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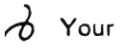
	TAKE OUT CHARACTER INDICATED	 Your proof.
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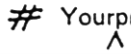
	INSERT SPACE	 Yourproof.
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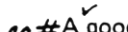
	EVEN SPACE	 A good proof.
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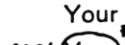
	CAPITALS	Your proof.  Your proof.
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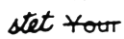
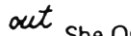
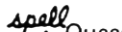
	DELETE, SEE COPY	 She Our proof.
	SPELL OUT	 Queen Eliz.
	START PARAGRAPH	 read. Your
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Understanding Heterologous Protein Overproduction under the T7 Promoter

A PRACTICAL EXERCISE

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Because various genome projects have been advanced many genes are known, and large amounts of proteins are required to elucidate their function. Most biomolecular research laboratories have a need to overexpress a certain gene, or a part of it, in eukaryotic or prokaryotic expression systems. It is therefore important for young students to become familiar with the technology of heterologous gene expression systems. Gene expression in eukaryotic cells is rather complicated and costly and is therefore not ideally suited to exercises for students. The goal of this paper is to describe an experimental example of a well known and broadly used prokaryotic system, the pET system, that works under the strong T7 promoter. The clones described in this paper are suitable for the practical exercise and are available upon request.

Keywords:—Bacteria, heterologous protein production, T7 promoter, gene overexpression, high levels of protein production.

[1]A large number of vector systems have been developed for the expression of cloned genes. Nowadays, methods for producing proteins from a cloned gene introduced in *Escherichia coli*, in yeast, or in eukaryotic cells have proved to be invaluable for the purification and functional analysis of proteins or their domains [1].

AQ: A

In this paper, we describe a practical exercise designed to provide an opportunity for bioscience students to carry out gene expression experiments and to quantify the overproduction of a protein under various cell growth and induction conditions. The target gene proposed encodes the HU protein from *Bacillus stearothermophilus*. The advantages of using the HU gene are as follows: (*a*) it shows a clear expression that can be detected by SDS-PAGE analysis and can be easily quantified, (*b*) it follows exactly the properties of the expression system, (*c*) it requires minimum experimental skills and equipment, (*d*) it can be carried out within a period of 3 to 4 days, and (*e*) it is inexpensive.

AQ: B

HU is a major component of the prokaryotic cell nucleoid that contains several abundant, low molecular weight, and positively charged proteins. HU has been classified as histone-like DNA-binding proteins. HU from *B. stearothermophilus* is a homodimer with 90 amino acid residues for the monomer. Further information about the function of the HU proteins can be found in Ref. 2.

MATERIALS AND METHODS

Construction of the Plasmids and Clones Available for this Exercise

The cloning of the gene encoding for the HU protein from *B. stearothermophilus* into the pET-3a vector leads to the construction of the pCV*hubst* expression plasmid as described [3, 4]. The pCV*hubst* plasmid (ampicillin-resistant) was introduced into three *E. coli* host strains, BL21(DE3), BL21(DE3)pLysS, and BL21(DE3)pLysE, that have different properties as discussed previously [3]. The transformation was carried out as described [4]. The three expression bacterial strains are as follows: (*a*) BL21(DE3)-pCV*hubst*, which grows at 37 °C in LB medium (Luria-Bertani medium: 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, pH 7.0) in the presence of 50 μg/ml ampicillin; (*b*) BL21(DE3)pLysS-pCV*hubst*, which grows at 37 °C in LB medium in the presence of 50 μg/ml ampicillin and 35 μg/ml chloramphenicol; and (*c*) BL21(DE3)pLysE-pCV*hubst*, which grows at 37 °C in LB medium in the presence of 50 μg/ml ampicillin and 35 μg/ml chloramphenicol. In cases where the clones are unstable and the plasmids are rejected, higher ampicillin concentrations, up to 100–200 μg/ml, can be used.

