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Controlled Expression of Cholera Toxin B Subunit from *Vibrio cholerae* in *Escherichia coli*

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Abstract: The *ctxB* gene, the causative agent of cholera epidemic was successfully cloned from *V. cholerae* in *E. coli*. The insertion of the gene was confirmed by PCR as well as restriction digestion analyses. The sequencing results for the gene confirmed that the insert was in the correct orientation and in-frame with the P_{BAD} promoter and it showed that the gene was 99% homologous to the published *ctxB* sequence. The CTB protein was successfully expressed in *E. coli* using the pBAD/His vector system. The expected protein of ~14 kDa was detected by SDS-PAGE and Western blot. The use of pBAD/His vector to express the cholera toxin gene in *E. coli* would facilitate future study of toxin gene products.

Key words: *ctxB* gene, cloning, *E. coli*, expression, *Vibrio cholerae*

INTRODUCTION

Vibrio cholerae is a well-known human pathogen that has caused cholera epidemics worldwide. Sanchez and Holmgren (2005) have reported that enteric infections causing diarrheal disease remain a leading global health problem. It has been estimated that 2 to 4 billion episodes of infectious diarrhea occur annually in developing countries, resulting in 3 to 5 million deaths, with the highest incidence and case fatality rates in children below the age of 5 years.

The symptoms of cholera are caused by cholera toxin (CT), an 85 kDa protein composed of A (CTA) and B (CTB) subunits combined in an AB₅ holotoxin. CT function as a mucosal adjuvant but it's used in human is not possible because of its toxicity (Snider *et al.*, 1994; Tamura *et al.*, 1994). Bergquist *et al.* (1997) reported the used of nontoxic B subunits to resolve the problem. CTB has been described as a potent immunogen in the intestinal and nasal mucosal sites (Tamura *et al.*, 1988; Rudin *et al.*, 1999), a mucosal adjuvant for oral and nasal vaccines (Pascale *et al.*, 1999; Briles *et al.*, 2000) and a transmucosal carrier delivery system for induction of oral tolerance when conjugated to auto antigens and allergens (Bergerot *et al.*, 1997; Wiedermann *et al.*, 1999).

The pBAD/His (Invitrogen) expression vector was used in this report. This system offers very tight regulation of gene expression (Lobell and Schleif, 1990). In the presence of arabinose, the P_{BAD} promoter is turned on, while in its absence, very low or undetectable levels of transcription occur (Lee *et al.*, 1991; Johnson and Schleif, 1995). The system has been demonstrated to be an attractive choice for expression of toxic proteins in *E. coli* due to its stringent control under uninduced conditions (Guzman *et al.*, 1995).

The aim of this study was to construct an efficient expression system of CTB using *E. coli* bearing the pBAD vector. We report the amplification of the complete CTB gene of *V. cholerae*, cloning and expression of the protein under optimum conditions.

MATERIALS AND METHODS

Bacterial strains and media used: The study was conducted during December 2006 until August 2007 at the Department of Cell and Molecular Biology, Universiti Putra Malaysia, Malaysia. The *E. coli* was grown in Luria-Bertani (LB) medium (0.5% yeast extract, 1% tryptone, 1% NaCl) at 37°C with agitation. The *E. coli* TOP10 (Invitrogen) cells were used as host for gene cloning

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while *E. coli* BL21(DE3) pLysS (Invitrogen) cells were used as a host for over expression of protein. Ampicillin was used at the final concentration of 50 $\mu\text{g mL}^{-1}$ and chloramphenicol at 35 $\mu\text{g mL}^{-1}$.

