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Evaluation of different promoters and host strains for the high-level expression of collagen-like polymer in *Escherichia coli*

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Abstract

The increased expression of collagen-like polymer, CLP3.1-his which consists of 52 repeating peptide (GAP-GAPGSQGAPGLQ), in *Escherichia coli* was investigated. The effects of three promoters, thermally inducible promoter, T7 promoter and T7lac promoter, and three *Escherichia coli* host strains, BL21, BL21(DE3) and BL21(DE3)[pLysS] which differ in stringency of suppressing basal transcription, were compared. Based on the CLP3.1-his expression level, solubility of CLP3.1-his in cells and basal transcription that occurred in the absence of induction, two expression systems, BL21(DE3) containing plasmid pJY-2 with T7lac promoter and BL21(DE3)[pLysS] containing plasmid pJY-1 with T7 promoter, were selected. With these two expression systems, CLP3.1-his expression levels greater than 40% (g/g) of total cellular proteins and CLP3.1-his concentrations of 0.1–0.2 g l^{-1} can be achieved by using Luria–Bertani medium in shake flask batch cultures. The CLP3.1-his accumulated in the cells is totally soluble and no basal transcription was found before induction. These two high-level expression systems are promising for use in scale-up production.

Keywords: Collagen-like polymer; High-level expression; *Escherichia coli*; Promoter

1. Introduction

Collagen is a family of extracellular matrix proteins found in all connective tissues such as basement membranes, tendons, ligaments, cartilage, bone and skin (Jenkins and Raines, 2002; Schlosser et al., 1998). Various types of collagen molecules have in common a triple helical domain, in which each of the three α-helices contains the repeating triple sequence Gly–X–Y and the X and Y are frequently proline and hydroxyproline residues respectively (Schlosser et al., 1998; Cappello and Ferrari, 1998; Ruggiero et al., 2000a,b). The mechanical properties and biological characteristics of collagen render it suitable for natural-based biomaterial use (Ruggiero et al., 2000a,b).

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Table 1
Escherichia coli strains and plasmids used in this study

	Relevant properties	Sources or references
Strains		
DH5α	F$^-$ ϕ80d*lac*ZΔM15, Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_k^-,m_k^+) *pho*A *sup*E44λ$^-$ *thi*-1 *gyr*A96 *rel*A1	GibcoBRL[a]
BL21	F$^-$ *ompT hsdS*$_B$($r_B^-m_B^-$) *gal dcm*	Novagen[b]
BL21(DE3)	F$^-$ *ompT hsdS*$_B$($r_B^-m_B^-$) *gal dcm* (DE3)	Novagen[b]
BL21(DE3)[pLysS]	F$^-$ *ompT hsdS*$_B$($r_B^-m_B^-$) *gal dcm* (DE3) pLysS (Cm^R)	Novagen[b]
Plasmids		
pPT0296	Kan^R, cI-P$_R$	Cappello and Ferrari (1998)
pJHL	Kan^R, cI-P$_R$, His·Tag	This study
pET-14b	Amp^R, T7, His·Tag	Novagen[b]
pET-15b	Amp^R, T7*lac*, His·Tag	Novagen[b]
pJY-1	Amp^R, T7, His·Tag	This study
pJY-2	Amp^R, T7*lac*, His·Tag	This study

[a] GibcoBRL Life Technologies, Inc., 9800 Medical Center Drive, Rockville, MD 20850.
[b] Novagen, Inc., 601 Science Drive, Madison, WI 53711.

However, there are potential risks in term of viruses and other infectious agents pertaining to the use of collagen from animal sources. It is therefore useful to consider recombinant technology as a valuable alternative for collagen production (Ruggiero et al., 2000a,b).

During the past decade, different cellular systems such as mammalian cells (Fertala et al., 1994), insect cells (Nokelainen et al., 1996) and yeasts (Vaughan et al., 1998; Toman et al., 2000; Olsen et al., 2001; Nokelainen et al., 2001; Myllyharju et al., 2000), and transgenic organisms such as mice (Toman et al., 1999; Tomita et al., 1999) and plants (Ruggiero et al., 2000b) have been developed to produce stable triple helical collagens type I–III. However, the production levels of collagens were only about 0.6–20 mg l^{-1} for mammalian cells, 15–600 mg l^{-1} for yeasts, 10–60 mg l^{-1} for insect cells and 30 mg kg^{-1} for plants (Ruggiero et al., 2000a,b; Bulleid et al., 2000; Myllyharju et al., 2000). The most promising expression system might be the mouse mammary glands, which can produce up to 8 mg ml^{-1} type I procollagen (Toman et al., 1999).

