

Construction and Expression of a Fused Gene for B Subunit of the Heat-Labile and a Truncated Form of the Heat-Stable Enterotoxins in *Escherichia coli*

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ABSTRACT

Elaboration of different toxins by enterotoxigenic *E. coli* has been considered as one of the main virulence factors contributing to the manifestation of disease caused by these microorganisms. Various strategies have been employed to raise antibodies against these toxins as a line of defense. In this study, the 3' terminus of the gene that codes for the binding subunit of the heat-labile enterotoxin of *E. coli* (LTB) was fused to the 5' terminus of a truncated heat-stable enterotoxin of *E. coli* (ST) with a region coding for 7 amino acids separating the two moieties. The fused gene was sequenced and subsequently subcloned in the pET23a (+) expression vector in the *EcoRI/HindIII* sites. The construct was transferred into the *E. coli* strain, BL21 (DE3) pLysS and isopropyl- β -D-thiogalactopyranoside (IPTG) was used for induction. The expression of the LTB and ST in the fused protein was assessed using two commercially available kits. SDS-PAGE and Western blot were also used to examine the expressed protein. The results indicated the low expression of both toxin moieties that were recognizable by the commercially available antibodies and the expressed protein was non-toxic as indicated by suckling mouse assay. *Iran. Biomed. J. 3 (3 & 4): 83-86, 1999*

Keywords: Heat-labile enterotoxin, Heat-Stable enterotoxin, *E. coli*

INTRODUCTION

Enterotoxigenic *E. coli* (ETEC) causes diarrheal disease in developing countries as well as among travelers in such areas [1,2]. These organisms elaborate a high molecular weight heat-labile toxin (LT) or a heat-stable enterotoxin (ST) or both [2].

LT is a bacterial ADP-ribosylating exotoxin composed of six non-covalently linked polypeptides, including a single A subunit (27 kDa) with ADP-ribosyltransferase activity and five monomeric B subunits (11.6 kDa) which is immunologically and physiochemically related to cholera toxin [3-5]. The binding of the toxin to eukaryotic cell membrane is mediated by the interaction of the B subunit (LTB) with GM1 ganglioside. LT holotoxin and its B subunit are highly immunogenic and the role of antitoxic immunity to these has been the subject of many studies [6-8]. On the other hand, the ST molecule, because of its small size, tends to be poorly immunogenic, therefore, the elaboration of protective antibodies against ST requires the coupling of this toxin or its derivatives to larger

carrier molecules. Several proteins including BSA, the B-subunits of cholera toxin and LT have been coupled chemically to ST for this purpose [8-11]. The ST molecule has also been linked genetically in various combinations with LTB and CTB [12-14]. In this study, a synthetically assembled ST was placed in frame downstream of LTB in order to produce a fusion peptide carrying antigenic determinants of both LT and ST. The expressed LTB-ST fusion protein can be used to stimulate an immune response effective against both enterotoxins of *E. coli*.

MATERIALS AND METHODS

Bacterial strains and plasmids. For initial cloning and maintenance of the DNA fragments pUC18 vector (Pharmacia), together with *E. coli* strain XL-1 blue and for protein production, expression vector pET23a (+) (Novagen) and bacterial strain BL21 (DE3) pLysS were used. Bacterial transformation and DNA manipulations were performed as described by Sambrook *et al.* [15].

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Polymerase Chain Reaction. PCR was performed using two sets of primers designed for the amplification of LT-B gene from the standard strain H10407 with the following sequences: 5'CCGAATTCGGGATGAATTATGAATAAAG3' and 5'AGCTGCAGAGTTTTCCATACTGATTGCCGC3' containing EcoRI and PstI sites respectively. Ventä DNA polymerase (New England Biolabs) was used for amplification and the PCR conditions were as follows: 94°C 1 min, 60°C 45s, 72°C 30s. This program was performed for 25 cycles. DNA sequencing was performed by the dideoxy chain termination technique of Sanger et al. [16].

Construction of synthetic ST gene. The gene encoding ST was assembled synthetically using two sets of overlapping oligomers 5'CGGGATCCTGCTGTGAACTTTGTTGTAATCCT3' and 5'CGGGATCCACATCCTGTACAGGCAGGATTACA3' dNTPs and Taq DNA polymerase. The size of the fragment was assessed by SDS-PAGE and the sequence was verified by DNA sequencing.