DNA manipulation and cloning of the *V. cholerae*, *ctxB* gene:

The *ctxB* gene from *V. cholerae* was PCR amplified and inserted into *SacI* and *PstI* sites of plasmid pBAD/His (Invitrogen, USA). To amplify the *ctxB* gene, primers pBADctxF and pBADctxR were used. The pBADctxF is a 31 mer forward primer with the sequence of 5'- GC ***GAGC*** TCG ATG ATT AAA TTA AAA TTT GGT G-3'. The pBADctxR is a 32 mer reverse primer of 5'- GGC ***CTG CAG*** TTA ATT TGC CAT ACT AAT TGC GG -3'. The restriction enzyme sites of *SacI* and *PstI* were indicated in bold and italics. PCR profiles included an initial step at 95°C for 5 min. Then, 25 cycles of denaturing at 95°C for 1 min, followed by annealing at 60°C for 1 min and extension of the primers at 72°C for 1 min. A final extension was performed at 72°C for 5 min. Plasmid DNA from *E. coli* was isolated using protocol from Birnboim and Doly (1979) with minor modifications. DNA ligation, DNA transformation, restriction endonuclease digestions and agarose gel electrophoresis were performed by standard techniques (Sambrook *et al.*, 1989).

The amplified *ctxB* gene was purified by using Qiagen PCR purification kit (Qiagen) and cloned into pBAD/HisB (Invitrogen). The ligation was then transformed into *E. coli* TOP10 host strains. Clones were selected on LB plates containing ampicillin as the selective agent. After overnight incubation at 37°C, 10 putative colonies were selected and grown in 5 mL of LB broth supplemented with ampicillin with vigorous shaking at 37°C for 18 h. The *E. coli* recombinants that were positive by PCR and restriction enzyme analyses were sent for sequencing. The selected clones were subsequently retransformed into *E. coli* BL21(DE3) pLysS and assayed for expression of the protein.

Expression of the CTB: For optimal production of the protein, the condition such as arabinose (Sigma) concentration and time course induction were conducted. The constructs were grown in LB medium supplemented with ampicillin (50 $\mu\text{g mL}^{-1}$) and chloramphenicol (35 $\mu\text{g mL}^{-1}$) at 37°C until the Optical Density (OD) at 600 nm reached ~0.6 (mid-log phase), and protein expression was induced at 37°C for 3 h by the addition of various concentrations of arabinose ranging from 0.0002 to 0.2% (w/v). After the optimum arabinose concentration was determined, the time course study of 0-5 h was carried out to find the best condition for protein induction. After induction, cells were harvested by centrifugation and

bacterial pellets were lysed with 2-fold SDS-PAGE sample buffer (0.125 M Tris, 4% SDS, 0.2 M DDT, 0.02% bromophenol blue, 20% glycerol) and heated at 95°C for 5 min. Aliquots of the total cellular extracts were analyzed by 12.5% SDS-PAGE according to the methods of Laemmli (1970) and Western blot assay.

SDS-PAGE and Western blotting analyses of CTB:

The total protein extracts (approximately 15 μg) of the recombinant *E. coli* were migrated by 12% SDS-PAGE gel and the protein bands were stained with Coomassie brilliant blue. For Western blot assay, the protein bands were electro-blotted onto PVDF membrane (Amresco) by semi-dry blotter (CBS, Inc). The membrane was then blocked with 1% (w/v) BSA in DBT buffer (Amresco) for 30 min, followed by incubation in 10 mL of DBT (Amresco) containing 10 μL of anti- His-Tag antibody (Novagen) for 1 h. The conjugated membrane was washed three times with DBT, and then was incubated with HRP conjugated goat anti-mouse antibody (50 mU mL⁻¹ in DBT) for 1 h. After washing the membrane with three times DBT, the bound secondary antibody was detected by freshly prepared DAB substrate for brown colour development within 20 min.

