Expression and Secretion of Cholera Toxin B Subunit in Lactobacilli

Takahiro Okuno,^a Nobuhiro Kashige,^a Tomomitsu Satho,^a Keiichi Irie,^a Yukihiro Hiramatsu,^a Tanjina Sharmin,^a Yuki Fukumitsu,^a Saori Uyeda,^a Seitaro Yamada,^b Tetsuya Harakuni,^b Takeshi Miyata,^b Takeshi Arakawa,^b Masumi Imoto,^c Akihisa Toda,^c Yukihiro Nakashima,^a and Fumio Miake^{*,a}

^aFaculty of Pharmaceutical Sciences, Fukuoka University; 8–19–1 Nanakuma, Jonan-ku, Fukuoka 814–0180, Japan:

^bMolecular Microbiology Group, Department of Tropical Infectious Diseases, COMB, Tropical Biosphere Research Center, University of the Ryukyus; 1 Senbaru, Nishihara, Okinawa 903–0213, Japan: and ^cDepartment of Hygienic Chemistry, Daichi University of Pharmacy; 22–1 Tamagawa-cho, Minami-ku, Fukuoka 815–8511, Japan.

Received November 26, 2012; accepted March 6, 2013

Lactic acid bacteria (LAB) are used in various fields, including in food and medical supplies. There has been a great deal of research into vaccine development using LAB as carriers due to their “generally recognized as safe” status. Cholera is an infectious disease that causes diarrhea due to cholera toxin (CT) produced by *Vibrio cholerae*. The pentameric cholera toxin B (CTB) subunit has no toxicity, and is used as an antigen in cholera vaccines and as a delivery molecule in vaccines to various diseases. In this study, we generated recombinant LAB expressing and secreting CTB. Here, we first report that CTB expressed and secreted from LAB bound to GM1 ganglioside. The secreted CTB was purified, and its immunogenicity was determined by intranasal administration into mice. The results of the present study suggested that it may be useful as the basis of a new oral cholera vaccine combining LAB and CTB.

Key words cholera toxin B subunit; GM1 ganglioside; lactic acid bacteria; expression; secretion; oral cholera vaccine

Lactic acid bacteria (LAB) constitute a group of Gram-positive bacteria that generally produce large quantities of lactic acid by fermenting with sugar and are used in the production of fermented foods, including milk, alcohol, and plant. LAB are used for the production of pharmaceutical products as probiotic and generally recognized as safe (GRAS) organisms.^{1,2} In general, LAB colonize mucosal surfaces, particularly the gastrointestinal tract.³ Among them, *Lactobacillus* species are present in large numbers in the human gut. Lactobacilli are resistant to gastric acid and bile acid, and arrive alive in the intestinal tract following ingestion.⁴ Furthermore, as lactobacilli do not have lipopolysaccharide (LPS), they could be safely expressed and secreted heterologous proteins. Taking advantage of these characteristics, previous studies using recombinant DNA technology have used lactobacilli to express or secrete useful proteins.^{3,5}

Cholera is a diarrheal infection disease caused by *Vibrio cholerae*, with an estimated 3–5 million cholera cases and 100000–120000 deaths due to cholera every year.⁶ Cholera toxin (CT) is comprised of a toxic A (CTA) subunit and a pentameric non-toxic B (CTB) subunit. CTB binds to GM1 ganglioside present on the membranes of intestinal epithelial cells. This binding is very specific because of the high affinity of the interaction.⁷ Many studies are currently directed toward the development of cholera vaccines using CTB and plants, as exemplified by tomato, rice seed, and carrot.^{7–9} However, there are a number of problems in these approaches, such as the vast areas of land and long times required for cultivation of plants. In this respect, lactobacilli have an advantage in that they can be obtained in large quantities within a short time under standard laboratory conditions. In addition, administration of yogurt supplemented with LAB was reported to enhance mucosal and systemic immunoglobulin A (IgA)

responses to the CT immunogen.¹⁰ Slos *et al.* reported the successful production of CTB in lactobacilli; however, the secreted CTB did not form a pentamer and did not bind to GM1 ganglioside in their study.¹¹

