Journal of Genetic Engineering and Biotechnology (2017) **15**, 179–185

HOSTED BY

ELSEVIER

Academy of Scientific Research & Technology and National Research Center, Egypt

Journal of Genetic Engineering and Biotechnology

www.elsevier.com/locate/jgeb

ORIGINAL ARTICLE

Chemical complexity of protein determines optimal *E. coli* expression host; A comparative study using Erythropoietin, Streptokinase and Tumor Necrosis Factor Receptor

Subramani Ramkumar *, **Vaishnavo Rabindranath Pai, Chinnathambi Thangadurai, Vidhya Priya Murugan**

Genetic Engineering Unit, Centre for Biotechnology, Anna University, Sardar Patel Road, Guindy, Chennai 600 025, India

Received 8 August 2016; revised 3 November 2016; accepted 19 December 2016
Available online 13 January 2017

KEYWORDS

Erythropoietin;
Streptokinase;
TNFR;
Expression;
Recombinant;
Inclusion bodies

Abstract High throughput expression of proteins is often hampered by the failure of certain proteins to express in the particular *E. coli* host strain used for the study. The identification of a host strain compatible for a wide variety of proteins is desirable. In this study, the recombinant expression of therapeutic proteins Erythropoietin (EPO), Streptokinase (SK) and Tumor Necrosis Factor Receptor Extra cellular domain (TNFR ED) that vary widely in their chemical nature was studied in four different strains of *E. coli* namely BL21 (DE3), BL21 (DE3) pLys S, BL21 (DE3) Rosetta pLys S and GJ1158. Since there are no previous report for the analysis of expression and solubility of the above mentioned proteins we studied the same in various *E. coli* stains. Here we report that *E. coli* strain GJ1158 which uses salt induction was found to be the most suitable for overexpression of all the three proteins. Interestingly rare codons were found not to play any significant role in the expression. Protein toxicity and aggregation propensity were also studied. One of the major factors influencing expression was the tendency of the protein to aggregate which in turn influences folding and toxicity levels. The solubility of the proteins was inversely proportional to aggregation. Expression levels were in the order of TNFR ED < EPO < SK. In conclusion, it was observed that *E. coli* GJ1158, a strain known to decrease aggregation of proteins was found to be more suited for expression. This is the first time GJ1158 has been included in this kind of analysis for comparison of protein expression in various *E. coli* hosts.

1. Introduction

An efficient recombinant protein production strategy determines the friendliest host for the protein of interest in order to

* Corresponding author.
E-mail address: ramelango2002@gmail.com (S. Ramkumar).
Peer review under responsibility of National Research Center, Egypt.

http://dx.doi.org/10.1016/j.jgeb.2016.12.006

achieve high yields. When a large number of recombinant proteins are being screened for expression, the choice of the host for expression is of major importance. A host that is compatible for majority of the proteins is desired.

In our study, we have compared the expression of three major therapeutic proteins namely Streptokinase (SK), Erythropoietin (EPO) and Tumor Necrosis Factor Receptor extracellular domain (TNFR ED) in four different strains of *E. coli*. Streptokinase is a thrombolytic agent, its source being *Streptococcus*. TNFR ED is a human transmembrane receptor used in treatment of rheumatoid arthritis [2]. EPO is a human secretory glycoprotein used in chronic anemia therapy [9]. In this study on recombinant protein synthesis, a construct carrying the first domain of TNFR II namely, TNFR-ED was used for expression analysis. A number of differences are present between the proteins for eg. Post-translational modifications such as glycosylation, presence of disulfide bonds, number and composition of rare codons (Table 1). There are no disulfide bonds in SK, since there are no cysteine residues present in this protein. On the other hand human TNFR ED, which is pretty rich in cysteine residues, contains 12 disulfide bonds. Bridging these two above-mentioned proteins is human EPO having two disulfide bonds. Both TNFR ED and EPO are known to be glycosylated in humans; however in *E. coli* this particular posttranslational modification is not possible. Based on these modifications and composition of amino acids, the proteins can be said to be in the following order of chemical complexity – TNFR ED > EPO > SK. It is therefore interesting to analyze the heterologous expression behaviors of the above-mentioned proteins in different *E. coli* host strains. Interestingly, there are no previous reports of such comparison for the above-mentioned proteins.