RESULTS AND DISCUSSION

The 1% agarose gel electrophoresis of PCR amplification revealed the presence of the 450 bp of *ctxB*-encoding gene (Fig. 1). The amplified PCR product was then purified and introduced into the expression vector, pBAD/HisB. The amplified DNA fragment was cloned under the control of P_{pBAD} promoter in pBAD/His vector, carrying the ampicillin resistant marker and the protein expression was performed in the *E. coli* BL21 (DE3) pLysS

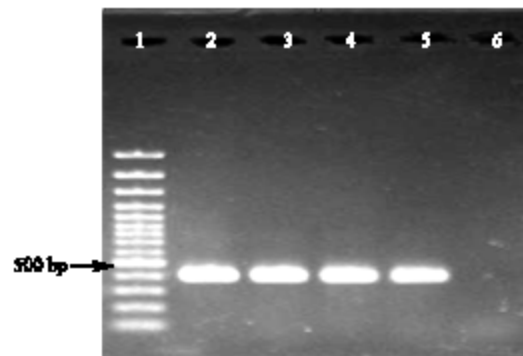


Fig. 1: Ethidium bromide stained 1.0% agarose gel electrophoresis of PCR of *ctxB* gene, Lane 1: GeneRuler™ 100 bp DNA Ladder Plus (Fermentas), Lane 2-5: Amplified *ctxB* gene, Lane 6: Negative control

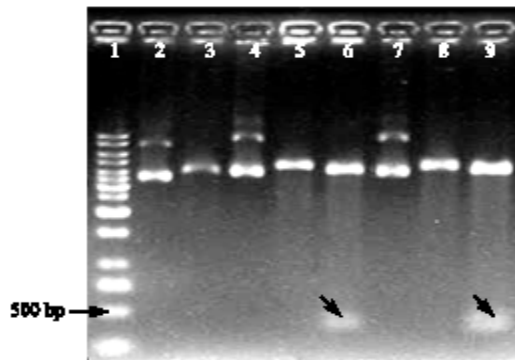


Fig. 2: Ethidium bromide stained 1.0% agarose gel electrophoresis of restriction enzyme digestion analysis of pBAD/His-ctxB, Lane 1: Undigested pBAD/His, Lane 2: pBAD/His digested with SacI, Lane 3: Undigested pBAD/His- ctxB, Lane 4: pBAD/His-ctxB digested with SacI, Lane 5: pBAD/His-ctxB digested with SacI and PstI, Lane 6: Undigested pBAD/His- ctxB, Lane 7: pBAD/His- ctxB digested with SacI, Lane 8: pBAD/His- ctxB digested with SacI and PstI and Lane 9: GeneRuler™ 1 kb DNA ladder (Fermentas)

strain PCR amplification and restriction enzyme digestion of plasmid isolated from the positive clone confirmed the presence of the insert indicated by the arrow (Fig. 2). The correct cloning of the recombinant plasmid was further confirmed by DNA sequencing. The similarity of the sequence was found to be 99% homology to the published sequence of the *ctxB* gene in GenBank assessed using BLAST (<http://www.ncbi.nlm.nih.gov>). The DNA sequence also indicated that the gene was successfully cloned in the correct orientation and in-frame with the *araBAD* promoter of the vector. The physical map of the recombinant plasmid of pBAD/His-ctxB was shown by Fig. 3.

Approximately 15 μ L of the total protein extracts of the recombinant *E. coli* BL21 (DE3) pLysS harbouring the CTB protein were analysed by SDS-PAGE and Western blotting. The expression of CTB in small scale was verified by 12% SDS-PAGE. Expression of the cholera toxin B subunit was stimulated by the addition of arabinose, which induces the *araC* gene promoter of the pBAD system. To achieve the optimal expression of the recombinant protein, we have tested condition such as the induction time and different arabinose concentration.

