In these cases, the thermoinducible cI857-$p_{R}$ or cI857-$p_{I}$ promoter-repressor systems derived from bacteriophage lambda serve as alternatives (8). Transcription from the powerful $p_{R}$ promoter is repressed by the mutated lambda repressor encoded by the cI857 gene in cells growing at 30°C. At an incubation temperature of 42°C, the thermolabile repressor is inactivated, and transcription is enabled. Induction of the λ-$p_{R}$ promoter can also be achieved by an alkaline pH shift of the growth medium (6), a method that can easily be used in fermentation bioreactors. These expression systems are suitable for high-level expression of heterologous proteins in *E. coli* leading up to 40% of total protein synthesized (8). However, a decreased growth rate of recombinant cells is often observed when expressing a cloned gene encoded by a multicopy plasmid and induced from a strong promoter (1). Respectively, high-level production of even a normal nontoxic protein can be disadvantageous and can lead to decreased stability of the particular host-vector system (3). Because plasmid stability is obviously an essential prerequisite for successful expression of heterologous proteins, efforts were made to achieve stable host-vector associations.

However, only a few expression vectors based on low-copy-number plasmids are available for moderate overexpression in *E. coli* (4,12), and none use a thermoinducible promoter-repressor system and show an increased plasmid stability.

Therefore, we have constructed a set of low-copy-number vectors with different unique restriction sites suitable for moderate overexpression of bacterial genes in strains of *E. coli*. These pSC101-derived plasmids combine the advantages of the thermoinducible λ-cI857-$p_{R}$ promoter-repressor system with an increased plasmid segregation stability. In addition, they are useful as co-plasmids for ColE1- and p15A-derived vectors in complementation studies. The cI857 gene encoding the thermolabile lambda repressor is located on the expression plasmids to increase the versatility of the usable host-vector associations. In contrast to most of the other λ-cI857-$p_{R}$ expression vectors, the $p_{R}$ promoter lacks the ribosomal binding site and the start codon of the λ-cro gene. The exclusion of these features avoids the translation of a fusion...
protein between λ-cro and the bacterial gene to be expressed. Improved segregational stability of the recombinant plasmids was obtained by use of the pSC101 replication origin, including the par locus. This 375-bp DNA fragment is critical for plasmid segregation (5) and partially reverses the negative effect of prolonged expression of genes encoded in high-copy-number vectors on the cell growth (2). Because of the stringent mode of replication, these vectors are maintained at about 6–8 copies per genome (11).

All DNA manipulations during construction of the low-copy-number expression vectors were performed using standard methods (9), except for ligations for which Ready-To-Go™ T4 DNA Ligase (Pharmacia Biotech, Stockholm, Sweden) was used according to the manufacturer’s instructions. Cells harboring λ-cI857-par plasmids were always grown at an incubation temperature of 30°C or below to keep the par promoter in a repressed state.

For construction of the described expression vectors, we isolated the λ-cI857-par cassette from pCQV2 (7). Two oligonucleotide primers were designed based on the nucleotide sequence of bacteriophage lambda and used to amplify an 816-bp product containing the cI857 repressor gene and the par promoter using polymerase chain reaction (PCR). The sequence of primers used is as follows: pCQV2-85C, 5′-GCCGAGATATATTACGCCG3′ and pCQV2-4155, 5′-CGACCAGAACCTTGCCCA3′. These primers were chosen to amplify a product that contains the cI857 gene controlled by its natural promoter and the strong par promoter but lacks the ribosomal binding site and the start codon of the cro gene. The PCR product was cloned into the TA Cloning® Vector pCR® II (Invitrogen, Carlsbad, CA, USA). The obtained plasmid was termed pCG13. It contains a restriction site of the rare cutting enzyme NotI downstream of the par promoter, which is suitable for the insertion of genes under control of the λ-cI857-par expression system. The pSC101 replication origin and the genes coding for tetracycline resistance were isolated from pLG339 (11). This plasmid possesses a functional par locus at a minimized vector size. pLG339 (Accession No. 37131; ATCC, Rockville, MD, USA) was digested with EcoRI and PvuII, and the 3.7-kb fragment was isolated after filling in the EcoRI site with the Klenow fragment of DNA polymerase I. This was ligated to the 1.3-kb PvuII fragment isolated from pCG13, obtaining the low-copy-number expression vector pCG19 (Figure 1). To increase the number of unique restriction sites, a 230-bp PvuII/KpnI fragment was removed from pCG19 by re-ligation after DNA polymerase I exonuclease treatment. This plasmid was then digested with XhoI, treated with the Klenow fragment of DNA polymerase I and ligated to a 322-bp PvuII fragment from pUC18 containing the multiple cloning site. The obtained λ-cI857-par expression vector was termed pCG30 (Figure 1).

