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Expression of Functional Pentameric Heat-Labile Enterotoxin B Subunit of *Escherichia coli* in *Saccharomyces cerevisiae*

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Although the *Escherichia coli* heat-labile enterotoxin B subunit (LTB) has already been expressed in several different systems, including prokaryotic and eukaryotic organisms, studies regarding the synthesis of LTB into oligomeric structures of pentameric size in the budding yeast *Saccharomyces cerevisiae* have been limited. Therefore, this study used a functional signal peptide of the amylase 1A protein from rice to direct the yeast-expressed LTB towards the endoplasmic reticulum to oligomerize with the expected pentameric size. The expression and assembly of the recombinant LTB were confirmed in both the cell-free extract and culture media of the recombinant strain using a Western blot analysis. The binding of the LTB pentamers to intestinal epithelial cell membrane glycolipid receptors was further verified using a GM1-ganglioside enzyme-linked immunosorbent assay (GM1-ELISA). On the basis of the GM1-ELISA results, pentameric LTB proteins comprised approximately 0.5–2.0% of the total soluble proteins, and the maximum quantity of secreted LTB was estimated to be 3 mg/l after a 3-day cultivation period. Consequently, the synthesis of LTB monomers and their assembly into biologically active oligomers in a recombinant *S. cerevisiae* strain demonstrated the feasibility of using a GRAS microorganism-based adjuvant, as well as the development of carriers against mucosal disease.

Keywords: Enterotoxin B subunit, GM1-ELISA, *Saccharomyces cerevisiae*

Enterotoxigenic bacteria generate two types of toxin: heat-stable and heat-labile toxins. The heat-labile enterotoxin of *Escherichia coli* (LT) is the principal disease agent of enterotoxigenic *E. coli* and the most common cause of diarrhea [20, 44]. LT is composed of one copy of the A subunit (LTA), which includes ADP-ribosylation activity, and a homopentamer of B subunits (LTB), which binds to the cell receptors on the surface of eukaryotic cells [28, 40]. The LT homopentamer consists of five identical 103-amino-acid B peptides (11.6 kDa), which form a donut-shaped pentamer *via* noncovalent associations [41]. LT is an immunogenic molecule that stimulates both systemic and mucosal immune system responses [12], and it has also been identified as a potent immunoadjuvant, which has been demonstrated to enhance mucosal IgA as well as systemic antibody responses against co-administered or coupled antigens [42]. However, the use of LT as an adjuvant remains limited, mainly due to the toxicity of the LTA subunit [47]. Therefore, based on establishing the importance of the adjuvant activity of LTA [7, 27], strategies to separate the effect of the adjuvant from the toxicity have resulted in the production of LTB lacking the A subunit. In addition, various previous studies have shown that LTB alone is a potent mucosal immunogen that induces systemic and mucosal responses [5, 43], and has a profound adjuvant activity as a carrier protein for co-administered unrelated antigens [45]. Although the exact mechanism by which the adjuvant effects of LTB are exerted is not yet fully understood, it has been suggested that the stimulatory activity of LTB depends on its ability to bind to cell receptors, most commonly GM1, which requires the pentameric association of B subunits. Thus, several expression systems have already been applied for the production of recombinant LTB, including *E. coli* [4], *Mycobacterium bovis* [14], *Lactobacillus brevis* [10], *Staphylococcus xylosus* [22], *Pichia pastoris* [8], *Saccharomyces cerevisiae* [33], and plants [13].

The *S. cerevisiae*-based expression system is unique among expression systems, most notably because it combines the

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advantages of a prokaryotic system, high expression levels, easy scale-up, and inexpensive growth media with the inherent advantages of a eukaryotic system, thereby enabling the execution of the majority of posttranslational modifications [3]. Moreover, yeast is a GRAS (generally recognized as safe) organism with an extensive history of applications for the production of rich biomass yields of high-quality proteins and vitamins, and this allows it to be utilized in pharmaceutical, livestock feed, and food industry applications. *S. cerevisiae* has already been successfully used live and orally for the treatment of *Clostridium difficile* diarrhea in humans [35], and has been determined to protect mice against mild influenza virus infections [6]. In addition, the results of several studies have also supported the use of yeast expression systems for recombinant vaccine formulations, due to the adjuvant properties of yeast derivatives, which enhance the immune response [37]. Thus, along with these characteristics of *S. cerevisiae*, the biochemical characteristics of LTB, showing resistance to proteolytic degradation and the persistence of its pentameric structure at pH values as low as 2.0, render the combination of a *S. cerevisiae* expression system with LTB suitable for the development of mucosal vaccines.

