The Journal of Applied Pharmacology, **12**, 179-184 (2004)

Optimized Culture Conditions for Production of the chimaeric protein, Uropathogenic *Escherichia coli* Adhesin - Cholera Toxin A2B Subunits, in *Escherichia coli* TB1

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(Received July 3, 2004; Accepted September 1, 2004)

Abstract – The FimH subunit of type 1-fimbriated *Escherichia coli* has been determined as a major cause for urinary tract infections. In our previous study, the Adhesin/CTXA2B was expressed as soluble recombinant chimaeric protein derived from the uropathogenic *Escherichia coli* adhesin genetically coupled to cholera toxin A2B (CTXA2B) subunit in *Escherichia coli*. Since it is very important to optimize IPTG concentration and culture temperature to maximize cell growth and productivity, these optimal culture factors were determined to increase the productivity of the expressed Adhesin/CTXA2B chimaeric protein in *Escherichia coli* TB1 carrying pMAL*fimH/ctxa2b*. Our data demonstrate that optimal concentration of IPTG for increased production of chimaeric protein was 0.5 mM. Additionally, culture time was 10 hours and temperature, 37°C.

Keywords □ Uropathogenic *Escherichia coli*, fimH, CTXA2B, culture conditions

INTRODUCTION

The enterobacteria *Escherichia coli* (*E. coli*) are the main causative agents of the urinary tract infections (UTIs) and account for the occurrence of over 85% of acute cystitis and pyelonephritis cases, 60% or more of recurrent cystitis, and at least 35% of recurrent pyelonephritis (Langermann *et al.*, 1997; Sauer *et al.*, 2000; Uehling *et al.*, 1994). An attractive approach to eradicate this infection has been suggested by the therapeutic use of vaccine. The mouse model of persistent infection by mouse-adapted uropathogenic *E. coli* strains allowed assessment of the potential of vaccine candidate such as FimH, the adhesin present at the tip of type I pili (Knudsen *et al.*, 1998). Antibodies directed at the amino terminal region of *E. coli* FimH have been found to specifically block attachment to epithelial cells of various *E. coli* strains and, even more remarkably, block attachment to several other enterobacteria. Our previous study showed the expression of Adhesin/CTXA2B chimaeric protein in *E. coli* TB1 carrying pMAL*fimH/ctxa2b* that is *E. coli* adhesin molecular genetically coupled to cholera toxin A2B subunits (Harokopakis *et al.*, 1998; Kim *et al.*, 2001; Lee *et al.*, 2003a; Lee *et al.*, 2003b; Tochikubo *et al.*, 1998; Verweij *et al.*, 1998).

Methods for expressing large amounts of protein from a cloned gene introduced into *E. coli* have proven invaluable in functional analysis of proteins. In addition, irrespective of the expression system used, dramatic differences in expression levels are often realized in different growth conditions (Weikert *et al.*, 1998). Therefore, one can approach to various strategies to produce enough protein in *E. coli*.

Inasmuch as it is hoped that the Adhesin/CTXA2B chimaeric protein can be highly expressed by optimization of various cultures conditions such as IPTG concentration and culture temperature, the present study was undertaken to optimization culture conditions or production of the chimaeric protein by the microorganism. Here, our results demonstrated the effect of cultivation temperature and IPTG concentration on the protein production by the *E. coli* TB1.

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MATERIALS AND METHODS

Chemicals and bacterial strains

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Co. (St Louis, MO). *E. coli* strain DH5a was used as the host for cloning and *E. coli* strain TB1 was used for expression of cloned DNA.