In the present study, we first constructed the LAB-*Escherichia coli* shuttle vector pHL253. Second, we produced plasmids encoding CTB, which we used to produce *Lactobacillus casei* ATCC27092 and *Lactobacillus reuteri* ATCC23272 expressing or secreting CTB. Here, we first report that CTB expressed and secreted from lactobacilli bound to GM1 ganglioside. In addition, we purified CTB secreted into the culture supernatant and confirmed the immunogenicity of purified recombinant CTB (rCTB). As rCTB bound to GM1 ganglioside and retained immunogenicity, our study suggested that it may be useful as the basis of a new cholera vaccine LAB and CTB.

MATERIALS AND METHODS

Bacterial Strains, Media, and Growth Conditions The bacterial strains used in this study are shown in Table 1. *L. casei*, *L. reuteri*, and recombinant strains were grown in de Man-Rogosa-Sharpe (MRS) medium at 37°C to obtain samples of intracellular extract and in MRS/K (MRS in 0.2M potassium phosphate buffer) medium at 30°C to obtain samples of supernatant. Erythromycin (5 µg/mL) was added to MRS or MRS/K medium for selection of recombinant strains. The *E. coli* strain DH5α used as a host for cloning was grown in LB with or without ampicillin (100 µg/mL).

Mice Four-week-old female Balb/c mice weighing 13–16g were purchased from Kyudo Co., Ltd. (Saga, Japan). Mice were kept in plastic cages under a 12h light/dark cycle (lights on from 07:00 to 19:00) in an air-conditioned room (23±2°C) and allowed free access to water. All animal care and use procedures were performed in compliance with the regulations established by the Experimental Animal Care and Use Com-

The authors declare no conflict of interest.

*To whom correspondence should be addressed. e-mail: fmiake@fukuoka-u.ac.jp

Table 1. Bacterial Strains and Plasmids Used in This Study

Bacterial strains	Characteristics	Source
<i>E. coli</i> DH5a		TaKaRa Bio Inc.
<i>L. casei</i> ATCC27092	Wild-type strain isolated from human feces, plasmid-free, Em ^s	ATCC*
<i>L. reuteri</i> ATCC23272	Wild-type strain isolated from human gut, plasmid-free, Em ^s	ATCC*
Vectors/Plasmids		
pIL253	pAM β 1 derivative, Em ^r	Provided by Dr. Seiya Ogata formerly of Kyushu Univ.
pHY300PLK	Shuttle vector, <i>E. coli</i> - <i>B. subtilis</i> , Am ^r and Tc ^r	TaKaRa Bio Inc.
pHYEMI	pHY300PLK added Em ^r	This study
pHIL253	Shuttle vector, LAB- <i>E. coli</i> , Am ^r and Em ^r	This study
pUC19	<i>E. coli</i> vector, Am ^r , <i>lacZ</i>	TaKaRa Bio Inc.
pET-43.1a (+)/CTB	pET-43.1a (+) with added CTB gene at <i>KpnI</i> and <i>StuI</i> sites	Provided by Dr. Takeshi Arakawa of Ryukyu Univ.
pLDH	pHIL253 with added LDH	This study
pLglnAcase	pLDH with added <i>glnAcase</i>	This study
pLST	pLDH with added SS and Term	This study
pCTB	pSCTB with SS removed	This study
pSCTB	pLST with added CTB gene and His-tag	This study

*ATCC: American Type Culture Collection.

mittee of Fukuoka University following the Guidelines of the Science Council of Japan.

