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Research Article

Development of a novel vector for cloning and expressing extremely toxic genes in *Escherichia coli*

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ABSTRACT

Background: Escherichia coli has been widely used as a host to clone and express heterologous genes. However, there are few vectors available for cloning and expressing extremely toxic genes, which limits further basic and applied research on extremely toxic proteins.
Results: In this study, a novel vector pAU10 was constructed in *E. coli*. pAU10 utilizes the combination of the efficient but highly repressible *T7-lacO* promoter/operator and the strong *rrnBT2* transcriptional terminator upstream of the *T7* promoter to strictly control unwanted transcription of the extremely toxic gene; in addition, the *trp* promoter/operator is oriented opposite to the *T7* promoter to control the production of the antisense RNA that may block the translation of leaky mRNA. Without the supplementation of IPTG and L-tryptophan in the culture medium, transcription of the extremely toxic gene by the *T7* promoter is highly repressed, and the *trp* promoter produces the antisense RNA, which strictly prevents unwanted expression of the extremely toxic protein in *E. coli*. With the supplementation of IPTG and L-tryptophan, the *T7* promoter efficiently transcribes the extremely toxic gene, and the *trp* promoter does not produce the antisense RNA, ensuring efficient expression of the extremely toxic protein in *E. coli*. Tight regulation and efficiency of expression of an extremely toxic gene cloned in the vector pAU10 were confirmed by cloning and expressing the restriction endonuclease-encoding gene *bamHI* without its corresponding methylase gene in *E. coli* JM109(DE3).
Conclusion: pAU10 is a good vector used for cloning and expressing extremely toxic genes in *E. coli*.

1. Introduction

Escherichia coli has been widely used as a host to clone and express heterologous genes because of its many advantages such as rapid growth kinetics [1], high cell density of cultivation [2], easy transformation of the exogenous DNA [3], and high level of expression of recombinant proteins [4]. However, cloning and expressing extremely toxic genes is difficult in *E. coli* because their encoding products are lethal to *E. coli* at an extremely low quantity of molecules per cell [5]. To achieve the cloning and expression of an extremely toxic gene, unwanted expression of the extremely toxic protein must be strictly prevented during culture propagation, while the desired expression should be efficient at the stage of protein production in *E. coli*.

Leaky transcription of the extremely toxic gene under the control of a repressible transcription system is the main reason why unwanted expression of the protein occurs in *E. coli* [6,7]. Another reason may be read-through transcription from other promoters located upstream of the repressible transcription system [8,9]. Therefore, to prevent unwanted expression of the protein, the first step should be to strictly control leaky and read-through transcription of the extremely toxic gene. In addition, blocking translation of mRNA through the use of antisense RNA complementary to the mRNA is another critical measure to prevent unwanted expression [10,11]. There are few vectors available for cloning and expressing extremely toxic genes [12,13,14], which limits further basic and applied research on extremely toxic proteins. Therefore, it is of importance to develop novel vectors that enable the stable maintenance and efficient but tightly regulated expression of extremely toxic genes in *E. coli*.

In this study, we constructed a novel vector pAU10 that is used for cloning and expressing extremely toxic genes in *E. coli*. pAU10 utilizes the combination of the efficient but highly repressible *T7-lacO* promoter/operator and the strong *rrnBT2* transcriptional terminator upstream of the *T7* promoter to control unwanted transcription of the extremely toxic gene strictly; in addition, the *trp* promoter/operator oriented opposite to the *T7* promoter to control the production of the antisense RNA that may block translation of the leaky mRNA. Tight regulation and efficiency of expression of an extremely toxic gene

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cloned into pAU10 were confirmed by cloning and expressing the restriction endonuclease-encoding gene *bamHI* without its corresponding methylase gene in *E. coli* JM109(DE3).

2. Materials and Methods

2.1. Strains, plasmids, and growth conditions

All bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* BL21 was grown in LB medium [15]. *E. coli* JM109(DE3) and its derivants were grown in M9-S medium [M9 minimal medium [15] supplemented with 4 g L^{-1} glucose, 1.2 g L^{-1} $MgSO4 \cdot 7H_2O$, 0.1 g L^{-1} thiamine, and 2 g L^{-1} Dropout Supplement-Trp (containing all essential amino acids except L-tryptophan)] at 200 rpm and 37°C. The final concentration of kanamycin used in the study was 50 μg/mL for *E. coli* strains harboring the plasmid.

2.2. DNA preparation and PCR techniques

Restriction enzymes, T4 DNA ligase, DNA ladder, and λ DNA were purchased from Takara (Dalian, China). Plasmid DNA was prepared using the Tianprep Mini Plasmid Kit (Tiangen Biotech, Beijing, China). *E. coli* BL21 genomic DNA was prepared using the Bacterial Genome Extraction Kit from Tiangen Biotech. A prestained protein molecular weight marker was purchased from Promega (Madison, USA). DNA synthesis and sequencing were performed by Sangon (Shanghai, China). *E. coli* cells were transformed using the standard $CaCl_2$ heat shock method [16].