Construction, cloning and expression of the fused gene. The LT-B gene was cloned in pUC18 digested with SmaI (pUC-LTB); the resulting construct was digested with BamHI and filled in with Klenow. The blunt ended ST gene was cloned in this construct (pUC-LTB-ST) and transformed into *E. coli* strain XL-1 blue. For expression, the fused genes were digested with EcoRI and HindIII and cloned into pET23a (+) digested with the same enzymes.

E. coli BL21 (DE3) pLysS transformed with pET-LTB/ST was cultured in LB supplemented with ampicillin (100 mg/ml) at 37°C with good aeration to early exponential phase (OD 600 approximately 0.2-0.5). IPTG was added to a final concentration of 0.5 mM and the culture was allowed to continue growing for 3-4 h. Bacteria were harvested by centrifugation and LTB/ST was released using sonication or 8 M urea [17].

Detection of the expressed fusion peptide. The reversed Passive Latex Hemagglutination kit and Colist EIA kit (Denka Sieken co. Ltd.) were used according to manufacturer's instruction to detect LTB and ST respectively. SDS-PAGE, Western blot and dot blot immunoassay using antibodies provided

in the kits were also used for analysis of the expressed protein [14].

Toxicity test. The suckling mouse assay for ST was performed essentially as described by Giannella [18]. Newborn suckling mice (1 to 4 days old) were randomly divided into the groups of three. Each mouse was inoculated intragastrically with 1, 100, 1000 ng of the LTB/ST fusion polypeptide stained with 2% Evans blue. At 3h postinoculation, the mice were killed, the abdomens were opened, and the entire intestine from each mouse was removed and the ratio of the gut weight to that of the remaining carcass (G/C) was calculated. The mean G/C ratio was then calculated for each group. The G/C ratios of >0.09 are considered positive. A wild ETEC isolate producing ST was used as positive and PBS as negative control. One ng of the toxins produces positive reaction in infant mice [12].

RESULTS AND DISCUSSION

The B subunit of heat-labile enterotoxin of *E. coli* is part of a contiguous operon encoding a single polycistronic mRNA for synthesis of both A- and B-subunit precursor polypeptides that contain N-terminal hydrophobic signal sequences [12]. In the present study, the full coding region of LTB including the signal sequence, which could direct the expressed protein to the bacterial preplasmic space was amplified. On the other hand, the human ST is synthesized as a 72 amino acids (AA) residue precursor, which is proteolytically processed to produce a 19 AA peptide constituting the mature toxin [19]. In this study, the constructed gene coded for a truncated form of the toxin with only 13 AA and it was cloned after LTB with a segment coding for 7 AA separating the two genes.

Previously, It had been shown that the alteration of the carboxyl terminus of the LTB affects its receptor-binding properties, therefore ST had been fused to the 5' end of the LTB or CTB [21]. However, Clements [12] showed that the fusion of the gene for mature ST to the carboxyl terminus of LTB would be functional if a 7 AA linker containing two prolines separates the two moieties. But, in the absence of the linker, no ST antigen could be detected. Therefore, in the present study, we used 7 AA linker containing 2 prolines to separate the two

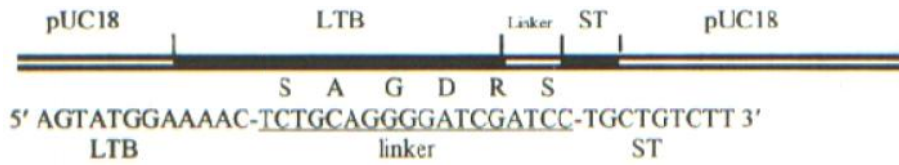


Fig. 1. Construction of the LTB-ST gene fusion. The S, A, G, D, R and S represent the amino acid sequences of the linker.

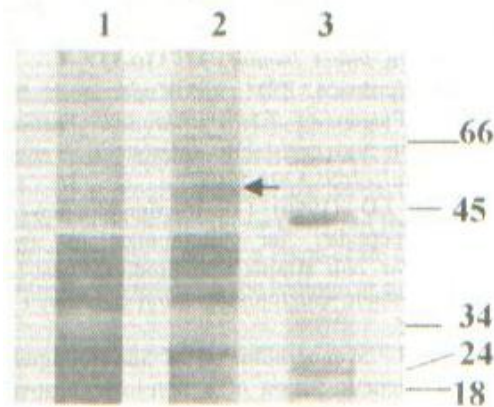


Fig. 2. SDS-PAGE analysis of expressed LT::ST. (1) uninduced, (2) induced and (3) M.W.