Growth and Induction Experiments of the Three Bacterial Clones

Each of the above three bacteria strains can be activated from a bacterial colony from agar plates, stabs, or glycerol stocks by inoculating a 5–10-ml culture of LB growth medium supplemented with antibiotics as described above and incubated at 37 °C overnight. Under these conditions the cultures usually reach an $A_{600\ nm}$ of about 2.0. The cell density of each individual culture has to be determined, because the next step is to dilute each culture to 50 ml with fresh LB medium, plus proper antibiotics, to a final $A_{600\ nm}$ of 0.2, and incubated until it reaches cell density of about 0.6. At this point each culture can be divided in four parts, and isopropyl-β-D-thiogalactopyranoside (IPTG)[1] can be added at four different final concentrations between 0 and 1 mM, to initiate the induction of the gene expression. It is recommended to try 0, 0.1, 0.25, 0.5, and 1 mM IPTG to study the effect of the IPTG concentration on the expression level of the HU gene in the three different expression plasmid constructs. A time ki-

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[1] The abbreviation used is: IPTG, isopropyl-β-D-thiogalactopyranoside.

netics analysis is also recommended after addition of IPTG. Usually 0, 1, 2, 3, 6, and 24 h after induction is sufficient to provide to the students good information to understand the function of this particular expression system. If the laboratory is well equipped, a few bacterial growth temperatures can also be tried, *e.g.* 19, 25, 30, and 37 °C. During the course of this study the optical density at 600 nm of each sample can be determined to follow the growth of the cells under various temperature and IPTG conditions. Additionally, a rich medium, LB, and a minimal medium, M9 (1 g/l NH_4Cl, 3 g/l KH_2PO_4, 6 g/l Na_2HPO_4, 0.4% glucose, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1% NaCl, pH 7.0), can also be tried. From each kinetic point, cells of a 1-ml bacteria culture can be pelleted in a table top centrifuge at 10,000 rpm for 10 min. After careful removal of the supernatant, the cell pellet can be stored frozen at −20 °C. When the induction kinetics is completed, the induced clones can be analyzed by 15% SDS-PAGE and stained with Coomassie Blue G-250, as described below, to analyze the total protein profile of the cell and the overproduction of the target protein under various conditions.

Preparation of the Protein Samples and Analysis by SDS-PAGE

Each individual pellet of a 1-ml bacteria culture (stored at −20 °C) can be dissolved in SDS loading buffer and proteins run on an SDS-PAGE gel as described by Laemmli [6] and Sambrook *et al.* [7]. The amount of the sample that can be applied depends on the dimensions of the casted plates. For a 10 × 12-cm slab gel with 6–8-mm slots, application of a bacterial pellet from 1 ml of culture at 0.1 optical density dissolved in 10 μl of SDS buffer is recommended.

Organization of the Practical Exercise

The overproduction experiments described in this paper can be performed in a 3–4-day practical exercise as described below.

Day 1—The first day requires 2–3 h to organize the experiments and to decide on the kinetics that can be carried out. The following three parameters have to be considered: (*a*) IPTG concentration, in a range from 0 to 1 mM; (*b*) bacterial growth temperature, in a range from 19 to 37 °C (*e.g.* 19, 25, and 37 °C); and (*c*) time of gene expression after IPTG induction in the range from 0 to 24 h (usually 0, 1, 2, 3, 6, and 24 h are sufficient). The size of the group and the available equipment will define the number of the experiments that can be carried out. The first day ends with the inoculation of bacteria culture either from frozen glycerol stock or preferably from already activated cultures on agar plates.

Day 2—The bacterial cultures are diluted as described above to the right bacterial density, and IPTG is added to start the induction of the target gene. The second day is fully occupied.

Day 3—The induction kinetics have been completed after the 24-h samples have been withdrawn. Meanwhile the SDS-PAGE have been prepared to run (requires 1–1.5 h). After completion of the sample preparation (requires 2–3 h) the SDS-PAGE protein analysis is carried out (requires 4 h).

Day 4—The SDS-PAGE results can be evaluated and discussed. If a gel densitometer is available the overproduction of the target protein, HUBst, can be quantified.

During the performance of the exercise the students have to keep records concerning bacterial growth, amounts of samples withdrawn during the kinetics studies, and, if possible, quantification of the overproduced HU protein under various growth and induction conditions. The final evaluation of the exercise can be performed by comparing various induction conditions with the growth rate of the cells and the produced amount of the target protein.