Molecular Techniques Plasmids were isolated from *E. coli* using the Wizard *Plus* SV Minipreps DNA Purification System (Promega, Tokyo, Japan) according to the manufacturer's protocol. Digestion with restriction enzymes was conducted according to the supplier's instructions (Toyobo Co., Ltd., Osaka, Japan; TaKaRa Shuzo, Tokyo, Japan; New England Biolabs Japan Inc., Tokyo, Japan). Plasmids were introduced into lactobacilli by electroporation using a gene pulser (Bio-Rad, Hercules, CA, U.S.A.). Transformation of plasmids into *E. coli* was performed according to the method of Senba *et al.*¹²⁾ The DNA fragments were purified using a QIAEX II Gel Extraction kit as described by the manufacturer (Qiagen, Valencia, CA, U.S.A.). DNA was ligated with T4 DNA ligase (TaKaRa Bio, Shiga, Japan) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed using KOD-Plus (Toyobo). Protein volume assay was performed using a Coomassie (Bradford) Protein Assay kit (Thermo Scientific, Waltham, MA, U.S.A.) according to the manufacturer's instructions.

Vector and Plasmid Construction First, we constructed a new vector for efficient expression or secretion of target molecules in lactobacilli. The erythromycin resistance gene (*Em*^r) from the plasmid pIL253 (kindly provided by Dr. Seiya Ogata formerly of Kyushu University), was digested with *HhaI* and fused with the plasmid pHY300PLK (TaKaRa Bio) that had been digested with *SmaI*. The new construct, designated pHYEMI, was digested with *EcoRI* and *AatII* and fused with pIL253, digested with *HpaI* and *SmaI*, to construct the LAB-*E. coli* shuttle vector pHIL253. Second, we produced plasmids for expression or secretion of CTB. The LDH promoter fragment was obtained from the lactate dehydrogenase gene of *L. casei* ATCC393¹³⁾ and terminator (Term) and secretory signal sequence (SS) fragments were obtained from the β -N-acetylglucosaminidase gene (*glnAcase*) of *L. casei* ATCC27092.¹²⁾ pET-43.1a (+)/CTB was a kind gift from Dr. Takeshi Arakawa of Ryukyu University. All primers used are shown in Table 2. The LDH promoter fragment was obtained by PCR using the primers LDH-Fw and LDH-Rv and sub-

cloned into the *SmaI* site of pUC19. A fragment bounded by *BamHI* and *BglII* restriction sites was amplified by PCR using the primers LDH-Fw-*BamHI* and LDH-Rv-*BglII*. The product was then cloned into the corresponding restriction sites of pHIL253, and the resultant plasmid was named pLDH. Next, to insert Term and SS fragments, the *glnAcase* gene was amplified by PCR using the primers Glc-*BglII*-Fw and Glc-*XbaI*-Rv and inserted into the *BglII* and *XbaI* restriction sites of pLDH; the resulting construct was named pLglnAcase. As Term and SS fragments were located on downstream and upstream in a *glnAcase* gene, to remove the unnecessary compartment between these fragments, PCR was performed using the primers SS-*StuI*-Fw and SS-*KpnI*-Rv with pLglnAcase as the template; the resulting construct was named pLST. Then, the CTB gene was amplified by PCR from pET-43.1a (+)/CTB using the primers CTB-*KpnI*-Fw and CTB-*StuI*-Rv. The product was inserted at the *KpnI* and *StuI* sites of pLST to yield the CTB secretion plasmid, pLDH-SS-CTB-Term. A histidine tag (His-tag) was added to allow purification of CTB secreted into the culture supernatant. PCR was performed using the primers SSCTB-*BglII*-Fw and CTB-Rv-His4 to obtain the CTB-His4 domain. This domain was then amplified by PCR using the primers SSCTB-*BglII*-Fw and CTB-His6-*StuI*-Rv, digested with *BglII* and *StuI* and inserted into the corresponding restriction sites of pLDH-SS-CTB-Term to yield pLDH-SS-CTB-His-Term (pSCTB). To produce the CTB expression plasmid, pLDH-CTB-His-Term (pCTB), pSCTB was digested with *BglII* and *StuI*, and the CTB gene and His-tag were amplified by PCR from pSCTB with the primers CTB-*BglII*-Fw and CTB-His-*StuI*-Rv. This fragment was then inserted into the *BglII* and *StuI* sites of pSCTB to yield pCTB. The integrity of DNA amplification at each step of the cloning process was confirmed by sequence analysis. The plasmids pHIL253, pCTB, and pSCTB were introduced into *L. casei* and *L. reuteri* by electroporation.