Apart from the advantageous properties and usages of native fibrillar collagen, gelatin, the denatured and partially degraded collagen, is also widely used in medical and industrial areas such as intravenous infusions, matrix implants, injectable drug delivery microspheres and photographic film (Werten et al., 1999, 2001; Djagny et al., 2001). However, these industrial and medical applications are restricted to the type and availability of natural gelatins. To create new materials with novel properties and extend the utilization of gelatin, recombinant technology has been used to produce collagen-like polymers (CLPs) or gelatin-like polymers. By contrast with natural forms of collagen, recombinantly-produced CLP may consist exclusively of a single repeating tripeptide sequence selected from a wide variety of Gly–X–Y, where X and Y can be any amino acid and therefore additional chemical or biological functionality could be introduced (Cappello and Ferrari, 1998). Several systems such as *Pichia pastoris* (Werten et al., 1999, 2001), *Bacillus brevis* (Kajino et al., 2000) and *Escherichia coli* (Goldberg et al., 1989; Schlosser et al., 1998; Cappello and Ferrari, 1998) have been used to express CLPs. Kajino et al. (2000) reported the use of *Bacillus brevis* to secrete artificially designed gelatins derived from human αI collagen at 0.5 g l^{-1} in a 6-day culture. Werten et al. (1999) used *Pichia pastoris* to secrete recombinant non-hydroxylated gelatins based on mouse type I and rat type III collagen sequences.