Genetic construction

fimH/ctxa2b fused gene was constructed as described previously by Lee *et al* (2003a). Briefly, amplified *E. coli* adhesion gene (*fimH*) and *V. cholera ctxa2b* genes digested with *Bam*H and *Hin*dIII and inserted into the expression vector pMAL-p2E (New England Biolabs, Inc) to construct the pMAL*fimH* and pMAL*ctxa2b*, respectively. pMAL*ctxa2b* was digested with *Hin*dIII and *Bam*H, then amplified 576 bp DNA fragment was ligated into the pMAL*fimH* vector. The resulting construct was named pMAL*fimH/ctxa2b*. The correct nucleotide sequence and inframe sequence were verified by nucleic acid sequencing [Sequenase version 2.0 DNA sequencing kit (USB^{TM})]. The plasmid constructed by insertion of the *fimH* gene genetically coupled to *ctxa2b* gene into pMAL-p2E was used to transform the *E. coli* TB1 expression host (Fig. 1).

Growth conditions for increased expression of Adhesin/CTXA2B chimaeric protein

E. coli TB1 was grown from 1 ml seed culture (1×10^6 CFU/) in 50 ml LB (Luria-Bertani) with various IPTG concentrations and culture temperatures. LB medium was consisted of bacto peptone (10 g/L), yeast extract (5 g/L), sodium chloride (5 g/L), glucose or glycerol (5 g/L), and antifoam-289 (0.5 g/L). Ampicillin stock solution (12.5 μg/ml) was also added to media and

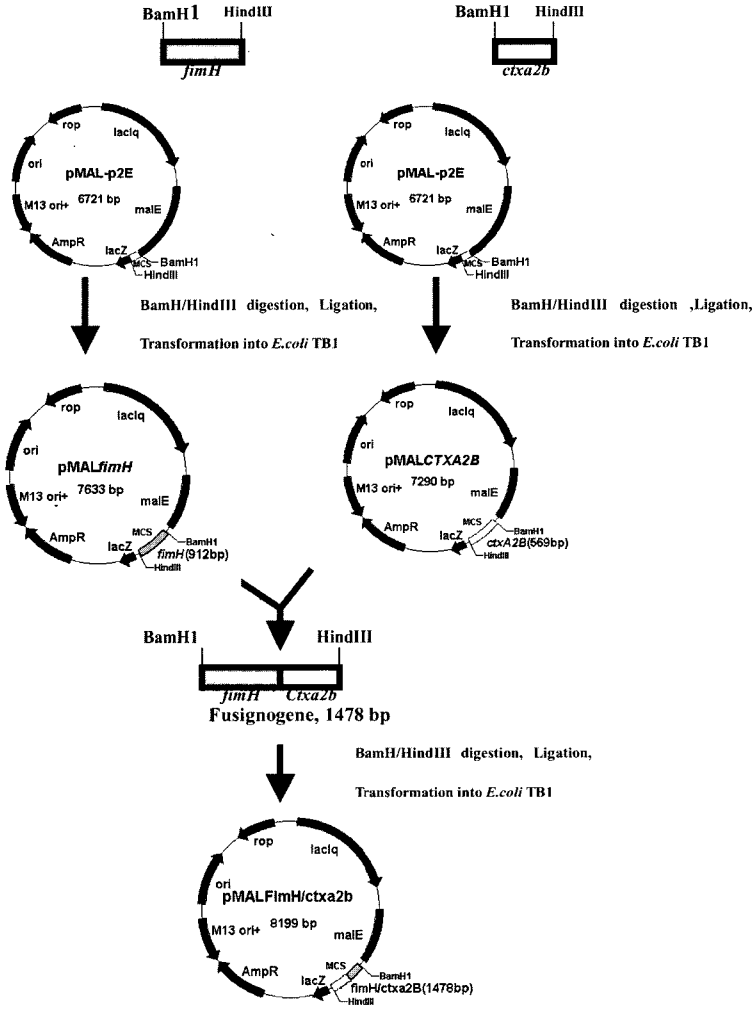

Fig. 1. Structure of the pMAL*fiimH/ctxa2b* expression vector. Abbreviations: malE, maltose binding protein; mcs, multi cloning sites; ori, origin of replication; Amp, ampicillin.

final concentration was 12.5 μg/ml. IPTG concentration was determined by serial concentrations (0.01, 0.03, 0.05, 0.08, 0.1, 0.3 0.5, 0.8, and 1.0 mM). Bacteria cultures were shaken for 24 hrs at 200 rpm and different culture temperature such as 25°C, 30°C, and 37°C. Cell growth was determined by reading OD at 600 nm using 10 × 15 mm culture tube as a cuvette directly.