Preparation of Protein Extracts and Supernatant Concentration. Protein Extracts Bacterial cells were collected by centrifugation (6000rpm, 4°C, 15min) after growth at 37°C until the optical density at 600nm (OD₆₀₀) reached 2.0 in MRS medium. Collected bacterial cells were washed twice

Table 2. Primers Used in This Study

Primers	Sequence (5'→3')
LDH-Fw	CTTGTCACAGGATTACAAGCTTGTCTATTG
LDH-Rv	ATCTATCATCCTTTCTTATGTGCATGCAAACTG
LDH-Fw- <i>Bam</i> HI	CGGGATCCCTTGTCACAGGATTACAAGTC
LDH-Rv- <i>Bgl</i> II	GAAGATCTATCTATCATCCTTTCTTATGTGCATG
Glc- <i>Bgl</i> II-Fw	GAAGATCTATGAAGAAGCACGGGTTTCGAT
Glc- <i>Xba</i> I-Rv	GCTCTAGACCGCTGACCTTTATTTGTAAA
SS- <i>Sna</i> I-Fw	GAAGGCCTTCTTCTGACAATAATCAAAAATAC
SS- <i>Kpn</i> I-Rv	GGGGTACCCGAGCAGCAGCTACCTGCTTGGG
CTB- <i>Kpn</i> I-Fw	GGGGTACCGACACCTCAAAATATTACTGAT
CTB- <i>Sna</i> I-Rv	GAAGGCCTTTAATTTGCCATACTAATTGCCG
SSCTB- <i>Bgl</i> II-Fw	GAAGATCTATGAAGAAGCACGGGTTTCG
CTB-Rv-His4	GTGGTGGTGGTGATTTGCCATACTAATTG
CTB-His6- <i>Sna</i> I-Rv	GAAGGCCTTTAGTGGTGGTGGTGGTGGTGATT
CTB- <i>Bgl</i> II-Fw	GAAGATCTATGACACCTCAAAATATTACTG

Restriction enzyme recognition sites are underlined.

with phosphate-buffered saline (PBS, pH 7.4), followed by addition of 2 mL of PBS, protease inhibitors (aprotinin 2 μ g/mL, leupeptin 2 μ g/mL, phenylmethylsulfonyl fluoride 1 mM), and glass beads, and vortex mixing 10 times for 1 min. After centrifugation (10000 rpm, 4°C, 10 min), the supernatants were collected as protein extracts and stored at -80°C until analysis.

Supernatants Culture supernatants were collected by centrifugation (6000 rpm, 4°C, 15 min) after growth at 30°C until OD₆₀₀ reached 2.0 in MRS/K medium. After collecting supernatants, they were inspissated with Amicon Ultra centrifugal filter units (10 kDa; Merck Millipore, Tokyo, Japan) and stored at -80°C until analysis.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting Protein extracts, concentrated supernatants, and rCTB were subjected to Western blotting. These were separated by 17% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Buckinghamshire, U.K.). For analysis of monomeric CTB proteins, sample buffer (2-mercaptoethanol, 12% SDS, 40% glycerol, 0.27 M Tris-HCl buffer, BPB solution) was added to the samples (protein extract: 1 μ g/ μ L, rCTB and CT: 25 ng/ μ L, concentrated supernatants: 20 μ L) and heated at 95°C for 3 min prior to SDS-PAGE analysis. The CTB moieties of blotted protein bands were recognized using rabbit anti-cholera toxin antiserum (1:4000 dilution; Sigma-Aldrich, St. Louis, MO, U.S.A.) and alkaline phosphatase (AP)-conjugated anti-rabbit IgG (1:4000 dilution; Cell Signaling Technology, Danvers, MA, U.S.A.). Binding was detected with a chemiluminescent substrate for AP (CDP-Star reagent; Biolabs, Beverly, MA, U.S.A.).

