J. Microbiol. Biotechnol. (2003), **13**(4), 552–559

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

© The Korean Society for Microbiology and Biotechnology

Expression and Characterization of Uropathogenic *Escherichia coli* Adhesin Protein Linked to Cholera Toxin A2B Subunits in *Escherichia coli* TB1

LEE, YONG-HWA, DONG-KYUN RYU, BYUNG-OH KIM, AND SUHKNEUNG PYO*

Division of Immunopharmacology, College of Pharmacy, SungKyunKwan University, Suwon, Kyunggi-do 440-746, Korea

Received: November 29, 2002
Accepted: June 1, 2003

Abstract The FimH subunit of type 1-fimbriated *Escherichia coli* (*E. coli*) has been determined as a major cause for urinary tract infections. Thus, to produce a possible vaccine antigen against urinary tract infections, the *fimH* gene was genetically coupled to the *ctxa2b* gene and cloned into a pMAL-p2E expression vector. The chimeric construction of pMAL*fimH*/*ctxa2b* was then transformed into *E. coli* K-12 TB1 and its nucleotide sequence was verified. A fusion protein, based on fusing adhesin to the cholera toxin subunit A2B (CTXA2B), was induced with 0.01 mM isopropyl-β-D-thiogalactoside (IPTG) for 4 h at 37°C to yield a soluble fusion protein. The fusion protein was then purified by affinity chromatography. The expressed fusion protein was confirmed by SDS-PAGE and Western blotting using antibodies to the maltose binding protein (MBP) or the cholera toxin subunit B (CTXB), plus the N-terminal amino acid sequence was also analyzed. The orderly-assembled fusion protein was confirmed by a modified G_{M1}-ganglioside ELISA, using antibodies to adhesin. The results indicated that the purified fusion protein was an adhesin/CTXA2B protein containing *E. coli* adhesin and the G_{M1}-ganglioside binding activity of CTXB. Accordingly, this adhesin/CTXA2B protein may be a potential antigen for oral immunization against uropathogenic *E. coli*.

Key words: *E. coli* adhesin, adhesin/CTXA2B, CTXA2B, FimH

The enterobacteria *Escherichia 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, 60% or more of recurrent cystitis, and at least 35% of recurrent pyelonephritis [30]. The reservoir for uropathogenic *E. coli* is fecal flora, from which the bacteria spread to the urogenital mucosa, ascend into the bladder, and adhere to the bladder epithelium. Once established in the bladder, the bacteria multiply and establish a local infection (cystitis) and can further ascend to the ureters and kidneys (pyelonephritis) [4, 18]. While many factors contribute to the acquisition and progression of *E. coli* UTIs, colonization of the urogenital tract by pathogenic bacteria would appear to be a prerequisite for the disease [23].

Recent *in vivo* studies in mice demonstrated that colonization of the bladder by pathogenic *E. coli* requires the mannose-sensitive binding of FimH, the adhesin present at the tip of type 1 pili, to the bladder epithelium [7]. This evidence suggests that the determinant in *E. coli* type 1 fimbriae responsible for mediating mannose-specific adherence is its FimH subunits, and that the presence of this fimbrial moiety is important for initiating bacteria infections in the urinary tract [10–12]. Antibodies directed at the amino terminus 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 [4, 29]. One method of eliminating acute UTIs is the use of regular or intermittent antimicrobial prophylaxis. However, concern about the emergence of antibiotic-resistant bacterial strains limits the long-term feasibility of this approach [13, 21].

Type 1 fimbriae were first described by Brinton in 1955 and represent the most widely studied fimbriae of bacteria. Each type 1 fimbrial filament is 1–2 µm long with a diameter of 7 nm [7] and is a heteropolymer comprised of a major subunit FimA and three minor subunits of FimF, FimG, and FimH. The FimA subunits constitute over 95% of the total fimbrial proteins which are arranged in a tight right-handed helix forming a central axial hole [17]. The FimH subunit is critical for cystitis to occur in both mouse and non-human primate models of the disease. Furthermore, FimH interacts specifically with mannosylated glycoproteins (uroplakins) that line the bladder mucosa, eventually leading to bacteria colonization [12].

*Corresponding author
Phone: 82-31-290-7713; Fax: 82-31-292-8800;
E-mail: snpyo@skku.ac.kr

Aside from its role in colonization, FimH has also been implicated in the subsequent stage of pathogenesis, including the stimulation of proinflammatory cytokines and chemokines by uroepithelial cells [24], leading to neutrophil recruitment and pyuria [28], the induction of uroepithelial cell exfoliation by an apoptosis-like mechanism [18], and invasion of bladder cells [2].