The selected clone was grown in LB broth with ampicillin and various concentration of arabinose ranging from 0-0.2% (w/v). The optimal arabinose concentration was found to be at 0.2%. The results suggest that the optimum production of CTB was after 2 h induction with

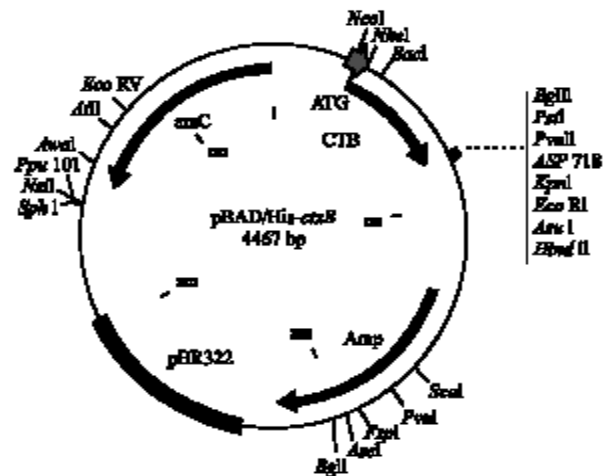


Fig. 3: Map of pBAD/His-ctxB indicating the site of the insertion of the *ctxB* gene and the orientation of the gene

0.2% arabinose concentration at 37°C. Compared to other researchers, Clark *et al.* (1997) have reported the expression of tetanus toxin (TT) under the control of P_{BAD} was obtained at low temperature (20°C) after 3 h of induction with 0.2% arabinose. Whereas, in a study done by Tang-Feldman *et al.* (2002), the optimal concentration for expression of toxin B of *C. difficile* was found to be at 0.02%.

The SDS-PAGE analysis profile (data not shown) of the CTB revealed the expected protein band at ~14 kDa, which corresponded approximately to the size of CTB protein. The recombinant CTB was further characterized by immunoblot (Fig. 4) using anti His-Tag antibody. The immunoblotting result confirmed that the rCTB was specifically and strongly recognized by the antibody. A single protein band of expected size of ~14 kDa was observed in Western blot analysis which is in agreement with the previous works described by others (L'hoir *et al.*, 1990; Lebens *et al.*, 1996; de Mattos Areas *et al.*, 2002). No reactivity was observed in the negative control (vector alone). The result confirmed that the construct was successfully expressed in *E. coli*. In contrast, Hagiwara *et al.* (1999) and Kozuka *et al.* (2000) reported CTB protein size of 11.6 kDa. Our protein band was slightly higher than the exact monomer CTB of the protein size due to the fusion of 6XHis tag present in the expression vector. The His-tag fusion will be an added advantage for the detection of recombinant protein using anti His-Tag antibody. The study showed that the anti His-tag antibody used was specific enough to detect the recombinant protein.

In this study, we have demonstrated the efficient expression of CTB protein in *E. coli*. The araBAD

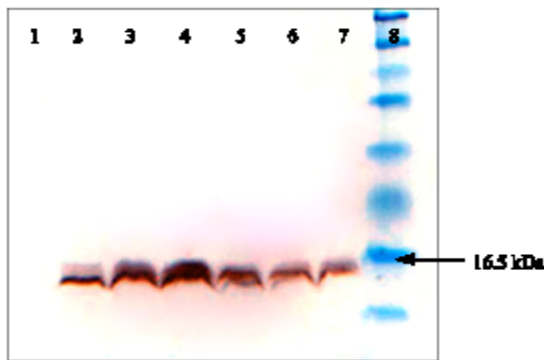


Fig. 4: Western blotting of time course expression of recombinant *E. coli* harbouring CTB, Lane 1: negative control, Lane 2: Uninduced rCTB, Lane 3-7: Induced rCTB 1-5 h and Lane 8: Prestained Protein Marker (NEB)

promoter of this vector provides a distinct advantage over the most commonly used protein over expression systems in bacteria (e.g. in pET vectors: T7lac), as it provides much tighter control over basal expression (Boomershine *et al.*, 2003). The expression of proteins using *E. coli* system remains one of the most attractive organisms for recombinant protein production compared to other bacteria such as *Salmonella* because its genetics and physiology are well understood and quantities up to 50% of the total cell protein can be produced (Lewis *et al.*, 2004).

CONCLUSION

In this study of pBAD/His, we have found that the *ara*-specific promoters are able to express high CTB protein in *E. coli*. For future study the expressed CTB protein purified and utilized as could subunit vaccine for cholera disease.

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