GM1-Enzyme-Linked Immunosorbent Assay (GM1 ELISA) GM1 ELISA was performed to determine the expression and secretion levels and specific binding affinity of CTB and its fusion proteins for GM1 ganglioside, a natural receptor of cholera toxin. Briefly, a 96-well microtiter plate (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) was coated with 5 μ g/mL of monosialoganglioside GM1 (Sigma-Aldrich) diluted in bicarbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6), and incubated overnight at 4°C. After incubation, wells

were washed with PBS supplemented with 0.05% Tween-20 (PBS-T) three times and rinsed with PBS three times. The wells were then blocked with PBS containing 1% bovine serum albumin (BSA) at 37°C for 2 h. After washing and rinsing, boiled (100°C, 10 min) or unboiled protein extracts (1 μ g/ μ L), concentrated supernatants (100 μ L), rCTB (0.5 ng/ μ L), CT (0.5 ng/ μ L) as a positive control, and PBS as a negative control were applied to wells and incubated for 2 h at 37°C. After washing and rinsing, the same primary and secondary antibodies used for detection of CTB by Western blotting were used. Plates were incubated at 37°C for 20 min after the addition of alkaline phosphatase substrate (Sigma-Aldrich) to the wells, and OD₄₀₅ was measured with a microplate reader (ImmunoMini Nj-2300; Nunc, Rochester, NY, U.S.A.).

Purification of CTB in the Culture Supernatant To confirm its immunogenicity, CTB was purified from the culture supernatant of *L. casei* transformed with pSCTB. The culture supernatant was collected by centrifugation (8000 rpm, 4°C, 30 min) after growth at 30°C until OD₆₀₀ reached 2.0 in MRS/K medium. After filtering at 0.22 μ m Rapid Filter Max Set (TPP Techno Plastic Products AG, Trasadingen, Switzerland), 2 M imidazole was added to the culture supernatant to make the final concentration 20 mM, then the culture supernatant was adjusted to pH=7.0. Nickel resin (Ni Sepharose High Performance; GE Healthcare Japan, Tokyo, Japan) was added to the culture supernatant and agitated gently overnight at 4°C. After filling the open column with resin and passage of washing buffer (10 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole), CTB was eluted with elution buffer (10 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole). Eluted CTB was inspissated and replaced with PBS with Amicon Ultra centrifugal filter units (10 kDa; Merck Millipore), and rCTB was achieved. The purity of CTB was confirmed by SDS-PAGE on 17% polyacrylamide gels with the protein bands stained with Coomassie Brilliant Blue staining (CBB staining), Western blotting, and GM1 ELISA.

Immunization of Mice and Sample Collection Female Balb/c mice (4 weeks old), were housed 5 mice per group in the same cage for 7 d prior to commencement of the experiment. Mice were immunized intranasally (i.n.) with 3 or 30 μ g of rCTB or PBS as a negative control 3 times on weeks 0, 2, and 4. Blood collection was performed 4 times on weeks 0, 1, 3, and 5. Sera were collected by centrifugation (10000 rpm, 4°C, 15 min) after incubation for 2 h at room temperature and inactivated for 30 min at 50°C.