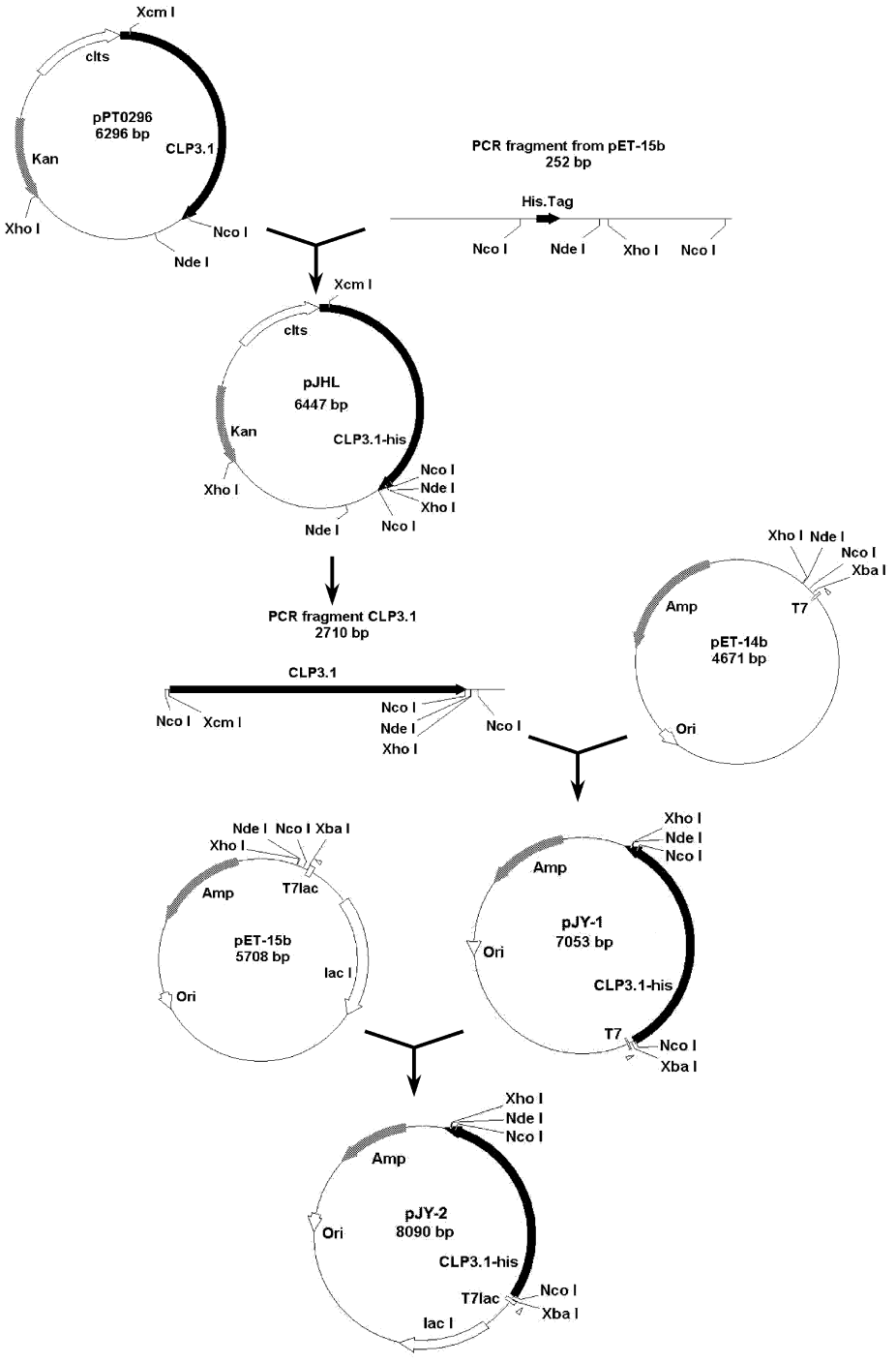

Fig. 1. Scheme for the construction of expression plasmids.

In a 94-h culture, 4–5 g l^{-1} recombinant gelatin was produced at a biomass wet-weight of 300–500 g l^{-1} when single-copy transformant was used. By using 15-copy transformant, up to 14.8 g l^{-1} recombinant gelatin was obtained (Werten et al., 1999). *Escherichia coli* is potentially a cost-effective, quick and technically straightforward prokaryotic expression system and has been frequently used for the high-level production of heterologous proteins. By using this system, protein expression levels can reach up to 50% of the total cellular proteins and cell concentration up to 180 g dry cell weight l^{-1} of fermentation broth can be obtained (Lee, 1996; Hannig and Makrides, 1998). Despite the fact that CLPs can be produced by recombinant *Escherichia coli* (Goldberg et al., 1989; Schlosser et al., 1998; Cappello and Ferrari, 1998), little effort for the efficient production of CLPs has been reported. Therefore, we explored the possibility of high-level production of CLPs by using *Escherichia coli* as the expression system. Since CLPs are intracellular products, their volumetric yields depend on both cell concentrations in bioreactors and protein expression levels in cells. Here, we report a strategy focusing on how to achieve high-level expression of CLPs in *Escherichia coli* cells by the selection of suitable promoters and host strains.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

The strains and plasmids used in this study are listed in Table 1.

Plasmid pPT0296 was obtained from Protein Polymer Technology, Inc. (San Diego, USA). This plasmid, with a thermally inducible promoter, can be used to express one type of CLP (CLP3.1) which consists of 52 repeating peptide (GAP-GAPGSQGAPGLQ) (Cappello and Ferrari, 1998). Plasmids pJHL, pJY-1 and pJY-2 were constructed in this study. They can be used to express CLP3.1-his in which a six-histidine tag is attached at the C-terminus of CLP3.1. This six-histidine tag renders CLP3.1 measurable by the immunodetection method and purified by metal–chelate affinity chromatography.

Escherichia coli strain DH5α was used as the recipient for all subclonings, and strains BL21, BL21(DE3) and BL21(DE3)[pLysS] were used for the expression studies. Cells were grown in Luria–Bertani (LB) liquid or on LB solid medium, as described by Sambrook et al. (1989). All media were supplemented, when necessary, with 50 μg ml^{-1} ampicillin (sodium salt) (Ap), 34 μg ml^{-1} chloramphenicol (Cm) and 30 μg ml^{-1} kanamycin (sulfate) (Kan), respectively.

2.2. Recombinant DNA methods

For most of the experiments, the standard techniques in recombinant DNA as described by Sambrook et al. (1989) were employed. All enzymes used for recombinant DNA techniques were from New England BioLabs Inc. or GibcoBRL Life Technologies. All primers were synthesized at Integrated DNA Technologies, Inc. All other chemicals were from Bio-rad, Sigma, Promega, Qiagen or Pierce.

Competent *Escherichia coli* DH5α was from GibcoBRL Life Technologies and competent *Escherichia coli* BL21, BL21(DE3) and BL21(DE3)[pLysS] were from Novagen. Large-scale preparation of plasmid DNA or purification of DNA fragment was achieved with Qiagen columns following the manufacturer's instructions.

2.3. Construction of plasmids

The scheme for the construction of the expression plasmids pJHL, pJY-1 and pJY-2 is shown in Fig. 1.

The gene of His·Tag was amplified by PCR using plasmid pET-15b as a template (primer 1, forward: 5′-cgcgaaattaatacgactca-3′; primer 2, reverse: 5′-atccatggccaaggggttatgctagt-3′). The restriction site *Nco* I was inserted into primer 2. The PCR product was digested with *Nco* I and the resulting 151 bp fragment was inserted into plasmid pPT0296, which had been digested with *Nco* I. The resulting plasmid was called pJHL.