However, a recent study of LTB expression using *S. cerevisiae* found that, although yeast-derived LTB was antigenically indistinguishable from bacterial LTB, its intracellular presence was monomeric and it failed to assemble into its pentameric form [33]. Furthermore, in the presence of a bacterial signal peptide, the recombinant LTB from *S. cerevisiae* assembled into a pentameric form, although it remained associated with the membrane fraction and was not secreted into the media [36]. Yet, the secretion of target protein has several advantages over an intracellular location, including easy purification. Accordingly, this study attempted to produce and secrete pentameric LTB using *S. cerevisiae* as the host.

MATERIALS AND METHODS

Chemicals and Enzymes

All the chemicals utilized in this study were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.) unless indicated otherwise. The media and enzymes were obtained from Difco Laboratories (Detroit, MI, U.S.A.) and Boehringer Mannheim (Mannheim, Germany), respectively.

Strains and Culture Conditions

The plasmids were maintained and propagated in *E. coli* TOP10 in accordance with the methods described by Sambrook *et al.* [34]. The pMYO50 plasmid harboring the *E. coli* heat-labile enterotoxin B subunit gene (*sLTB*) was utilized [18], and *S. cerevisiae* 2805 (*MATα pep4::HIS3 prb1-δ Can1 GAL2 his3 ura3-52*) was used as the recipient cell for LTB production [39].

The *S. cerevisiae* culture was maintained in a YEPD medium (1% yeast extract, 2% peptone, and 2% dextrose), whereas a uracil-deficient selective medium (0.67% yeast nitrogen base without amino acids, 0.003% adenine and tryptophan, 0.5% casamino acid, 2% dextrose, and 2% agar) was used to screen the transformants at 30°C. The primary inoculum was prepared from 5 ml of the uracil selective medium and cultured for 24 h, and then a total of 1×10^7 cells was inoculated into 40 ml of the YEPD medium in a 300-ml Erlenmeyer flask. The cultures were grown at 30°C with continuous agitation (200 rpm), following which the culture was assayed for the presence of recombinant LTB.

Construction of Plasmid and Transformation of Yeast

The amylase 1A (*Ramy*1A) signal peptide (ASP) and LTB gene, which have already been described in several previous studies, were fused *via* an overlap extension PCR to create BamHI and SalI restriction sites (underlined) at the 5' and 3' ends, respectively, using the following primers: forward 5'-GGATCCGCATCCAGGTGCTG-AAC-3' and reverse 5'-GGTCGACTCAGTTCTCCATGCTGATAGC-

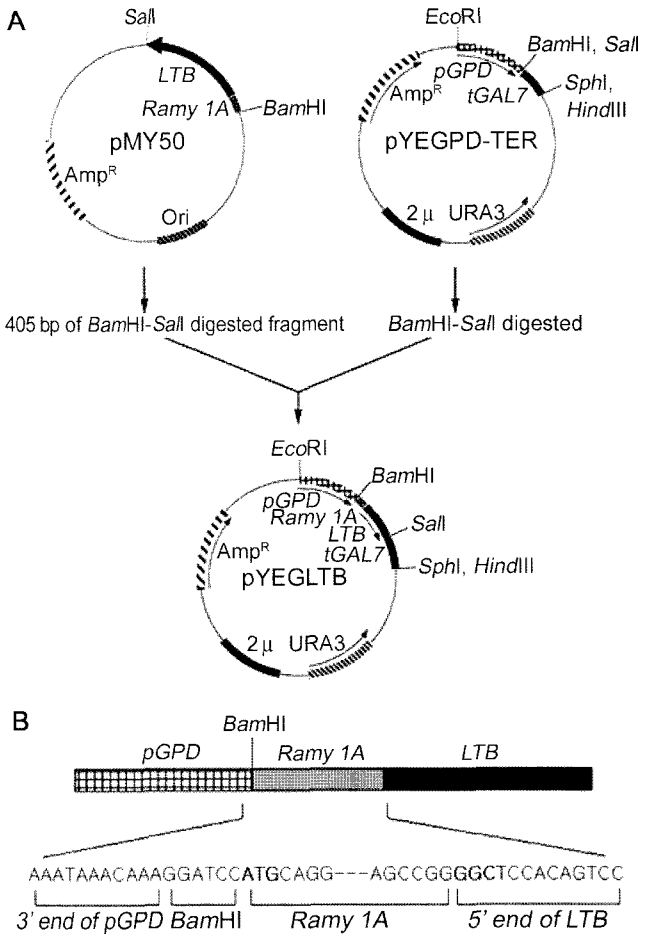

Fig. 1. A. Schematic diagram of pYEGLTB yeast expression vector. The boxes represent genes or their corresponding functional domains. **B.** Schematic diagram of the fusion construct cloned into pYEGPD plasmid and sequence covering links of the *GPD* promoter-rice *Amy*1A signal peptide-*LTB*.

