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Nisin-induced expression of recombinant T cell epitopes of major Japanese cedar pollen allergens in *Lactococcus lactis*

Vinh Van Hoang[1,2] · **Takahumi Ochi**[1] · **Kentaro Kurata**[1] · **Yutaka Arita**[1] · **Yusuke Ogasahara**[1] · **Keiichi Enomoto**[1]

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Abstract Japanese cedar pollinosis is a seasonal allergic disease caused by two major pollen allergens: Cry j 1 and Cry j 2 antigens. To develop an oral vaccine to treat pollinosis, we constructed recombinant *Lactococcus lactis* harboring the gene encoding fused T cell epitopes from the Cry j 1 and Cry j 2 antigens. The recombinant T cell epitope peptide was designed to contain the fused cholera toxin B subunit as an adjuvant and a FLAG tag at the C-terminus. An expression plasmid was constructed by inserting the T cell epitope peptide gene into the multiple cloning sites of plasmid pNZ8148, an *Escherichia coli-L. lactis* shuttle vector. The constructed plasmid was transformed into *L. lactis* NZ9000 for expression induced by nisin, an antibacterial peptide from *L. lactis*. The expression of the epitope peptide was induced with 10–40 ng/mL nisin, and the expressed T cell epitope peptide was detected by western blot analysis using an anti-FLAG antibody and an antibody against the T cell epitopes. The concentration of the epitope peptide was estimated to be ~ 22 mg/L of culture in the presence of 40 ng/mL nisin, although it varied depending on the nisin concentration, the culture time, and the bacterial concentration when nisin was added. The expression of the recombinant epitope peptide in *L. lactis*, an organism generally recognized as safe, as demonstrated in this study, may contribute to the development of an oral vaccine for the treatment of pollinosis.

✉ Keiichi Enomoto
enomoto.keiichi@kochi-tech.ac.jp

[1] School of Environmental Science and Engineering, Kochi University of Technology, 185 Miyanokuchi, Tosayamada, Kami, Kochi 782-8502, Japan

[2] Present address: Institute of Microbiology and Biotechnology, Vietnam National University, Hanoi, 144 Xuan Thuy St., Cau Giay Dist., Hanoi, Vietnam

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Introduction

Pollinosis, caused by Japanese cedar (*Cryptomeria japonica*) pollen, is a common allergic disease that is prevalent in Japan from February to April each year (Saito 2014). Pollinosis has been shown to be induced by Cry j 1 and Cry j 2 antigens, two major allergens in Japanese cedar pollen (Yasueda et al. 1983; Sone et al. 1994; Sakaguchi et al. 1990; Komiyama et al. 1994; Namba et al. 1994). Although approximately 27% of the population in Japan has suffered from this disease for decades (Saito 2014), causal therapies for pollinosis have only recently become available. One such treatment is repeated sublingual administration of the pollen extract containing the Cry j 1 and Cry j 2 antigens, which has been shown to ameliorate the symptoms of pollinosis by inducing immunological desensitization (Okamoto et al. 2015; Sakashita et al. 2015). However, careful administration is necessary to avoid anaphylaxis because the Cry j 1 and Cry j 2 antigens contain epitopes that react with IgE antibodies in addition to the T cell epitopes for immunotherapy. To overcome this problem, recombinant antigen vaccines that contain only T cell epitopes have been designed and expressed in *Escherichia coli*, rice seed, and other organisms (Hoang et al. 2015; Takagi and Takaiwa 2016; Takaiwa and Yang 2014; Kawabe et al. 2012).