The gene of CLP3.1 was amplified by PCR using plasmid pJHL as a template (primer 3, forward: 5′-taatccatggcagcacatccccctttc-3′; primer 4, reverse: 5′-tcagaggttttcaccgtcatc-3′). The restriction site *Nco* I was inserted into primer 3. The PCR product was digested with *Nco* I and the resulting 2382 bp fragment was inserted into plasmid pET-14b, which had been digested with *Nco* I. The resulting plasmid was called pJY-1.

A 2486 bp *Xho* I-*Xba* I fragment containing CLP3.1-his was excised from pJY-1 and cloned into the Xho I-*Xba* I sites of pET-15b. The resulting plasmid was called pJY-2.

2.4. Expression of recombinant collagen CLP3.1-his

Plasmid pJHL was transformed into *Escherichia coli* strain BL21(DE3) and plasmid pJY-1 or pJY-2 was transformed into *Escherichia coli* strains BL21, BL21(DE3) and BL21(DE3)[pLysS], respectively. *Escherichia coli* BL21(DE3) with pJHL was first grown at 30 °C, 220 rpm until the optical density (OD at 600 nm) reached 0.6–0.9, and then the temperature was increased to 41 °C to induce expression. For *Escherichia coli* BL21, BL21(DE3), BL21(DE3)[pLysS] with pJY-1 or pJY-2, the strain was grown at 37 °C, 220 rpm until the optical density (OD at 600 nm) reached 0.6–0.9, and then IPTG was added (final concentration 0.4 mM for pJY-1 and 1 mM for pJY-2) to induce expression. Five milliliter of cells were harvested by centrifugation for 10 min at 4000 rpm, 4 °C and then stored in a −20 °C freezer.

2.5. Small-scale purification of CLP3.1-his

CLP3.1-his in *Escherichia coli* cells was purified with metal–chelate affinity chromatography by the use of poly-his protein purification kit (Roche) following the manufacturer's instruction. SDS-PAGE (7.5% Tris–HCl gel) was performed to estimate the purity and molecular weight of CLP3.1-his. The commercially available bicinchoninic acid (BCA) protein assay (Pierce) was used to estimate the concentration of purified CLP3.1-his by using analytical grade gelatin as a reference.

2.6. Mass spectrometry

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed at the MIT Biopolymers Laboratory, using a Voyager-DE^{TM} STR Bio-Spectrometry Workstation (PerSepctive Biosystems). Sample was prepared by the dried droplet method, using sinapinic acid dissolved in 30% (v/v) acetonitrile, 0.07% (v/v) trifluoroacetic acid as matrix. Measurement was made in the positive, linear mode and the accelerating voltage was 25 000 V. Bovine serum albumin was used as external calibrant.

2.7. Measurement of CLP3.1-his by the immunodetection method

The concentration of CLP3.1-his was determined by the immunodetection method. This method was performed according to the description in 'QIAexpress Detection and Assay Handbook' (Qiagen). Proteins were separated using SDS-PAGE (7.5% Tris–HCl gel) and transferred electrophoretically onto a nitrocellulose membrane in a tank-blotting system (Bio-rad). The primary antibody was penta·his (Qiagen) and the secondary antibody was alkaline phosphatase conjugated anti-mouse IgG(H + L) (Promega). The bands of CLP3.1-his in the membrane were developed by reaction with BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium salt) (Sigma). The amount of CLP3.1-his was quantified by the densitometric scanning (Personal Densitometer SI, Molecular Dynamics Inc.) of the stained membrane. In the same membrane, the scanning volume of the band is proportional to the amount of CLP3.1-his. In each of our experiments, a known amount of purified CLP3.1-his was used as a standard for quantifying the recombinant collagen.

2.8. Determination of CLP3.1-his expression levels in Escherichia coli cells

Cell pellets were resuspended in 2 ml lysis buffer (8 M Urea, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 8.0). Disruption of the cells was conducted on ice using