In general, protein antigens that may be highly immunogenic by parenteral routes are usually ineffective when administered orally [15], probably because of intestinal digestion and lack of uptake by the gut-associated lymphoid tissue. A notable exception is the cholera toxin (CTX), whose strong enteric immunogenicity depends in part on its affinity for the G_{M1}-ganglioside and consequent tropism for gut-associated lymphoid tissue [5]. CTX (87 kDa) is composed of toxic A1 subunits of CTX (23 kDa), A2 subunits (5 kDa), and five identical subunits of CTXB (11.8 kDa each) that bind to the G_{M1}-ganglioside receptors of the intestinal cell [20]. The A2B subunit of CTX has adjuvant activity, and serves as a carrier protein for orally administered vaccine [6, 15]. At the present time, the cholera toxin (CTX) and the *E. coli* heat-labile toxin (LTX) have both been used as mucosal adjuvants [20]. To stimulate a mucosal immune response, CTX exhibits the most effective adjuvant properties for mucosal immunity. CTX cannot be used in humans due to its toxic effects, yet CTXA2B (i.e., a protein consisting of the A2 and B subunits of CTX) is nontoxic and can be used in humans [5, 8, 32]. A protein antigen that is linked genetically or chemically to CTXA2B confers on them the ability to bind the G_{M1} ganglioside and to elicit the secretory (S)-IgA antibodies, while it provides protection for mucosal surfaces by interfering with microbial adherence, colonization, and invasion [5].

In addition to the targeting effect of coupling antigens to CTXB, which is caused by the binding properties of CTXB, intact CT has adjuvant properties, which seem to depend on the enzymatic toxin activity of the CTX A1 subunit. Such adjuvant activity is obtained when relatively large doses of antigens are coadministered with CTX and may be caused by several mechanism of action. These include increased permeability of mucosal epithelia, stimulation of antigen presentation by enhancement of MHC class II expression and IL-1 production, and promotion of B cell isotype switch differentiation toward IgG and IgA [1, 5, 8].

Accordingly, to produce a possible vaccine antigen against urinary tract infections [13, 16, 26, 31], the current paper reports on the cloning and sequencing of the *fimH* gene and a fusion gene of *fimH* genetically linked to the *ctxa2b* gene. The expression, purification, and characterization of a recombinant protein containing adhesin/CTXA2B are also described.

MATERIALS AND METHODS

Bacterial Strains, Plasmid, Enzymes, and Growth Conditions

The uropathogenic strain *E. coli* J96 (O4:K6) was used as the source of chromosome DNA for constructing the recombinant plasmid; *E. coli* K12 strain DH5α [*F⁻ lacZ_ M15 hsdR17*(*r⁻m⁻*)*gyrA36*] was used as the host for maintaining the plasmid clones; *E. coli* K12 TB1 [*ara* Δ(*lac proAB*) *rpsL thi*[80 *dlac*]^{q} Δ(*lacZ*) *M15*] *hsdR* ($r_k^- m_k^+$) (New England Biolabs, Inc, MA, U.S.A.)] was used for the gene expression of the cloned DNA. The plasmid pMAL-p2E (New England Biolabs, Inc) was used for cloning, amplifying, and expressing the adhesin/*ctxa2b*, *fimH*, and *ctxa2b* genes. The restriction enzymes were purchased from Boehringer-Mannheim (Indianapolis, U.S.A.) and were used according to the recommendation of the supplier. The plasmid DNA was purified by using a QIAEX II gel extraction kit (Qiagen, Germany). The other DNA manipulation experiments were all performed as described by Sambrook *et al.* [25].

For the plasmid isolation and recombinant DNA experiments, *E. coli* J96 and DH5α were grown in a Luria-Bertani (LB) medium (DIFCO, Maryland, U.S.A.) containing 100 μg/ml of ampicillin. The culture was incubated at 37°C with vigorous shaking. For expression of the cloned DNA, recombinant *E. coli* TB1 carrying pMAL*fimH/ctxa2b* was grown in an LB medium supplemented with 0.2% of glucose containing 100 μg/ml of ampicillin at 37°C, with vigorous shaking. This transformant was grown to 0.5-0.6 OD_{600} at 37°C in shaken flasks.

Oligonucleotide and Polymerase Chain Reaction

The oligonucleotide primers were synthesized using an Applied Biosystems DNA Synthesizer (model 3100). The primers used to amplified the *fimH* gene were 5'-CGAAGGATCCATTGTAATGAAACGAGTTATT-ACC-3' (underline containing *Hin*dIII site) and 5'-TGTGAAGCTTTTATTGATAAACAAAAGTCACG-3' (underline containing *Bam*HI site). The primers used to amplify the *ctxa2b* gene were 5'-AGCTGGATCCG-AAGAGCCGTGGATT-3' (underline containing *Hin*dIII site) and 5'-TTTTAAGCTTTTAATTTGCCATACTAAT-TGC-3' (underline containing *Bam*HI site). The *fimH* and *ctxa2b* genes were amplified from *E. coli* DH5α and plasmid pRIT10814 (clone 39052 containing the *ctx* gene: American Type Culture Collection, Rockville, U.S.A.) by a PCR, respectively. The amplifications of the *fimH* and *ctxa2b* genes were performed as previously described and verified by digestion of the amplified DNA with several restriction endonucleases, which were chosen according to the published *fimH* and *ctxa2b* nucleotide sequences [3, 16].