In a previous study, we successfully constructed recombinant *E. coli* that expressed a peptide containing multiple T cell epitopes from Cry j 1 and Cry j 2 (Hoang et al. 2015). Four epitopes from Cry j 1 and six from Cry j 2 identified by Sone et al. (1998) were selected to ensure that the epitopes were presented by various MHC (HLA) class II types and, therefore, applicable to a large population of patients. However, it

is essential to express the peptide in an organism generally recognized as safe (GRAS) if the whole organisms are to be used as an oral vaccine to cure pollinosis. In the present study, we constructed recombinant *Lactococcus lactis* that contained the gene encoding the T cell epitope peptide, using a plasmid in which expression of the inserted gene is induced with nisin, an antibacterial peptide from *L. lactis* used as a preservative in food. Nisin activates the two-component system in *L. lactis*, which is composed of a sensor protein and a response regulator, thereby inducing transcription from the nisin promoter. Therefore, the nisin-controlled gene expression system (NICE) described by Mierau and coworkers (Mierau et al. 2005; Mierau and Kleerebezem 2005) is proposed to be an excellent tool for the production of oral vaccines. Here, we investigate the properties required for optimal expression of the recombinant T cell epitope peptide in *L. lactis* for potential future vaccine production.

Materials and methods

Plasmids and bacterial strains

E. coli-L. lactis shuttle vector pNZ8148, *E. coli* MC1061 for the construction of an expression vector, and *L. lactis* NZ9000 for the expression of the recombinant peptide were purchased from MoBiTec (Göttingen, Germany). The plasmid pET28a-*BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-HindIII* constructed in the previous study (Hoang et al. 2015), containing the cholera toxin B subunit gene (*ctb*) fused with the cry j 1 and cry j 2 epitope genes and the FLAG tag sequence located at the 3′-end, was used as a source of the recombinant T cell epitope peptide gene. *E. coli* BL21(DE3) harboring this recombinant plasmid has been deposited in the NITE Biological Resource Center (NBRC) and assigned accession number NBRC 112070. The DNA sequence of the insert in this plasmid has been deposited in the DDBJ database under accession number AB978369.

PCR amplification of the recombinant epitope gene

The recombinant T cell epitope gene was amplified by PCR using the plasmid pET28a-*BamHI-ctb-linker-cry j 1 epi-cry j 2 epi-flag-HindIII* as a template for the first round of PCR. PCR was conducted using forward primer His-CTB-F (5′-CATCATCATCATCATCATACACCTCAAAATAT TACTGA-3′) and reverse primer *Hin*dIII-FLAG-R (5′-CGCA AGCTTCTTGTCATCGTCGTCTTTGTA-3′) to add a histidine tag to the 5′-end. The resultant PCR product was used as a template for the second round of PCR conducted using forward primer *Nco*I-add-His-CTB-F (5′-GCGC CATGGGTCATCATCATCATCATCATAC-3′) and reverse primer *Hin*dIII-FLAG-R to add a *Nco*I restriction site at the 5′-end and insert nucleotides (*add*) to maintain the correct reading frame. The purified PCR product (*NcoI-add-his-ctb-linker-cry j 1 epi-cry j 2 epi-flag-HindIII*) was used in the construction of an expression vector, as described below. All PCRs were performed using high fidelity KOD-Plus DNA polymerase (Toyobo, Osaka, Japan).

Construction of the expression vector

The recombinant T cell epitope gene, amplified as described above, and a shuttle vector pNZ8148 were separately digested with restriction enzymes, *Nco*I and *Hin*dIII, and then ligated using a DNA ligation kit (Mighty Mix) purchased from Takara Bio (Kusatsu, Japan).

Electroporation of the recombinant pNZ8148 plasmid into *E. coli* MC1061 was conducted in a 2-mm-width electrocuvette at a voltage of 2.0 kV and 186 Ω. *E. coli* cells containing the plasmid were selected on an agar plate containing chloramphenicol (10 μg/mL), and colonies were checked for the presence of the recombinant T cell epitope gene by colony PCR using forward primer pNZ8148-F (5′-AACG CGAGCATAATAAACGGCTCTG-3′) and reverse primer pNZ8148-R (5′-GCTTTTTGGCTATCAATCAAAGCAAC AC-3′). The DNA sequence of the plasmid insert (Fig. 1) was determined using the dye terminator method by Bio Matrix Research (Nagareyama, Japan).

Electroporation of the purified recombinant plasmid into *L. lactis* NZ9000 was conducted by the method described above. Transformants were selected on agar plates containing chloramphenicol and further examined for the presence of the recombinant plasmid by colony PCR as described above (Fig. 2).

