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Prophylactic effect of *Lactobacillus* oral vaccine expressing a Japanese cedar pollen allergen

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Lactic acid bacteria (LAB) represent an attractive delivery vehicle for oral allergy vaccine because of their safety as a food microorganism as well as their potent adjuvant activity triggering anti-allergic immune response. Here, we report the generation of recombinant LAB expressing a major Japanese cedar pollen allergen Cry j 1 (Cry j 1-LAB), and their prophylactic effect *in vivo*. To facilitate heterologous expression, the codon usage in the Cry j 1 gene was optimized for the host LAB strain *Lactobacillus plantarum* by the recursive PCR-based exhaustive site-directed mutagenesis. Use of the codon-optimized Cry j 1 cDNA and a lactate dehydrogenase gene fusion system led to a successful production of recombinant Cry j 1 in *L. plantarum* NCL21. We also found that oral vaccination with the Cry j 1-LAB suppressed allergen-specific IgE response and nasal symptoms in a murine model of cedar pollinosis.

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[**Key words:** Allergen; Codon optimization; IgE; Japanese cedar pollen; *Lactobacillus*]

Allergic rhinitis is the most prevalent type I allergy in the developed countries (1). In Japan, seasonal rhinitis caused by Japanese cedar (*Cryptomeria japonica*) pollen represents the most serious nation-wide allergic disease, whose morbidity has reached 26.5% in 2008 (2). Current treatments of Japanese cedar pollinosis are medication with corticosteroids, antihistamines, or leukotriene antagonists, but these symptomatic therapies have no curative effect. In contrast, allergen-specific immunotherapy (SIT) is recognized as an only promising treatment of allergy (3), and its long-term clinical efficacy has also been reported against pollinosis (4). However, SIT for Japanese cedar pollinosis has yet to be accepted in Japan, because the subcutaneous vaccination with crude allergen extract has a risk of anaphylaxis.

Allergen vaccination *via* mucosal route is a new alternative instead of the subcutaneous injection. Especially, sublingual immunotherapy (SLIT) has been developed as a safer protocol in the United States and Europe, and two Japanese clinical trials have shown that SLIT is also effective against cedar pollinosis (5,6). Another option for the development of mucosal vaccine is oral administration of sensitized allergens. In this regard, lactic acid bacteria (LAB) have recently been focused as a new and safe biotherapeutic vehicle that delivers recombinant allergens to the gastrointestinal mucosa (7,8). LAB are a group of Gram-positive, non-sporulating bacteria including *Lactobacillus*, *Leuconostoc*, *Lactococcus*, *Pediococcus*, *Streptococcus* and so on. The use of dietary LAB for the therapeutic vehicles has several advantages. First, safety of those LAB is guaranteed through a long-term consumption of fermented foods, and some LAB strains are approved as probiotics possessing health-promoting activity. Another advantage is their potent adjuvant effect to inhibit allergic inflammation, mainly attributable to their up-modulatory effect on type 1 helper T cell (Th1) response (9,10) as well as on the development of torelogenic immune response (11,12). Several papers have reported the generation of recombinant LAB carrying airborne allergens including a T cell epitope peptide from house dust mite allergens Der p 1 (13), a proform Der p 1 polypeptide (14), or birch pollen allergen Bet v 1 (15). However, these recombinant LAB have only been tested as a nasal vaccine, and LAB-based oral vaccines remains to be developed for the treatment of allergic rhinitis. In addition, none of recombinant oral LAB vaccine has been generated for Japanese cedar pollinosis because of the technical difficulties in heterologous expression of Japanese cedar pollen allergens.

In the present study, we show for the first time a successful production of a major Japanese cedar pollen allergen Cry j 1 in *Lactobacillus plantarum* by means of the exhaustive codon optimization and a lactate dehydrogenase (LDH)-fusion protein expression system. Moreover, we also demonstrate that oral vaccination with the LAB carrying Cry j 1 suppresses nasal clinical symptoms as well as allergen-specific IgE response in a murine model of cedar pollinosis.

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MATERIALS AND METHODS

Bacterial stains and culture media *Escherichia coli* DH5α used for plasmid construction was grown at 37°C in LB medium. *Lactobacillus plantarum* NCL21 (16) is a derivative obtained from *L. plantarum* L-137, which has been isolated from a traditional fermented food prepared in the Philippines (17). The NCL21 was cultured in *Lactobacilli* MRS Broth (BD Biosciences, San Jose, CA, USA) at 30°C. For antibiotic selection and culture of transformants, medium was supplemented with ampicillin (50 μg/ml, Sigma-Aldrich, St. Louis, MO, USA) for *E. coli* DH5α or erythromycin (5 μg/ml, Sigma-Aldrich) for *L. plantarum* NCL21.