Construction of *fimH* and *ctxa2b* Fusion Gene Expression Vector

The *E. coli* J96 chromosomal DNA was isolated as described by Majewski and Goodwin [14]. The amplified *fimH* and *ctxa2b* genes were gel purified by using a QIAEX II gel extraction kit (Qiagen), digested with *Bam*HI and *Hin*dIII (Boehringer Mannheim), and the purified *fimH* and *ctxa2b* genes were inserted into the expression vector, pMAL-p2E. The resulting constructs were named pMAL*fimH* and pMAL*ctxa2b*, respectively. The correct nucleotide sequence and in-frame sequence were verified by nucleic acid sequencing (Sequenase version 2.0 DNA sequencing kit; USB™), and the amino acid sequences of the *fimH* and *ctxa2b* genes were deduced. pMAL*ctxa2b* was digested with *Hin*dIII and *Bam*HI, then the 576 bp DNA fragment generated by the restriction endonucleases was ligated into the pMAL*fimH* vector. The resulting construct was named pMAL*fimH/ctxa2b* and used to transform *E. coli* K12 TB1. The transformation was carried out by electroporation according to the manufacturer's directions (Gibco BRL Life Technologies, Inc., Gaitherburg, U.S.A.).

Synthesis of Peptide for Receptor-Binding Pocket of Adhesin and Production of Antiserum

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 in Freund's complete adjuvant. After receiving two booster injections of 100 µg of the synthetic peptide, the antibody was isolated [29].

Cell Fractionation and Purification of Fusion Protein

The target gene expression was induced in the mid-log phase by the adding isopropyl-β-D-thiogalactoside (IPTG) (Sigma, St. Louis, MO, U.S.A.) to a final concentration of 0.01 mM. The cells were grown for an additional 4 h at 37°C and harvested by centrifugation at 4,000 ×g for 20 min and treated in one of the following ways.

Periplasmic Fraction. Bacteria were resuspended in 1/20 of the original culture volume with ice-cold 100 mM Tris-HCl buffer, pH 8.0, containing 20% sucrose, and 5 mM EDTA. After 20-min at 4°C, $MgSO_4$ (50 mM) was added to stabilize the spheroplasts, which were removed by centrifugation. The supernatant was then passed through a 0.45-µm syringe filter [19].

Cytoplasmic Fraction. The spheroplasts were re-suspended in 1/50 of the original culture volume of ice-cold lysis buffer containing 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM of NaCl, and 100 µM PMSF per g wet weight of bacterial pellet. Cells were lysed by sonication at 50% duty cycle for four 40s bursts with 1 min intervals. The soluble fraction was then separated by centrifugation at 12,000 ×g for 20 min at 4°C.

Whole-Cell Lysates. Cells were resuspended in 3 ml of an ice-cold column buffer containing 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 100 mM NaCl per g wet weight of the bacterial pellet. PMSF (1 mM) and lysozyme (0.25 mg/ml) were added, and, after a 20-min incubation period at 4°C, the suspension was treated with sodium deoxycholate (1.33 mg/ml) at room temperature until it became viscose. DNase I (7 µg/ml) was used to digest DNA and to reduce viscosity, and soluble extracts were obtained by centrifugation of the cell lysate [27]. Soluble *E. coli* TB1 extracts were subjected to fractional 20%, 40%, and 60% (of saturation) ammonium sulfate precipitation. The chimeric protein was completely precipitated with 60% saturated ammonium sulfate, whereas the 20% saturation step precipitated essentially the irrelevant proteins. The supernatant containing fusion protein was further purified by affinity chromatography on amylose resin in a 2.5×10 cm column. Purification was carried out using a pMAL™ Protein Fusion and Purification System according to the manufacturer's directions (New England Biolabs).

SDS-PAGE and Western Blotting Analysis of Fusion Protein

SDS-PAGE and Western blotting were used to examine the cell protein expression. The soluble fractions were resuspended in a gel-loading buffer (0.5 M of Tris-HCl, pH 6.8, 2% glycerol, 10% SDS, 0.1% bromophenol blue) to give a final concentration of 10 µg/ml. The proteins were then resolved by electrophoresis in 7% and 15% (w/v) SDS-PAGE gels and stained with Coomassie blue. For the Western blots, a parallel gel run was electrotransferred onto nitrocellulose and PVDF membranes. The blots were probed with antibodies to CTXB (List Biologic Laboratories, Campbell, U.S.A.) and the maltose binding protein (MBP) (New England Biolabs). The proteins on the blot were detected by using an alkaline phosphatase-conjugated goat antiserum to mouse IgG (Caltag Laboratories Inc., South San Francisco, U.S.A.), and developed by the use of a BCIP/NBT (Sigma, St. Louis, U.S.A.) substrate system [12].

Modified G_{M1}-Ganglioside ELISA

To determine a properly assembled fusion protein, binding to G_{M1}-ganglioside (from Bovine Brain) on an ELISA plate was performed. Ninety-six-well microplates 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. Thereafter, 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 (paranitrophenyl phosphate) solution, and absorbance was read at 405 nm using an ELISA reader [8].

N-Terminal Amino Acid Sequence

The purified fusion proteins were resolved by electrophoresis in SDS-PAGE gels. The protein bands corresponding to adhesin/CTXA2 and CTXB were transferred to a PVDF membrane and subjected to NH_2-terminal sequencing (Model Precise 491 sequencer, Applied Biosystems, Inc., Foster City, U.S.A.).

