Medical Journal of the
Islamic Republic of Iran

Volume 13
Number 1
Spring 1378
May 1999

ESCHERICHIA COLI HEAT-LABILE TOXIN B SUBUNIT: CONSTRUCTION AND EVALUATION OF PLASMIDS PROVIDING CONTROLLED HIGH LEVEL PRODUCTION OF THE PROTEIN

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ABSTRACT

With the plasmid DNA from a clinical isolate of enterotoxigenic *Escherichia coli* (ETEC) H10407 as template, PCR-mediated cloning of the sequence encoding the heat-labile toxin B subunit (LT-B) has been carried out. Then this sequence was recloned into the pTrc 99A and pET23a expression vectors to give the plasmids pTRCLTB and pETLTB, respectively. After induction, the former plasmid provides for the production of rLT-B in a yield of up to 15 mg per liter of bacterial culture. The recombinant protein was shown to be structurally and immunologically identical with the native LTB. High titer antibodies capable of neutralizing the native toxin were raised in mice by oral administration of the rLT-B. Hence the constructed plasmids provide the basis for an oral ETEC vaccine, as well as for genetic fusion of foreign antigens with the aim of developing polyvalent vaccines.

MJIRI, Vol. 13, No. 1, 55-60, 1999

Keywords: ETEC, Recombinant LT-B, Oral vaccine, PCR.

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) is a major causative agent of diarrhea among children in the developing world as well as travelers to such areas. 650 million diarrheal episodes and 800,000 deaths are reported annually among children in developing countries.[2,3] Heat-labile enterotoxin (LT) is one of the most important pathogenic factors of ETEC which is immunologically and physiologically closely related to cholera toxin. LT is composed of one A subunit (LT-A, 28 kDa) that has toxic (ADP ribosyltransferase) activity, and five identical B subunits (LT-B), responsible for binding the toxin to ganglioside GM1 and other ganglioside receptors on the surface of eukaryotic cells and promoting the entry of the A subunit into the cells.[17] LT-B is a strong immunogen and protecting factor against ETEC mediating diarrhea,[1,7,9,12,19] as well as a carrier for delivery of genetically or chemically coupled antigens or epitopes.[4,5,10,13,16] Moreover, LT-B has a significant immunoregulatory potential, not only as a means of preventing the induction of tolerance but also as an adjuvant for orally administered antigens.[8,17,20-22] For these reasons, cloning and expression of the LT-B gene was carried out and bioactivity of the recombinant LT-B (rLT-B) was studied.

We report here the construction of plasmids providing for controlled production of rLT-B and purification of the protein, as well as its immunochemical and immunogenic

characterization. The biological activity of rLT-B was shown by neutralization of the native toxin activity with antibodies raised against the recombinant protein.

MATERIALS AND METHODS

Bacterial strains and plasmids

Plasmid DNA from a clinical isolate of enterotoxigenic *Escherichia coli* H10407 serotype 078:H11 (obtained from Persian type culture collection PTCC 1287) was used as a source of the LT-B gene. *E.coli* strains JM105 (Pharmacia), BL21 (DE3) (Novagen) and MM294 (ATCC) used as hosts were cultured in LB medium (Bacto-tryptone 10 g, yeast extract 5 g and NaCl 10 g per liter) supplemented with 100 µg/mL ampicillin to propagate plasmids and stored at -70°C in the same medium containing 15% (v/v) glycerol. A common pUC18 (Pharmacia) vector was used for initial cloning of the LT-B gene, and the p*Trc* 99A (Pharmacia) and pET23a (Novagen) vectors were used to express it.

DNA manipulation

DNA manipulation, including plasmid preparation, use of restriction enzymes, DNA ligation, plasmid transformation, and agarose gel electrophoresis, was done according to standard procedures, essentially as described by Sambrook et al.[14] Restriction endonucleases, T4DNA ligase and *Taq* DNA polymerase were purchased from Gibco-BRL. Vent DNA polymerase and ganglioside GM1 were obtained from Biolab and Sigma respectively. DNA sequencing was performed by the dideoxy chain termination technique of Sanger et al.[15] Polymerase chain reaction (PCR) was carried out in a Pharmacia LKB-Gene ATAQ thermocycler under standard buffer conditions using oligonucleotide primers prepared on an Applied Biosystems DNA synthesizer model 360B. Amplified products were analyzed by agarose gel electrophoresis, and the desired DNA fragments were isolated from the low melting agarose gel (Sigma, Type VII).