The PCR experiments were performed using a Mastercycler (Eppendorf, Hamburg, Germany). The amplification of DNA was conducted using PrimeSTAR™ HS DNA Polymerase according to the manufacturer's protocol (Takara). The primer sequences are listed in Table 2. Generally, the PCR reaction mixture was 50 μl, which contained 10 μl 5× PrimerSTAR buffer (Mg^{2+} plus), 4 μl dNTP mixture (2.5 mM each), 1 μl plasmid template (100 ng/μl), 1 μl forward primer (20 μM), 1 μl reverse primer (20 μM), and 0.5 μl PrimeSTAR™ HS DNA Polymerase. Each reaction mixture was first heated to 94°C for 5 min, and 35 cycles of denaturation were run at 94°C for 30 s, annealing was for 15 s at the corresponding temperature, and elongation at 72°C for the corresponding time followed by a 10-min extension at 72°C. PCR products were separated on agarose gels and purified using the Tiangen Midi Purification Kit from Tiangen Biotech.

Table 1
Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Sources
Strains		
E. coli BL21	F⁻, *ompT*, *hsdS*(*rBB*⁻ *mB*⁻), *gal*, *dcm*	Stratagene
E. coli JM109(DE3)	*endA1*, *recA1*, *gyr96*, *thi*, *hsdR17*(rk⁻, mk⁺), *relA1*, *supE44*, λ⁻, Δ(*lac-proAB*), [F′, *traD36*, *proAB*, *lacI*^{q}ZΔM15], DE3	Promega
Plasmids		
pKK233-3	An expression vector in *E. coli*, Amp^r	Pharmacia
pET-28a	An expression vector in *E. coli*, Km^r	Novagen
pAU7	pKK223-3 *rep* (pMB1) (900 bp) plus pET28a *aphI* (1023 bp), a mini plasmid in *E. coli*, Km^r	This work
pAU8	pAU7 (*Kpn*I/*Afl*II) Ω *lacI*^{q} (*Kpn*I/*Afl*II, 1207 bp, pET28a), a plasmid in *E. coli*, Km^r	This work
pAU9	pAU8 (*Bam*HI/*Pst*I) Ω *trpR* (*Bam*HI/*Pst*I, 558 bp, *E. coli* BL21 genome), a plasmid in *E. coli*, Km^r	This work
pAU10	pAU9 (*Nde*I/*Spe*I) Ω expression/repression cassette (*Nde*I/*Spe*I, 574 bp, artificial synthesis), an expression vector in *E. coli*, Km^r	This work
pAU10-*bamHI*	pAU10 (*Nhe*I/*Hin*dIII) Ω *bamHI* (*Nhe*I/*Hin*dIII, 642, artificial synthesis), a recombinant plasmid harboring the *bamHI* gene	This work

2.3. Construction of plasmids

The detailed procedure for the construction of the vectors pAU7, pAU8, pAU9, and pAU10 is shown in Fig. 1. First, the replication origin *rep* (pMB1) (900 bp) of the *E. coli* plasmid was PCR amplified from the plasmid pKK223-3 using the primers *oriE*-F and *oriE*-R (annealing temperature, 61°C; elongation time, 1 min). The *KpnI* and *AflII* restriction sites were introduced at the 5′ end of *rep* (pMB1), and the *Bam*HI restriction site was introduced at the 3′ end. The kanamycin-resistance gene *aphI* (1023 bp) was PCR amplified from plasmid pET28a using the primers *kan*-F and *kan*-R (annealing temperature, 68°C; elongation time, 75 s). The *Bam*HI and *Pst*I restriction sites were introduced at the 5′ end of the *aphI* gene, and the *Kpn*I restriction site was introduced at the 3′ end. The PCR products of *rep* (pMB1) and *aphI* were digested with *Kpn*I and *Bam*HI, respectively, and ligated together, generating an *E. coli* mini plasmid pAU7 (1962 bp). Second, the *lacI*^{q} gene (1207 bp) was PCR amplified from plasmid pET28a using the primers *lacI*^{q}-F and *lacI*^{q}-R (annealing temperature, 68°C; elongation time, 75 s), and *Kpn*I and *AflII* restriction sites were introduced at the 5′ and 3′ ends of the PCR product, respectively. The PCR product of *lacI*^{q} was digested with *Kpn*I and *AflII*, and ligated into pAU7, which was also digested with *Kpn*I and *AflII*, generating the plasmid pAU8 (3161 bp). Third, the *trpR* gene (558 bp) was PCR amplified from *E. coli* BL21 genomic DNA using the primers *trpR*-F and *trpR*-R (annealing temperature, 62°C; elongation time, 40 s). The *Bam*HI restriction sites were introduced at the 5′ end of the *trpR* gene, and *Pst*I, *Spe*I, and *Nde*I restriction sites were introduced at the 3′ end. The PCR product of *trpR* was digested with *Bam*HI and *Pst*I and ligated into pAU8, which was also digested with *Bam*HI and *Pst*I, generating the plasmid pAU9 (3733 bp). Finally, the expression/repression cassette (574 bp, flanked by *Nde*I restriction sites at the 5' end and *Spe*I restriction site at the 3' end) was artificially synthesized by Sangon. The cassette was digested with *Nde*I and *Spe*I and ligated into pAU9, which was also digested with *Nde*I and *Spe*I, generating the target vector pAU10 (4297 bp).