The *E. coli* expression hosts that have been used are also different in that they have their own special purpose in recombinant protein expression. *E. coli* BL21 (DE3) is the commonly used host for expression of non-toxic proteins. *E. coli* BL21 (DE3) pLys S is also a common host, used however preferentially for the expression of toxic proteins. [16]. *E. coli* BL21 (DE3) Rosetta pLys S is also a stringent host providing tRNAs for rare codons. *E. coli* GJ1158 is unique in that it contains the T7 RNA Polymerase gene under the control of pro U promoter, which is induced by increased salt concentrations [14]. There have been many reports comparing expression of particular proteins in different hosts [3,4], however this is the first time *E. coli* GJ1158 has been included in this kind of analysis. Here we have attempted to analyze the different expression levels of the therapeutic proteins EPO, TNFR ED and SK in different hosts and of the different proteins in same host.

2. Materials and methods

2.1. Bacterial strains and plasmids

For initial transformation of the ligation mixtures, the maintenance strain *E. coli* DH5α was used. The bacterial strains used to study expression were *E. coli* GJ1158, *E. coli* BL21 (DE3), *E. coli* BL21 (DE3) pLys S, *E. coli* BL21 Rosetta (DE3) pLys S. *E. coli* GJ1158 [14], derived from *E. coli* B strain BL21, is a salt inducible strain with a pro U promoter (Table 2). The strain was obtained from the Centre for Cellular and Molecular Biology (CCMB), India. *E. coli* BL21 (DE3), *E. coli* BL21 (DE3) pLys S, *E. coli* BL21 Rosetta (DE3) pLys S (Novagen,) are IPTG inducible strains in which the T7 RNA Polymerase gene is under the control of a Lac UV5 promoter.

The expression vector used in this study is pRSET A obtained from Invitrogen Life Technologies, USA. pRSET is a high copy number plasmid with a pUC origin of replication.

2.2. Recombinant plasmid construction

Standard recombinant DNA techniques were used for the cloning of human EPO gene, Streptokinase and TNFR ED in pRSET vector. Genes were cloned in the *Bam* HI-*Hind* III

Table 1 Characteristics of the proteins used in this study.

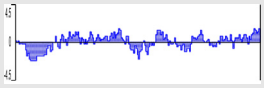
	Streptokinase	Erythropoietin	TNFRII-ED
Origin	Prokaryotic	Eukaryotic	Eukaryotic
Molecular weight (kDa)	45	18.6	25
No of amino acids in protein used in this study	414	166	235
Rare codons	9 leucine 8 arginine 2 proline 2 isoleucine No rare codon repeats	8 arginine 1 prolines 	13 prolines 6 arginines Arg-Pro doublet In two positions
Disulfide bonds	No cysteines	2	12
Glycosylation	None	4 sites	4 sites
Protein localization	Secretory in *Streptococcus*	Secretory in humans	Either part of receptor or shed into blood stream
Hydrophilicity(Kyle and Do Little)			
Insolubility index http: //www.biotech.ou.edu/	57.3% chance of solubility	72.6% chance of insolubility	87.5% chance of insolubility

Table 2 Primers and Strains used in this study.

S. No	Primer used	
1	TNFR ED Forward	5′cccggatcc
2	TNFR ED Reverse	5′accaagctt
3	EPO Forward	5′cccggatccgccccaccacgcctcatctgtgac3′
4	EPO Reverse	5′ accaagctttcatcttgtcccctgtc3′
5	Streptokinase Forward	5′cccggatcc
6	Streptokinase Reverse	3′cccgaattc
	Host strains	Genotype
1	*E. coli* DH5α	F^- Φ80dlacZΔM15, Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(r_k^-/m_k^-) phoA supE44 l^-thi-1 gyrA96 relA1
2	*E. coli* BL21 (DE3)	F^- ompT hsdSB (r^- B/m^-B) gal dcm (DE3)
3	*E. coli* BL21 (DE3) pLys S	F^- ompT hsdSB (r^-/B/m^-B) gal dcm (DE3) pLysS (CmR)
4	*E. coli* BL21 (DE3) Rosetta pLys S	F^- ompT hsdSB(rB^-mB^-) gal dcm lacY1 (DE3) pLysSRARE6 (CmR)
5	*E. coli* GJ1158	ompT hsdS gal dcm DmalAp510 malP::(proUp-T7 RNAP) malQ::lacZhyb11 D(zhf-900::Tn10dTet

sites using standard recombinant techniques (for primers used refer Table 2). All the three proteins are therefore fusion proteins with an N terminal His tag, Gene 10 leader sequence express epitope region, Enterokinase cleavage site contributing an additional 4 kDa to their molecular weight.