The translation start codon and first codon of LTB are shown in bold letters. p*GPD*, promoter of glyceraldehyde-3-phosphate dehydrogenase; ASP, rice Amylase1A signal peptide; *LTB*, mature peptide of heat-labile enterotoxin B subunit of *E. coli*; t*GAL7*, terminator of galactose-1-P uridyl transferase.

3', and overlap-forward 5'-TCTAACTTGACAGCCGGGGCTCCA-CAGTCCATTACA-3' and overlap-reverse 5'-TGTAATGGACTGT-GGAGCCCCGGCTGTCAAGTTAGA-3' [9, 18, 23, 30]. The amplified gene was cloned into a pGEM-T Easy vector (Promega, Madison, WI, U.S.A.), analyzed *via* restriction enzyme digestion, and confirmed *via* DNA sequencing. To construct the yeast expression vector, the ASP/LTB fusion fragment, excised from the pGem T-Easy vector by digestion with BamHI and SalI, was inserted into a pYEGPD-TER vector that harbors the same restriction enzyme sites between the glyceraldehyde-3-phosphate dehydrogenase (*GPD*) promoter and the galactose-1-P uridyl transferase (*GAL7*) terminator [23]. The resultant plasmid was designated as pYEGLTB (Fig. 1).

The constructed recombinant vector was then introduced into *S. cerevisiae* 2805 using the lithium acetate procedure [17]. The stability of the plasmids introduced into the yeast was measured as follows: samples grown in the nonselective YEPD medium were serially diluted with sterile H_2O to an expected 50 colony-forming units (CFUs) per plate, plated on ura$^-$-selective and nonselective plates, and the relative number of CFUs was determined.

Northern Blot Analysis

The transformed yeast cells were lysed with glass beads and the total RNA was extracted using a previously described procedure [30]. The RNA was then quantified *via* UV-spectrophotometry, and total RNA (10 μg per lane) fractionated on a 1% formaldehyde-containing agarose gel. Prior to blotting, the gel was stained with ethidium bromide to confirm that similar quantities of RNA were loaded for each sample. The RNA was then transferred onto Hybond membranes, in accordance with the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). The hybridization was conducted in a church buffer [7% (w/v) SDS, 1% bovine serum albumin (BSA), 1 mM EDTA, 250 mM $NaPO_4$, pH 7.2] at 65°C with a α-[^{32}P]-labeled probe using a random labeling kit (Amersham Pharmacia Biotech). The blots were washed and exposed to X-ray film for autoradiography.

Western Blot Analysis

The preparation of the cell-free extracts (CFE) was conducted as previously described [29]. The cells were grown for three days, harvested, washed twice in an extraction buffer (50 mM Tris-HCl, 2 mM EDTA), and homogenized seven times in a vortex for 1 min each, with 1-min intervals of ice-cooling. The homogenates were observed under a microscope to confirm that the cells had been broken and centrifuged (10 min at 10,000 ×*g*). The resultant supernatants were then filtered through a cellulose filter (pore size 0.4 mm) to prepare the cell-free extracts. To obtain concentrates of the culture supernatants, the culture supernatants of the recombinant *S. cerevisiae* were collected by centrifugation at 3,000 ×*g*, concentrated using acetone [31], dialyzed against phosphate-buffered saline (PBS) twice for 4 h at 4°C, and sterilized by passing through a 0.4-mm syringe filter. The protein concentrations in the filter-sterilized cell-free extracts and culture supernatants were measured *via* a Bradford assay using a Bio-Rad Protein Assay Kit (Bio-Rad Inc., Hercules, CA, U.S.A.) [2].