The functionality of our low-copy-number expression vectors was ensured by expression of chloramphenicol acetyltransferase (CAT) under control of the λ-cI857-par expression system. A promoterless cat indicator gene (Chloramphenicol Acetyltransferase Gen-Block®; Pharmacia Biotech, Piscataway, NJ, USA) was cloned into the NotI restriction site after treatment with the Klenow fragment of DNA polymerase I. E. coli DH5α™ (Life Technologies, Gaithersburg, MD, USA) was transformed with the ligation mixture, and the temperature-inducible CAT expression of the obtained strains was determined using the CAT enzyme-linked immunosorbent assay (ELISA; Boehringer Mannheim GmbH, Mannheim, Germany). The results of the expression studies are shown in Table 1 and demonstrate that because of the sensitivity of the immunological test, it is possible to determine the amount of CAT produced even when the promoter is in a repressed state. At the time of temperature shift, less than 0.01% of total cellular protein is represented by CAT, indicating that the lambda repressor tightly regulates the par promoter. Incubation of the cultures at 42°C for 1 h results in more than 100-fold induction of CAT expression. These expression studies under non-optimized conditions demonstrate that pCG19 and pCG30 are thermoiducible expression vectors suitable for medium overexpression of bacterial genes in E. coli.

The improved plasmid segregation stability of the constructed expression vectors was determined under selective and nonselective conditions. Cultures were grown in LB medium with 1 g/L glucose with and without addition of tetracycline (5 mg/L) at incubation temperatures of 30°C and 42°C for more than 40 generations. The ratio of cells harboring plasmids was determined by comparison of colony-forming units (cfu) on agar plates containing or lacking tetracycline every 5 generations. However, no plasmid segregation was detected, even in cultures incubated at 42°C without addition of tetracycline. This implies that because of the par locus located on the expression vectors, transcription from the powerful par promoter does not lead to increased

### Table 1. Induced Expression of CAT Protein by pCG30cat in E. coli Strain DH5α

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>OD&lt;sub&gt;660&lt;/sub&gt;</th>
<th>Plating Efficiency (%)</th>
<th>Protein Concentration (µg/mL)</th>
<th>CAT Concentration (µg/mL)</th>
<th>CAT/Total Protein (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.3</td>
<td>100</td>
<td>365</td>
<td>0.04</td>
<td>0.01</td>
</tr>
<tr>
<td>1</td>
<td>0.8</td>
<td>100</td>
<td>381</td>
<td>3.45</td>
<td>0.9</td>
</tr>
<tr>
<td>2</td>
<td>1.6</td>
<td>100</td>
<td>436</td>
<td>6.05</td>
<td>1.4</td>
</tr>
</tbody>
</table>

Cells were grown in 100 mL LBG medium (Luria broth with 20 g/L glucose and 5 mg/L tetracycline) at 30°C until an optical density (OD<sub>660</sub>) of 0.3 was reached. The incubation temperature was then shifted to 42°C, and the amount of CAT in the cells was measured 0, 1 and 2 h after temperature shift. CAT concentration in the lysed cells was determined using the CAT ELISA according to the manufacturer's instructions. Total protein concentration was measured using the Coomassie® Protein Assay (Pierce Chemical, Rockford, IL, USA). Plating efficiency was determined by comparison of cfu on agar plates containing or lacking antibiotics. Similar results were obtained for DH5α/pCG19cat (data not shown).
plasmid segregation even under nonselective conditions.

Also, derivatives of the described low-copy-number vectors were successfully used for moderate overexpression of E. coli homoserine kinase and aspartokinase in strains of E. coli (data not shown).

REFERENCES


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Chemiluminescence-Based RNase Protection Assays for Simultaneous Quantification of Procollagen mRNAs Containing AU-Rich Regions


Ribonuclease protection assays (RPAs) offer several advantages over other methods for the detection and quantification of specific mRNAs such as Northern or dot blots. They are highly sensitive and specific because cross-hybridizations and nonspecific binding of labeled probes are eliminated by the RNase digestion step (1). An RPA allows simultaneous detection and quantification of several different transcripts in one sample by using a set of multiple antisense probes of different characteristic sizes. We are primarily interested in the regulation of collagen metabolism in the University of California at Davis line 200 (UCD-200) chickens, an animal model of human systemic sclerosis, and our test system was designed to simultaneously quantify procollagen transcripts of various types in chicken tissues.

Because we used combinations of up to five antisense probes specific for different transcripts in one lane, some of the probes yielded unexpected band patterns on the RPA blot. There appeared to be enzyme-digested regions within some of the antisense probes that did not hybridize completely to the target mRNA, causing loss of specific signals, bands of unexpected size and increased background. To overcome this problem, we performed the recommended modifications, such as reduction of hybridization and digestion temperature and the use of T1 RNase, which only cleaves next to GC residues (Figure 1). Decreasing hybridization and RNase digestion temperature was not sufficient because a second, in part even more pronounced, band appeared below the 406-bp α1(VI) band, obviously resulting from incomplete alignment of the α1(VI) antisense probe. The signals specific for type I collagen transcripts were diffuse and overlayed