Table I. Expression of rLT-B controlled by a number of promoters.

Host strain	Plasmid	Promoter	Amount of rLT-B (mg/L)
MM 294	pUC18	lac	ND*
	pUCLTB		2.5
	pTrc 99A	trc	ND
	pTRCLTB		3.6
JM105	pUC18	lac	ND
	pUCLTB		2-2.5
	pTrc 99A	trc	ND
	pTRCLTB		13-15
BL21 (DE3)	pET23a	T7	ND
	pETLTB		10-12

*ND= Not detected

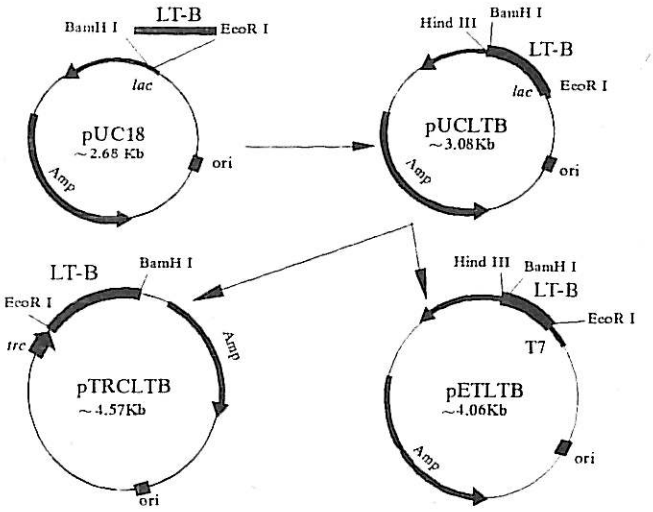

Fig. 1. Construction of plasmids encoding LT-B. The amplified LT-B gene was cloned into pUC18 digested with *Eco*RI and *Bam*HI (pUCLTB). The LT-B gene was further recloned from pUCLTB into pTrc 99A and pET23a digested with *Eco*RI/*Bam*HI and *Eco*RI/*Hin*dIII, respectively.

Large plasmid purification

The 62.7Kb (42×106 Dalton molecular weight) plasmid DNA was purified from a clinical enterotoxigenic isolate, *E. coli* H10407 23 by SDS-lysis method which is suitable for extraction of large plasmids. Further purification of the plasmid was carried out by ethidium bromide-cesium chloride gradient centrifugation (final density, 1.55 g/mL) for 20 h at 70,000 rpm (20°C) using a Beckman VTi90 rotor.[14] The H10407 plasmid was cut with the restriction endonuclease *Pst*I, and the *Pst*I DNA fragment (5.2Kb) containing the LT operon was isolated by preparative gel electrophoresis, and the product was used as the template for PCR amplification.

PCR amplification of the LT-B gene

On the basis of the known nucleotide sequence of the LT-B gene[6,11,24] (Gene Bank accession number: J01646, we designed a pair of oligonucleotide primers annealing to opposite ends of the LT-B gene. The 5'-terminal (forward) primer was 5'-ACAGAATTCGGAATGAATTATG-3' and the 3'-terminal (reverse) primer was 5'-CGCGGATCCCTAGTTTTCCATACTGATTGCC-3' with the first 9 nucleotides in the latter primer being extrinsic to the target sequence and creating the unique *Bam* HI site for ease of cloning. With this pair of primers, a 403-bp fragment was prepared by a 30-cycle amplification from the 5.2 Kb *Pst*I fragment containing the LT operon as template. The PCR reaction mixture contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgSO_4$, 200 mM