Results and Discussion

Cloning and Expression of *fimH/ctxa2b* Gene

The *fimH* gene was amplified by a PCR by using *E. coli* J96 chromosomal DNA as the template. The amplified *fimH* gene fragment, containing *Hin*dIII and *Bam*HI restriction sites, was gel-purified by using a QIAEX II gel extraction kit, digested with *Hin*dIII and *Bam*HI, and inserted into pMAL-p2E yielding pMAL*fimH*. The nucleotide sequences of both strands were determined based on a

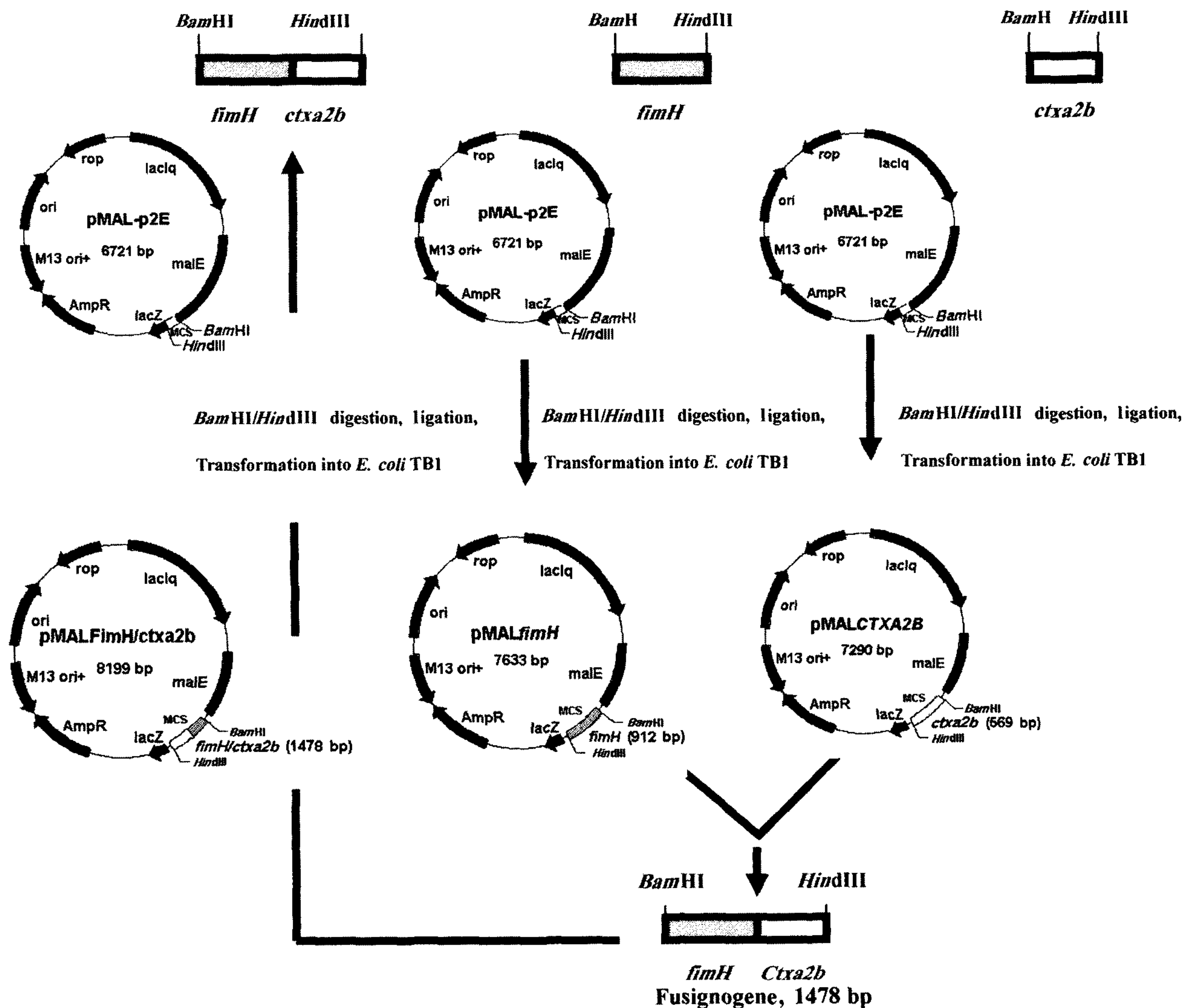

Fig. 1. Scheme of construction of the pMAL*fimH/ctxa2b* expression vector.
The pMAL*fimH* vector was constructed by tranferring the PCR products of the *fimH* gene from *E. coli* J96 chromosomal DNA into pMAL-p2E. The pMAL*ctxa2b* vector was constructed by transferring the PCR products of the *ctxa2b* gene from pRT10814 into pMAL-p2E. The pMAL*fimH/ctxa2b* vector was constructed from the *fimH* gene from pMAL*fimH* and *ctxa2b* gene from pMAL*ctxa2b*. The *fimH/ctxa2b* gene was cloned into *Hin*dIII and *Bam*HI sites of the pMAL-p2E vector. Abbreviations: malE, maltose binding protein; mcs, multicloning sites; ori, origin of replication; Amp, ampicillin.