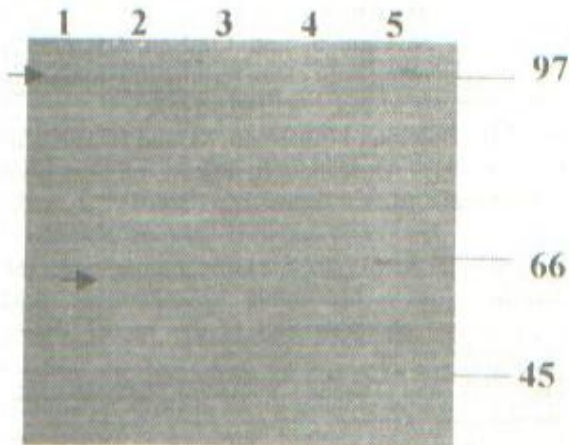


Fig. 3. Western blot analysis of expressed LT::ST. (1) holotoxin, (2) urea-extracted protein, (3) sonicate, (4) uninduced and (5) M.W.

genes. Although, this coded AAs were different from those reported by Clements [12].

The resulting construct was induced and expression of LTB/ST was evaluated using two commercially available kits, which showed the low level of the expression for both LTB and ST. Crude extract was further analyzed by SDS-PAGE and Western blot, both of which showed a band with the apparent molecular weight of about 60 kDa which

could correspond to the pentameric form of LTB/ST (Fig. 2 and 3).

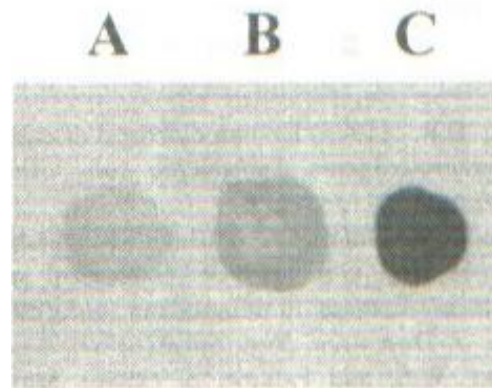


Fig. 4. Dot-Blot analysis of extracts from (A) uninduced, (B) induced and (C) induced using urea for extraction.

The monomeric form was not detected in either SDS-PAGE or Western blot, which could be due to the low expression or the lack of recognition by the antibody raised against the pentameric form as suggested by Sewani *et al.* [20]. Therefore, urea was used for extraction since it had been shown [17] that urea could facilitate the release and help antigen-antibody recognition of the LT from clinical isolates. The immunodot blot assay was performed on the supernatants obtained after urea extraction and by sonication. As it is shown in Fig. 4, the urea-extracted protein produced much stronger signals.

Sandkvist *et al.* [21] had shown that two constructs, in which 12 and 17 AA had been added to the carboxyl-end of the LTB, the fused proteins were not transported to the preplasmic space and the expression level was low. In the present study, the carboxyl terminus of LTB had been altered by the addition of 24 AA (ST plus the flanking AA), which might have resulted in the low expression of the fused protein or its anchorage to the cell membrane resulting in its low recovery when sonication had been used. However, it must be noted that Aitken *et al.* [14] reported no such difficulties with the fused protein obtained from the fusion of LTB with a gene

coding for a precursor of human ST.

The fused protein was tested in the suckling mouse assay for toxicity and the amount of up to 1m g of the fused protein failed to elicit any reaction. Whether this non-toxic fusion protein can give rise to protective antibody needs to be studied.