Conditions of large scale-up culture for adhesin/CTXA2B chimaeric protein

In fermentor experiments, 80 ml seed suspension was transferred into a 5-liter jar fermentor containing 4 liters of the culture medium. The culture was carried out for 10 hrs after various concentration of IPTG induction at various temperatures with an airflow rate of 4.0l/min. The cells were grown for an additional 8-10 hours at 37°C and harvested by centrifugation at 4,000 × g for 20 min. As the majority of G_{M1}-ganglioside ELISA binding activity of Adhesin/CTXA2B protein occurred in periplasmic fraction, which was separated by method of osmotic shock (Neu and Heppel, 1965). The supernatant containing fusion protein was purified by affinity chromatography on amylase resin in a 2.5 × 10 cm column (Slos *et al.*, 1994). The purification was carried out using a pMAL™ Protein Fusion and Purification System according to the manufactures directions (New England Biolabs, Inc). The Protein yield was estimated by the Bradford assay method using BSA (bovine serum albumin) as the standard.

Characterization of adhesin/CTXA2B chimaeric protein

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were used to examine the expressed Adhesin/CTXA2B chimaeric protein from recombinant *E. coli* TB1 carrying pMAL*fimH/ctxa2b*. The blots were probed with antibodies to CTXB (List Biologic Laboratories, Campbell, U.S.A) and the maltose binding protein (MBP) (New England Biolabs). Growth of recombinant *E. coli* TB1 was conducted by method described previously (Lee *et al.*, 2003a). Target gene expression was induced at midlog phase by various concentrations of isopropyl-β-D-thiogalactoside (IPTG). The cells were harvested by centrifugation (6,000 × g for 10 min) and subsequently lysed by osmotic shock.

Synthesis of the peptide for receptor-binding motif of Adhesin

A peptide corresponding to the putative mannose-binding pocket at the tip of the Adhesin domain (Phe-Ile-Asn-Asp-Tyr-Ile-Asp-Gln-Asn-Tyr-Asn-Asp-Phe) was synthesized by Multiple Peptide Systems (TAKARA SHUZO Co., Ltd. Japan). Rabbits were injected with 100 μg of the protein carrier (keyhole limpet hemocyanin)-conjugated peptide. After receiving two booster injections of 100 μg of synthetic peptide, the antibody was isolated. A protocol similar to that described by Evans and coworkers was applied (Evans *et al*, 1993).

Measurement of modified G_{M1}-ganglioside binding activity

To determine of chimaeric protein with G_{M1} ganglioside binding activity and adhesion antigenicity, binding to G_{M1}-ganglioside (from Bovine brain, Sigma) in an ELISA plate was performed (Hajishengallis *et al.*, 1995). Wells were coated overnight at room temperature with 100 μl/well of 1.5 μM G_{M1}-ganglioside in PBS and then washed with PBS. The purified fusion protein was diluted with an assay buffer (PBS containing 0.05% Tween-20 and 0.5% BSA), transferred to the coated wells of the plates, and incubated at 37°C for 120 min. After the wells were washed three times and the plates were incubated with the rabbit antibody to the peptide containing the receptor-binding pocket of adhesin at 37°C for 120 min followed by the alkaline phosphatase-conjugated goat antiserum to rabbit IgG. After incubation for 120 min at 37°C, the plates were developed with a pNPP (para-nitrophenyl phosphate) solution and the absorbance read at 405 nm in a Molecular Device microplate reader (Menlo Park, CA).