2.3. Plasmid stability test

Freshly transformed *E. coli* BL21 (DE3) harboring the recombinant plasmids was used. A single colony was picked up and inoculated in 3 ml LB–ampicillin (100 μg/ml) medium. The culture was incubated at 37 °C with shaking until the turbidity reached 0.6 O.D at 600 nm. Cultures were induced by the addition of 1.0 mM IPTG for 3 h. Following measurement of optical density of the cultures at 600 nm of uninduced and induced cells, a series of dilution was prepared and for each dilution, 150 μl was immediately plated on LB-agar plates containing ampicillin (100 μg/ml).

To determine the fraction of cells that carry the plasmid before induction and to test for unstable target plasmids, uninduced cultures (150 μl of each dilution) was plated in LB Agar plates supplemented with ampicillin and 1 mM IPTG. The plates were then incubated at 37 °C overnight. The next day the number of colonies was counted.

2.4. Expression studies of human EPO, SK and TNFR ED from pRSET vector

A single colony of *E. coli* harboring the recombinant plasmid was inoculated in 3 mL of LB medium and grown to 0.6–1.0 O D_{600}, induced with 1 mM IPTG and incubated for 3 h at 37 °C in a shaker at 180 rpm. Prior to induction, 1 mL of cells were aliquoted to use as control for uninduced cells. The cells were harvested by centrifuging at 6000 rpm for 10 min**.** Cell pellets of both induced and uninduced cell cultures were then suspended in 1X PBS and vortexed. Later proteins are estimated by the Bradford method [11], and 4× sample solubilizing buffer was added then boiled for 10 min at 100 °C. The protein samples were then taken for SDS–PAGE analysis for assaying protein amounts.

In case of *E. coli* GJ1158, the cells were grown as usual to 0.6–1.0 O D_{600} in LBON medium and induced with 0.1 M NaCl for 3hours at 37 °C.

2.5. Determination of protein in soluble and insoluble (aggregated) fractions

50 mL shake flask cultures of *E. coli* GJ1158 harboring the recombinant plasmids were induced at the optimal 0.6 OD_{600} for EPO and 1.0 OD_{600} for TNFR ED and Streptokinase with 0.3 M NaCl for 3 h. The cell pellet of the induced cultures was then suspended in 3 mL of 1XPBS. Samples were sonicated using Branson sonicator, by applying a 30 s on/ off cycle for 5 times at amplitude of 60 and the frequency 0.5.

2.6. Western blot analysis

Expression of the three proteins from *E. coli* BL21 (DE 3) in all the expression strains was analyzed by western blotting [11] using 1/2000 dilution of anti His monoclonal antibodies (as recommended by manufacturer, Sigma Aldrich, USA). The blot was incubated in primary antibody overnight or alternatively for 2 h at room temperature and in alkaline phosphatase conjugated secondary *mouse anti human IgG* for 1 h at room temperature with gentle shaking. The blot was developed using Nitroblue Tetrazolium and Bromo Chloro Indolyl Phosphate.

3. Results and discussion

The influence of the 5′ region of the coding sequence of proteins on heterologous expression levels has been well documented [3]. To compare the expression levels of these proteins in different *E. coli* expression strains, a common downstream box would be therefore essential in order to exclude any potential effects of the N terminal region of the native protein on its expression behavior.

In this study, the coding sequences of these proteins have been introduced in the *Bam* HI – *Hind* III/*Eco* RI sites of the pRSET vector. By doing so, all the proteins will be made