Plasmid construction To make a basal *L. plantarum* expression vector, LDH promoter (pLDH) gene from *L. plantarum* L-137 genomic DNA was amplified by PCR using following primers (forward: GGAAGAGCTCAGTCGTATTGGCAC, reverse: GATTGGATCCGCTTGACAAAATAAGTCAT, *Sac* I and *Bam* HI sites are underlined). Then the pLDH gene was inserted into the *Sac* I/*Bam* HI site of the *E. coli*/*L. plantarum* shuttle vector pRN14 (18) to make a pLDH-driven expression vector, pLDH/pRN14.

To construct a positive control vector expressing LDH, LDH coding sequence deleting the ATG initiation codon was first obtained from L-137 genomic DNA by PCR with specific primers (forward: GTCAGGTACCCCAAATCATCAAAAAGTT, reverse: TGATGAGCTCTTATTTATTTTCTAATTCAG, *Kpn* I and *Sac* I sites are underlined). To detect a recombinant protein, a *Bam* H1-ATG-FLAG-*Kpn* I DNA fragment was fused at the 5′-termini of the *Kpn* I-LDH-*Sac* I PCR fragment. This FLAG-LDH fusion gene was used as a PCR template to make a *Bam* HI-ATG-FLAG-LDH-*Pst* I gene fragment using the following primers (forward: AAAGGGATCCATGGACTATAAGGATGA, reverse: TTGGCTGCAGTATTTATTTTCTAATTCA, *Bam* HI and *Pst* I sites are underlined) to add the *Bam* HI/*Pst* I site for subcloning into pLDH/pRN14. Resulting FLAG-LDH gene was then inserted into the pLDH/pRN14 vector to generate a LDH expression vector, pLDH/pRN14-LDH.

An expression vector carrying a Japanese cedar pollen allergen (Cry j 1) was constructed as a fusion gene with a FLAG-tagged LDH fragment (amino acid numbers 2–155). First, we obtained a wild type (WT) Cry j 1 cDNA encoding a mature polypeptide (amino acid numbers 22–375) with specific primers (forward: CTTGACGCGTTGATAATCCCATAGAC, reverse: TATCTGCAGTCAACAACGTTTAGAGAGAGAG, *Mlu* I and *Pst* I sites are underlined). This Cry j 1 PCR fragment was inserted into the *Mlu* I/*Pst* I site of the pLDH/pRN14-LDH to generate an expression vector, pLDH/pRN14-LDH-WT-Cry j 1. This construct enables to express a FLAG-tagged LDH fragment (2–155)-Cry j 1 fusion protein, since a *Mlu* I/*Pst* I digestion of pLDH/pRN14-LDH vector will result in a 3′-deletion of LDH gene.

We also generated another Cry j 1 expression vector carrying codon-optimized Cry j 1 cDNA to facilitate its heterologous overexpression in *L. plantarum*. To this end, we compared the codon usage in the Cry j 1 gene with that of *L. plantarum* in reference to a codon usage database (http://www.kazusa.or.jp/codon/), and found 58 codons that are rarely used in *L. plantarum* (less than 1% frequency). To optimize them, we thoroughly replaced all the 58 codons (in total of 88 nucleotides) with those most frequently used in *L. plantarum* by the recursive PCR-based site-directed mutagenesis (see following section for methodological details). The codon-optimized Cry j 1 cDNA added the *Mlu* I/*Pst* I sites by PCR was then inserted into the pLDH/pRN14-LDH to generate a codon-optimized Cry j 1 expression vector, pLDH/pRN14-LDH-Opt-Cry j 1.

Recursive PCR-based site-directed mutagenesis To optimize codon usage in the Cry j 1 sequence, we prepared 22 pairs of recursive PCR primers (F1/R1 to F22/R22), which not only yielded 22 Cry j 1 PCR fragments that entirely encompassed the full-length mature Cry j 1 cDNA, but also introduced exhaustive mutation in the 58 rare codons (primer sequences are listed on Table S1). Recursive PCR (19) for the codon optimization was performed in three separated steps as follows. In the first round PCR, we got 22 primary PCR fragments (fragments nos.1–22) using corresponding primer pairs (F1/R1 to F22/R22, shown in Table S1). For the second PCR, we pooled 5 or 7 successive PCR fragments to make four template cDNA pools (the template pools nos.1–5, nos.6–10, nos.11–15, and nos.16–22), and amplified those four recursive PCR products using corresponding primer pairs F1/R15, F6/R10, R11/R15, and F16/R22. Finally, we synthesized a codon-optimized mature Cry j 1 cDNA using primers F1/R22 and a template cDNA made of a pool of the four Cry j 1 fragments obtained by the secondary PCR. Correct introduction of mutation was verified by DNA sequencing with the ABI PRIZM 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The optimized Cry j 1 cDNA was then inserted into the *Mlu* I/*Pst* I site of the pLDH/pRN14-LDH vector as described earlier.