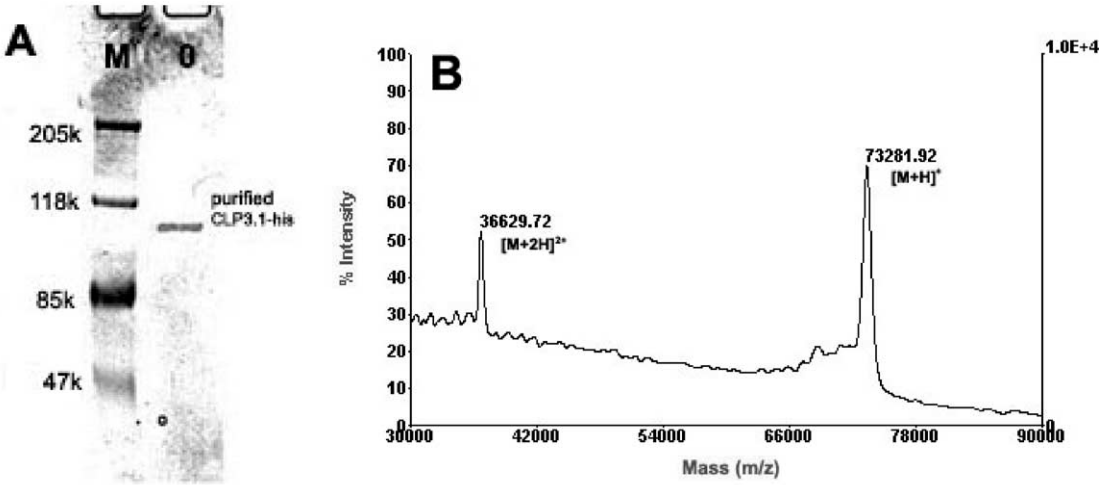

Fig. 2. SDS-PAGE (A) and Mass Spectrometry (B) analyses of CLP3.1-his purified from *Escherichia coli* BL21(DE3)[pJHL]. Lane M: molecular weight marker; lane 0: purified CLP3.1-his.

VirSonic Sonicator at level 3 for 12×10 s. Cell debris was removed by centrifugation at 12 000 rpm for 20 min and supernatant was collected. The amount of total proteins in the supernatant was determined by BCA protein assay using bovine serum albumin as a standard. The amount of CLP3.1-his in the supernatant was determined by immunodetection method. The expression level of CLP3.1-his in cells was expressed with ratio of CLP3.1-his to total proteins in the supernatant.

2.9. Determination of CLP3.1-his solubility

Cell pellets were resuspended in 2 ml buffer (50 mM Tris–HCl, 1 mM EDTA, 100 mM NaCl, pH 8.0). Disruption of cells was conducted on ice using VirSonic Sonicator at level 3 for 12×10 s. Supernatant (crude extract A, soluble CLP3.1-his) was collected by centrifuging the cell lysate at 12 000 rpm for 20 min. The sediment was resuspended in 2 ml lysis buffer (8 M Urea, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 8.0) and the suspension (crude extract B, insoluble CLP3.1-his) was disrupted on ice again by using VirSonic Sonicator at level 3 for 12×10 s. The amount of CLP3.1-his in either crude extract A or B was measured by immunodetection method.

3. Results and discussion

3.1. Characterization of CLP3.1-his

To qualitatively and quantitatively characterize the CLP3.1-his expressed by different *Escherichia coli* expression systems, a small amount of pure CLP3.1-his from *Escherichia coli* BL21(DE3)[pJHL] cells was first purified by metal–chelate affinity chromatography. The purity and molecular weight of the purified CLP3.1-his were first estimated by SDS-PAGE. As shown in Fig. 2(A), no other proteins were found in the purified CLP3.1-his product. However, by SDS-PAGE (Fig. 2(A)) the molecular weight of CLP3.1-his was estimated to be about 100 kDa, which is greater than 73.3 kDa deduced from the DNA sequence in plasmid pJHL. This difference may be due to the fact that the mobilities of CLPs on SDS-PAGE gels are lower than those of common proteins used as molecular weight marker (Kajino et al., 2000; Hayashi and Nagai, 1980). To confirm whether the CLP3.1-his has in fact the correct molecular weight, mass spectrometry was used. The observed molecular weight of $73\,268.18 \pm 12.74$ Da by mass spectrometry (Fig. 2(B)) agreed well with the theoretical value of 73 274 Da.