Sample aliquots of the CFE and culture media were separated *via* SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and subsequently blotted onto nitrocellulose filters. After blocking, the filters were incubated with an anti-LTB antiserum (Immunology Consultants Laboratory Inc., Newberg, OR, U.S.A.), followed by binding to anti-rabbit IgG conjugated to alkaline phosphatase as a secondary antibody (Promega). The color was developed using BCIP/NBT (USB, Cleveland, OH, U.S.A.) in a TMN buffer (100 mM Tris, pH 9.5, 5 mM $MgCl_2$, and 100 mM NaCl) in accordance with the methods described by Kang *et al.* [18].

ELISA Quantification of Recombinant LTB Protein

The quantity of the expressed LTB protein in the cell-free extracts and culture media was determined using a quantitative ELISA, as previously described [19]. In brief, 10-fold dilutions of the cell-free extracts were utilized to coat a 96-well microtitration plate at a concentration of 100 μl per well in a bicarbonate buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$) and incubated overnight at 4°C. The wells were washed three times with PBS containing 0.05% Tween-20 (PBST), blocked for 2 h at 37°C *via* the addition of 300 μl of 1% BSA in PBS, and washed three times again with PBST. Thereafter, the plates were incubated with a 1:4,000 dilution of an anti-LTB antiserum (Immunology Consultants Laboratory Inc.) in 0.01 M PBS containing 0.5% BSA for 2 h at 37°C and washed three times with PBST. The plates were then incubated with a 1:7,000 dilution of anti-rabbit IgG conjugated with horseradish peroxidise (Promega) in 0.01 M PBS containing 0.1% BSA and washed three times with the PBST buffer. The plates were developed *via* the addition of 100 μl of a TMB substrate kit for peroxidase (PharMingen, Fallbrook, CA, U.S.A.) for 30 min at room temperature in darkness. The plates were then read at 405 nm using an ELISA reader (Packard Instrument MRA-006, Meriden, CT, U.S.A.) and quantified *via* comparison with a known quantity of purified bacterial LTB. The purified bacterial LTB, obtained from a previous study [18], was serially diluted based on a range of concentrations from 0.1 ng to 10 ng.

GM1-Ganglioside Binding Assay

GM1-ganglioside has been shown to be a receptor for the CTB protein *in vivo* [11], and for appreciable receptor binding, a pentameric structure of LTB is required [46]. Thus, GM1-ganglioside receptor binding *in vitro* has been used to assess the native pentameric form of the recombinant protein. The affinity of the yeast-derived LTB towards the GM1-ganglioside receptors was determined *via* a GM1-ELISA [21]. In brief, microtiter plates were coated with 0.3 μg of monosialoganglioside GM1 (Sigma) dissolved in a bicarbonate buffer and incubated overnight at 4°C. The wells were then washed, blocked, washed three times again with PBST, blocked for 2 h at 37°C *via* the addition of 300 μl of 1% BSA in PBS, and washed three times again with PBST. The wells were then loaded with 250 ng of protein preparations of either the cell-free extracts or the concentrated culture supernatants and incubated overnight at 4°C. Thereafter, the wells were washed three times with PBST, loaded with 100 ml per well of a 1:4,000 dilution of a rabbit anti-LTB primary antibody (Immunology Consultants Laboratory Inc.), and incubated for 3 h at room temperature, followed by three washes with PBST. The remaining steps were conducted as described in the ELISA quantification section above.

RESULTS

Analysis of Transformed *S. cerevisiae*

The pYEGLTB plasmid was constructed as described in the Materials and Methods section (Fig. 1) and used for the

transformation of the *S. cerevisiae* 2805 strain. pYEGLTB was a multicopy episomal plasmid for the expression of the LTB gene harboring the secretion signal sequence of rice amylase 1A (*Ramy*1A), instead of the endogenous bacterial signal sequence of 21 residues [48], under the control of the *GPD* promoter. Over 20 colonies were selected randomly on a ura$^-$ medium, and then examined for the presence of pYEGLTB *via* plasmid extraction, followed by PCR amplification of the LTB gene and reintroduction into *E. coli*. All the sample preparations from the selected colonies showed the presence of a 417-bp PCR amplicon corresponding to the LTB gene amplified from the plasmid pYEGLTB and demonstrated the capability of transformation of *E. coli*. Thus, the results suggested that pYEGLTB existed in the transformants, yet not in the untransformed cells.

The plasmid stability of the introduction of pYEGLTB to the selected transformants was good, as more than 82% of the cells retained the plasmid up to 72 h without selective pressure.