2.4. Cloning and expression of the bamHI gene

The plasmid pAU10-*bamHI* was constructed as follows: the ORF of the *bamHI* gene encoding the *Bam*HI restriction endonuclease (642 bp, flanked by a *Nhe*I restriction site at the 5' end and a *Hin*dIII restriction site at the 3' end) was artificially synthesized by Sangon. The *bamHI gene* was digested with *Nhe*I and *Hin*dIII and ligated into pAU10, which was also digested with *Nhe*I and *Hin*dIII. The ligation mixture was used to transform to *E. coli* JM109(DE3), which contains a chromosomal copy of the gene for T7 RNA polymerase, and the transformants were plated on M9-S media.

Single colonies of JM109(DE3)/pAU10 and JM109(DE3)/pAU10-*bamHI* from M9-S agar plates were inoculated into 10 mL of M9-S liquid media in 50-mL flasks to prepare the seed cultures. After overnight growth, approximately 0.5 mL of the JM109(DE3)/pAU10 and JM109(DE3)/pAU10-*bamHI* seed cultures were inoculated into 50 mL of M9-S liquid media in 250-mL flasks and cultivated for monitoring the bacterial growth tendency.

IPTG and L-tryptophan (final concentrations, 1 mM each) were added to the JM109(DE3)/pAU10-*bamHI* culture when its OD_{600} reached approximately 0.9. After further cultivation for 2 h, the cells were harvested by centrifugation at 13680 xg for 10 min. The cell pellets from 50 mL of culture were washed and resuspended in 0.75 mL Tris-HCl buffer (100 mM, pH 8.5). The cell suspension was disrupted using ultrasound disruption systems (JY98-III DN systems, Ningbo, China), and the debris was removed by centrifugation at 4°C. The resulting cell homogenate supernatant was used for SDS-PAGE,

Table 2
Sequences of primers used for PCR experiments in this study. The restriction sites are underlined.

Names	Sequences	Restriction sites
oriE-F	atatggtaccattcaacacttaagtcactcaaaggcggtaatacggtta	*Kpn*I, *Afl*II
oriE-R	agctggatccgcctcactgattaagcattggtaa	*Bam*HI
kan-F	aatcggatccaactttctctgcagctttgatcttttctacggggtctgacg	*Bam*HI, *Pst*I
kan-R	agcaggtacctcaggtggcacttttcggggaa	*Kpn*I
lacI^q-F	atatggtaccgacaccatcgaatggtgcaaaaccttcgcggtatggcatgatagc	*Kpn*I
lacI^q-R	agcacttaagcaaaaaggccatccgtcaggatggccttacattaattgcgttgcgctcactgc	*Afl*II
trpR-F	agctggatccaatggggacgtcgttactgatc	*Bam*HI
trpR-R	atcactgcagactagttaatcctaaacatatgttatcatgcctaccaaacatattgaaat	*Pst*I, *Spe*I, *Nde*I