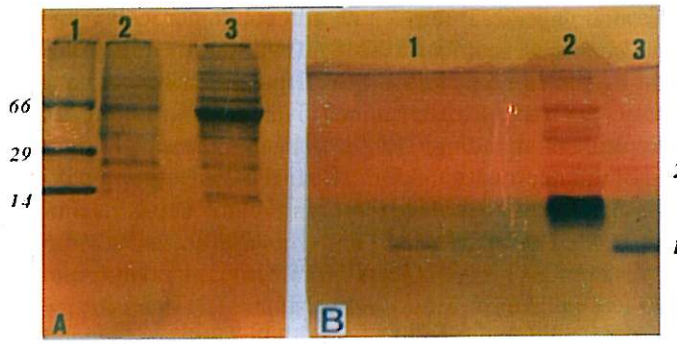

Fig. 2. SDS polyacrylamide gel analysis of crude extract and affinity purified rLT-B.

A: lane 1: Molecular weight standards (66, 29 and 14 kDa); lane 2: unboiled crude lysate of induced JM 105 (pTRCLTB) (Negative control); lane 3: unboiled crude lysate of induced JM 105 (pTRCLTB).

B: lane 1: Affinity purified rLT-B; lane 2: molecular weight standards (66, 45, 29, 18 and 14 kDa); lane 3: boiled native LT (LT-A 28 kDa and LT-B 11.8 kDa).

each of dNTP, 1.25unit of Vent DNA polymerase and 0.4 µL of the LT-B primers. The tube was initially heated to 94°C for 5 min to denature the DNA. The LT-B gene amplification profile was as follows: 94°C for 1 min, 55°C for 1 min and 72°C for 1 min with a final 5-min incubation at 72°C.

Production of the recombinant LT-B

Plasmid-containing *E.coli* cells (Table I) were cultured in LB medium supplemented with ampicillin (100 µg/mL) at 37°C with vigorous shaking (150 rpm), and when cultures reached an optical density of 0.5 (OD 600 nm), 0.5 mM isopropyl thiogalactopyranoside (IPTG, Sigma) was added to induce the production of LT-B. After overnight incubation, cells were harvested by centrifugation (5000 rpm, 10 min) at 4°C and resuspended in TEAN buffer (0.05 M Tris, 0.001 M EDTA, 0.003M NaN_3, 0.2 M NaCl [pH 7.5]). After sonication the crude lysate was clarified by centrifugation and precipitated by adding sodium hexametaphosphate to 2.5 mg/mL and adjusting the pH to 4.5 with HCl. The precipitate was harvested, resuspended in, and dialyzed against, TEAN buffer.

GM1-ELISA

Recombinant LT-B was detected and measured by the GM1-ganglioside enzyme-linked immunosorbent assay (GM1-ELISA)[4,7,10,18] using anti-LTB monoclonal antibodies LT-39 (a gift from Sevenerholm A.M.) or D15-8 (kindly provided from the Pasteur Institute, Paris). ELISA plates were precoated with 5 µg of mixed ganglioside GM1 type III per well in 100 µL of 0.05 mol carbonate buffer, (pH 9.6) for 6 h at room temperature and kept at 4°C until use. Coated plates were first washed three times with PBS-

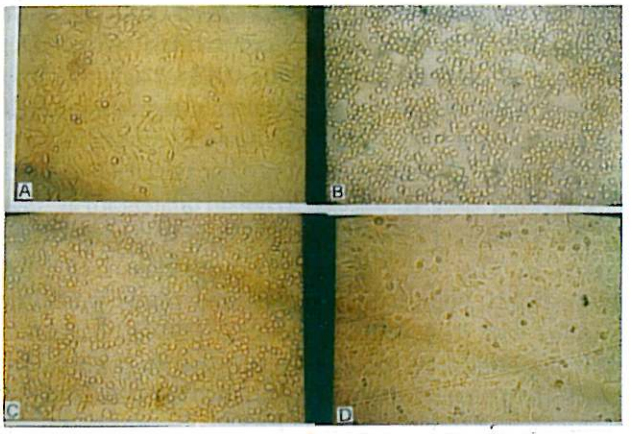

Fig. 3. Toxin neutralization a say on mouse Y1 adrenal tumor cell.