AQ: C

RESULTS AND DISCUSSION

The expression system used for this study is a rather complicated system, because it is based on a direct control of the T7 RNA polymerase, which is incorporated into the chromosome via IPTG and not on the plasmid where the target gene is cloned under the T7 promoter. Briefly, the bacterial host for overproduction of the HU protein pCV*hubst* construct is *E. coli* B strain BL21 [1]. Bacteriophage DE3 is a λ derivative that has the immunity region of phage 21 and carries a DNA fragment containing the *lac*I gene, the *lac*UV5 promoter, the beginning of the *lac*Z gene, and the gene for T7 RNA polymerase. Once a DE3 lysogen is formed, the only promoter known to direct transcription of the T7 RNA polymerase gene is the *lac*UV5 promoter, which is inducible by IPTG. Addition of 0.1–1 mM IPTG to a growing culture of the BL21(DE3) induces T7 RNA polymerase, which in turn transcribes the target DNA in the pET plasmid. Target genes whose products are sufficiently toxic cannot be established in BL21(DE3), because the basal level of T7 RNA polymerase activity will promote some transcription of the target toxic gene in the uninduced cell, and the cells will either not grow or reject the plasmid. One way to reduce this basal activity is to use the T7 lysozyme. T7 lysozyme is a natural inhibitor of T7 RNA polymerase and can be provided to the cell from a clone of the T7 lysozyme gene in another plasmid (pLyS) where the T7 lysozyme gene is cloned in two different orientations. In pLysS the T7 lysozyme is expressed moderately whereas in plasmid pLysE it is expressed highly. These plasmids confer resistance to chloramphenicol and are compatible with pET vectors. The presence of either pLysS or pLysE increases the tolerance of BL21(DE3) for toxic target plasmids. This is because of the presence of lysozyme. In the strain that contains the pLysS, a small amount of T7 lysozyme is produced, and therefore lower inhibition of the T7 RNA polymerase occurs. In the strain that contains pLysE, a higher amount of lysozyme is produced, and therefore higher inhibition of the RNA polymerase occurs. Therefore, unstable constructs become stable in the presence of pLysS plasmid, whereas constructs that are too toxic to be established in the presence of pLysS but are able to be established in the presence of pLysE. Only a few genes are too toxic even in the presence of pLysE. BL21 cells are suitable as an expression strain, because they are deficient in the *lon* protease and also lack the *ompT* outer membrane protease that can degrade proteins during purification.

In the gene overexpression (or protein overproduction) experiments for HU the results follow exactly the general rule of the system described briefly above. In the BL21(DE3) cells no induction of HU occurred even after 24 h, because HU has an homologous partner in *E. coli*, and therefore overproduction of this protein causes lethal problems for the bacteria (Fig. 1, *lanes 1* and *2*). The BL21(DE3)pLysS-pCV*hubst* clone was induced by IPTG up to 24 h. The cells had difficulty growing to higher densities in the absence of IPTG but showed significant synthesis of HU protein even 1 h after IPTG induction and reached a maximum within the first 3 h at 1.0 $A_{600\ nm}$ units of cell density (Fig. 1, *lanes 3* and *4*). In the next few hours the cells grow more slowly, reaching a plateau after 6 h, when HU overproduction stops. 24 h after IPTG induction HU synthesis is reduced to 30% of its maximum value. Upon long induction the cells do not survive and are lysed. There-

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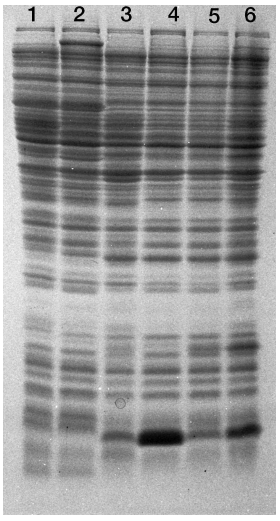