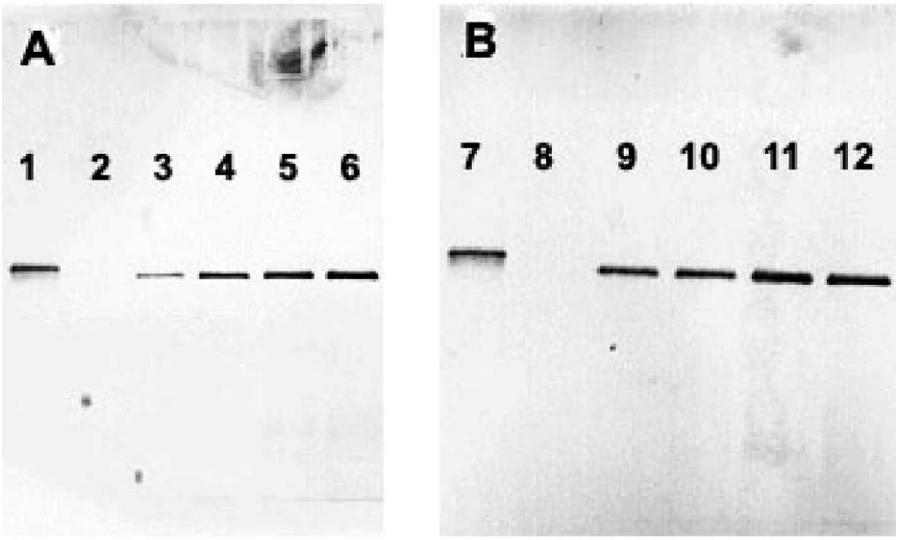

Fig. 3. Immunodetection analysis of CLP3.1-his contained in cells of *Escherichia coli* BL21(DE3)[pLysS][pJY-1] (A) and BL21(DE3)[pJY-2] (B) at different induction times. Lanes 1 and 7: pure CLP3.1-his purified from *Escherichia coli* BL21(DE3)[pJHL]; lanes 2–6: samples of BL21(DE3)[pLysS][pJY-1] cells at an induction time of 0, 1, 2, 3 and 4 h (all samples have same dilution factor); lanes 8–12: samples of BL21(DE3)[pJY-2] cells at an induction time of 0, 1, 2, 3 and 4 h (all samples have same dilution factor).

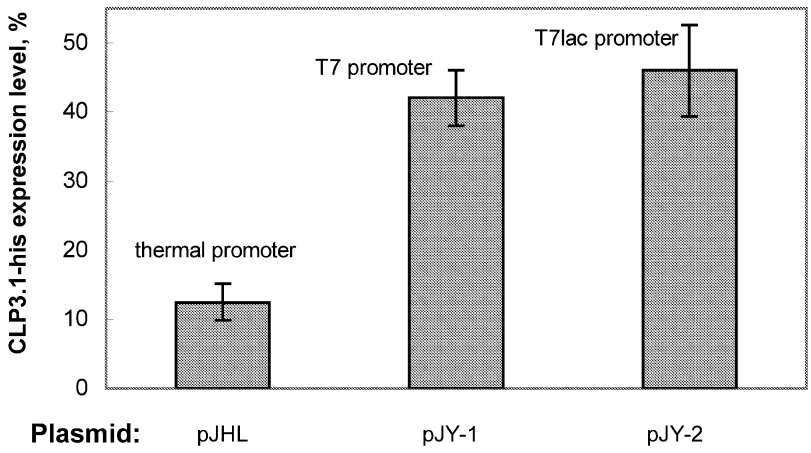

Fig. 4. Comparison of different promoters on the expression levels of CLP3.1-his in *Escherichia coli* BL21(DE3). The induction times for maximum expression level of CLP3.1-his were 3 h for pJHL, 1 h for pJY-1 and 3 h for pJY-2.

The concentration of the purified CLP3.1-his was further determined by BCA protein assay with analytical grade gelatin as the reference. This provided the known concentration for CLP3.1-his and was then used as standard in our immunodetection assay. Fig. 3(A) and (B) are examples of immunodetection analysis of CLP3.1-his expressed in cells of BL21(DE3)[pLysS][pJY-1] and BL21(DE3)[pJY-2] at different induction times. The molecular weight of CLP3.1-his deduced from the DNA sequence in plasmids pJY-1 and pJY-2 is about 70.8 kDa which is a little lower than 73.3 kDa of plasmid pJHL. This can explain why in Fig. 3(A)