Expression of the recombinant peptide

A single colony of *L. lactis* containing the recombinant plasmid was selected and pre-cultured at 30 °C overnight in 10 mL of G-M17B broth. Four milliliters of the culture was then inoculated into 100 mL of G-M17B broth and incubated at 30 °C until the OD_{600} reached ~ 0.4. Then, nisin at 5 to 160 ng/mL was added to 11 mL aliquots of the culture in 50-mL tubes and further incubated for 2–10 h at 30 °C, with shaking at 130 rpm to maintain constant culture conditions by preventing cell precipitation.

Western blot analysis

The recombinant *L. lactis* cells harvested by centrifugation from 10 mL of the culture were mixed with 500 μL xTractor buffer (Takara Bio) and sonicated briefly to disperse the cells. After incubation at room temperature for 15 min with 30 μg/mL DNase I and 550 μg/mL lysozyme, the cell lysate was mixed with 4× SDS sample buffer at a ratio of 3:1 and then

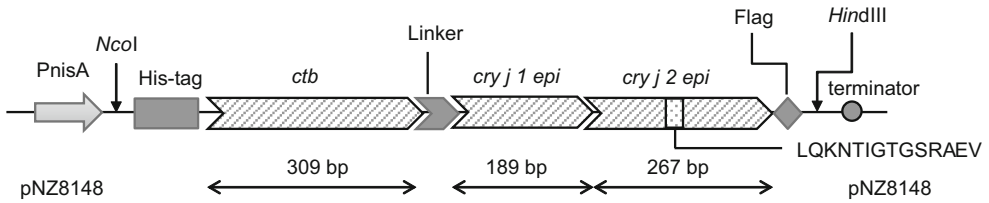

Fig. 1 Schematic structure of the T cell epitope peptide gene. PnisA: nisin A promoter region including the ribosomal binding site. The regions, *cry j 1 epi* and *cry j 2 epi*, contain sequences for the four and six T cell epitopes from Cry j 1 and Cry j 2 antigens, respectively. The indicated amino acid sequence (LQKNTIGTGSRAEV) encoded by *cry j 2 epi* was used to prepare an antibody for western blotting

heated at 95 °C for 5 min. The supernatant after centrifugation (12 μL) was subjected to SDS-PAGE on a commercial 12.5% polyacrylamide gel (Atto, Tokyo, Japan), and then, proteins were electroblotted onto a Hybond polyvinylidene fluoride membrane (GE Healthcare Japan, Hino, Japan). After blocking with skim milk powder, the membrane was incubated at 4 °C overnight with rabbit anti-FLAG antibody (Sigma-Aldrich Japan, Tokyo, Japan) or rabbit anti-Cry j epi antibody that was raised against the partial amino acid sequence (LQKNTIGTGSRAEV) in the epitope region of the recombinant peptide and prepared by Eurofins Genomics (Tokyo, Japan). The membrane was then incubated with horseradish peroxidase-linked anti-rabbit IgG antibody (Cell Signaling Technology Japan, Tokyo, Japan) at room temperature for 1 h. Can Get Signal Immunoreaction Enhancer Solutions 1 and 2 (Toyobo, Osaka, Japan) were added to the incubations with primary and the secondary antibodies, respectively. Affinity-purified T cell epitope peptide from the recombinant *E. coli* was prepared according to the method of Hoang and coworkers (2015) and was used as a standard in western blot analysis.