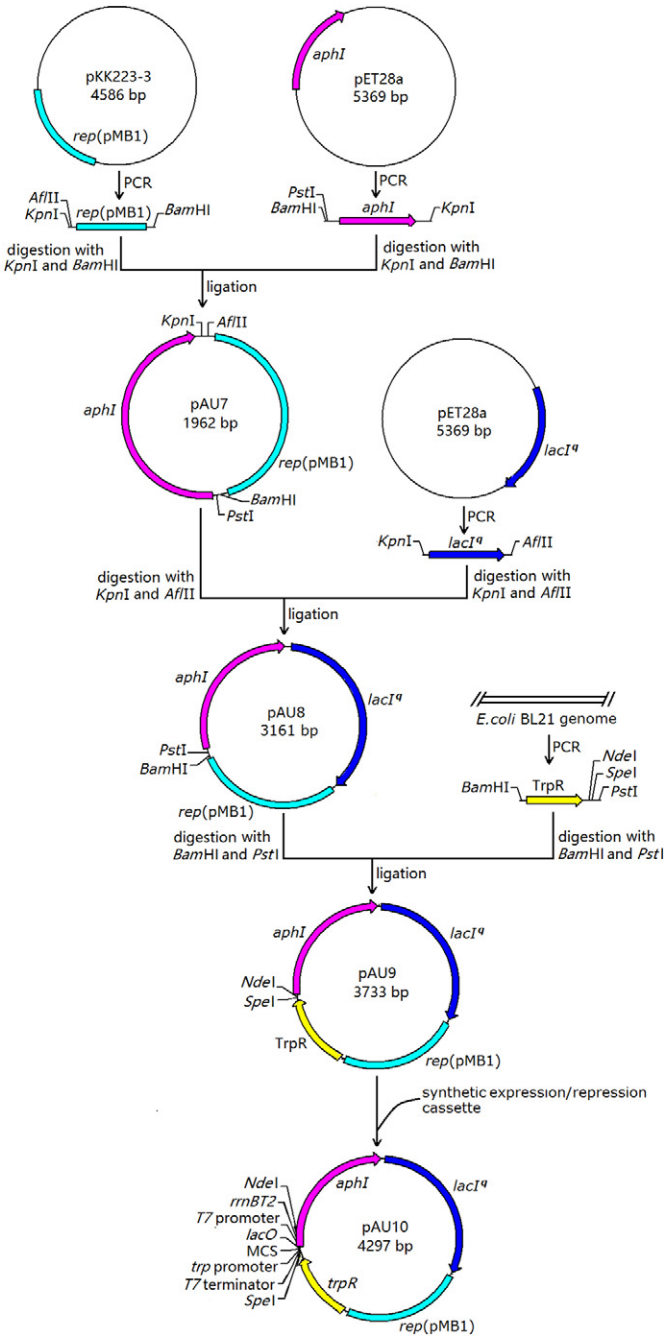

Fig. 1. Construction map of the gene expression vector pAU10. *rep* (pMB1), the origin of *E. coli* plasmid pBR322; *aphI*, kanamycin resistance gene; *lacI*^{q}, the LacI repressor protein-encoding gene; *trp R*, the TrpR repressor protein-encoding gene; MCS, multiple cloning sites.

Western blot, and endonuclease activity analysis. The total protein concentration of the cell homogenate supernatant was determined using the Protein dotMETRIC^{TM}Kit (Sangon, Shanghai, China). To quantify the BamHI protein band, the SDS-PAGE gel was scanned using a gel scanner (GS800, Bio-Rad) and analyzed using Quantity One software (Bio-Rad).

2.5. SDS-PAGE and Western blot analysis of BamHI expression in E. coli

SDS-PAGE was performed on 12% polyacrylamide gels. In total, 50 μg of total protein of the cell homogenate supernatant was loaded in each lane. According to the experimental design, the recombinant BamHI protein was fused with 6×His-tag at its both C-terminus and N- terminus, and its expression could be tested by Western blot analysis using anti-6×His tag antibody. Following SDS-PAGE, all protein bands were transferred onto a polyvinyl difluoride membrane (Invitrogen, Carlsbad, USA) for 60 min at 100 mA using the Bio-Rad Trans-Blot apparatus (Bio-Rad, Hercules, USA). The membrane was incubated with a blocking solution 1% (w/v) bovine serum albumin in TBST-buffered saline (24.7 mM Tris, 137 mM NaCl, 2.7 mM KCl, and 0.5% Tween-20, pH 7.5) for 1 h and then incubated with a mouse anti-6× His tag polyclonal antibody (BioDev-Tech, Beijing, China) for the immunodetection of 6× His-tagged protein for 1 h. After washing with TBST, the membrane was incubated with a goat anti-mouse polyclonal antibody labeled with alkaline phosphatase (BioDev-Tech, Beijing, China) for the immunodetection of His-antibody. The membrane was washed with TBST again and incubated with color-substrate solution (100 mM Tris, 100 mM NaCl,50 mM $MgCl_2$, 0.01 mM NBT, 0.01 mM BCIP, and pH 9.5) to observe a color reaction.

2.6. Analysis of the recombinant BamHI activity

The restriction endonuclease activity of the recombinant BamHI in the cell homogenate supernatant was determined by cleaving DNA *in vitro* [17]. The 10 μL reaction system contained 8.5 μL cell homogenate supernatant (55 μg total cell homogenate supernatant protein, approximately 2 μg BamHI protein), 1 μL buffer K (Takara), and 0.5 μL λ DNA (Takara, 300–500 μg/mL). The control reaction system contained 0.25 μL commercial enzyme BamHI (Takara, 15 U/μL), 1 μL buffer K, 0.5 μL λ DNA (Takara, 300-500 μg/mL), and 8.25 μL H_2O. The reaction mixtures were incubated at 37°C for 1 h, and the digestion products were analyzed by agarose gel electrophoresis.