912 bp *Hin*dIII/*Bam*HI *fimH* fragment of pMAL*fimH*. The amino acid sequence FINDYIDQNYNDF corresponded to the putative mannose-binding sequence, previously described by Sauer and coworkers [24]. The *ctxa2b* gene was amplified by a PCR using the plasmid pRIT110814 as the template, then the PCR product, containing *Hin*dIII and *Bam*HI restriction sites, was gel-purified, digested with *Hin*dIII and *Bam*HI, and cloned into pMAL-p2E yielding pMAL*ctxa2b*. pMAL*ctxa2b* was then excised with *Hin*dIII and *Bam*HI, and the 576 bp DNA fragment was ligated into the pMAL*fimH* vector. The resulting construct was named pMAL*fimH/ctxa2b*, and transformed into *E. coli* K12 TB1. The *fimH/ctxa2b* gene fragment generated by digesting pMAL*fimH/ctxa2b* with *Hin*dIII and *Bam*HI was 1,478 bp. The structure of the plasmid encoding the fusion protein is shown in Fig. 1.

The cell proteins expressed in *E. coli* TB1 harboring pMAL*fimH/ctxa2b* were examined by SDS-PAGE, as shown in Figs. 2A and 2C. Since a high level of a fusion protein can lead to the formation of insoluble aggregates, lowering the expression temperature and/or the level of induction of Ptac sometimes yields a soluble fusion protein (e.g., induction with 10 to 100 μM IPTG at ≤30°C) [9, 22]. As such, induction with low and high concentrations of IPTG (0.01 mM, 0.05 mM, 0.1 mM, 0.5 mM, and 1.0 mM) was compared at 37°C (data not shown). Induction with 0.01 mM IPTG of the *E. coli* transformants harboring the plasmids resulted in the highest expression level of the cell protein. To obtain a more soluble form, the protein expression was compared at culture temperatures at 25°C, 30°C, and 37°C with 0.01 mM IPTG concentration. A higher expression level of the fusion protein was achieved at a culture temperature of 37°C than at 25°C (data not shown). The highest protein expression of fusion protein was achieved by shifting the culture temperature of the log-phase culture of *E. coli* TB1 from 28°C to 37°C with 0.01 mM IPTG concentration (data not shown). The protein expression at various times was also investigated (2 to 48 h). Cells grown for 4 and 6 h at 37°C with 0.01 mM IPTG produced the highest protein expression level (Fig. 2). According to 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 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 (data not shown).

SDS dissociated the protein into MalE/adhesin/CTXA2B and CTXB, thereby disrupting the pentameric structure. The coexpressed MalE/Adhesin/CTXA2 and CTXB were specifically detected by a Western blot analysis by using antibodies to MBP and CTXB (Figs. 2B and 2D), plus the Western blotting analysis confirmed that adhesin/CTXA2B represented a protein with a molecular weight of approximately 82 kDa and 14 kDa, respectively (Figs. 2B and 2D). 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 [16]. A fusion protein consisting of the vector-derived MalE, adhesin, 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.

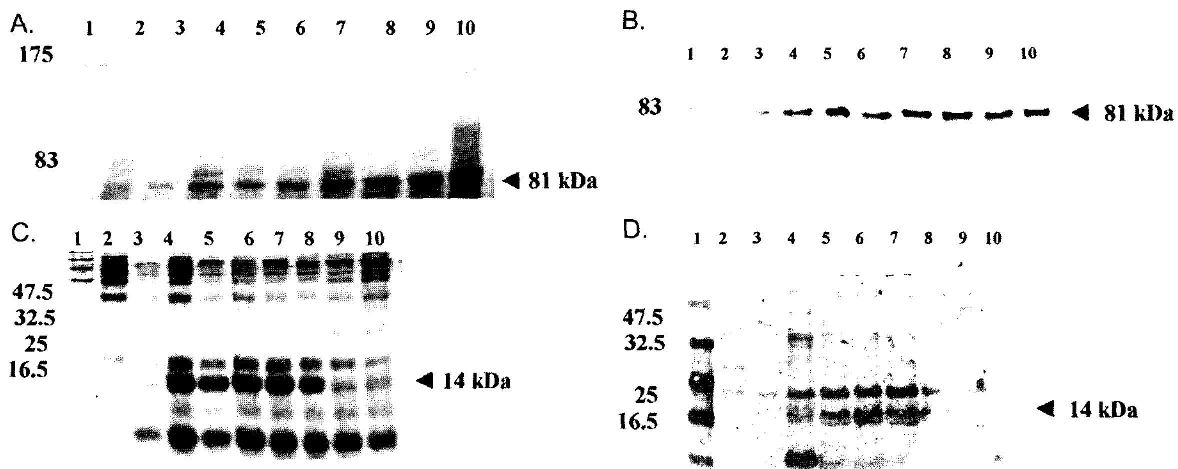

Fig. 2. SDS-PAGE of total proteins from *E. coli* TB1 carrying pMAL*fimH/ctxA2B* (A & C) and Western blots using polyclonal antibodies to MBP and CTXB (B & D).