Expression of Recombinant LTB Gene

The level of accumulation of the recombinant LTB transcripts was evaluated using a Northern blot analysis, the results of which revealed that all the transformants expressed the recombinant LTB transcript. However, variations were detected in the transcriptional levels of the recombinant LTB genes among the strains transformed with the same expression construct (Fig. 2). A wide variation in the expression level of the heterologous gene in *S. cerevisiae* is not unusual when using episomal 2μ ori-based plasmids, possibly due to variations in the plasmid copy numbers between different transformants [25, 26, 32]. Thus, the transformant with the highest expression level, TYEGLTB-4, was selected, and then employed for the production and assembly of the recombinant LTB.

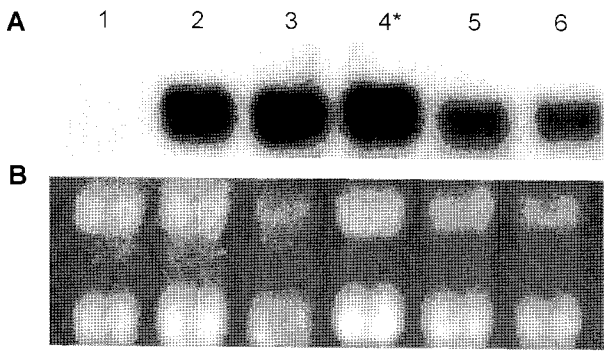

Fig. 2. A. Northern blot analysis of accumulation of *LTB* transcript in transformed yeasts.
The yeasts transformed with pYEGLTB were probed with labeled *LTB*. Lane 1 contains the RNA of the recipient strain, and lanes 2–6 show those of five different transformants. The asterisk indicates the selected transformant TYEGLTB-4. Prior to blotting, the gel used for the blot in the upper panel was stained with ethidium bromide (**B**), to confirm that similar quantities of RNA had been loaded for each sample.

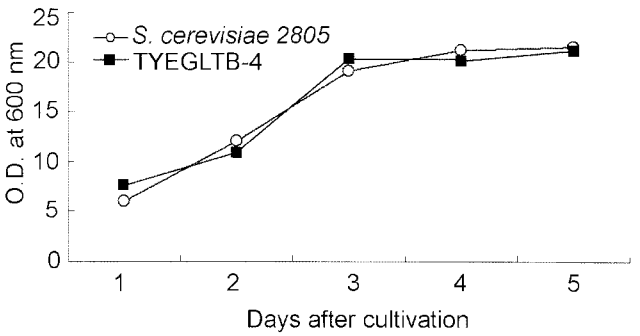

Fig. 3. Time course of cell growth of recipient strain 2805 and TYEGLTB-4.
The cell growth was monitored by measuring the OD_{600} using a spectrophotometer. The data are shown as a representative profile based on duplicate experiments with three replications.

To attain better production of the recombinant LTB, the TYEGLTB-4 strain was cultured in the nonselective YEPD medium for five days and the growth pattern compared with that of the control. The growth curve of TYEGLTB-4 in the YEPD broth exhibited canonical lag, log, deceleration, stationary, and decline phases (Fig. 3). The growth pattern of the selected transformant was similar to that of the control strain, and appeared to be similar to those determined in other previous studies [24, 30, 38]. No growth abnormalities were observed in conjunction with the LTB expression.

The temporal expression pattern of the recombinant LTB gene in TYEGLTB-4 was analyzed further *via* a

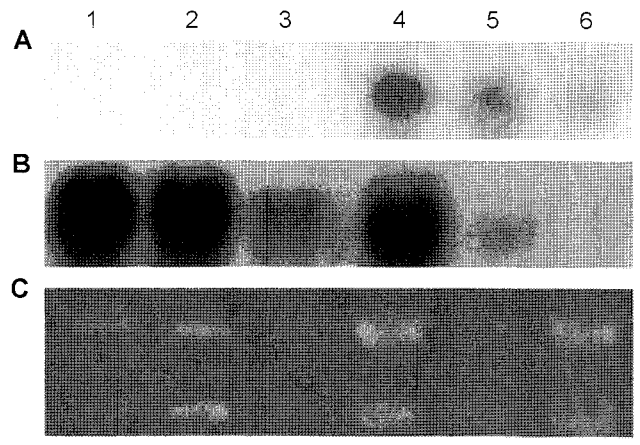

Fig. 4. Expression pattern of *LTB* in TYEGLTB-4.
Northern blot analysis using *LTB* (**A**) and *GPD* (**B**) as probes. Lanes 1, 2, and 3 contain the total RNA from 1-, 3-, and 5-day-old cultures of the recipient strain, respectively, and lanes 4, 5, and 6 contain the total RNA from 1-, 3-, and 5-day-old transformant cultures, respectively. **C.** Ethidium bromide-stained gel, indicating similar amounts of RNA loaded for each sample.