Transformation of *L. plantarum* Transformation of *L. plantarum* NCL21 was performed as described (20) with minor modifications. Briefly, a preculture of NCL21 was seeded in a fresh MRS Broth (50 ml) supplemented with 1% glycine at a cell density shown by the optical density at 660 nm (OD 660) was 0.1, and cultured at 30°C until OD 660 reached 0.4–0.6. After cell harvest and three times washing with sterile distilled water, cells were resuspended in 500 μl of 40% polyethylene glycol 1000 (Nakalai Tesque, Kyoto, Japan), and resulting competent cell suspension was stored in a 40 μl aliquot at −80°C. For transfection, a 40 μl NCL21 competent cells was mixed with plasmid DNA (1 μg) and salmon sperm single stranded DNA (10 μg, Cosmo Bio, Tokyo, Japan), and the mixture was then transferred into an ice-cold BTX electroporation cuvette (2 mm gap, Harvard Apparatus, Holliston, MA, USA). Electroporation was performed using BTX ECM 600 (Harvard Apparatus) at following conditions: electric field strength, 1.25 kV; capacitance, 10 μF; resistance, 129 Ω. After electroporation with a single pulse, the cuvette was immediately put on ice, and supplemented with fresh MRS Broth (360 μl) containing 5 μg/ml erythromycin, and then incubated at 30°C for 2 h. Finally, the cell suspension was seeded on a MRS/erythromycin agar plate, and incubated at 30°C for three days to obtain transformants.

SDS-PAGE and immunoblot analyses Cell cultures of NCL21 transformants were centrifuged at 4°C, 7500 rpm for 10 min. The cells were then resuspended in phosphate-buffered saline (PBS), and sonicated on ice for 5 min. The bacterial cell lysates were mixed with equal volume of ×2 Laemmli's sample buffer (21), and boiled for 5 min. Protein samples were then resolved by SDS-PAGE on a 12.5% slab gel, and protein bands were visualized by Coomassie Brilliant Blue (CBB) staining using reagents from Nakalai Tesque. For immunoblotting, resolved proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon P, Millipore, Bedford, MA, USA). After blocking with PBS supplemented with 3% skim milk/1% bovine serum albumin (BSA, Nakalai Tesque)/0.05% Tween 20 (Nakalai Tesque), the PVDF membrane was incubated with a primary mouse anti-FLAG M2 monoclonal antibody (1:10,000 dilution, Sigma-Aldrich) or rabbit anti-Cry j 1 polyclonal antibody (1:1200, Seikagaku Biobusiness, Tokyo, Japan) at room temperature for 1 h. After washing with 0.05% Tween 20/PBS, the membrane was further incubated with secondary horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (for detection of anti-FLAG signal, 1:20,000, Sigma-Aldrich) or goat anti-rabbit IgG-HRP (for immunoblotting with anti-Cry j 1, 1:20,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein bands were visualized using the ECL-Plus Western Blotting Detection Reagent (GE Healthcare Bio-Sciences, Uppsala, Sweden) on a medical X-ray film (Fuji Photo Film, Tokyo, Japan).

Oral vaccination with LAB expressing Cry j 1 (Cry j 1-LAB) to murine model of Japanese cedar pollinosis Six-week-old female BALB/c mice (Charles River Japan, Yokohama, Japan) were housed in specific pathogen-free conditions kept at 22 ± 3°C under a 12 h light/12 h dark cycle, and were fed with CRF-1 diet (Oriental Yeast, Tokyo, Japan) and sterile distilled water. To test the prophylactic effect of Cry j 1-LAB *in vivo*, BALB/c mice were intragastrically (*i.g.*) administered with sonicated Cry j 1-LAB (5.9×10^7 cfu, containing 1 μg of recombinant Cry j 1) using an intragastric probe for seven consecutive days (days 1–7, see Fig. 2). Control mice were given the same cell number of LAB expressing recombinant LDH (LDH-LAB). Then the mice were intraperitoneally (*i.p.*) immunized with 10 μg of Cry j 1 (Seikagaku Biobusiness) emulsified in alum adjuvant (Inject Alum, Thermo Fisher Scientific, Rockford, IL, USA). Immunization was carried out twice (days 8, and 22) with a 12-day interval. Sham control groups were immunized with PBS/alum. Twelve days after the second immunization (day 34), peripheral blood was collected from all groups of mice for immunoglobulin analysis. Then the mice were intranasally (*i.n.*) sensitized with Cry j 1 solution (1.25 μg/μl solution in PBS, 12.5 μg/head) by daily instillations (days 36–42). To analyze cedar pollinosis-like nasal symptoms, the mice were placed into an observation cage (one animal/cage) immediately after each daily *i.n.* challenge with Cry j 1, and numbers of sneezing behavior were counted for 5 min under blinded conditions. All animal studies were carried out using protocols reviewed and approved by the Committee on Animal Experimentation of Hiroshima University.