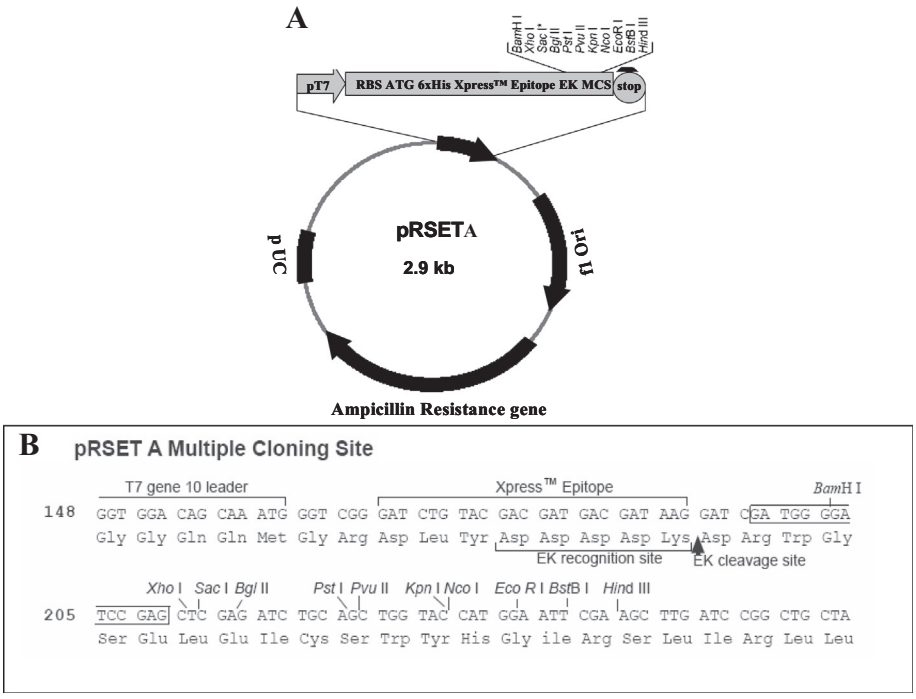


Fig. 1 A: pRSET vector map, B: pRSET A multiple cloning site.

as fusion proteins carrying a N terminal-His tag, gene 10 leader sequence, X press epitope and a enterokinase cleavage site (Fig. 1) adding an extra 4 kDa to the molecular weight. No differences therefore exist in the backbone of the constructs; particularly in the downstream region of the start site, whatever differences in expression levels reflect the compatibility levels between the protein and the hosts employed. We therefore choose to analyze the results based on protein-host combinations using the above-mentioned constructs.

3.1. Effect of common downstream box on expression

Previous *in vitro* protein expression studies using cell free systems showed that the use of a common downstream box such as Chloramphenicol Acetyl Transferase (CAT) leader sequence or the 6X His tag repeat led to equal expression of two different proteins [7]. In our study, we found that this doesn't extrapolate to *in vivo* expression experiments. In spite of using a common downstream box (pRSET Histidine fusion tag), uniform expression of all the three proteins in any single *E. coli* host strain was not observed. One of the possible explanations for this effect could be due to variation in the stability of the specific mRNAs and proteins in different *E. coli* strains. Additionally there might be differences in the decoding rates of mRNAs due to formation of secondary structure etc. therefore resulting in the synthesis of various amounts of proteins in different host strains.

3.2. Expression in E. coli BL21 (DE3) and plasmid stability

TNFR ED showed moderate expression levels in *E. coli* BL21 (DE3) on induction at 0.6 and 1.0 OD_{600} (Fig. 3). Streptokinase showed very high expression levels compared to TNFR at both the induction points (Table 3). While leaky expression

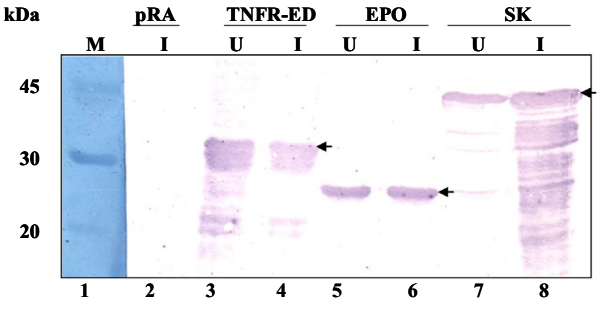


Fig. 2 Immunoblot analysis of total protein of induced *E. coli* BL21 (DE3) cellsharboring the recombinant plasmids using anti-His antibodies**.** Lanes 1 – Protein marker, 2 – pRSET A vector control induced, 3 – pRSET-TNFR ED uninduced, 4 – pRSET-TNFR ED induced, 5 – pRSET EPO-uninduced, 6 – pRSET EPO-induced, 7 – pRSET SK uninduced, 8 – pRSET SK induced.

was not visible by SDS PAGE for TNFR ED, it was clearly seen for streptokinase as a prominent band in uninduced total protein (Table 3). In fact in the case of EPO, at 0.6 OD_{600} no additional band was visible whereas in the 1.0 OD_{600} samples equal amount of EPO protein was seen in uninduced and induced (Table 3). Western blot analysis for all three proteins showed high concentration of protein in uninduced total protein samples as well (Fig. 2). The high levels of protein in uninduced sample may be due to auto induction by low levels of lactose in LB medium as reported earlier [17].