A: Monolayer of Y1 cells.

B: Monolayer of Y1 cells after treatment with trypsin-activated LT.

C: Monolayer of Y1 cells after treatment with trypsin-activated LT in the presence of a negative control antiserum.

D: Monolayer of Y1 cells after treatment with trypsin-activated LT in the presence of anti-rLT-B antiserum.

0.05% Tween 20 (PBS-T), blocked with PBS 0.1% (w v) bovine serum albumin (BSA) for 30 min at 37°C. 100 µL of serial sample dilutions were added to each well and incubated at 37°C for 1h. Plates were washed three times with PBS-T and 100 µL of anti-LT-B monoclonal antibody was added to each well and incubated for 1.5 h at 37°C for 1 h, and then washed. A chromogenic substrate, ortho-phenylenediamine (OPD) and H_2O_2 in citrate buffer (pH 4.5) were added. The rLT-B was measured using the calibration curve plotted against the standard heat-labile toxin (LT).

Immunization

Groups of five C57/BL mice were immunized intraperitoneally (i.p.), intramuscularly (i.m.) and intradermally (i.d.) with 0.1 mL of crude lysate from *E. coli* strain JM 105 (pTRCLTB). The crude lysate was filter sterilized through a 0.22 µm pore-size filter and injected three times (days 0, 14, 28). The primary doses of i.d. and i.m. immunization were given with Freund's complete adjuvant and booster doses were given with Freund's incomplete adjuvant. Intraperitoneal immunization was done with no adjuvant. For oral immunization, mice were first gavaged with 0.5 mL of a solution of 8 parts Hank's balanced salt solution and 2 parts 7.5% sodium bicarbonate and then orally immunized three times (days 0,14,28) with filter sterilized crude lysate. Additionally, two rabbits were intramuscularly immunized three times with 50 µg reference LT in each dose. The first dose was given with Freund's

complete adjuvant, and booster doses with Freund's incomplete adjuvant. Sera were collected two weeks after the final booster, and analyzed for antibody against LT by GM1-ELISA and toxin neutralization assay in mouse Y1 adrenal cells.

Serum titer determination

Sera obtained from immunized mice and rabbits were tested by GM1-ELISA as previously described.[4,7,10,18] Immediately before use, GM1-coated plates were first washed with PBS and blocked with PBS-0.1% BSA at 37°C for 30 min. 100 µL of LT (10 µg/mL was added to the wells, the plates were incubated at room temperature for 1 h and washed three times with PBS-T.

Immunized mice or rabbit sera titrated out with serial dilutions was added and the plates were incubated at room temperature for 1h. After three washes with PBS-T, LT bound antibodies were assayed by incubation of appropriate anti-mouse or anti-rabbit IgG conjugated with peroxidase. The chromogenic substrate was OPD, and titers were determined using absorbance of 0.4 at 490 nm after a reaction time of 15 min as baseline.

Purification of rLT-B

For purification of rLT-B, an affinity column was prepared. At first the IgG fraction of anti-LT rabbit antiserum was purified by protein A chromatography.

Subsequently, it was covalently bound to activated Sepharose-4B (Sigma). The column was equilibrated with TEAN buffer, and after applying the sample was extensively washed with the same buffer. The rLT-B was eluted with 0.2 M glycine hydrochloride (pH 2.6) containing 0.2 M NaCl. The eluate was analyzed by GM1-ELISA and by SDS-PAGE after concentration by trichloroacetic acid.