Analysis of Cry j 1-specific antibody response Plasma Cry j 1-specific IgE, IgG1, and IgG2a titers were analyzed by enzyme-linked immunosorbent assay (ELISA) according to previously described methods (22) with modifications. Briefly, 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated with Cry j 1 solution (50 μg/ml). These plates were incubated with diluted plasma samples (1:10 dilution for IgE, 1:20,000 for IgG1, and 1:400 for IgG2a), and then applied to a further incubation with secondary biotinylated rat anti-mouse IgE/IgG1/IgG2a monoclonal antibodies (1:250 dilution, BD Biosciences). Immunofluorescence detection was carried out using streptavidin-conjugated alkaline phosphatase (Jackson Immuno Research, West Grove, PA, USA) and the AttoPhos Substrate System (Promega, Madison, MI, USA). Fluorescence intensity was analyzed by the Wallac 1420 ARVO Multilabel Counter (Perkin Elmer Life Sciences, Boston, MA, USA). Cry j 1-specific IgE titer (1 unit/ml) was defined as a parallelly-performed sandwich ELISA value corresponding to 1 ng/ml mouse IgE isotype control monoclonal antibody (mAb), while those of specific IgG1 and IgG2a (1 unit/ml) were defined as sandwich ELISA values of 1 μg/ml isotype IgG1 and 1 ng/ml IgG2a mAbs, respectively.

Statistical analysis Statistical analysis was done by unpaired Student's *t*-test, and *P* value less than 0.05 was evaluated as the difference being statistically significant.

RESULTS AND DISCUSSION

Generation of recombinant LAB expressing a major Japanese cedar pollen allergen Cry j 1 To construct a Cry j 1 expression vector, we first made a pRN14 plasmid-based LDH expression vector pLDH/pRN14-LDH, which enabled to express a FLAG-tagged LDH under the control of LDH promoter. Then we inserted wild type (WT) Cry j 1 cDNA into this pLDH/pRN14-LDH to make a Cry j 1 expression vector pLDH/pRN14-WT-Cry j 1. In this construct, a FLAG-LDH fragment fusion gene was introduced in frame with the 5′-termini of WT Cry j 1 cDNA to facilitate heterologous expression. We chose the LDH fragment as an *N*-terminal expression tag not only because it was a constitutively expressed molecule in *Lactobacillus* species, but also because its promoter was one of the strongest promoters known in

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the *L. plantarum* host-vector system (23). Furthermore, we optimized codon usage in the Cry j 1 gene for the maximal expression in the host LAB strain *Lactobacillus plantarum*. To this end, we introduced exhaustive mutations at the 58 rare codons in the Cry j 1 cDNA by the recursive PCR-based site-directed mutagenesis. The codon-optimized Cry j 1 cDNA was inserted into the pLDH/pRN14-LDH plasmid to make another Cry j 1 expression vector, pLDH/pRN14-Opt-Cry j 1. Then we obtained three *L. plantarum* NCL21 transformants carrying LDH, WT-Cry j 1, or codon-optimized Cry j 1 genes.

Immunoblot analysis of those transformants with anti-FLAG antibody indicated that 56 kDa FLAG-tagged LDH-Cry j 1 fusion protein was detected in that carrying codon-optimized Cry j 1 cDNA (Opt-Cry j 1-LAB; lane 3 in the right panel of Fig. 1A). By contrast, we failed to detect recombinant Cry j 1 band in that obtained using the WT Cry j 1 construct (WT-Cry j 1-LAB; lane 2 in the right panel of Fig. 1A). Equal protein loading of this experiment was confirmed by CBB staining (the left panel of Fig. 1A), ruling out the possibility that the lack of anti-FLAG signal in the WT-Cry j 1-LAB was due to a low amount of sample application. We also found that a control transformant generated using LDH expression vector (LDH-LAB) showed a nice production of FLAG-tagged LDH protein (35 kDa, lane 1 in the right panel of Fig. 1A) whose anti-FLAG signal intensity was comparable to that of the LDH-Cry j 1 band (lane 3). This indicated that our LDH expression system *per se* was properly worked in the *L. plantarum* NCL21. The successful expression of recombinant Cry j 1 by codon-optimization was also confirmed by another immunoblot analysis with anti-Cry j 1 antibody (Fig. 1B), which revealed that the codon-optimized Cry j 1-LAB but not those carrying WT-Cry j 1 cDNA showed a positive signal of recombinant Cry j 1. We also observed another anti-Cry j 1 smear signal around high molecular weight ranges in the Opt-Cry j 1-LAB. Since this signal was not observed in the control LDH-LAB, the anti-Cry j 1-reactive molecules were not from the bacterial cell component, but rather might be polymerized recombinant Cry j 1 produced *via* unusual post-translational modifications (*e.g.,* inter-molecular disulfide linkages and/or rigid aggregation) that cannot be reduced and/or dissociated in the Laemmli's SDS-PAGE sample buffer.