RESULTS AND DISCUSSION

The cell lysates of *E. coli* TB1 producing chimaeric protein was separated by SDS-PAGE (Fig. 2A and 2C). The Adhesin/CTXA2B chimaeric protein was specifically detected by Western blot analysis using antibody to peptide containing the mannose receptor-binding motif of *E. coli* Adhesin and to CTXB (Fig. 2B and 2D). As shown in Fig. 2B, the expression of Adhesin protein was increased several-fold following the induction with 0.5 mM IPTG after 10 hour. The expression of CTXB protein also was increased compared to uninduced condition. Additionally, SDS-PAGE and Western blots were used to analyze the purified fractions by affinity chromatography (Fig. 3). The blotting analysis confirmed that adhesion/CTXA2B represented a protein with a molecular weight of approximately 81 kDa and 14 kDa, respectively (Fig. 3B and Fig. 3D). It was expected that the pMAL*fimH/ctxa2b* construct would be transcribed as one message and translated as two proteins, as in the case with the native ctx operon. A fusion protein consisting of

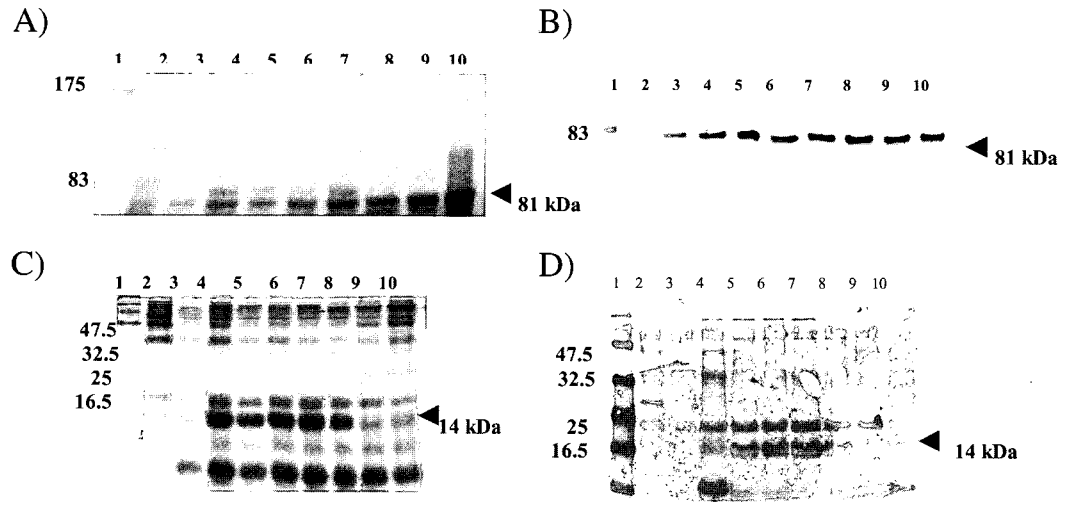

Fig. 2. SDS-PAGE of crude extract from recombinant *E. coli* TB1 carrying pMAL*fimH/ctxA2B* (A & C) and western blots using polyclonal antibodies to MBP and CTXB (B & D). Lane 1, prestained molecular weight marker. The cell lysate proteins (lane 2-10) from *E. coli* TB1 carrying pMAL*fimH/ctxa2b* after 0, 2, 4, 6, 8, 10, 12, and 24 hours of induction with 0.5 mM of IPTG were resolved by electrophoresis on 7% (A & B) and 15% (C & D) polyacrylamide gels. The gels were stained with Coomassie blue (A & C) and immunoblotted on nitrocellulose(B) and PVDF membrane (D), respectively. The nitrocellulose membrane was exposed to a 1:10,000 dilution of a polyclonal antibody to MBP. The PVDF membrane was exposed to a 1:1,000 dilution of a polyclonal antibody to CTXB. In all cases, the samples applied to the gel were equivalent to 10 μg of cell lysates. Right arrowhead indicates Adhesin/CTXA2 (A&B) and CTXB (C&B).