FIG. 1. **0.1% SDS-15% PAGE of the induction and overproduction experiments of HU as described under "Materials and Methods."** Each *lane* contains total protein extract of each clone at time 0 and 3 h after induction. *Lanes 1* and *2*, BL21(DE3)-pCV*hubst* total bacterial proteins after 0- and 3-h inductions, respectively. *Lanes 3* and *4*, BL21(DE3)pLysS-pCV*hubst* total proteins after 0- and 3-h inductions. *Lanes 5* and *6*, BL21(DE3)pLysE-pCV*hubst* total protein profile at 0 and 3 h post-induction, respectively. In each slot 0.1 $A_{600\,nm}$ units of the bacteria were supplied after having been resuspended in loading buffer, boiled for 3 min, and briefly centrifuged at 15,000 rpm. The electrophoresis was carried out for 2.5 h at 30 mA at room temperature. The gel was soaked for 45 min in staining solution and then in destaining solution for 3–5 h [7].

fore it is advisable to perform short inductions, usually no more that 3 h. The BL21(DE3)pLysE-pCV*hubst* cells showed slower HU synthesis that reaches half the expression level compared with pLysS clone within 3 h (Fig. 1, *lanes 5* and *6*). It has to be pointed out that in BL21(DE3)pLysS-pCV*hubst* and BL21(DE3)pLysE-pCV*hubst* clones, even at time 0 of induction there is still low protein production (basal T7 RNA polymerase activity) because of the high potential of the T7 promoter (Fig. 1, *lanes 3* and *5*). In the BL21(DE3)-pCV*hubst* we have no expression and background activity, because the HU gene is toxic for the *E. coli*, and the cells have difficulty growing.

Induction kinetics were performed to estimate the optimum final IPTG concentration. Various concentrations used were in the range of 0 to 1 mM IPTG. In all cases, the optimum IPTG concentration proved to be 0.4 mM with a 3-h induction in the BL21(DE3)pLysS cells. In addition different growth media were tested for the induction of HU. Among various media tested, LB and M9 [7] are the most efficient.

One of the major problems associated with production of foreign protein in non-physiological amounts in bacteria is the correct folding and the solubility of the final product, the protein. In many cases, high protein overproduction drives to a large population of misfolded protein molecules, their aggregation, and the formation of inclusion bodies. There are several ways to minimize this problem, but not all of them are successful. Many laboratories and the industry search for conditions to refold and activate these proteins; this approach is not always successful. An alternative approach is to optimize the overproduction system by selecting conditions that lower or even prevent the formation of inclusion bodies. Because each protein has its own features, a search for various host cells with different phenotypes, as well as cell growth temperatures, growth medium, cell density, and induction conditions, are parameters that have to be considered. The effect of the post-translational modifications on the folding, solubility, stability, and activity of the target protein or eukaryotic origin also have to be taken into account.

During the course of this practical exercise, bioscience students have the chance to try three different expression bacterial clones with different properties for the same target gene. They can test different induction conditions and quantify the overproduced protein and therefore understand the mechanism of this expression system. The pET expression system is a rather complicated expression system based on a delicate balance between the high activity of the T7 RNA polymerase and its inhibitor T7 lysozyme. This is regulated experimentally by controlling the expression of the T7 RNA polymerase using various concentrations of IPTG and regulating the cell activity via growth temperature. The students will become familiar with the handling of bacteria cells that can produce a target protein, analysis, and quantification of a protein using SDS-PAGE and will gain experience searching for conditions for optimal expression of particularly genes that are toxic for the host cells. The students are encouraged to study Studier *et al.* [1] to be able to understand and successfully carry out the experiments. The proposed system gives students an opportunity to use three different expression bacterial clones that exhibit different properties and capabilities to overproduce the same target protein. The quantification of the results is focused on the correlation of several parameters like initial cell densities for induction, IPTG concentration, length of induction, and growth temperature. These parameters can be correlated with the amount of the HU protein produced, which is the final goal of this exercise.

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AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES **1**

A—The genus was added per journal style; the genus must be written out the first time in the title, Summary, and text, otherwise it must be abbreviated.

B—If 'it is inexpensive' not as meant please reword 'has a low budget.'

C—Moved up to avoid a one-sentence paragraph per journal style.

D—Please cite Ref. 5 in text, or it will be replaced with 'Deleted in proof.'