Taken together, these results suggested that the codon optimization was critical for the ectopic expression of Cry j 1 in *L. plantarum*. Fuglsang (24) has pointed out that LAB including *L. plantarum* are a prime candidate host for codon optimization because of their strong correlation of codon bias with empirical expressivity. However, actual attempts to ectopically produce therapeutic molecules by codon engineering are still very limited in the *Lactobacillus* host-vector system (25,26). To our knowledge, this is the first reported case of the heterologous production of recombinant Japanese cedar pollen allergen in the *Lactobacillus* species.

Detailed molecular mechanism underlying the increased heterologous Cry j 1 expression through codon optimization is still unknown. It has long been proposed in the prokaryote system that improved translational efficiency is a key mechanism of increased heterologous protein production (27), while Wu et al. (28) have reported that RNA stability and its secondary structure are critical determinants for high level protein expression achieved by codon optimization. Tokuoka et al. (29) have demonstrated in an *Aspergillus* expression system that codon optimization increases steady-state mRNA levels of the heterologous gene, which is in line with the conclusion of Wu et al. (28) that the transcript-coupled mechanism is involved in the increased heterologous protein expression. Although we have not checked Cry j 1 mRNA levels in our recombinant Cry j 1-LAB, we have preliminarily observed in our anti-FLAG western analysis that a very faint recombinant Cry j 1 signal is occasionally seen in the unoptimized WT-Cry j 1-LAB, and that its expression level is further up-modulated in the codon-optimized Cry j 1-LAB (data not shown). This suggests that recombinant Cry j 1 expression is indeed facilitated upon optimization of codon usage. Further investigation is needed to know whether translational efficiency and/or stability of Cry j 1 mRNA are actually up-modulated in our codon-optimized Cry j 1-LAB.

Oral vaccination with LAB expressing Cry j 1 (Cry j 1-LAB) suppresses allergen-specific IgE response in a murine model of cedar pollinosis We next sought to see whether oral administration with Cry j 1-LAB could prevent allergic immune response and nasal symptoms in a murine model of cedar pollinosis (experimental procedures are summarized in Fig. 2). We orally administered sonicated Cry j 1-LAB (5.9×10^7 cfu/head/day, containing 1 μg r-LDH-Cry j 1 fusion protein) to BALB/c mice for seven days. Then the mice were *i.p.* immunized with Cry j 1/alum on days 8 and 22. For immunoglobulin analysis, peripheral blood was collected twelve days after the second immunization (day 34). ELISA revealed that oral administration with Cry j 1-LAB significantly suppressed Cry j 1-specific IgE response as compared with control mice fed LDH-LAB (Fig. 3). By contrast, the oral Cry j 1-LAB administration had no effect on the development of Cry j 1-specific IgG1 response (Fig. 4A). We also found a significant increase in Cry j 1-specific IgG2a antibody titer in Cry j 1-LAB fed mice (Fig. 4B). The results indicate that oral Cry j 1-LAB vaccination prevents the development of allergic antibody response.

We also tested whether a mixture of soluble Cry j 1 and *L. plantarum* NCL21 cells showed IgE suppression in our model, but

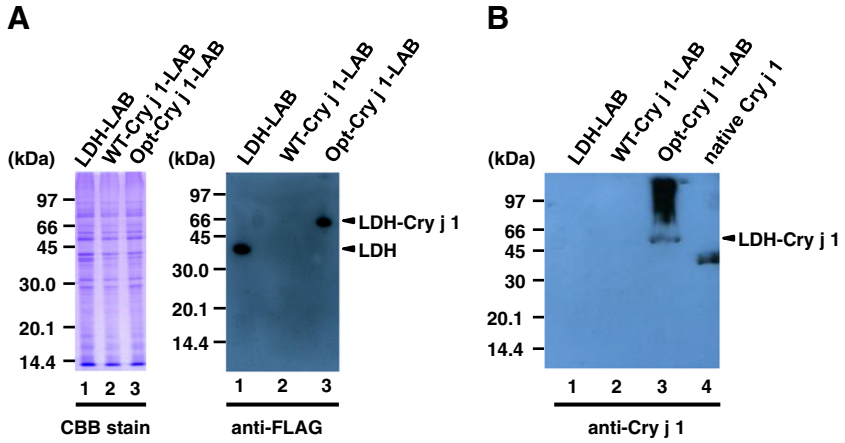


FIG. 1. Expression of recombinant Cry j 1 in *L. plantarum* NCL21 by codon optimization. (A) Left panel; Equal protein loading of this experiment was confirmed by CBB staining. Right panel; Detection of FLAG-tagged LDH-Cry j 1 fusion protein (56 kDa) by immunoblotting with anti-FLAG monoclonal antibody. (B) Expression of recombinant Cry j 1 in the Opt-Cry j 1-LAB was re-confirmed by immunoblotting with anti-Cry j 1 polyclonal antibody (lane 3). Lane 4 shows a positive control staining of native Cry j 1.