Lane 1, prestained molecular weight marker. The cell lysate proteins (lanes 2- 10) from *E. coli* TB1 carrying pMAL*fimH/ctxa2b* after 0, 2, 4, 6, 8 10, 12, 24, and 48 h of induction with 0.01 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 (C) and PVDF membranes (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) and CTXB (C).

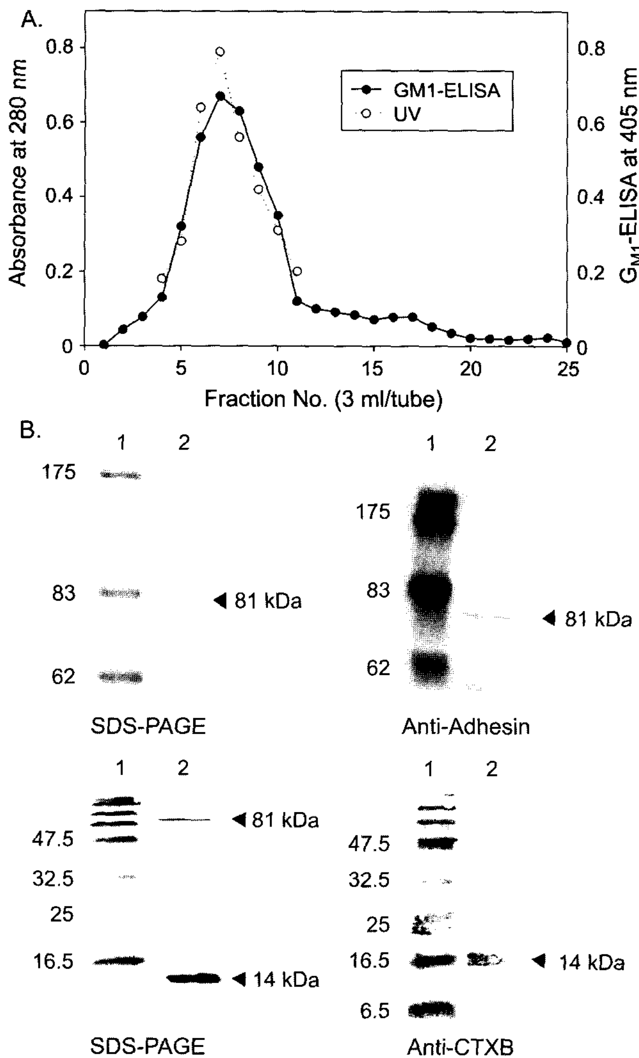

Fig. 3. Affinity chromatography of adhesin/CTXA2B proteins on amylose column (A). SDS-PAGE and Western blots for purified fractions (B).

A crude extract from *E. coli* TB1 containing a plasmid that expresses a maltose-binding protein-β-galactosidase fusion protein 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. ○ represents the absorbance level at 280 nm, while ● represents the absorbance level at 405 nm. SDS-PAGE and Western blots were performed as described in Materials and Methods.

Purification of Fusion Protein

The fusion proteins were purified by affinity chromatography on an amylose resin. The correct assembly of adhesin/CTXA2B was monitored by a G_{M1}-ganglioside ELISA, developed using an antibody to the peptide containing the receptor-binding pocket of adhesin (Fig. 3A). SDS-PAGE and Western blots were used to analyze the purified fractions (Fig. 3B). The purified adhesin/CTXA2B was estimated to be 80% pure according to a densitometric assay. The molecular weight of the adhesin/CTXA2 and CTXB predicted by the amino acid sequence was 97 kDa (MalE/adhesin/CTXA2B). To estimate the molecular weight, the purified fusion protein was subjected to size-exclusion chromatography on an HPLC column calibrated with molecular standard markers. The eluted proteins indicated a 190 kDa molecular weight which was approximately twice the expected value. Whether the fusion proteins could be associated to create a dimetric molecule or the molecular shape of adhesin was responsible for the anomalous elution is still not known.

Characterization of Fusion Protein

The purified fusion proteins were confirmed by an N-terminal amino acid sequence analysis of the adhesin/CTXA2 and CTXB bands resulting from the SDS-PAGE (data not shown). The N-terminal amino acid sequences of the adhesin protein and CTXB were Ile-Val-Met-Lys-Arg-Val-Ile-Thr-Leu-Phe and Met-Ile-Lys-Leu-Lys-Phe-Gly-Val-Glu-Phe, respectively, which were the signal peptides of MalE/adhesin/CTXA2 and CTXB, respectively. To properly determine the assembled fusion protein, the purified adhesin/CTXA2B molecule was tested in a modified G_{M1}-ganglioside ELISA, developed using polyclonal antibodies to the peptide containing the receptor-binding pocket of adhesin. As shown in Fig. 4, the purified adhesin/CTXA2B was able to bind G_{M1}-ganglioside, suggesting that it possesses adhesin epitopes. The data also indirectly showed that the CTX bound to G_{M1}-ganglioside no longer possessed adhesin epitopes and did not exhibit any affinity for CTXB binding sites. There is evidence to suggest that the conjugation of antigens to CTXB improves the immunogenicity of