During the course of the experiments it was observed that expression of Streptokinase in BL21 DE 3 was reproducible and stable. But in case of TNFR ED and EPO, consistent expression was not observed. Only freshly transformed cells were capable of expression and even the moderate expression

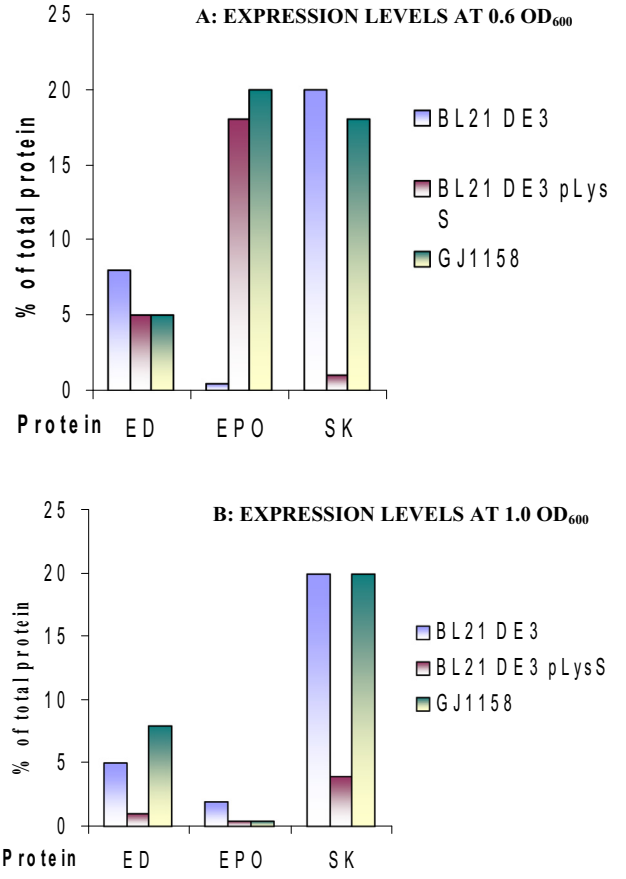


Fig. 3 Comparison of expression levels of TNFR ED, EPO and SK in different hosts at 37 °C on induction with 1 mM IPTG for *E. coli* BL21 (DE3) and BL21 (DE3) pLys S strains and 0.3 M NaCl for *E. coli* GJ1158 strains at (A) 0.6 OD_{600}, (B) 1.0 OD. Basal level expression was given a minimal value of 0.5%.

was lost within a week on repeated attempts. This kind of inconsistency in expression levels when using *E. coli* BL21 DE 3 has been reported before [6].

When plasmid stability was tested by plating uninduced cells in LB Amp plates, almost equal numbers of colonies were formed with all three proteins. However on checking for unstable target plasmid by plating induced culture on LB Amp agar, there was a drastic difference, with the highest number of colonies formed by ED and the least by EPO and SK. Those plasmids which are regarded as unstable will grow in LB Amp IPTG plates. Only those cells harboring the plasmid but unable to express the protein will form colonies in LB Amp IPTG plates. In case of Streptokinase, there is successful induction of target protein by IPTG. Although there is plasmid stability, due to induction, there is no growth of cells and this leads to few colonies on LB Amp IPTG plates (Table 4).

E. coli BL21 DE 3 is regarded generally to be a suitable host for the expression of non toxic proteins [6]. Based on the above results, it was inferred that Streptokinase is not toxic to *E. coli* whereas EPO and TNFR ED pose some level of toxicity to the host that is reflected by their lowered expression levels in spite of equal plasmid stability.

Table 3 Results of Plasmid stability and Plasmid toxicity tests.

Plasmid	LBAmp	LBAmp IPTG
pRA-ED	Many	Many (40–50)
pRA-EPO	Many	Few (5–10)
pRA-SK	Many	Few-none or < 5