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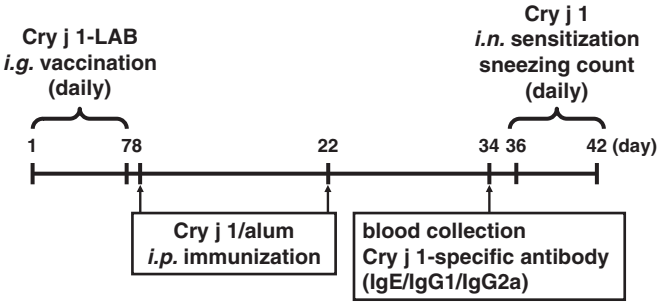


FIG. 2. Experimental schedule of oral vaccination with Cry j 1-LAB to a murine model of cedar pollinosis. BALB/c mice were orally administered with Cry j 1-LAB for seven days (days 1–7). Then the mice were *i.p.* immunized with Cry j 1/alum (days 8 and 22) followed by daily *i.n.* challenge with Cry j 1 to induce nasal hyperreactivity (day 36–42). On day 34, peripheral blood was collected for analysis of Cry j 1-specific IgE/IgG1/IgG2a responses. Nasal symptoms were evaluated by counting daily sneezing behavior triggered upon *i.n.* challenge with Cry j 1.

no inhibitory effect on the Cry j 1-specific IgE response was observed (data not shown). This suggests that anti-allergic potency of our recombinant Cry j 1-LAB cannot be reconstituted by a simple mixing of allergen and parental LAB. We have observed that our recombinant Cry j 1 was expressed in an insoluble form in the transformant (data not shown). We now assume that those insoluble Cry j 1 together with insoluble LAB materials (cell wall and/or membrane components) could have worked as a vaccine adjuvant that effectively activate mucosal innate immunity to elicit anti-allergic immune response.

Although it is a widely-accepted idea that dietary LAB by themselves have anti-allergic potency, we have found that oral PBS administration in our model results in a Cry j 1-specific IgE response comparable to that seen in the LDH-LAB-fed group (data not shown). This demonstrates that LAB *per se* do not affect on the IgE response in our experimental settings. One plausible explanation of this observation is that we have administered a much lower LAB cell number (5.9×10^7 cfu/head) than those reported in the recent literatures (*e.g.*, 2×10^9 or 1×10^{10} cfu/head) showing IgE-inhibitory effect of dietary LAB (30,31).

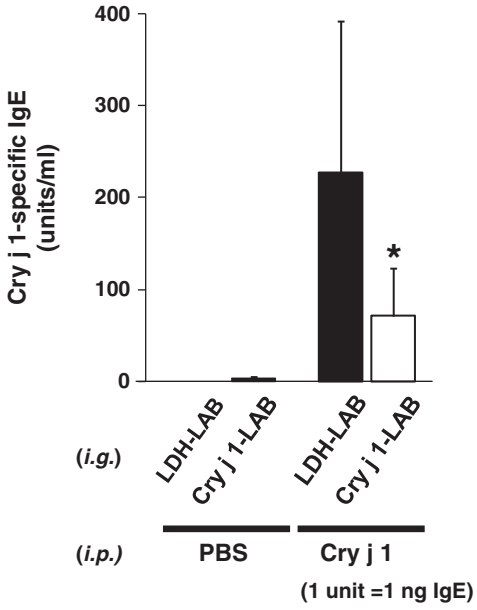


FIG. 3. Oral vaccination with Cry j 1-LAB suppresses Cry j 1-specific IgE response in a murine model of cedar pollinosis. After the second *i.p.* immunization with Cry j 1, peripheral blood samples were collected from all groups of mice. Plasma Cry j 1-specific IgE response was analyzed by ELISA. The specific IgE titer (unit) was defined as a parallel sandwich ELISA value corresponding to 1 ng mouse IgE isotype control antibody. The number of mice in each group is as follows: *i.g.* LDH-LAB/*i.p.* PBS (n = 6), *i.g.* Cry j 1-LAB/*i.p.* PBS (n = 6), *i.g.* LDH-LAB/*i.p.* Cry j 1 (n = 10), and *i.g.* Cry j 1-LAB/*i.p.* Cry j 1 (n = 10). $^*P<0.05$ (as compared with the control 'LDH-LAB' fed group upon *i.p.* immunization with Cry j 1).

Oral vaccination with Cry j 1-LAB prevents pollinosis-like clinical symptoms We next tested the effect of Cry j 1-LAB administration on the development of nasal symptoms. To this end, Cry j 1-LAB fed mice given *i.p.* immunization with Cry j 1 were nasally challenged with Cry j 1, and their clinical symptoms were evaluated by counting sneezing behavior for seven days (days 36–42, Fig. 2).