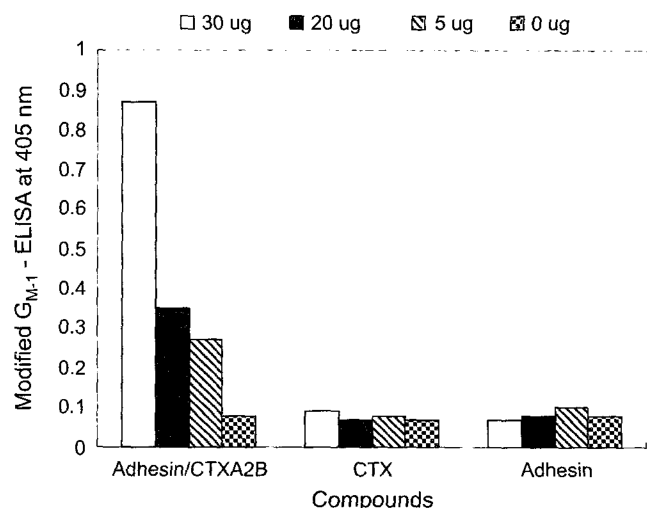

Fig. 4. Comparison of affinity of adhesin/CTXA2B, CTX, and adhesin for G_{M1}-ganglioside by ELISA.

A modified G_{M1}-ganglioside ELISA was performed as described in Materials and Methods. This experiment was repeated three times with similar results.

such preparations, when they are administered orally [15]. Accordingtly, based on previous data, the adhesin/CTXA2B protein may be a good candidate antigen for oral immunization against uropathogenic *E. coli.*

Acknowledgments

The authors would like to thank Dr. Eugene P. Mayer (University of South Carolina) for his helpful discussions and for critically reviewing the manuscript. This work was supported by the Samsung Research Fund, Sungkyunkwan University, 2001.