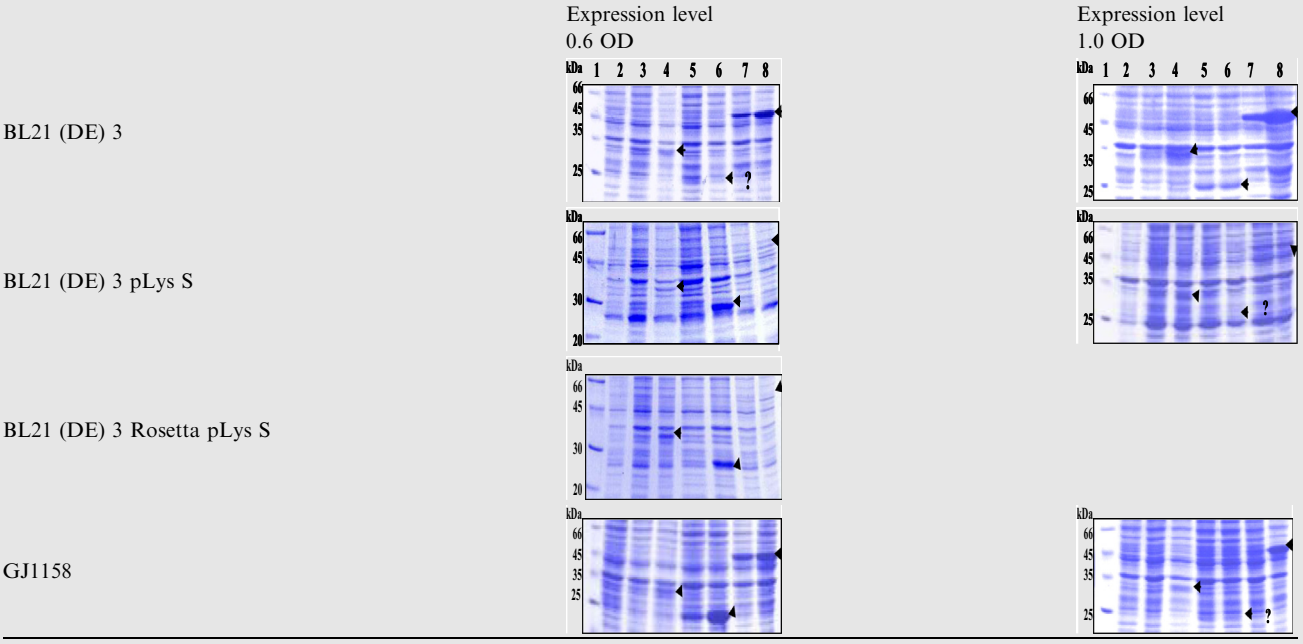
3.3. Rare codons – no role in expression

In pLys S expression host, Streptokinase expression was completely suppressed. On the other hand, EPO (0.6 OD_{600} alone) and TNFR (0.6 and 1.0 OD_{600}) showed better expression levels in pLys S host (Table 4). The results are therefore reversed in the case of BL21 (DE3) pLys S, a strain designed to express toxic proteins. The expression results were reproducible for TNFR ED and EPO establishing the fact that they are indeed toxic. Streptokinase, expressed to very high levels in *E. coli* BL21 (DE3) is completely down regulated in pLys S (0.6 and 1.0 OD_{600}) (Table 4). The suppressing nature of pLys S strain on otherwise proteins well expressed in BL21 DE 3 has been documented before [16]. Such instances wherein a protein expressed at high levels in BL21 DE3, expected to be at the same or higher level in pLys S strain but fail to do so have been reported before (Wu et al. 2004). In other words, less toxic proteins are suppressed in expression under stringent conditions.

The Rosetta pLys S strain supplies the following rare codons-CCC for proline, AGG, AGA, CGA for arginine, ATA for Isoleucine, CTA for leucine. It is to be noted that all three proteins have more or less the same number of rare arginine codons, Streptokinase has more of rare leucine, and TNFR is richer in prolines encoded by rare CCC. EPO contains only one rare proline codon apart from the arginine (Table 1).

However, when *E. coli* BL21 DE3 Rosetta pLys S was used, TNFR and Streptokinase showed only slightly enhanced levels of expression compared to that in BL21 (DE3) pLys S (Table 4). As Rosetta supplies tRNAs for rare codons, the natural conclusion arrived was that TNFR and Streptokinase comprise of rare codons that lead to decreased expression levels in BL21 (DE3) pLys S. For TNFR ED slightly better expression levels were obtained in Rosetta. TNFR ED is quite rich in rare proline codons with 13 out of the 26 being codon biased, the use of Rosetta may be helping to enhance

Table 4 Total Protein profile of different *E. coli* host strains harboring the recombinant plasmids under uninduced and induced conditions.



Lanes 1: Protein marker, 2: vector – Induced, 3: pRA-TNFR ED – uninduced, 4: pRA-TNFR ED – induced, 5: pRA-EPO – uninduced, 6: pRA-EPO – induced, 7: pRA-SK – uninduced, 8: pRA-SK – induced. Arrow mark indicates target protein. Question mark shows absence of expression

expression slightly though not significantly. The concept does take a doubtful turn when the expression results in BL21 (DE3) for Streptokinase are considered. We can say that the rare codons do not play any significant role in influencing expression levels for Streptokinase. Wu et al., 2004 have also reported that the amount of rare codons does not necessarily correlate with expression levels.

Levels of expression of EPO in *E. coli* BL21 (DE3) pLys S and its Rosetta counterpart were somewhat equal. The presence of arginine rare codons does not play any role in expression level of EPO.

3.4. Salt induction for expression

In BL21 DE 3, it was observed that consistent expression was not observed for TNFR ED and EPO. Only Streptokinase was found to show uniform over expression every time induction was carried out. In pLys S, although EPO and TNFR ED performed well, Streptokinase suffered low expression levels.