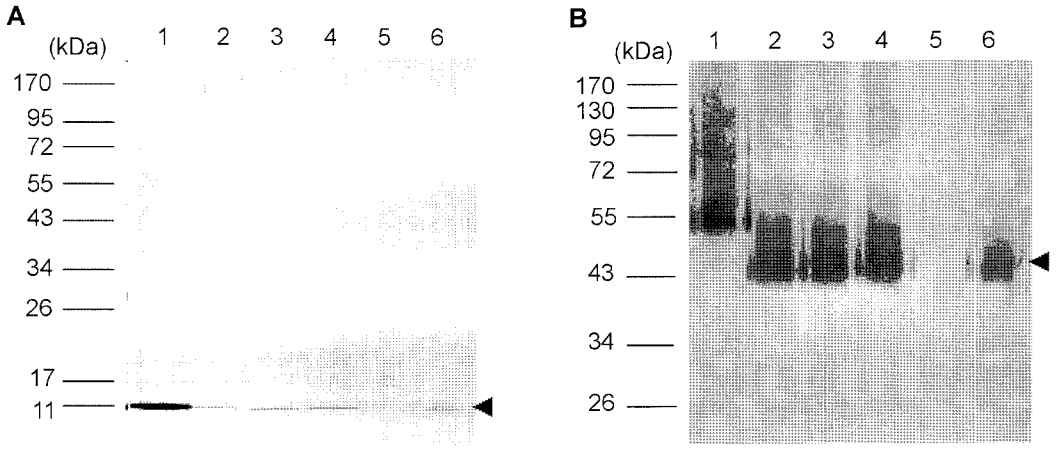

Fig. 5. Western blot analysis of recombinant LTB in CFE and culture media.
Antigen-antibody reaction of sample aliquots after (**A**) and prior to (**B**) boiling treatment. Lane 1 contains 1.0 μg of bacterial LTB. Lanes 2, 3, and 4 contain 20 μg of protein extracts from 1-, 3-, and 5-day-old cultures of the transformant, respectively. Lane 5 contains 20 μg of protein extracts from a 3-day-old culture of the recipient strain. Lane 6 contains 20 μl of 5-times-concentrated 3-day-old culture media for the transformant strain. The numbers on the left refer to the estimated sizes in kDa, and the arrowheads on the right indicate the recombinant LTB protein.

Northern blot analysis (Fig. 4). As shown in Fig. 4, the accumulation of LTB transcripts peaked after 24 h of cultivation, and then decreased until only small amounts of transcripts were observed after 5 days of cultivation. When compared with the growth curve (Fig. 3), the expression of the LTB gene directed by the *GPD* promoter in TYEGLTB-4 peaked 3 days prior to that of cell growth. As a comparison, the expression pattern of the *GPD* gene was assessed in the transformed and nontransformed yeast cells. In the recipient strain, the expression of *GPD* followed a similar pattern to that of cell growth, reaching a maximum after 3 days of cultivation, and then gradually decreasing. However, in the tranformant TYEGLTB-4, it followed a similar pattern to that of the LTB gene in TYEGLTB-4. These early peaks and rapid declines for both the recombinant and endogenous gene transcripts derived from the *GPD* promoter were also demonstrated in previous studies [24] and appeared to reflect the characteristics of the *GPD* promoter under the present culture conditions. Again, the titration effect induced by the dilution of the necessary transcription factors resulting from the presence of a multicopy promoter may explain the observed rapid decline of *GPD* and LTB expressions in the transformants [24].