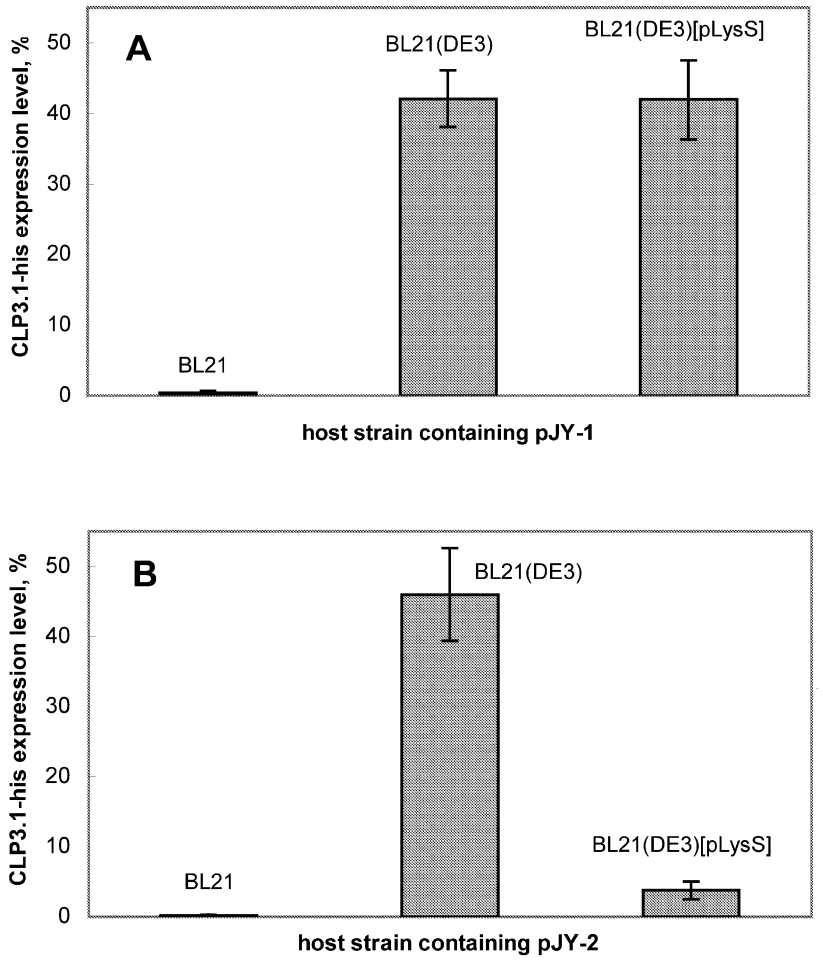

Fig. 5. Comparison of different host strains on the expression levels of CLP3.1-his by the use of (A) T7 promoter in plasmid pJY-1 and (B) T7lac promoter in plasmid pJY-2. The induction times for maximum expression level of CLP3.1-his were 1 h for BL21[pJY-1] and BL21(DE3)[pJY-1], and 3 h for BL21(DE3)[pLysS][pJY-1], BL21[pJY-2], BL21(DE3)[pJY-2] and BL21(DE3)[pLysS][pJY-2].

and (B), the bands (lanes 1 and 7) of pure CLP3.1-his from BL21(DE3)[pJHL] are a little bit higher than those (lanes 2–6, 8–12) of the CLP3.1-his from BL21(DE3)[pJY-2] and BL21(DE3)[pLysS][pJY-1] samples.

3.2. Effect of different promoters on the expression levels of CLP3.1-his

A fundamental factor influencing the expression of heterologous genes in *Escherichia coli* is the level of transcription provided by the promoter. Strong promoters have often been chosen for efficient foreign protein production. In this study, three promoters (Table 1), thermal promoter cI-P_R in plasmid pJHL, T7 promoter in plasmid pJY-1 and T7lac promoter in plasmid pJY-2, were compared. The host strain was BL21(DE3). As shown in Fig. 4, the maximum CLP3.1-his expression levels for these three promoters are approximately 13% (thermal promoter), 42% (T7 promoter) and 46% (T7lac promoter), respectively. These results demonstrate that using T7-based promoter to replace the thermal promoter can effectively increase the expression level of CLP3.1-his from 13% to greater than 40%.

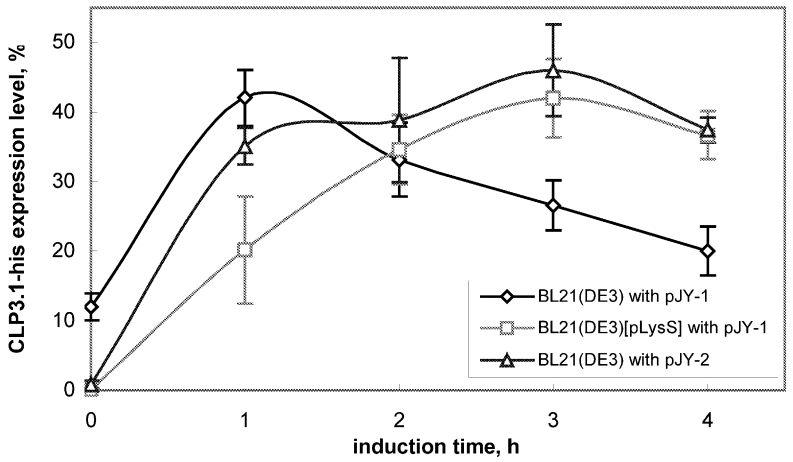

Fig. 6. Kinetics of CLP3.1-his expression levels in BL21(DE3)[pJY-1], BL21(DE3)[pJY-2] and BL21(DE3)[pLysS][pJY-1].