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REFERENCES

1. Sack, B.R. (1975) Human diarrheal disease caused by enterotoxigenic *Escherichia coli*. *Annu. Rev. Microbiol.* 29: 333-353.
2. Katouli, M., Jafari, A., Farhoudi-Moghadam, AA. And Ketabi, G.R. (1990) Etiological studies of diarrheal diseases in infants and young children in Iran. *J. Trop. Med. Hyg.* 93: 22-27.
3. Dallas, W.S. and Falkow, S. (1980) Amino acid sequence homology between cholera toxin and heat-labile toxin. *Nature* (London) 288: 499-501.
4. Gill, D.M., Clements, J.D., Robertson, D.C. and Finkelstein, R.A. (1981) Subunit number and arrangement in *Escherichia coli* heat-labile enterotoxin. *Infect. Immun.* 33: 677-682.
5. Moss, J.H. and Richardson, S.H. (1978) Activation of adenylate cyclase by heat-labile enterotoxin. Evidence of ADP-ribosyltransferase activity similar to that of cholera toxin. *J. Clin. Invest.* 62: 281-285.
6. Kaper, J.B. and Levine, M.M. (1988) Progress towards a vaccine against enterotoxigenic *Escherichia coli*. *Vaccine* 6: 197-199.
7. Levine, M.M., Kaper, J.B., Black, R.E. and Clements, M.L. (1983) New knowledge on pathogenesis of bacterial enteric infection as applied to vaccine development. *Microbiol. Rev.* 47: 510-550.
8. Svennerholm, A.M., Jertborn, M., Gothefors, L., Karim, A.M., Sack, D.A. and Holmgren, J. (1984) Local and systemic antibody response and immunological memory in humans after cholera disease and after immunization with a combined B subunit-whole cell vaccine. *J. Infect. Dis.* 149: 884-893.
9. Svennerholm, A.M., Wikstrom, M., Lindblad, M. and Holmgren, J. (1986) Monoclonal antibodies against *Escherichia coli* heat-stable toxin and their use in a diagnostic ST ganglioside GM1-enzyme linked immunosorbent assay. *J. Clin. Microbiol.* 24: 585-590.
10. Klipstein, F.A., Engert, R.F. and Clements, J.D. (1982) Development of a vaccine cross-linked heat-stable and heat-labile enterotoxins that protects against *Escherichia coli* producing either enterotoxin. *Infect. Immun.* 37: 550-557.
11. Guzman-Verduzco, L.M. and Kupersztoch, Y.M. (1987) Fusion of *Escherichia coli* heat-stable enterotoxin and heat-labile enterotoxin B subunit. *J. Bacteriol.* 169:5201-5208.
12. Clements, J.D. (1990) Construction of a non-toxic fusion peptide for immunization against *Escherichia coli* strains that produces heat-labile and heat-stable enterotoxins. *Infect. Immun.* 58: 1159-1166.
13. Sanchez, J., Svennerholm, A.M. and Holmgren, J. (1988) Genetic fusion of a non-toxic heat-stable enterotoxin-related decapeptide antigen to cholera toxin B subunit. *F.E.B.S. Lett.* 241: 110-114.
14. Aitken, R. and Hirst, T.R. (1993) Recombinant enterotoxins as vaccines against *Escherichia coli*-mediated diarrhea. *Vaccine* 11: 227-233.
15. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular cloning: a laboratory manual. 2nd. Ed., Cold Spring Harbor, N.Y.
16. Sanger, F., Nicklen, S. and Coulson, A.R. (1997) DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci.* 74: 5463-5467.
17. Qu, Z.H., Boesman-Finkelstein, M. and Finkelstein, R.A. (1991) Urea-induced release of heat-labile enterotoxin from *Escherichia coli*. *J. Clin. Microbiol.* 29: 773-777.
18. Giannella, R.A. (1976) Suckling mouse model for detection of heat-stable *Escherichia coli* enterotoxins: characteristics of the model. *Infect. Immun.* 14: 95-99.
19. Nair, G.B. and Takeda, Y. (1998) The heat-stable enterotoxins. *Microb. Pathog.* 24: 123-131.
20. Sewani, C.R., Bagdasarian, M.M., Ireland, J.J. and Bagdasarian, M. (1998) Display of an inhibin epitope in a surface-exposed loop of the *E. coli* heat-labile enterotoxin B subunit. *Vaccine* 17: 1611-1619.
21. Sandkvist, M., Hirst, T.R., Bagdasarian, M. (1987) Alteration at the carboxyl terminus change assembly and secretion properties of the B subunit of *Escherichia coli* heat-labile enterotoxin. *J. Bacteriol.* 169: 4570-4576.