Antibody Analysis For CTB-specific serum antibody analysis, a 96-well microtiter plate (Sumitomo Bakelite Co., Ltd.) was coated with 5 μ g/mL of rCTB diluted in bicarbonate buffer, and incubated overnight at 4°C. After incubation, wells were washed three times with PBS-T and rinsed three times with PBS. The wells were then blocked with PBS containing 1% BSA at 37°C for 2 h. After washing and rinsing, sera diluted 100-fold with PBS were applied to wells and incubated for 2 h at 37°C. After washing and rinsing, anti-mouse IgG AP-conjugated antibody (Cell Signaling Technology) was added to wells and incubated for 2 h at 37°C. Finally, after washing and rinsing, plates were incubated at 37°C for 20 min after the addition of alkaline phosphatase substrate (Sigma-Aldrich) to the wells, and the OD₄₀₅ was measured using a microplate reader (ImmunoMini Nj-2300; Nunc).

Statistical Analysis All statistical analyses were per-

Fig. 1. Formulation of LAB-*E. coli* Shuttle Vector and Plasmids for CTB Expression and Secretion

(A) Construction of the LAB-*E. coli* shuttle vector, pHIL253. (B) The pCTB and pSCTB cassettes are shown. LDH: the promoter from the lactate dehydrogenase gene of *L. casei* ATCC393; SS: the secretory signal sequence from β -*N*-acetylglucosaminidase gene of *L. casei* ATCC27092; CTB: CTB gene; His-tag: histidine tag; Term: the terminator from the β -*N*-acetylglucosaminidase gene of *L. casei* ATCC27092; aa: amino acids.

formed using OriginPro 8.1 (OriginLab, Northampton, MA, U.S.A.). Values are presented as the means $\pm$ standard error of independent experiments. One-way analysis of variance (ANOVA) was used to calculate the significance of differences. In all analyses, $p < 0.05$ was taken to indicate significance.

RESULTS AND DISCUSSION

Expression and Secretion of CTB in Lactobacilli LAB are present in large numbers in the human intestinal tract, and *L. casei* and *L. reuteri* used in this experiment are from human feces and human intestinal tract, respectively. LAB in general and strains of *Lactobacillus* in particular have a number of properties that make them attractive candidates for oral vaccination purposes, e.g., GRAS status, adjuvant properties, mucosal adhesive properties, and low intrinsic immunogenicity.¹⁴ Furthermore, they are used in clinical and experimental studies for their immunomodulatory properties, inhibition of allergic reaction, and downregulation of inflammatory signals in inflammatory bowel disease.^{15–17}

In the present study, the LAB-*E. coli* shuttle vector pHIL253 was constructed for expression and secretion of CTB. The pHIL253 vector contains ori-177 from pPHY300PLK and pAMβ1 from pIL253, along with the ampicillin resistance gene of pPHY300PLK and the erythromycin resistance gene of pIL253 (Fig. 1A). The plasmid allows replication and selection in *E. coli* and LAB. This shuttle cloning vector was successfully introduced into *E. coli* DH5α, *L. casei*, and *L. reuteri* strains. Similarly, pCTB and pSCTB plasmid vectors (Fig. 1B) were successfully introduced into *E. coli*, *L. casei*, and *L.*

reuteri strains. We chose the LDH promoter not only because it is constitutively expressed in *Lactobacillus* species,¹⁸ but also because this is one of the strongest and highest efficiency promoters known in the *L. casei* vector system.¹⁹

pCTB was designed for intracellular expression of CTB in LAB. As shown in Fig. 2A, CTB expression was detected in the intracellular extract of *L. casei* and *L. reuteri* carrying pCTB by Western blotting. pSCTB was designed for secretion of CTB into the culture supernatant of LAB. As shown in Fig. 2B, secretion of CTB was confirmed in the culture supernatant of *L. casei* and *L. reuteri* carrying pSCTB by Western blotting. CT was used as a positive control; the band of about 27 kDa represents CTA and the band of about 11 kDa represents monomeric CTB. Furthermore, as a His-tag was added to the C'-terminus of the CTB gene, expressed or secreted CTB bands were found at a slightly higher position than native CTB.

Interestingly, CTB was also expressed and secreted in *L. reuteri* in the present study. Taguchi and Ohta reported that L-lactate dehydrogenase (ldhL) showed homology of various lactobacillus species.²⁰ Thus, it is suggested that expressed and secreted vectors used here could be used to express and secrete heterologous proteins in various LAB.