In GJ1158, salt induced expression struck a common base for all the three proteins as they expressed very well at 0.6 OD_{600} (Table 4). At 1.0 OD_{600}, Streptokinase and TNFR ED expression was observed but not EPO. *E. coli* GJ1158 is derived from *E. coli* BL21 and contains T7 RNA polymerase under the control of pro U promoter. *E. coli* GJ1158 does not contain any additional plasmids or features for supplying rare codon tRNAs or controlling T7 RNA Polymerase levels, therefore the question arises of how it serves as a common host to express all three proteins to high levels? In *E. coli* GJ1158, the pro U promoter governs the expression of T7 RNA polymerase. The promoter is induced by addition of NaCl. Medium used for the growth of the strain is devoid of NaCl hence-hypoosmotic, on induction by the addition of NaCl becomes hyperosmotic. Under such conditions of hyperosmolarity, osmo responsive proteins that are involved in proline transport are induced and the intracellular levels of osmoprotectants such as proline, glycinebetaine have been known to increase in *E. coli* [10]. Such osmoprotectants have been known to improve the folding of proteins. Improperly folded proteins are toxic to the host and hence there is degradation of the protein (Baneyx and Mirna; [15]. Improper folding may be related to improper formation of disulfide bonds. A total of 12 disulfide bonds make TNFR a protein to reckon with in terms of folding and expression in *E. coli*. The GJ1158 host has been shown to improve folding and activity of recombinant proteins [19]. In the case of GJ1158, the environment of osmoprotectants like proline, glycinebetaine may help in TNFR expression by conferring better folding and stability. In fact hyperosmolarity has been shown to improve expression of many proteins not only in *E. coli* [8,12,19], but also in insect and mammalian expression systems [13].

The use of proU Operon system coupled with hyperosmotic shock therefore provides just the required environment for expression of proline-cysteine rich protein TNFR ED. A similar enhancement of expression is seen in the case of EPO also which has 2 disulfide bonds and is a heavily glycosylated protein in eukaryotes. The absence of glycosylation in prokaryotes makes this protein more susceptible to the formation of aggregates. This kind of aggregate formation by aglycosylated EPO has been reported before (Narhi L. O et al. 2001). The use of *E. coli* GJ1158 may therefore help to decrease the potential toxic nature of these EPO aggregates by its high NaCl concentration.

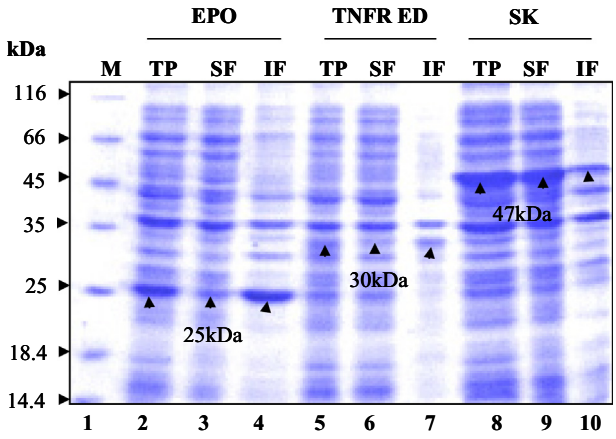


Fig. 4 Separation of induced cells of pRSET ED, pRSET EPO and pRSET SK intoinsoluble and soluble fractions; TP – total protein, IF – insoluble fraction, SF – soluble fraction. Lanes 1 – Marker; 2, 3, 4 – pRSET EPO; 5, 6, 7 – pRSET TNFR ED; 8, 9, 10 – pRSET SK.

Streptokinase expression levels and activity have shown to be improved by the use of sucrose-sorbitol system under hyperosmotic conditions and GJ1158 as host [19]. Streptokinase does not contain any disulfide bonds, hence improperly folded intermediates would be rare, hence threat of toxicity is less, and this may explain its easy expression in less stringent hosts.

The tendency to form improperly folded intermediates could be related to the probability to form inclusion bodies. Based on the Wilkinson Harrison model [18] for prediction of inclusion body formation, it was seen that ED has the highest probability of forming inclusion bodies, followed by EPO (Table 2). On the other hand, Streptokinase had slightly more than 50% chance at solubility. This index is therefore in correlation to our results obtained with *E. coli* GJ1158 (Fig. 4). EPO and ED were present mainly in the insoluble fraction while SK showed almost equal levels in the insoluble and soluble fractions.