3. Results and Discussion

3.1. Construction of a novel expression vector pAU10 in E. coli

Vectors that allow efficient but tightly regulated gene expression are necessary genetic tools in research on extremely toxic genes. In this study, first, the basic vector pAU7 was constructed by ligation of the high-copy plasmid origin *rep* (pMB1) [18] and the selection marker kanamycin resistance gene *aphI*; next, on the basis of vector pAU7, an *E. coli* expression vector pAU10 was constructed by successively introducing the LacI repressor protein-encoding gene *lacI*^{q}, the TrpR

repressor protein-encoding gene *trpR*, and the expression/repression cassette (Fig. 1). The various elements and their roles in the cassette are as follows: the strong *rrnBT2* terminator prevents read-through transcription from the upstream promoter; the efficient but highly repressible *T7-lacO* hybrid promoter/operator and the strong *T7* terminator control transcription initiation and termination of the target gene, respectively; the consensus ribosome binding site sequence of *E. coli* ensures efficient protein translation; the multiple cloning sites (*Not*I, *Eco*RI, *Bgl*II, *Nhe*I, *Xho*I, *Hin*dIII) ensure simple and rapid insertion of the target gene; the His-tag sequences enable the C-terminus and/or N-terminus of the protein to be fused with six histidines, which would be convenient for Western blot analysis and purification of the protein; the regulatory region of the *trp* operon of *E. coli*, composed of the *trp* promoter/operator (located in the interior of the promoter), leader peptide-encoding sequence, and *trp* attenuator [19], controls the production of antisense RNA (Fig. 2).

High efficiency of gene expression under the control of the *T7* promoter has been widely reported in *E. coli* [4]. To achieve efficient but highly repressible gene transcription, the *T7-lacO* transcription system, composed of the *T7* promoter, *lacO* operator, and $lacI^q$ gene, was introduced into pAU10. In this system, the $lacI^q$ gene possesses a strong promoter [20], which ensures high amount of the LacI repressor protein in *E. coli* cells. In the absence of the inducer IPTG, the *lacO* site is fully occupied by the LacI repressor protein, and the *T7* promoter cannot be recognized by T7 RNA polymerase and does not transcribe the extremely toxic gene [21] (Fig. 3A). In the presence of the inducer IPTG, the *lacO* site is empty because of LacI inactivation by IPTG, and the *T7* promoter is recognized by T7 RNA polymerase and efficiently transcribes the extremely toxic gene in *E. coli* [21] (Fig. 3A).

Although the *T7-lacO* transcription system is highly repressible, leaky transcription of the extremely toxic gene is still lethal to the host *E. coli* [12,14]. Therefore, it is necessary to take measures to prevent the translation of the leaky mRNA. Antisense transcription occurs counter to gene orientation, and promoters oriented opposite to genes can produce antisense RNA by directing antisense transcription [11]. Antisense RNA has been widely used to regulate gene expression [22]. The mechanism of antisense RNA regulation depends on RNA-RNA interaction, i.e., the binding of antisense RNA to its target mRNA forms an RNA secondary structure that inhibits ribosome binding and achieves regulation at the level of translation [23]. To block the translation of the leaky mRNA, the *trp* transcription system, composed of the *trp* promoter/operator, *trpR* gene, leader peptide-encoding sequence, and *trp* attenuator, was introduced into pAU10. In the absence of exogenous L-tryptophan, the *trpO* operator is empty because of the inactive TrpR repressor protein; in addition, the *trp* promoter is recognized by *E. coli* RNA polymerase and produces the antisense RNA [19], leading to the blocking of the leaky mRNA translation in *E. coli* (Fig. 3A). In the presence of exogenous L-tryptophan, the *trp* operator is fully occupied by the TrpR protein because of TrpR activation by the corepressor L-tryptophan, and the *trp* promoter cannot be recognized by *E. coli* RNA polymerase and does not initiate the antisense transcription [19] (Fig. 3A). Furthermore, for a very small number of initiation events of antisense transcription, the transcription elongation is aborted by an attenuation mechanism [19]. According to the attenuation mechanism, in the presence of enough L-tryptophan in *E. coli* cells, the leader peptide translation proceeds smoothly, and the translating ribosome permits the transcribed attenuator sequence to form an RNA hairpin structure. The interaction between the RNA hairpin and *E. coli* polymerase terminates the elongation of the antisense transcription.

Without the supplementation of IPTG and L-tryptophan in the culture medium, transcription of the extremely toxic gene by the *T7* promoter is highly repressed, and the *trp* promoter produces antisense RNA, which strictly prevents unwanted expression of the extremely toxic protein in *E. coli*. With the supplementation of IPTG and L-tryptophan, the *T7* promoter efficiently transcribes the extremely toxic gene, and the *trp* promoter does not produce the