Toxin neutralization assay

The ability of the raised antibodies to neutralize LT toxin was shown by toxin neutralization assay on mouse Y1 adrenal tumor cells as previously described.[7,8] In the initial experiments, different doses of LT were applied to determine the optimal dose for toxin activity. For this purpose, mouse Y1 adrenal tumor cells (ATCC CCL-79) were seeded in 96-well round-bottom plates at a concentration of 104 cells per well. Since it has been previously shown that trypsin-activated LT is more than 1000-fold more active on Y-1 adrenal cells than unnicked LT,[8] the toxin was activated with trypsin by a 45 min incubation at 37°C in the presence of 0.1µg trypsin in a final volume of 100 µL. The activated toxin was serially diluted prior to incubation with Y1 adrenal cells monolayer at 37°C in 5% CO_2 overnight. For the toxin neutralization assay a selected dose of activated toxin (approximately 10 rounding dose) was mixed with an equal volume of serially diluted pooled serum samples. After incubation for 1 h at 37°C, samples were applied to a monolayer of mouse Y-1 adrenal tumor cells, and incubation was continued for 18 h. Cells were then examined by light microscopy for typical cell rounding. The following day, the cells were examined by light microscopy for typical cell rounding. Titer is defined as the reciprocal of the minimum concentration of toxin required to give greater than 50% cell rounding.

RESULTS

Construction of LT-B expressing plasmids

The 62.7 Kb H10407 plasmid DNA was successfully isolated and purified by the method described earlier (data not shown) suitable for large plasmids.[14] In the PCR amplification experiments, the initial results indicated that the designed primers and conditions were suitable for the amplification. However, the system was further optimized for cloning purposes of the PCR product by substituting Taq DNA polymerase with Vent polymerase for its proof-reading characteristics.

The target PCR product was gel purified, cleaved with *Eco*RI and *Bam*HI, and ligated into pUC18 previously digested with the same enzymes. The structure of the resulting construct, named pUCLTB, was ultimately confirmed by direct nucleotide sequencing method.[15] The LT-B gene was further recloned from pUCLTB into pTrc 99 A and pET 23a yielding the plasmids named pTRCLTB and pETLTB, respectively (Fig.1). In the former case the gene expression is under the control of trc promoter, and in the latter under control of the T7 promoter.

Preparation and characterization of recombinant LT-B

Cells harbouring the different recombinant plasmids carrying the LT-B gene were cultured and induced as described in *Materials and Methods*. The level of LT-B was measured by GM1-ELISA with reference to the calibration standard curve obtained using standard LT.

As seen in Table I, the GM1-ELISA readings show that the rLT-B level ranges from 2 to 15 mg per liter of culture, the highest yield belonging to JM105 (pTRCLTB). The rLT-B was purified from *E. coli* JM 105 (pTRCLTB) in one step by affinity chromatography as described in *Materials and Methods*. The results of SDS-PAGE (see Fig. 2) and GM1-ELISA suggest that rLT-B has the same electrophoretic mobility as the native LT-B and is capable of binding to the ganglioside GM1. Moreover, in GM1-ELISA studies rLT-B reacted with both LT-39 and D15-8, the monoclonal antibodies specifically against the native LT-B. Hence it can be said with confidence that rLT-B is structurally and immunologically identical to the native LT-B.

Immunogenicity of rLT-B

Groups of mice were immunized by different routes–

i.p., i.m., i.d. and p.o. – with a filter sterilized crude cell lysate of *E. coli* JM105 (pTRCLTB) as described in *Materials and Methods*. Sera from immunized mice were able to recognize toxin in the GM1-ELISA, and high titer antitoxin antibodies (>24,000) were detected in all immunized groups. It was interesting to note that oral immunization could raise anti-rLTB antibody as high as other routes of immunization (data not shown). An even more important consideration in toxin neutralization studies was that anti-rLTB antiserum obtained from all groups of immunized mice was able to neutralize the native toxin. As may be seen in Fig. 3 the native toxin can really be neutralized by antibodies raised against rLT-B. The serum neutralization titer against LT was estimated at 1/400, whereas pooled sera from control mice had no effect on Y1 mouse adrenal cells.