As shown in Fig. 5, control mice fed LDH-LAB and *i.p.* immunized with Cry j 1 developed a prominent sneezing behavior upon nasal sensitization with Cry j 1 (shown open circles). We found that administration with Cry j 1-LAB prevented those clinical symptoms; significantly decreased sneezing counts were observed in the days 4, 6, and 7 (shown open triangles with asterisks in Fig. 5). No significant sneezing behavior was evident in the corresponding sham control groups immunized with PBS/alum (shown closed circles and triangles for the LDH-LAB and Cry j 1-LAB fed groups, respectively), confirming that our nasal sensitization model appropriately worked in an allergen-specific manner. These results indicate that oral vaccination with Cry j 1-LAB prevents the development of pollinosis-like clinical symptoms concomitant with the suppression of IgE response in this murine model of cedar pollinosis. Daniel et al. have reported that nasal application with recombinant LAB carrying birch pollen allergen Bet v 1 modulates allergic immune response and airway inflammation, but they have not addressed their effect on the clinical symptoms (15). Therefore, our data provide the first compelling evidence that LAB-based oral vaccine is effective for the prevention of allergic rhinitis.

Miyahara et al. have demonstrated in mice that IgE-mediated type I allergic response is essential for the pathogenesis of nasal allergy elicited upon *i.p.* immunization and *i.n.* sensitization with allergen (32). Thus, the suppression of Cry j 1-specific IgE response (Fig. 3) is the most plausible mechanism by which oral Cry j 1-LAB vaccination ameliorates pollinosis-like nasal symptoms (Fig. 5). How did Cry j 1-LAB suppress allergen-specific IgE response? The Th1-dominated cytokine milieu generally favors an IgG2a immune response, while such a Th1 microenvironment conversely suppresses both IgE and IgG1 antibody responses. The significant up-modulation of Cry j 1-specific IgG2a titer (Fig. 4B) suggests that Cry j 1-LAB may inhibit IgE production through induction of Th1 immune response. Murosaki et al. have reported that *L. plantarum* L-137, a parental LAB strain of NCL21 used in this study, is a potent inducer of IL-12, and that *i.p.* administration with L-137 suppresses casein-specific IgE response in a murine model of milk allergy (33). This implies that our NCL21-based oral vaccine may also negatively regulate IgE response by the induction of IL-12-driven Th1 response. However, the unchanged Cry j 1-specific IgG1 titer (Fig. 4A) argues that the IgE suppressive action of Cry j 1-LAB may not be simply explained by the regulation of Th1/Th2 cytokine balance. Indeed, we preliminary observed that oral Cry j 1-LAB vaccination had no effect on the IFN-γ/IL-4 secretion from cervical draining lymph node cells upon re-stimulation with Cry j 1 (data not shown), suggesting a complex immune regulation by which Cry j 1-LAB suppresses IgE response. Murosaki et al. also have reported a complicating observation in the milk allergy model that *i.p.* administration with the *L. plantarum* L-137 (parental strain of NCL21) unexpectedly induces casein-specific IgG1 response, even though it suppresses IgE response concomitant with the induction of pro-Th1 cytokine IL-12 (33). Further investigation is needed to know whether the induction of Th1-dominated cytokine response is actually involved in the down-modulatory effect of IgE response elicited by Cry j 1-LAB.

Another possible mode of IgE inhibitory action by Cry j 1-LAB is the induction of regulatory T cell (Treg) response. In this context, we found a significant increase in the secretion of IL-10 by Cry j 1-

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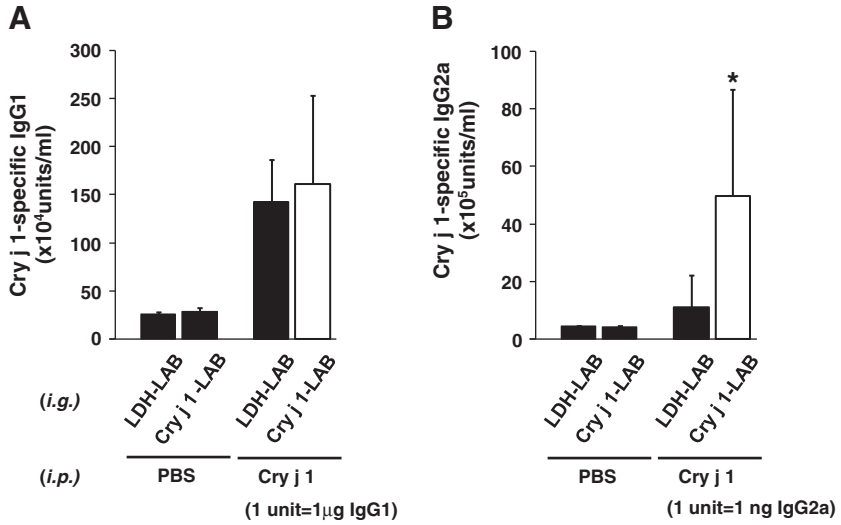