Immunoblot Analysis of Recombinant LTB

An immunoblot analysis was conducted to detect the presence of the LTB protein. As shown in Fig. 5A, when the samples were boiled to dissociate the pentameric structure into monomers prior to electrophoresis, an 11.6 kDa band was observed in the bacterial LTB control, and identified as a monomer. Corresponding immunoreactive bands were observed in boiled samples of the cell-free extract (CFE) of recombinant *S. cerevisiae*. In addition, a single band of 11.6 kDa was observed in the boiled sample of the culture media. Moreover, when the samples were loaded on a 12% denaturing SDS-polyacrylamide gel without boiling to avoid reduction of the pentameric structure into monomers, bands of a higher molecular mass of approximately 50 kDa were detected (Fig. 5B). The apparent molecular mass of the pentamer was less than that of the bacterial control, probably because the globular shapes of the oligomeric structure of the recombinant LTB subunits were not identical [18]. However, the immunoblot analysis strongly indicated that the yeast-derived LTB protein was antigenically indistinguishable from the bacterial protein. In addition, the results suggested the assembly of the corresponding recombinant LTB subunits into oligomeric structures resembling native pentamers. Moreover, the assembled pentameric form of the recombinant LTB appeared to be successfully secreted and stably preserved in the culture medium. No LTB proteins were detected from the nontransformed recipient strain, as expected.

Quantification of Recombinant LTB by ELISA

An ELISA was used to make quantitative estimates of the recombinant LTB. The quantity of the yeast-derived recombinant LTB was estimated *via* a comparison of the relative light units (RLU) from a known quantity of the bacterial LTB protein-antibody complex with that emitted from a known amount of the yeast CFE and culture medium. When the recombinant protein levels were plotted against dilutions of the CFE, a correlative increase in the LTB protein levels was observed within the optimal concentration range of the CFE (100 ng–500 ng). However, when the concentration of the CFE deviated from this range, the quantity of detected LTB protein decreased. This phenomenon can be ascribed to the binding characteristics

of the microtiter plates to the LTB protein in a mixture of total yeast proteins. When increasing the total protein levels, the increased amount of recombinant LTB may have been unable to bind to the wells and thus eventually lost through washing. Therefore, the quantitative ELISA revealed that the yields comprised 0.5–2.0% of the CFE, as inferred from the comparison with known amounts of LTB. Unlike the accumulation of the LTB gene transcript, the maximum yield of the recombinant LTB protein was 2.0% of the CFE detected during the early stationary phase, which occurred after 3 days of cultivation. Thus, since one liter of the transformant TYEGLTB-4 culture resulted in approximately 4.5 g of total soluble protein, this resulted in approximately 90 mg of LTB expressed in the CFE. In addition, the quantitative ELISA showed that the maximum amount of the LTB protein secreted into the culture media was estimated to be 3 mg/l of the culture media, which was also detected during the early stationary phase. Furthermore, the yield of the recombinant LTB was estimated *via* a direct comparison of the band intensities for known concentrations of bacterial LTB preparations with those for the yeast-derived recombinant LTB in a Western blot analysis. The results of the quantitative Western analysis provided us estimated amounts of 100 mg/l and 5 mg/l for the CEF and culture medium, respectively, which agreed well with the quantitative ELISA results. No rapid decline in the protein products was observed, although a slight decrease in the recombinant LTB was detected as the culture progressed, indicating that the expressed recombinant LTB appeared to be stable in terms of its protein level.

GM1-Ganglioside Binding Assay

The biological functions of LTB, including its binding to the GM1-ganglioside receptor, are dependent on the formation of a pentameric structure of LTB monomers, thus the ability of the recombinant LTB to bind to gangliosides was assessed using 96-well plates coated with GM1 gangliosides. As shown in Fig. 6, the yeast-derived recombinant LTB in both the CFE and the culture medium exhibited a profound affinity to the GM1 gangliosides, whereas no affinity to the GM1 gangliosides was observed for the recipient strain and mock transformant. Boiling the yeast-derived recombinant LTB for 10 min induced the denaturation of the pentameric structure and eliminated any GM1-ganglioside binding (data not shown). Therefore, since pentamerization is essential for binding to GM1 gangliosides, the results suggested that the intracellular LTB protein was able to assemble into its native conformation and the pentameric LTB remained in its native form in the culture medium, allowing interaction with the GM1 gangliosides. Thus, the recombinant LTB in both the CFE and the culture medium should exhibit antigenic properties similar to those expressed by the protein in bacteria.