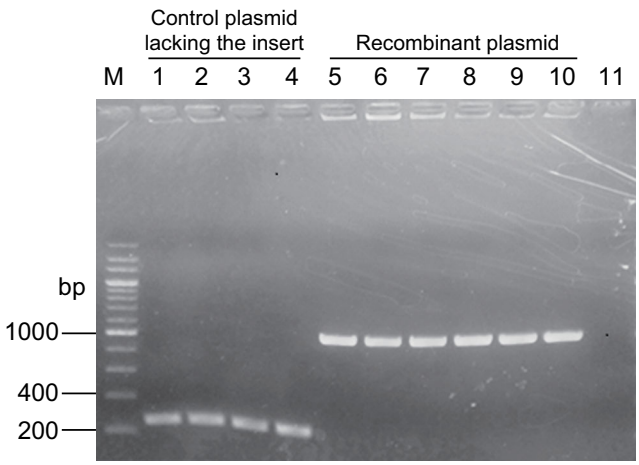

Fig. 2 Colony PCR of *L. lactis* NZ9000 transformed with the pNZ8148 plasmid. PCR products of *L. lactis* colonies were analyzed by electrophoresis on a 1% agarose gel. DNA marker (lane M); colonies transformed with untreated pNZ8148 lacking the insert (lanes 1–4); transformants with recombinant pNZ8148 containing the T cell epitope peptide gene (lanes 5–10); no template control PCR (negative control) (lane 11)

Detection of the signal was carried out using an ECL Select Western Blotting Detection Reagent (GE Healthcare Japan), Amersham Hyperfilm ECL (GE Healthcare), and a chemical luminescence imaging device Fusion FX7 (Vilber Lourmat, Marne-la-Valée, France).

Results

Construction of recombinant *L. lactis*

E. coli-L. lactis shuttle vector pNZ8148 and *L. lactis* NZ9000 were used for expression of the recombinant peptide containing four T cell epitopes from Cry j 1 and six from Cry j 2. The gene encoding the recombinant peptide was amplified by PCR from pET28a(+) plasmid containing the epitope peptide gene constructed in the previous study (Hoang et al. 2015). The PCR-amplified gene was inserted into the multiple cloning sites of pNZ8148, and then, *E. coli* MC1061 was transformed with the recombinant plasmid. The DNA sequence of the insert in the purified pNZ8148 plasmid was determined to be correct. Figure 1 shows the structure of the T cell epitope gene in plasmid pNZ8148. The cholera toxin B subunit gene (*ctb*) was fused with the *cry j 1* and *cry j 2* epitope genes, and the FLAG tag sequence was located at the 3′-end.

The recombinant pNZ8148 purified from *E. coli* was used to transform *L. lactis* NZ9000. Transformants were examined for the correct insert by colony PCR with primers flanking the open reading frame (Fig. 2). DNA bands corresponding to the expected size (1036 bp) were confirmed on an agarose gel (lanes 5–10, Fig. 2).

Nisin-induced expression of the recombinant T cell epitope peptide

The recombinant peptide expressed by *L. lactis* NZ9000 in the presence of nisin was detected by western blotting using two types of antibodies, anti-FLAG antibody and anti-Cry j epi antibody against the partial amino acid sequence in the epitope region of the recombinant peptide. The purified T cell epitope peptide produced by recombinant *E. coli* BL21(DE3) was