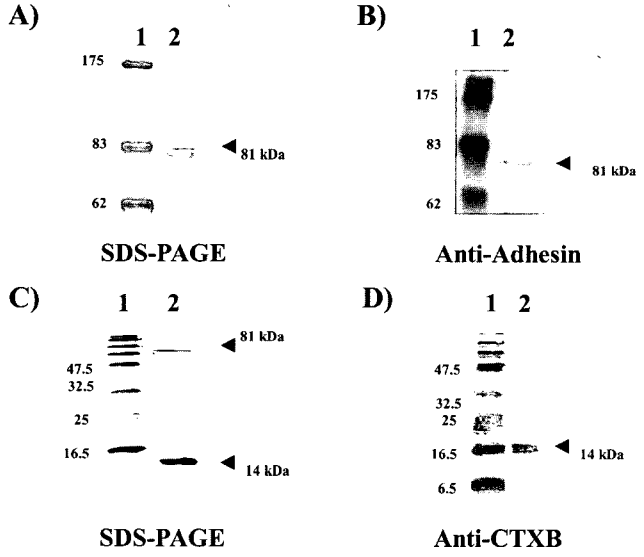

Fig. 3. SDS-PAGE and Western blots for purified fractions. A crude extract from *E. coli* TB1 containing Adhesin/CTXA2B was passed over a 15 ml column at 4°C. The column was then washed with 10 column volumes of 20 mM of Tris-HCl (pH 7.4), 0.2 M of NaCl, and 1 mM of EDTA. The protein was eluted using the above buffer and 10 mM of maltose at a flow rate of 1 ml/min. SDS-PAGE and western blots were performed as described in Materials and Methods. The gels were stained with Coomassie blue (A & C) and immunoblotted on nitrocellulose (B) and PVDF membrane (D), respectively. Right arrowhead indicates Adhesin/CTXA2 (A&B) and CTXB (C&B).

the vector derived MalE, adhesion, and CTXA2 peptide was expressed by means of the vector translation initiation signals and a stop codon at the 3' end of the CTXA2 coding sequence.

Effects of IPTG concentration on protein production in shaky culture were plotted. Induction of fusion protein with various concentrations of IPTG (0.01, 0.03, 0.05, 0.08, 0.1, 0.3. 0.5, 0.8, and 1.0 mM) was compared at 37°C. *E. coli* transformants cultured with 0.5 mM IPTG resulted in highest expression level of the protein (Fig. 4). Therefore, the suitable condition for IPTG concentration was determined on 0.5 mM. Since a high level of fusion protein can lead to the formation of insoluble aggregates, lowering the expression temperature and/or the level of induction of P*tac* (hybrid *trp-lac* (*tac*) promoter) sometimes yielded a soluble fusion protein (e.g., induction with 10 to 100 mM IPTG at $\leq$30°C) (Knudsen *et al*, 1998).

The protein expression was also investigated at various times (2 to 24 hours). The data indicated that cells grown for 10 hours at 37°C with 0.5 mM IPTG produced the level of highest protein expression (Fig. 5). In the next experiment, to obtain a more soluble form, the protein expression was compared at culture temperatures of 25°C, 30°C, and 37°C with a 0.5 mM IPTG concentration for 10 hours. The fusion protein was more expressed at a culture temperature of 37°C than at 25 and 30°C (Fig. 6). The highest protein expression of fusion protein was obtained by shifting the culture temperature of the log-phase

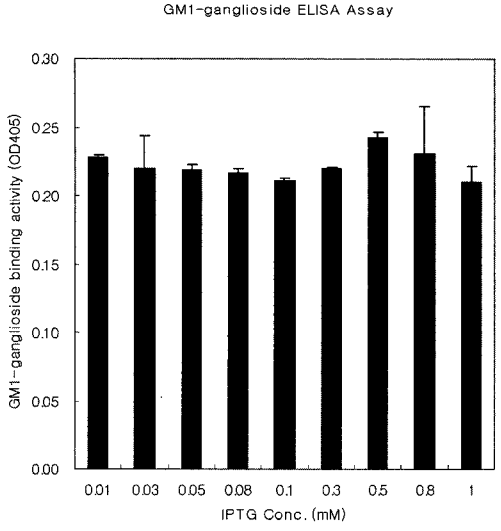

Fig. 4. The effect of IPTG concentrations on production of Adhesin/CTXA2B of E. coli carrying of pMAL*fimH/ctxa2b* after 10 hours induction with various IPTG concentrations after growth until reach to OD_{600} 0.5-0.6 at 37°C. Growth was determined by reading optical density (OD) at 600 nm and products of Adhesin/CTXA2B were measured by the GM1-ganglioside ELISA using Adhesin antibody at 405 nm.