3.5. The growth phase of the E. coli culture determines expression levels within the same strain

We noted that in the regimen that we followed for protein expression, the best results were obtained at different points of induction for each protein. Although Streptokinase expressed well at 0.6 OD_{600}, the levels were lower than EPO and higher than TNFR ED in *E. coli* GJ1158. At 1.0 OD_{600}, Streptokinase expression is highest while that of EPO is not seen at all. TNFR ED also gave slightly higher expression level at 1.0 OD_{600} than at 0.6 OD_{600}. Even in the other host strains with the exception of BL21 (DE3), it was seen that induction at 1.0 OD_{600} of culture fails to elicit EPO expression. For TNFR ED and Streptokinase, in all the host strains used, 1.0 OD_{600} was favorable over 0.6 OD_{600} (Table 4, Fig. 3). But in any case the levels of expression of TNFR ED mostly remained lower than that of the other two proteins. Although points of induction used are within log phase of bacterial growth, they very much affect overexpression (Fig. 3). Some protein character of EPO seems to suppress its expression when cells are induced in the late log phase (1.0 OD_{600}). [1], also observed similar influence by point of induction on expression of certain target proteins.

In conclusion we found that codon bias did not play any significant role in the expression of EPO, SK and TNFR ED in *E. coli*. By using stringent conditions such as pLys S hosts, better expression levels or rather consistent expression levels of EPO and ED were obtained. But the best expression results, in terms of consistency and yield were obtained for all three proteins in *E. coli* GJ1158. Perhaps osmoprotectant production induced by hyperosmolarity helps in toxic gene expression by supporting better folding of proteins, in turn reducing the stress on the host. Also, we found that the OD_{600} at the time of induction plays a significant role in success or failure of expression than the number of rare codons. We noted that the use of a common downstream box (pRSET Histidine fusion tag) did not cause a uniform expression of all the three proteins in any single *E. coli* host strain.

Acknowledgements (Funding Agenesis)

S.Ramkumar is a Senior Research Fellow of Indian Council of Medical Research (ICMR), India. R. Vaishnavo Pai is a Senior Research Fellow of Council for Scientific and Industrial Research (CSIR), India.

References

[1] N.S. Berrow, K. Büssow, B. Coutard, J. Diprose, M. Ekberg, G. E. Folkers, N. Levy, V. Lieu, R.J. Owens, Y. Peleg, C. Pinaglia, S. Quevillon-Cheruel, L. Salim, C. Scheich, R. Vincentellic, Didier Bussoh, J Biotechnol. 102 (3) (2006) 291–300.
[2] Charlotte Herman, Yuti Chernajovsky, J. Immunol. (1997) 2478–2487.
[3] E.I. Gonzalez de Valdivia, L.A. Isaksson, Nucleic Acids Res. 32 (17) (2004).
[4] A. Haitham, M. Hussain, John Ward, Appl. Environ. Microbiol. 69 (2003) 373–382.
[5] Jin Yin, Ju-hwa Lin, I.C. Wen-tyng Li, Wang Daniel, J. Biotechnol. 100 (2003) 81–191.
[6] Judy Furlong, Michelle Meighan, Joe Conner, Jill Murray, Barklie Clements, Nucleic Acids Res. 20 (1992) 4668.
[7] Jung-Won Keum, Jin-Ho Ahn, Cha-Yong Choi, Kyung-Ho Lee, Yong-Chan Kwon, Dong-Myung Kim, Biochem. Biophys. Res. Commun. 350 (2006) 562–567.
[8] N. Kagawa, Cao, Arch Biochem Biophys. 393 (2) (2001) 290–296.
[9] S.B. Krantz, Blood 77 (1991) 419–434.
[10] N. Laszlo Csonka, Microbiol. Rev. 53 (1989) 121–147.
[11] T. Mamiatis, E.F. Fritsch, J. Sambrook, Acta Biotechnol. 5 (1) (1982) 104–104.
[12] Natalia Oganesyan, Irina Ankoudinova, Sung-Hou Kim, Rosalind Kimb, Protein Expression Purif. 52 (2007) 280–285.
[13] A. Olejnik, W. Grajek, R. Marecik, J Biotechnol. 102 (3) (2003) 291–300.
[14] Poonam Bhandari, Gowrishankar, J. Bacteriol. 179 (1997) 4403–4406.
[15] C. Savvas Makrides, Microbiol. Rev. (1996) 512–538.
[16] Shi-hao Pan, Bruce Malcolm, Biotechniques 29 (2000) 1234–1238.
[17] F. William Studier, Protein Expression Purif. 41 (2005) 207–234.
[18] D.L. Wilkinson, R.G. Harrison, Biotechnology 9 (1991) 443–448.
[19] Yogender Pal, Jagdish Gupta, Mukherjee, Biotechnol. Lett. 23 (2001) 41–46.