GM1 Ganglioside-Binding Capability of Expressed and Secreted CTB GM1 ganglioside is a ubiquitously expressed glycosphingolipid on mammalian intestinal epithelial cells and many other cell types.^{21,22} CTB is known to combine with GM1 ganglioside.²² Therefore, we examined whether CTB expressed or secreted in LAB bind to GM1 ganglioside. GM1 ELISA was performed to determine the GM1 ganglioside-binding ability of the CTB protein. As shown in Fig. 3A, the

Fig. 2. Expression and Secretion of CTB in Lactobacilli

(A) Intracellular CTB monomer expression was analyzed by Western blotting of cellular extracts. (B) Extracellular CTB monomer secretion was analyzed by Western blotting from culture supernatants. a: Wild-type; b: pHIL253-transformed lactobacilli; c: pCTB-transformed lactobacilli; d: pSCTB-transformed lactobacilli; CT: Cholera toxin as a positive control. The 11kDa band is CTB monomer, and 27kDa is CTA. Data from a representative experiment are shown.

Fig. 3. Binding Capability of Expressed and Secreted CTB to GM1 Ganglioside

(A) Binding ability to GM1 ganglioside of intracellularly expressed CTB was analyzed by GM1 ELISA from cellular extracts. (B) Binding ability to GM1 ganglioside of extracellularly secreted CTB was analyzed by GM1 ELISA from culture supernatants. a: Wild-type; b: pHIL253-transformed lactobacilli; c: pCTB-transformed lactobacilli; d: pSCTB-transformed lactobacilli; CT: Cholera toxin as a positive control. Unboiled indicates no treatment, boiled indicates heat treatment at 100°C for 10min. Data represent the means $\pm$ S.E. ($n=6$ independent experiments).

intracellular extracts of *L. casei* and *L. reuteri* carrying pCTB bound to GM1 ganglioside. As shown in Fig. 3B, the culture supernatants of *L. casei* and *L. reuteri* carrying pSCTB showed GM1 ganglioside-binding capability.

Slos *et al.* reported the production of CTB in lactobacilli, but the secreted CTB did not bind to GM1 ganglioside.¹¹⁾ Based on this observation, they suggested that *L. plantarum* NCIMB8826 is limited in its ability to catalyze the formation of disulfide bonds. However, *L. casei* and *L. reuteri* used in the present study might not have this limitation. Alternatively, we also consider that CTB did not degrade since MRS/K medium was less likely to cause a decrease in pH due to lactic acid. So, this represents the first report of CTB expressed and secreted in LAB capable of binding to GM1 ganglioside.

Purification and Immunogenicity of rCTB To examine whether CTB secreted from *L. casei* produced an antibody reaction in mice, purification of CTB from pSCTB-transformed *L. casei* was performed for administration. A His-tag was added to the C'-terminus of the CTB gene as the His-tag combined easily with nickel and facilitates elution. Briefly, histidine has a high affinity for bivalent metal ions, such as Ni^{2+} , Zn^{2+} , and Cu^{2+} . Protein with an added His-tag binds to Ni^{2+} , and as the affinity of imidazole with nickel is higher than that of histidine, protein with an added His-tag is eluted by high-concentration imidazole. As shown in Fig. 4, rCTB migrated as a single band on Western blotting and CBB stain-

ing and bound to GM1 ganglioside. Dertzbaugh and Cox reported that specific histidine residue of CTB bind to Ni ions.²³⁾ Wherein, we constructed the plasmid not added to the His-tag, introduced into *L. casei* and performed purification of rCTB. As a result, the concentration of rCTB obtained was about 50 $\mu\text{g}/\text{L}$ of medium. In contrast, when His-tag was added, the amount of purified protein obtained was about 1 mg/L of medium in each case. Therefore, it is suggested that purification efficiency was higher by the addition of His-tag. Meanwhile, when comparing the toxin dose giving the half-maximum binding in ELISA using GM1-fixed microtiter plates of both of native CT and rCTB, rCTB was lower than native CT (data not shown). For this reason, SDS-PAGE of the unboiled rCTB was performed, as rCTB was also present in the monomer, it is considered that binding capacity to GM1 ganglioside was low (data not shown).