DISCUSSION

In various studies over the last decade it has been shown that LT-B is a strong immunogen and a protecting factor against ETEC diarrhea[1,7,12,19] and hence it is a suitable candidate for vaccine development. In 1983 Clements et al. constructed a plasmid containing the LT-B producing sequence and studied some molecular characteristics of the product. In various literature published since then, molecular and immunological characteristics of the LT-B produced in attenuated salmonella strains as host or conjugated with other immunogens have been reported.[5,7,10,16]

In this study, we have successfully purified the LT-B carrying plasmid from enterotoxigenic *E. coli* and the toxin B subunit sequence was amplified under optimized conditions. Furthermore, the PCR-amplified LT-B gene was cloned under the control of *lac*, *trc* and T7 promoters. Due to effectiveness of ribosome binding site (rbs) of LT-B gene, the PCR primers were designed so that the rbs and ATG initiation codon of the native gene were conserved.

An important consideration for the use of the rLT-B as an immunoprophylactic agent is that it is capable of eliciting antibodies which can recognize and, more importantly, neutralize the native toxin. The results presented in this paper indicate conclusively that rLT-B has the correct conformation since it can efficiently bind to the GM1-ganglioside and react with monoclonal antibodies, LT39 and D15-8. As expected, Coomassie stained SDS-PAGE showed that rLT-B is identical with the native LTB subunit in electrophoretic mobility (Fig. 2). It has been shown that oral administration of rLT-B to mice resulted in the induction of serum and mucosal anti-LT-B antibodies via Peyer's patch lymphocytes.[12] An important consideration for the use of this molecule as a vaccine was that oral administration of recombinant LT-B can elicite antibody titers as high as other routes of immunizaton. these antibodies were also able to recognize and, more importantly, neutralize the native toxin. This finding is in agreement with results reported by Lebens et al. and Clements et al.[7,10] It can, therefore, be concluded that rLTB with its immunological and molecular characteristics are of fundamental importance for vaccine development. Hence the constructed plasmids provide the basis for an oral recombinant ETEC vaccine, as well as for genetic fusion of foreign antigens, with the aim of developing polyvalent vaccines.

REFERENCES

1. Aitken R, Hirst TR: Recombinant enterotoxins as vaccines against *Escherichia coli*-mediated diarrhoea. Vaccine 11 (2): 227-233, 1993.
2. Black RE: The epidemiology of cholera and enterotoxigenic *E. coli* diarrheal diseases. In: Holmgren J, Lindberg A, Molby R. (eds.), Development of Vaccines and Drugs against Diarrhea. Proceedings of the 11th Nobel Conference. Student litterature, Lund, Sweden, pp. 23-32, 1986.
3. Black RE: Epidemiology of diarrheal disease: implications for control by vaccines. Vaccine 11: 100-106, 1993.
4. Clements JD: Construction of a nontoxic fusion peptide for immunization against *Escherichia coli* strains that produce heat-labile and heat-stable enterotoxins. Infect Immun 58 (5): 1159-1166, 1990.
5. Clements JD, El-Morshidy S: Construction of a potential live oral bivalent vaccine for typhoid fever and cholera - *Escherichia coli*-related diarrheas. Infect Immun 64 (2): 564-569, 1984.
6. Clements JD, Flint DC, Engert RF, Klipstein FA: Cloning and molecular characterization of the B subunit of *Escherichia coli* heat-labile enterotoxin. Infect Immun 40 (2): 653-658, 1983.
7. Clements JD, Lyon FL, Lowe KL, Farrand AL, El-Morshidy S: Oral immunization of mice with attenuated *Salmonella enteritidis* containing a recombinant plasmid which codes for production of the B subunit of heat-labile *Escherichia coli* enterotoxin. Infect Immun 53 (6): 685-692, 1986.
8. Dickinson BL, Clements JD: Dissociation of *Escherichia coli* heat-labile enterotoxin adjuvanticity from ADP-ribosyltransferase activity. Infect Immun 63 (5): 1617-1623, 1995.
9. Klipstein FA, Engert RF, Houghten RA: Immunization of volunteers with a synthetic peptide vaccine for enterotoxigenic *Escherichia coli*. The Lancet 1: 471-472, 1986.
10. Lebens M, Shahabi V, Backstrom M, Honze T, Lindblad M, Holmgren J: Synthesis of hybrid molecules between heat-labile enterotoxin and cholera toxin B subunits: potential for use in a broad-spectrum vaccine. Infect Immun 64 (6): 2144-2150, 1996.
11. Leong J, Vinal AC, Dallas WS: Nucleotide sequence comparison between heat-labile toxin B-subunit cistrons from *Escherichia coli of* human and porcine origin. Infect

Immun 48 (1): 73-77, 1985.