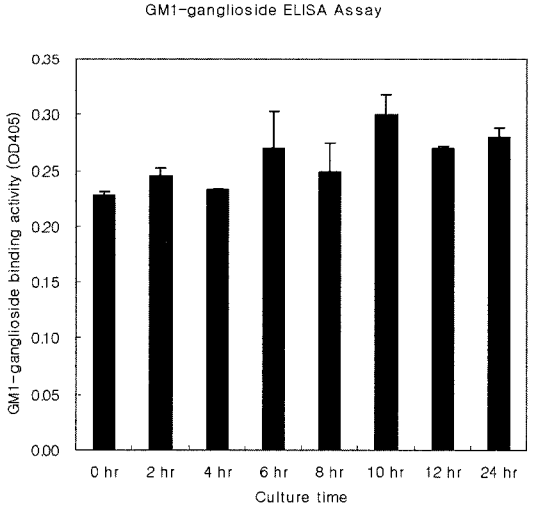

Fig. 5. The effect of culture times on production of Adhesin/CTXA2B of *E. coli* carrying of pMAL*fimH/ctxa2b* after induction with 0.5 mM IPTG after growth until reach to OD_{600} 0.5-0.6 at 37°C. Growth was determined by reading optical density (OD) at 600 nm and products of Adhesin/CTXA2B were measured by the G_{M1}-ganglioside ELISA using Adhesin antibody at 405 nm.

culture of *E. coli* TB1 from 30°C to 37°C with 0.5 mM IPTG concentration. According to a SDS-PAGE analysis, most of the Adhesin/CTXA2B protein was found to exist in a more soluble form of the total cellular protein than proteins expressed in the

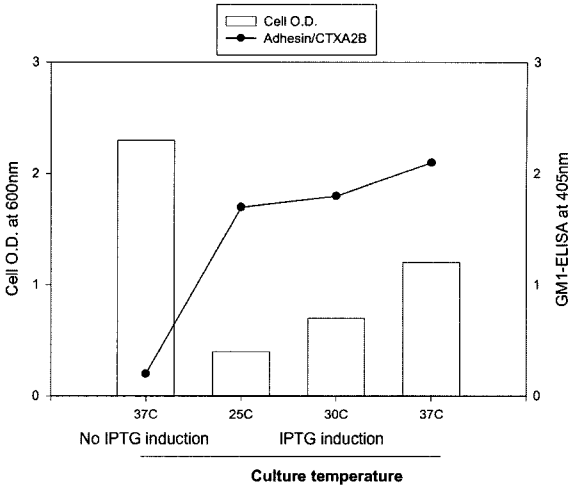

Fig. 6. The Effect of culture temperatures on changes of cell density, products of Adhesin/CTXA2B of *E. coli* carrying of pMAL *fimH/ctxa2b* after 10 hours induction with 0.5 mM IPTG after growth until reach to OD_{600} 0.5-0.6 at 37°C. Growth was determined by reading optical density (OD) at 600 nm and products of Adhesin/CTXA2B were measured by the G_{M1}-gangliosideELISA using Adhesin antibody at 405 nm.

form of inclusion bodies in the cytoplasm of *E. coli* (data not shown). The cell lysate proteins were also expressed more in periplasm than in cytoplasm, suggesting that proteins prepared from peiplasm were beneficial for production of possible vaccine antigen.

In conclusion, our data demonstrated that optimal IPTG concentration was 0.5 mM. In addition, culture time and temperature were 10 hour and 37°C, respectively.

ACKNOWLEDGMENTS

This work was supported by the Brain Korea 21 project in 2004.

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