FIG. 4. Effect of oral Cry j 1-LAB vaccination on the Cry j 1-specific IgG1 (A) and IgG2a (B) antibody responses. The specific IgG1 or IgG2a titers (unit) was defined as a parallel sandwich ELISA value of 1 μg mouse IgG1 or 1 ng IgG2a isotype control, respectively. The number of mice in each group is as follows: *i.g.* LDH-LAB/*i.p.* PBS (n = 6), *i.g.* Cry j 1-LAB/*i.p.* PBS (n = 6), *i.g.* LDH-LAB/*i.p.* Cry j 1 (n = 10), and *i.g.* Cry j 1-LAB/*i.p.* Cry j 1 (n = 10). $^*P<0.05$ (as compared with the 'LDH-LAB' fed group upon *i.p.* immunization with Cry j 1).

stimulated cervical lymph node cells from Cry j 1-LAB fed mice (Ohkouchi et al., unpublished data). Since IL-10 represents a key tolerogenic cytokine secreted by the Tr1 type of adaptive Tregs (34), the IL-10 secretion by Cry j 1-LAB-responsive Tregs might also play a part in the negative regulation of IgE response. Intriguingly, we observed that oral Cry j 1-LAB administration followed by *i.p.* immunization with Cry j 1 led to the significant, but transient increase in the cell number of $CD4^+Foxp3^+$ Treg subset in the spleen (data not shown), although neither IgE suppression nor IL-10 production by those Tregs remains to be investigated.

Mechanism by which Cry j 1-LAB acts on the gut mucosa to inhibit IgE response is also unknown. The key sentinel innate immune cells for sensing Cry j 1-LAB and sampling the delivered Cry j 1 are dendritic cells (DCs) in the intestinal lamina propria and Peyer's patch. These *in vivo*-primed DC subsets by Cry j 1-LAB may drive naïve T cell differentiation towards anti-allergic Th1 and/or inducible Treg phenotypes to suppress allergen-specific IgE production. Evidence is accumulated that *L. plantarum* L-137 tends to induce Th1 response by the induction of IL-12 from DCs (33), which is in line with our data that Cry j 1-LAB induces allergen-specific IgG2a response (Fig. 4B). Another possible model by which DCs suppress IgE production is their direct effect on B cells to inhibit IgE class switch recombination and/or IgE protein production (35). Analysis of the *in vivo* effect of Cry j 1-LAB on the intestinal DCs is a critical future experiment to address these issues as well as for the better design of oral LAB vaccine to prevent allergic inflammation.

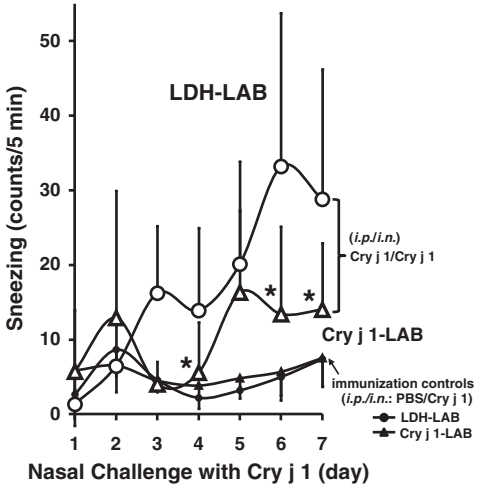


FIG. 5. Oral vaccination with Cry j 1-LAB ameliorates pollinosis-like nasal symptoms. The Cry j 1-immunized mice were given *i.n.* challenge with Cry j 1 to induce nasal hyperreactivity, and daily sneezing behavior (for 5 min) was counted for seven days. The number of mice in each group is as follows: LDH-LAB/*i.p.* PBS (n = 6, shown closed circles), Cry j 1-LAB/*i.p.* PBS (n = 6, shown closed triangles), LDH-LAB/*i.p.* Cry j 1 (n = 10, shown open circles), and Cry j 1-LAB/*i.p.* Cry j 1 (n = 10, shown open triangles). $^*P<0.05$ (as compared with the 'LDH-LAB' fed group upon *i.p.* immunization and *i.n.* sensitization with Cry j 1).

In conclusion, here we report for the first time to generate recombinant LAB expressing a major Japanese cedar pollen allergen (Cry j 1) by codon optimization. More strikingly, we also show that oral vaccination with the Cry j 1-LAB ameliorates the cedar pollinosis-like clinical symptoms and allergen-specific IgE response *in vivo*, providing the first experimental evidence that recombinant LAB-based oral vaccine is effective for the treatment of allergic rhinitis. Our *Lactobacillus* expression system offers a new platform for the development of safer, and more effective oral immunotherapy for Japanese cedar pollinosis.

Supplementary materials related to this article can be found online at doi:10.1016/j.jbiosc.2011.11.025.

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