12. Nakagawa I, Takahashi I, Kiyono H, McGhee JR, Hamada S: Oral immunization with the B subunit of the heat-labile enterotoxin of *Escherichia coli* induces early Th1 and late Th2 cytokine expression in Peyer's patches. J Infect Dis 173: 1428-36, 1996.
13. Nashar TO, Amin T, Marcello A, Hirst TR: Current progress in the development of the B subunit of cholera toxin and *Escherichia coli* heat-labile enterotoxin as carriers for the oral delivery of heterologous antigens and epitopes. Vaccine 11 (2): 235-240, 1993.
14. Sambrook J, Frisch EF, Maniatis T: Molecular Cloning, A Laboratory Manual. 2nd ed. New York: Cold Spring Harbor Laboratory Press, 1989.
15. Sanger F, Nicklen S, Coulson AR: DNA sequencing with chain-terminating inhibitors. Proc Nat Acad Sci USA 74: 5463-5467, 1977.
16. Schodel F, Will H: Construction of a plasmid for expression of foreign epitopes as fusion proteins with subunit B of *Escherichia coli*. Infect Immun 58 (4): 1347-1350, 1989.
17. Spangler BD: Structure and function of cholera toxin and related *Escherichia coli* heat-labile enterotoxin. Microbiol Rev 56: 622-647, 1992.
18. Svennerholm AM, Holmgren J: Identification of *Escherichia coli* heat-labile enterotoxin by mean of a ganglioside immunosorbent assay (GM1 ELISA) procedure. Curr Microbiol 1: 19-23, 1978.
19. Takahashi I, Kiyono H, Jackon RJ, Fujihashi K, Staats HF, Hamada S, Clements JD, Bost KL, McGhee JR: Epitope maps of the *Escherichia coli* heat-labile toxin B subunit for development of a synthetic vaccine. Infect Immun 64 (4): 1290-1298, 1996.
20. Tamura S, Asanuma H, Tomita T, Komase K, Kawahara K, Danbara H, Hattori N, Watanabe K, Suzuki Y, Nagamine T, Aizawa C, Oya A, Kurata T: *Escherichia coli* heat-labile enterotoxin B subunit supplemented with a trace amount of the holotoxin as an adjuvant for nasal influenza vaccine. Vaccine 12 (12): 1083-1089, 1994.
21. Tamura S, Hatori E, Tsuruhara T, Aizawa C, Kurata T: Suppression of delayed-type hypersensitivity and IgE antibody responses to ovalbumin by intranasal administration of *Escherichia coli* heat-labile enterotoxin B subunit-conjugated ovalbumin. Vaccine 15 (2): 225-229, 1997.
22. Tamura S, Yamanaka A, Shimohara M, Tomita T, Komase K, Tsuda Y, Kawahara K, Danbara H, Hattori N, Watanabe K, Suzuki Y, Nagamine T, Aizawa C, Oya A, Kurata T: Synergistic action of cholera toxin B subunit (and *Escherichia coli* heat-labile toxin B subunit) and a trace amount of cholera whole toxin as an adjuvant for nasal influenza vaccine. Vaccine 12 (5): 419-426, 1994.
23. Yamamoto T, Yokota T: Cloning of deoxyribonucleic acid regions encoding a heat-labile and heat-stable enterotoxin originating from an enterotoxigenic *Escherichia coli* induces early Th1 and late TH2 cytokine expression in Peyer's patches. J Infect Dis 173: 1428-36, 1996.
24. Yamamoto T, Yokota T: Sequence of heat-labile enterotoxin of *Escherichia coli* pathogenic for humans. J Bacteriol 155 (2): 728-733, 1983.