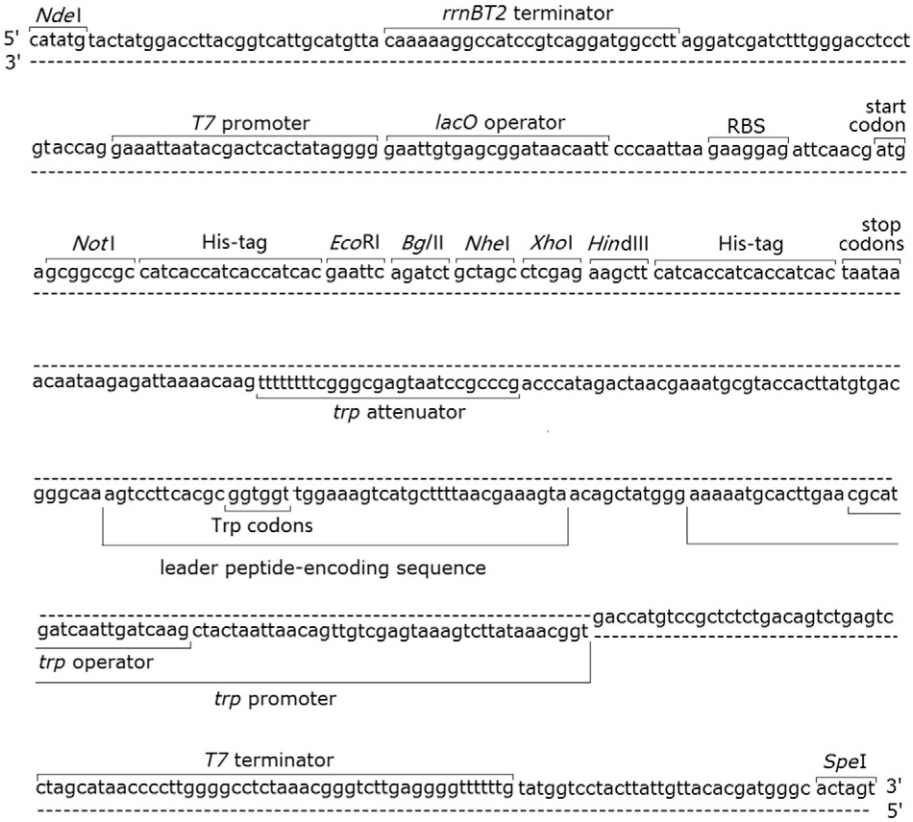

Fig. 2. Sequence of the expression/repression cassette of pAU10. Hyphens represent nucleotides pairing with the displayed ones.

