**Elucidating Bottlenecks to the Efficient Preparation of AB₅-Hexamer Mucosal Adjuvant Protein LTm by Genetic Engineering**

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**Introduction**

Heat-labile enterotoxin (LT) is an AB₅-type bacterial toxin produced by enterotoxigenic *Escherichia coli* [1]. The complete hexameric structure comprises one A subunit (LTA) (27 kDa) and a ring-like pentamer formed by five B subunits (LTB) (11.6 kDa each) [2, 3]. Both the LTA and LTB subunits contain 18 and 21 amino-acid-long signal peptides at the N-terminus after translation [4, 5]. Sequence analysis revealed that LT subunit-encoding genes, *eltA* and *eltB*, are in one operon, and both genes are transcribed as a single mRNA molecule. The open reading frames of *eltA* and *eltB* overlap, with a translational coupling between them [6]. After synthesis in the cytoplasm, both the LTA and LTB subunits translocate across the bacterial cytoplasmic membrane into the periplasmic space under the guidance of signal peptides. There, the leader of the subunits is removed and the AB₅ complex is assembled [7].

As a bacterial toxin, the LT non-toxic B subunit pentamer has the ability to bind to GM₁ gangliosides, which are expressed on the surface of many cell types; the LT toxic A subunit has ADP-ribosyltransferase activity, which causes
constitutive activation of adenylyl cyclase, and an increase in intracellular cAMP, leading to severe diarrhea [8]. As powerful mucosal adjuvants, LT and its non-toxic mutant (LTm) are capable of promoting strong mucosal immune responses to coadministered antigens [9–11]. Moreover, some types of non-toxic LTm adjuvant vaccines have entered clinical trials [12, 13].

However, the yield of LTm obtained from the wild-type or genetically engineered strain is quite low [14–16], which hinders extensive application in fundamental and clinical research. To overcome this difficulty, researchers are now exploring the use of expression hosts other than E. coli. Other host cells such as Bacillus [17], yeast [18, 19], and even plant cells [20, 21] have been used to produce LTm and its subunits. Despite the progress made by using different expression systems to express LTm, the question of why a protein derived from one type of E. coli cannot be produced at a high level by genetically engineered E. coli remains unanswered.

Therefore, in this study, to elucidate the expression bottlenecks of LTm in E. coli, we constructed a series of recombinant plasmids using different strategies. These recombinant plasmids were designed to express LTA, LTB, or both. After inducing the expression of target proteins under the same conditions, intracellular mRNA levels of a section of the strains were determined by quantitative PCR. Additionally, we identified the expression of target proteins by whole-cell protein electrophoresis analysis in all recombinant strains. Moreover, by comparing and analyzing those expression levels of strains, the factors were elucidated. Finally, the inclusion body form of the LTA and LTB subunits were purified and refolded, and the biological activity of the refolded proteins was determined. These results reveal the expression bottlenecks of LTm, provide the preparation strategy for LTm and its subunits, and promote efficient preparation of this adjuvant protein.

Materials and Methods

Chemicals and Reagents
DNA polymerase and restriction enzymes (Ndel, Ncol, Sall, Xhol) were purchased from Takara (China). FastDigest SapI was obtained from Thermo Fisher Scientific (China). Kanamycin, ampicillin, streptomycin, isopropyl-β-D-thiogalactopyranoside (IPTG), fluorescein isothiocyanate (FITC), and tetramethylrhodamine isothiocyanate (TRITC) were purchased from Sigma-Aldrich (China). Typtone and yeast extract were obtained from Oxoid (UK). GM3 ganglioside sodium salt and LTH-B Antibody (BB12) were purchased from SantaCruz (USA). Other routine analytical grade chemicals were obtained from local providers.

Strains, Plasmids, and Culture Media
Bacterial strains E. coli DH5α and BL21 (DE3) (Invitrogen, USA) were used as the hosts for cloning and expression. Plasmids pTYB11 (NEB, USA), pET24a(+), and pCDFDuet-1 (Novagen, Germany) were used as vectors to incorporate the target gene. Lysogeny broth (LB) medium (containing peptone 10 g/l, yeast extracts 5 g/l, and NaCl 10 g/l) was used for the growth of bacteria. Human colon cancer cells (HCT116) (CCTCC, China) culture medium McCoy’s 5A was supplemented with fetal bovine serum (15%) and penicillin-streptomycin (1%).

Molecular Cloning
The LT gene (Accession No. EU113245.1) with a R192G mutation site was derived from a laboratory reserved recombinant plasmid pET28a(+)-eltm, which was constructed based on the strain (E. coli O6:H16/LT+ ST+). Overlap extension PCR was used to eliminate the Ndel restriction site in the eltm sequence by site-directed mutagenesis (Fig. S1). This mutation product was used as the template for the subsequent gene cloning. The primers used for gene cloning are shown in Table S1. The amplified fragments were analyzed by agarose gel electrophoresis (1.5%) and purified with a DNA fragment purification kit (Takara). The cloned gene fragments were separately inserted into the multiple cloning sites of pET24a(+), pTYB11, and pCDFDuet-1. The details of the construction designs and processes are shown in Fig. 1 and Table S2. After being transformed into competent E. coli DH5α cells, the positive clones were identified by colony PCR and further confirmed by commercial DNA sequencing. The correct recombinant plasmid was transformed into E. coli BL21 (DE3) competent cells for protein expression.

Protein Expression and Electrophoresis Analysis
The recombinant E. coli BL21 (DE3) expression strain was cultured in a 10-ml sterile tube containing 5 ml of sterile LB and antibiotic (final concentration 50 μg/ml) with constant shaking at 200 rpm and 37°C overnight. The bacteria liquid (1 ml) was transferred into 100 ml of fresh medium for scale-up culture. When the optical density reached 0.7, IPTG (final concentration 0.6 mM) was added into the medium to induce protein expression. The bacteria were further cultivated for an additional 6 h at 37°C. The induced cells were harvested by centrifugation at 4°C for 15 min at 8,000 × g, and suspended with phosphate-buffered saline (PBS). Sodium dodecyl sulfate polyacylamide gel electrophoresis (SDS-PAGE) was utilized to check the expression of the desired protein. The gel images were captured, and electrophoresis bands were analyzed using the Bandscan software (Glyko, USA).

Quantitative PCR (qPCR)
Samples (5 ml) from bacterial cells (induced 5 h) were centrifuged, and total RNA was extracted using the RNAPrep pure cell/bacteria