3.3. Effect of different host strains on the expression levels of CLP3.1-his

In addition to the use of different promoters, another important factor that may affect protein expression is the host strain. To select an efficient CLP3.1-his expression system, three host strains, BL21, BL21(DE3) and BL21(DE3)[pLysS] which differ in their stringency of suppressing basal expression levels were compared. With T7 promoter (in plasmid pJY-1), the maximum CLP3.1-his expression level in either BL21(DE3) or BL21(DE3)[pLysS] is about 42%. This is much higher than that of in BL21 (0.5%) (Fig. 5(A)). With T7lac promoter (in plasmid pJY-2), the CLP3.1-his expression level in BL21(DE3) is about 46% while those in BL21 and BL21(DE3)[pLysS] are only about 0.2 and 3.8% (Fig. 5(B)). Therefore, three CLP3.1-his expression systems which were BL21(DE3) with plasmid pJY-1, BL21(DE3) with plasmid pJY-2 and BL21(DE3)[pLysS] with plasmid pJY-1 were first selected and all their CLP3.1-his expression levels are shown to be greater than 40% in LB medium.

3.4. Basal CLP3.1-his expression levels of different combinations of promoters and host strains

The accumulation of heterogenous protein in cells before induction may affect the growth of cells especially if the heterogenous protein might be toxic to the cells. Lower level of basal transcription that occurs in the absence of induction is usually preferred in order to develop an efficient inducible *Escherichia coli* expression system. The amount of basal transcription could vary with different promoters and host strains. In the above experiments, three CLP3.1-his expression systems where the CLP3.1-his expression levels were greater than 40% were first selected. To further select a practicable CLP3.1-his expression system from these three CLP3.1-his expression systems, their basal transcription levels were also examined.

Fig. 6 shows the kinetics of CLP3.1-his expression levels in BL21(DE3)[pJY-1], BL21(DE3)[pJY-2] and BL21(DE3)[pLysS][pJY-1]. When the induction time is zero, CLP3.1-his expression level is equal to CLP3.1-his basal transcription level. For BL21(DE3)[pJY-1], the basal transcription level is about 12% while for BL21(DE3)[pJY-2] and BL21(DE3)[pLysS][pJY-1] their basal transcription levels are only about 0.5% (Fig. 6). Therefore, we finally chose BL21(DE3)[pJY-2] and BL21(DE3)[pLysS][pJY-1] as the CLP3.1-his expression systems. With these two systems, CLP3.1-his expression levels greater than 40% of total cellular proteins and CLP3.1-his concentrations about 0.1–0.2 g l^{-1} can be achieved by using LB medium in shake flask batch cultures.

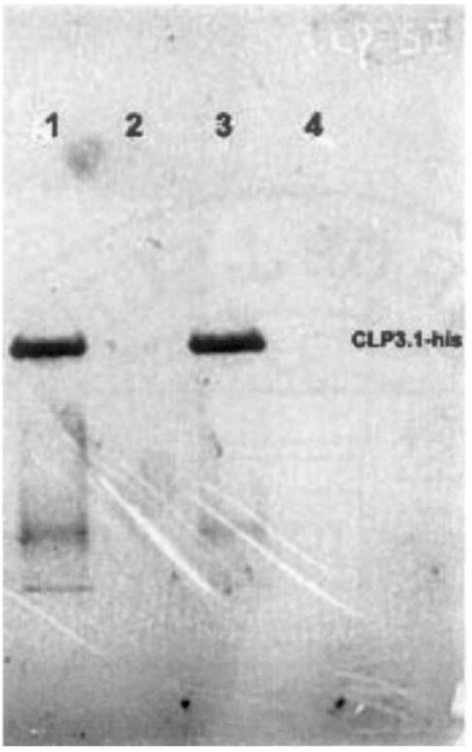

Fig. 7. Photograph from immunodetection method to determine soluble and insoluble CLP3.1-his in cells. Lanes 1 and 3 are for soluble CLP3.1-his. Lanes 2 and 4 are for insoluble CLP3.1-his. Lanes 1 and 2 are for BL21(DE3)[pJY-2] and lanes 3 and 4 are for BL21(DE3)[pLysS][pJY-1].

3.5. Soluble and insoluble CLP3.1-his in cells

Insoluble inclusion bodies are usually formed when heterogenous proteins are over-expressed in the host cells. To facilitate protein purification, it is desirable to express protein in the soluble state within the cells. Fig. 7 indicates that even CLP3.1-his expression levels are greater than 40%, CLP3.1-his in *Escherichia coli* cells are totally soluble. This demonstrates that the above two selected CLP3.1-his expression systems, BL21(DE3)[pJY-2] and BL21(DE3)[pLysS][pJY-1], are applicable to the potential production processes of producing a soluble CLP3.1-his protein.

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