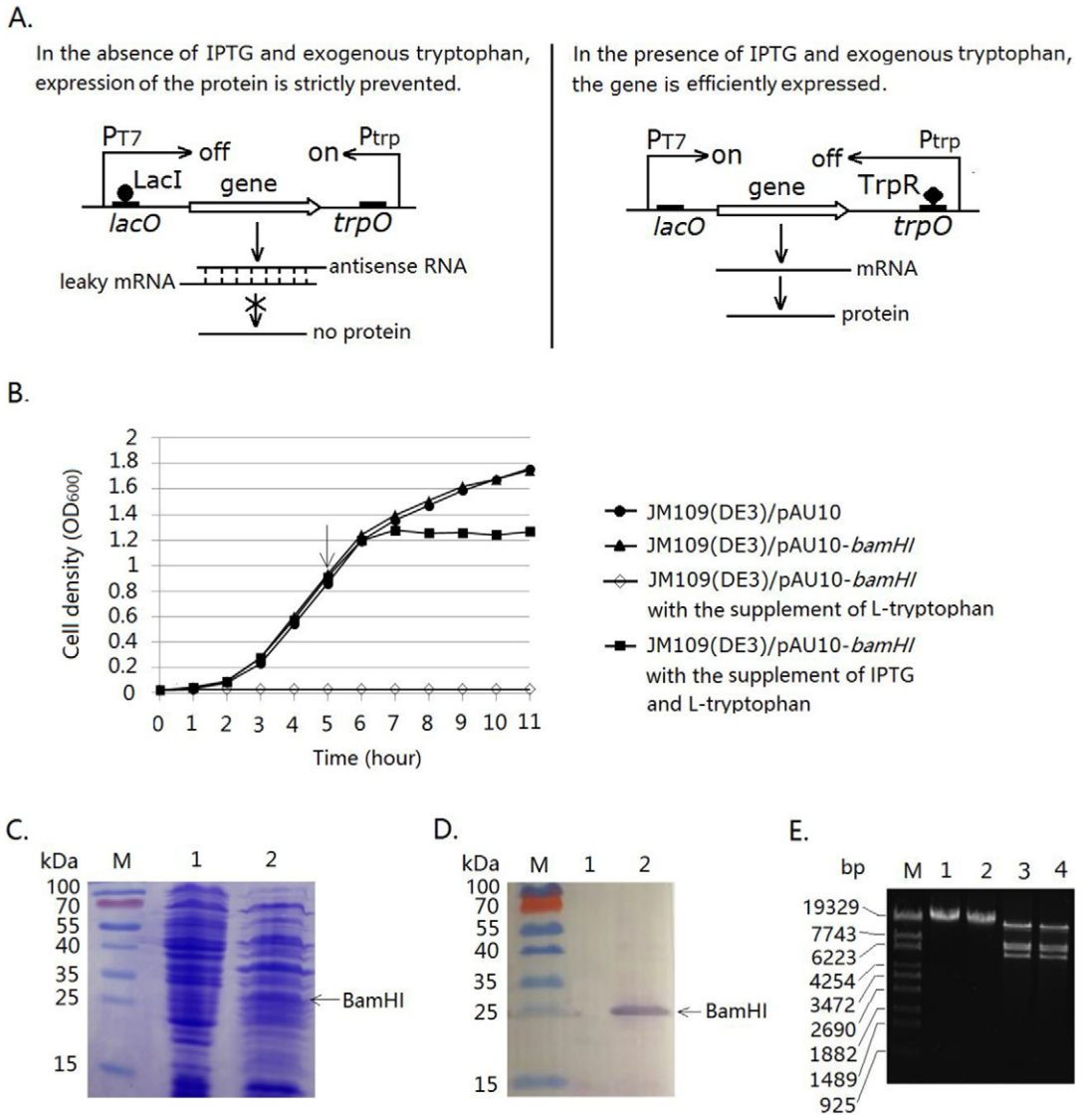

Fig. 3. Applicability of pAU10 in *E. coli* JM109(DE3). (a) Schematic map of expression of the extremely toxic gene in the absence and presence of IPTG and exogenous L-tryptophan. P_{T7}, the *T7* promoter; P_{trp}, the *trp* promoter; LacI and TrpR, repressor proteins binding to the *lac* and *trp* operators, respectively; *lacO*, the *lac* operator bound by LacI; *trpO*, the *trp* operator bound by TrpR. (b) Growth tendency of *E. coli* JM109/pAU10-*bamHI*. According to the same cell concentration at the initial stage of cultivation, the seed cultures of *E. coli* strains were inoculated into 50 mL M9-S media in 250-mL flasks. The cultivation was performed at 200 rpm and 37°C. Cell growth was monitored by measuring the OD_{600} of the culture. L-tryptophan (final concentrations, 1 mM) was added to the JM109(DE3)/pAU10-*bamHI* culture at the initial stage of cultivation. IPTG and L-tryptophan (final concentrations, 1 mM each) were added to the JM109(DE3)/pAU10-*bamHI* culture after 5 h of cultivation. The arrow indicates the time IPTG and L-tryptophan was added. (c, d) Coomassie blue-stained SDS-PAGE and Western blot analysis of BamHI protein expression, respectively. M, protein molecular weight marker; 1, 2, the cell homogenate supernatant of the JM109(DE3)/pAU10-*bamHI* culture without and with the supplementation of IPTG and L-tryptophan, respectively. (e) Agarose gel (1%) electrophoresis analysis of the digestion products of λ DNA. M, DNA ladder; 1, λ DNA; 2, 3, 4, λ DNA digested with the cell homogenate supernatant of the JM109(DE3)/pAU10-*bamHI* culture without and with the supplementation of IPTG and L-tryptophan, the commercial enzyme BamHI, respectively.

antisense RNA, ensuring efficient production of the extremely toxic protein in *E. coli*.

3.2. Analysis of tight regulation and efficiency of expression of an extremely toxic gene cloned in the vector pAU10

In the absence of the corresponding methylase, a restriction endonuclease of a Type II restriction-modification system is extremely toxic to its host cell because of its ability to cleave genomic DNA [24]. To detect the applicability of the vector pAU10, the *bamHI* gene, which encodes the Type II restriction endonuclease *Bam*HI of *Bacillus amyloliquefaciens* H [25], was used as the reporter gene. Lower concentration of exogenous L-tryptophan initially may function as corepressor but later, as tryptophan is depleted by bacterial growth, fails to repress the transcription of the *trp* operon [26]. Therefore, to ensure no production of the antisense RNA, L-tryptophan was added to the M9-S medium at a high final concentration of 1 mM when BamHI needed to be expressed. The OD_{600} of the JM109(DE3)/pAU10-*BamHI* culture with the supplementation of L-tryptophan maintained its initial value, while the growth tendency of JM109(DE3)/pAU10-bamHI without the supplementation of L-tryptophan was consistent with that of JM109(DE3)/pAU10 (Fig. 3B). These results suggested that leaky expression of the extremely toxic protein BamHI occurred in the presence of exogenous L-tryptophan, and the leaky expression was strictly prevented by the antisense RNA produced by the *trp* promoter in the absence of exogenous L-tryptophan. When the OD_{600} of the JM109(DE3)/pAU10-*BamHI* culture reached approximately 0.9, IPTG and L-tryptophan were added. After further cultivation for

approximately 2 h, the OD_{600} of the culture maintained a steady value (Fig. 3B), suggesting that abnormal death of the cells caused by BamHI expression occurred.