CT and CTB are used as mucosal adjuvants.^{24,25)} As described above, GM1 ganglioside is present on intestinal epithelial cells and many other cell types. Therefore, GM1 ganglioside is present in nasal mucosal epithelial cells. To check the immunogenicity of CTB expressed or secreted in lactobacilli, mice were immunized i.n. with rCTB, and antibody levels in serum were measured by ELISA. As shown in Fig. 5, the serum anti-CTB IgG level was increased significantly in the 30 μg rCTB i.n. group at 5 weeks. However, no increase in anti-CTB IgG level at 5 weeks was observed in

Fig. 4. Purification of CTB Secreted into Culture Supernatant

Purified rCTB was analyzed by (A) CBB staining and (B) Western blotting. a: Supernatant; b: Flow-through fraction; c: Wash fraction; d: Elution fraction. Data from a representative experiment are shown. (C) Binding ability to GM1 ganglioside of rCTB was analyzed by GM1 ELISA. PBS was used as a negative control, and CT was used as a positive control. Unboiled indicates no treatment, boiled indicates heat treatment at 100°C for 10 min. Data represent the means $\pm$ S.E. ($n=6$ independent experiments).

Fig. 5. Immunogenicity of rCTB in Mice

Serum antibody responses against CTB were examined in Balb/c mice immunized with rCTB through the intranasal route. Data represent the means $\pm$ S.E. ($n=5-6$ independent experiments).

the 3 μ g rCTB i.n. group, suggesting that production of CTB antibody in mice requires a certain level of CTB expression or secretion. In addition, we checked the antibody induced in immunized mice prevent the binding of rCTB to GM1 in ELISA. However, the antibody in the serum diluted 100 times could not prevent the binding of rCTB to GM1 (data not shown). In contrast, as anti-CT was used as a positive control, in the case of high concentration, could prevent the binding of rCTB to GM1, suggesting that anti-CTB antibody in the serum was more produced and obtained similar results to anti-CT by administering a high dose of rCTB.

We also checked immunogenicity of rCTB *via* intragastric (i.g., 300 μ g) administration according to the same schedule. In the 300 μ g i.g. group, the level was increased by about threefold at 5 weeks compared to PBS (data not shown) although this difference was not statistically significant. Taking into consideration protein breakdown by stomach acid with i.g. administration, stomach acid was neutralized 15 min before administration of rCTB. As rCTB was probably decomposed by stomach acid, this was thought to prevent the

increase in anti-CTB antibody level. As discussed above, lactobacilli are resistant to gastric acid. Therefore, it appears important to introduce CTB into LAB for production of an oral cholera vaccine.

CTB is used as mucosal adjuvants and delivery molecules. Therefore, CTB may be useful not only as a cholera vaccine antigen but also as a delivery molecule for vaccines against various diseases. Other antigens and useful proteins may be added to the CTB gene and introduced into LAB to facilitate the production of many oral vaccines and new medical supplies.

In conclusion, we constructed plasmid vectors for expression or secretion of CTB and confirmed expression and secretion of CTB in lactobacilli. The results reported here indicated that CTB expressed and secreted in lactobacilli bound to GM1 ganglioside. In addition, we showed that rCTB thus produced had immunogenicity. This study suggested that CTB may be useful as the basis of a new oral cholera vaccine and new medical supplies.

Acknowledgments This research was supported by the following Grants: Grants-in-Aid for Exploratory Research (No. 24659060 and No. 24658099) from the Ministry of Education, Culture, Sports, Science and Technology of Japan; the Central Research Institute (No. 126012) from Fukuoka University.

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