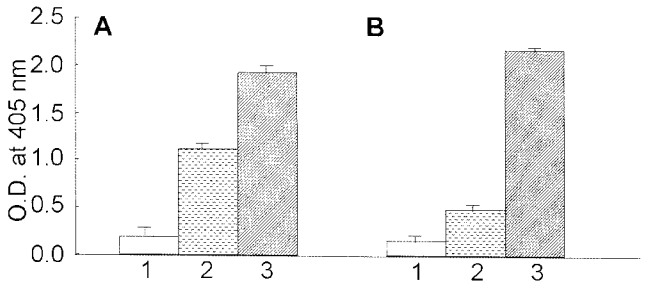

Fig. 6. GM1-ganglioside binding assay of yeast-expressed LTB. The ELISA assay was conducted by coating the plates with GM1 monosialoganglioside as the receptor molecule. Plates coated with GM1 ganglioside were incubated with the CFE (**A**) and culture media (**B**) of the recipient strain and transformant. Columns 1, 2, and 3 represent sample aliquots of the recipient strain, bacterial LTB, and transformant, respectively.

DISCUSSION

Because of such restrictions as glycosylation and oligomerization, the expression of prokaryotic proteins in *S. cerevisiae* has been relatively limited. However, this study directed the expressed LTB subunit into the lumen of the ER as an appropriate environment for oligomerization, based on replacing the endogenous signal peptide from the bacterial LTB with the signal peptide from the amylase1A protein from rice, which has already been proven to function well in a *S. cerevisiae* expression system [23]. The expressed oligomeric complex of recombinant LTB was found to retain its antigenicity, as indicated by a Western blot analysis. In addition, the current study demonstrated that the oligomeric complex appeared to have a pentameric form with the ability to bind to its natural receptors, and the functional pentameric form remained even after secretion into the culture media, thereby confirming *S. cerevisiae* as an eligible expression host for bacterial LTB.

In contrast to the results of a previous study, where the intracellular expression of LTB in *S. cerevisiae* did not produce a pentameric form [33], the current study demonstrated that the yeast-derived LTB in the CFE was assembled in its native pentameric form, allowing it to bind to GM1 gangliosides. Previous studies regarding the assembly of the pentameric form of LTB in *E. coli* have shown that oligomerization occurs after the liberation of the mature subunit into the periplasm [15]. Therefore, it has been postulated that the periplasm of gram-negative bacteria provides an environment favoring the spontaneous assembly of the B subunit monomers into oligomers [16]. Several expression systems, including *Pichia pastoris* [8] and plants [1], have already been found to direct heterologously expressed LTB into the lumen of the ER, which then functions in a similar manner to the periplasmic space of Gram-negative bacteria by providing an intracellular environment in which the monomeric subunits are concentrated

and assembled into an oligomeric form. Therefore, the present study attempted to direct the expressed LTB monomer into the lumen of the endoplasmic reticulum (ER) based on the presence of the well-known secretory sequence, *Ramy*1A, which would appear to facilitate the folding and assembling of the expressed LTB monomers into a biologically functional complex with the ability to bind to the GM1 receptors. In another previous study [36], a signal peptide of a bacterial lipoprotein was used to facilitate the assembly of the expressed recombinant subunit into a pentameric form, yet its cellular location was principally membrane-associated. In contrast, the present study used a Western analysis and GM1-ELISA assay using the culture media to demonstrate the media presence of the yeast-derived LTB in its native pentameric form, indicating that the recombinant LTB was secreted in a pentameric form and persisted in this form after secretion. Although differences in the host genetic background and failure of plant cells to remove the bacterial leader peptide [1] have been noted, further studies regarding the efficacy of signal peptides depending on their origins are required to explain the differences in the cellular locations.

S. cerevisiae as an expression system for vaccines and their related proteins has several advantages over other systems, including its status as a GRAS organism and that yeast confers a potent adjuvant property against the antigens utilized as an immunostimulus for a live vaccine, which renders a recombinant yeast in compliance with the requirements for the formulation of nontoxic and functioning vaccines without the need for additional adjuvants, yet which retains the ability to activate antigen-processing cells. In addition, this expression system is inexpensive and enables the production of fusion proteins that require posttranslational modifications in order to be immunogenic and elicit the production of neutralizing antibodies. LTB, as a nontoxic form for vaccination against diarrheal disease caused by enterotoxigenic *E. coli*, has an immunostimulatory function, which enables the use of LTB as an adjuvant in vaccine preparation. Moreover, the ability of LTB to cross the digestive tract raises the possibility of genetic or chemical protein fusion and its use for oral vaccinations. Therefore, a recombinant *S. cerevisiae* strain expressing LTB will facilitate the development of a live oral vaccine against the *E. coli* enterotoxin, and may also function as a carrier or adjuvant of other molecules for oral vaccinations.

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