SDS-PAGE and Western blot results showed that the specific protein bands, corresponding to the expected molecular mass of the recombinant BamHI protein (27.4 kDa), were observed in the cell homogenate supernatants of the JM109(DE3)/pAU10-*bamHI* cultures with the supplementation of IPTG and L-tryptophan and not observed in those without the supplementation of IPTG and L-tryptophan (Fig. 3C, D). The BamHI protein could be produced to a level corresponding to approximately 4% of the total cell homogenate supernatant protein. These results indicated that the pAU10 vector could tightly regulate the *bamHI* expression and enabled efficient expression of *bamHI* in the presence of IPTG and exogenous L-tryptophan in *E. coli*.

We initially transformed pAU10-*bamHI* into the *E. coli* BL21(DE3) strain. Agarose gel electrophoresis of the digestion products of λ DNA with the cell homogenate supernatant of the BL21(DE3)/pAU10-*bamHI* culture supplemented with IPTG and L-tryptophan showed a smear, without discrete bands, suggesting that λ DNA suffered non-specific digestion by nuclease in the cell homogenate supernatant. In contrast to *E. coli* BL21(DE3), the *endA1* gene encoding endonuclease I is inactivated in *E. coli* JM109(DE3), which eliminates non-specific digestion of the target DNA through endonuclease I and serve to investigate specific digestion of the target DNA by restriction endonuclease in cell homogenate supernatants. Therefore, *E. coli* JM109(DE3) is more suitable for expressing the BamHI protein. Restriction endonuclease activity analysis showed that the number and sizes of digestion fragments of λ DNA with the cell homogenate supernatant of the JM109(DE3)/pAU10-*bamHI* culture supplemented with IPTG and L-tryptophan were consistent with those of λ DNA with the commercial enzyme BamHI (Fig. 3E), further suggesting that the *Bam*HI protein was expressed well after the supplementation of IPTG and L-tryptophan in *E. coli*. These experimental results demonstrated tight regulation and efficiency of expression of the extremely toxic gene cloned in the vector pAU10.

To date, although there have been many vectors used for expressing toxic genes in *E. coli* [8,27], the reported vectors available for cloning and expressing extremely toxic genes only include pDOC55, pZA24, and pLT7K [12,13,14]. Tight repression of expression of extremely toxic gene cloned in the vector pDOC55 is achieved through the presence of the temperature-sensitive lambda promoter λ-P_L and the *lac* promoter. The vector pZA24 utilizes the combination of two repressor-operator systems (LacI-*lacO*, AraC-*araI1-I2*) near its promoter and low copy number (only 3–4 copies per cell) to tightly control leaky transcription of extremely toxic genes. The vector pLT7K uses the *T7-lacO* promoter to transcribe the target gene and the temperature-sensitive lambda promoter λ-P_R as an antisense promoter. pAU10 and three reported vectors can all strictly prevent the unwanted expression of extremely toxic proteins. In contrast to three reported vectors, pAU10 has the overall potential advantages that contribute to excessive yield of extremely toxic proteins. The first is that the *trp* promoter is used as an antisense promoter. To the best of our knowledge, this report is the first in which the entire regular region of the *trp* operon is applied in vector construction because it was identified by Yanofsky et al. [19]. The vector pDOC55 uses the *lac* promoter as an antisense promoter. In the *E. coli*/pDOC55 system, IPTG is present at the stage of protein production and the antisense RNA continues to be produced, which impairs the translation of the target mRNA. In contrast to the *lac* promoter regulated by the inducer IPTG, the *trp* promoter regulated by the corepressor L-tryptophan is more suitable for being used as an antisense promoter. In the *E. coli*/pAU10 system, L-tryptophan is added to the culture at the stage of protein production, which prevents the transcription of antisense RNA and removes the negative effect of antisense RNA on the translation of the target mRNA. The second is the use of the high-copy number plasmid origin *rep* (pMB1), resulting in high copy number of the cloned gene per cell. The third is the flexibility of cultivation temperature at the stage of protein production in the *E. coli*/pAU10 system. In the *E. coli*/pLT7K system, if cultivation temperature is above 30°C at the stage of protein production, the antisense RNA is produced by the λ-P_R promoter because of the inactivation of the cI857 repressor and thus affects the translation of the target mRNA. In the *E. coli*/pAU10 system, 37°C may still be used as the cultivation temperature at the stage of protein production, which especially contributes to the yield of proteins that do not form inclusion bodies [28]. The vector pAU10 will be highly useful for cloning and expressing extremely toxic genes in *E. coli*.

4. Conclusions

The vector pAU10 available for cloning and expressing extremely toxic genes was developed in *E. coli*. pAU10 is paired with an *E. coli* strain harboring a chromosomal copy of T7 RNA polymerase-encoding gene, forming a gene expression system. An extremely toxic gene can be cloned and stably maintained in the *E. coli*/pAU10 system in L-tryptophan-deficient medium, and the cloned gene can be efficiently expressed by adding IPTG and L-tryptophan to the medium. pAU10 is a good vector for cloning and expressing extremely toxic genes in *E. coli*.

Conflict of interest

The authors declare no conflict of interest.

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