References

1. Bromander, A., J. Holmgren, and N. Lycke. 1991. Cholera toxin stimulate IL-1 production and enhances antigen presentation by macrophages *in vitro. J. Immunol.* **146:** 2908-2915.
2. Beachey, E. H. 1981. Bacterial adherence: Adhesin-receptor interactions mediating the attachment of bacteria to mucosal surface. *J. Infec. Dis.* **143:** 325-345.
3. Blattner, F. R., G. Plunkett III, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau, and Y. Shao. 1997. The complete genome sequence of *Escherichia coli* K-12. *Science* **277:** 1453-1462.
4. Hamrick, T. S., E. A. Havell, J. R. Horton, and P. E. Orndorff. 2000. Host and bacterial factors involved in the innate ability of mouse macrophages to eliminate internalized unopsonized *Escherichia coli. Infect. Immun.* **68:** 125-132.
5. Hajishengallis, G., S. Hollingshead, T. Koga, and M. W. Russell. 1995. Mucosal immunization with a bacterial protein antigen genetically coupled to cholera toxin A2/B subunits. *J. Immunol.* **154:** 4322-4332.
6. Harokopakis, E., G. Hajishengallis, and S. M. Michalek. 1998. Effectiveness of liposomes possessing surface-linked recombinant B subunit of cholera toxin as an oral antigen delivery system. *Infect. Immun.* **66:** 4299-4304.
7. Jones, C. H., J. S. Pinkner, R. Roth, J. Heuser, A. V. Nicholes, S. N. Abraham, and S. J. Hultgren. 1995. FimH adhesin of type 1 pili is assembled into a fibrillar-tip structure in the *Enterobacteriaceae. Proc. Natl. Acad. Sci. USA* **92:** 2081-2085.
8. Kim, B. O., S. S. Shin, Y. H. Yoo, and S. Pyo. 2001. Peroral immunization with *Helicobacter pylori* adhesin protein genetically linked to cholera toxin A2B subunits. *Clin. Sci.* **100:** 292-298.
9. Kim, M. J., S. L. Park, S. K. Kim, and S. W. Nam. 2002. Overproduction of *Bacillus macerans* cyclodextrin glucanotransferase in *E. coli* by coexpression of GroEL/ES chaperone. *J. Microbiol. Biotechnol.* **12:** 1002-1005.
10. Knudsen, T. B. and P. Klemm. 1998. Probing the receptor recognition site of the FimH adhesin by fimbriae-displayed fimH-FocH hybrids. *Microbiology* **144:** 1919-1929.
11. Kobatake, E., Y. Ikariyama, and M. Aizawa. 1995. Production of the chimeric-binding protein, maltose-binding protein-protein A, by gene fusion. *J. Biotechnol.* **38:** 263-268.
12. Krogfelt, K. A., H. Bergmans, and P. Klemm. 1990. Direct evidence that the FimH protein is the mannose-specific adhesin of *Escherichia coli* type 1 fimbriae. *Infect. Immun.* **58:** 1995-1998.
13. Langermann, S., S. Palaszynski, M. Barnhart, G. Auguste, J. S. Pinkner, J. Burlein, P. Barren, S. Koenig, S. Leath, C. H. Jones, and S. J. Hultgren. 1997. Prevention of mucosal *Escherichia coli* infection by FimH-adhesin-based systemic vaccinaton. *Science* **276:** 607-611.
14. Majewski, S. I. H. and C. S. Goodwin. 1988 Restriction endonuclease analysis of the genome of *Campylobacter pylori* with a rapid extraction method: evidence for considerable genomic variation. *J. Infect. Dis.* **157:** 465-471.
15. Mckenzie, S. J. and J. F. Halsey. 1984. Cholera toxin B subunit as a carrier protein to stimulate a mucosal immune response. *J. Immunol.* **133:** 1818-1823.
16. Mekalanos, J. J., D. J. Swartz, G. D. N. Pearson, N. Harford, F. Groyne, and M. D. Wilde. 1983. Cholera toxin genes: Nucleotide sequence, deletion analysis and vaccine development. *Nature* **306:** 551-557.
17. Minion, F. C., S. N. Abraham, E. H. Beachey, and J. D. Goguen. 1986. The genetic determinant of adhesive function in type 1 fimbriae of *Escherichia coli* is distinct from the gene encoding the fimbrial subunit. *J. Bacteriol.* **165:** 1033-1036.
18. Mulvey, M. A., Y. S. Lopez-Boado, C. L. Wilson, R. Roth, W. C. Parks, J. Heuser, and S. J. Hultgren. 1998. Induction and evasion of host defenses by type 1-piliated uropathogenic *Escherichia coli. Science* **282:** 1494-1497.
19. Neu, H. C. and L. A. Heppel. 1965. The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. *J. Biol. Chem.* **240:** 3685-3692.
20. Pizza, M., M. M. Giuliani, M. R. Fontana, E. Monaci, G. Douce, G. Dougan, K. H. G. Mills, R. Rappuoli, and G. Del Giudice. 2001. Mucosal vaccines: Non toxic derivatives of LT and CT as mucosal adjuvants. *Vaccine* **19:** 2534-2541.
21. Russell, M. and H. Y. Wu. 1991. Distribution, persistence, and recall of serum and salivary antibody responses to peroral immunization with protein antigen I/II of *Streptococcus* mutants coupled to the cholera toxin B subunit. *Infect. Immun.* **59:** 4061-4070.
22. Ryou, C. S., T. Y. Chung, and M. S. Kwon. 2001. Molecular cloning and hyperexpression of a Bt Gene, *cryIAc*, in *Escherichia coli* DH5α: production and usage of anti-CryIAc antibody. *J. Microbiol. Biotechnol.* **11:** 1093-1098.
23. Sauer, F. G., M. A. Mulvey, J. D. Schilling, J. J. Marinez, and S. J. Hultgren. 2000. Bacterial pili: Molecular mechanism of pathogenesis. *Curr. Opin. Microbiol.* **3:** 65-72.
24. Sauer, F. G., M. Barnhart, D. Choudhury, S. D. Knight, G. Waksman, and S. J. Hultgren. 2000. Chaperon-assisted pillus assembly and bacterial attachment. *Curr. Opin. Struc. Biol.* **10:** 548-556.
25. Sambrook, J., E. F. Fritich, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., U.S.A.

26. Service, R. F. 1997. New vaccines may ward off urinary tract infections. *Science* **276:** 607–611.
27. Slos, P., D. Speck, N. Accart, H. V. J. Kolbe, D. Schubnel, B. Bouchon, R. Bischoff, and M. P. Kieny. 1994. Recombinant cholera toxin B subunit in *Escherichia coli*: High-level secretion, purification, and characterization. *Protein Expres. Purif.* **5:** 518–526.
28. Tewari, R., J. I. MacGregor, T. Ikeda, J. R. Little, S. J. Hultgren, and S. N. Abraham. 1993. Neutrophil activation by nascent FimH subunits of type 1 fimbriae purified from the periplasm of *Escherichia coli*. *J. Biol. Chem.* **268:** 3009–3015.
29. Thankavel, K., B. Madison, T. Ikeda, R. Malaviya, A. H. Shah, P. M. Arumugam, and S. N. Abraham. 1997. Localization of a domain in the FimH adhesin of *Escherichia coli* type 1 fimbriae capable of receptor recognition and use of a domain-specific antibody to confer protection against experimental urinary tract infection. *J. Clin. Invest.* **100:** 1123–1136.
30. Uehling, D. T., W. J. Hopkins, L. A. Dahmer, and E. Balish. 1994. Vaginal mucosal immunization in recurrent UTIs. *J. Urology* **152(6 Pt 2):** 2308–2311.
31. Wizemann, T. M., J. E. Adamou, and S. Langermann. 1999. Adhesin as targets for vaccine development. *Emerg. Infect. Dis.* **5:** 393–403.
32. Williams, N. A., T. R. Hirst, and T. O. Nashar. 1999. Immune modulation by the cholera-like enterotoxins: From adjuvant to therapeutic. *Immunol. Today* **20:** 95–101.