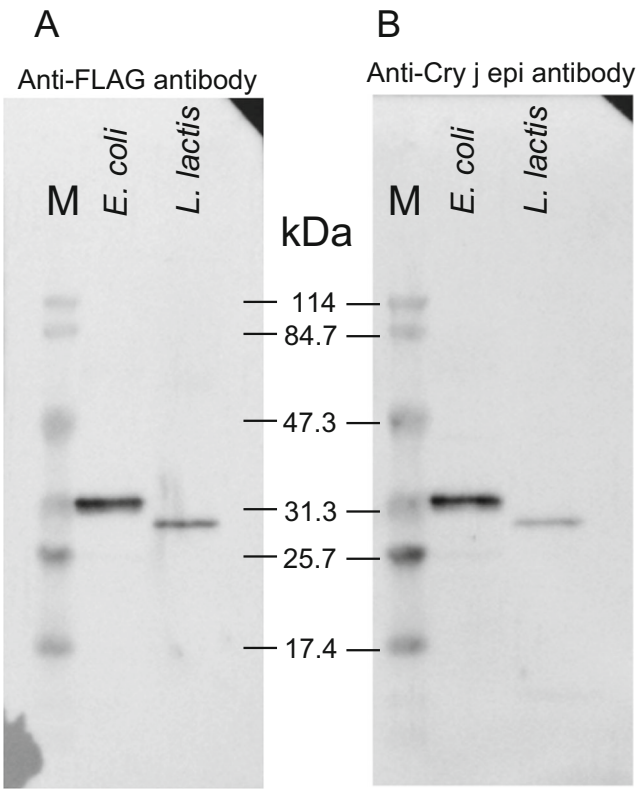

Fig. 3 Detection of the recombinant peptides from *E. coli* and *L. lactis* by western blot analysis. **a** Western blot with the anti-FLAG antibody. **b** Western blot with the anti-Cry j epi antibody against the partial amino acid sequence (LQKNTIGTGSRAEV) in the peptides. Molecular weight marker (lane M); purified peptide from the recombinant *E. coli* BL21(DE3) (lane *E. coli*); peptide expressed by the recombinant *L. lactis* NZ9000 with 40 ng/mL nisin (lane *L. lactis*)

used as a control. As shown in Fig. 3, both of the peptides from recombinant *E. coli* and *L. lactis* were detected by anti-FLAG antibody (Fig. 3a) or anti-Cry j epi antibody (Fig. 3b). The apparent size of the peptide from *L. lactis* on a polyacrylamide gel was ~ 29 kDa, corresponding to the molecular mass of the peptide (31 kDa) calculated from its deduced amino acid sequence. The peptide from *E. coli* was larger than that from *L. lactis* due to the different length of the open reading frames of the *E. coli* peptide (948 bp) and the *L. lactis* peptide (852 bp). The peptide from *E. coli* had additional amino acids encoded by the nucleotide sequence in pET28a(+).

Effects of nisin concentration on the expression of recombinant peptide

The epitope peptide expressed by *L. lactis* was successfully detected by western blotting. Then, the optimal conditions for expression by the recombinant *L. lactis* were examined. Firstly, the effects of nisin concentration on the expression were studied. Expression was induced by adding various concentrations of nisin (5–160 ng/mL) to the culture of *L. lactis* that had been pre-cultured until the OD_{600} reached ~ 0.4. After 2–4 h of culture at 30 °C for expression, the cells were harvested and the cell lysate was subjected to western blotting using an anti-FLAG antibody. As shown in the left lane of Fig. 4a, no peptide was detected in the absence of nisin, indicating that the anti-FLAG antibody used as a primary antibody did not cross-react with bacterial protein in the absence of nisin. The expression of the peptide increased dependent on the concentration of nisin, reaching a maximum after 2 h at a nisin concentration of 40 ng/mL (Fig. 4a). Maximal expression was achieved with 40 ng/mL nisin (Fig. 4b) when the bacteria were cultured for 4 h, with the expression slightly decreasing at higher concentrations of nisin. We also noticed that higher concentrations of nisin prevented bacterial growth. The OD_{600} after 4-h culture without nisin was 1.8, whereas it was 1.6 with 160 ng/mL nisin.

Time course of nisin-induced expression

The time course of nisin-induced expression during culturing for 0–10 h was monitored in the presence of 10 or 40 ng/mL nisin. The left lanes in Fig. 5a, b, which represent a culture time of 0 h, show no cross-reaction of the anti-FLAG antibody with bacterial protein, indicating that the anti-FLAG antibody is effectively specific to the expressed peptide. Maximal expression of the epitope peptide was achieved 2 h after nisin addition with both 10 and 40 ng/mL nisin (Fig. 5). Expression with 10 ng/mL nisin was stable up to 7 h but decreased at 10 h. In the presence of 40 ng/mL nisin, expression clearly decreased after 4 h, suggesting decomposition of the peptide in *L. lactis* cells.

Effect of the timing of nisin addition

In Figs. 4 and 5, nisin was added to the bacterial cells that had been cultured to an OD_{600} of ~ 0.4. However, it is well known that the physiological conditions can affect bacterial expression of recombinant peptides. Therefore, we studied the effects of bacterial concentration at the time of nisin addition on the expression of the recombinant peptide. Figure 6a shows the time course of bacterial growth, indicating that at an OD_{600} of 0.4 *L. lactis* is in logarithmic growth phase. In Fig. 6b, c, 10 or 40 ng/mL nisin was added to the culture at an OD_{600} of 0.2–1.8, and the expressed recombinant peptide was detected with anti-FLAG antibody. Maximal expression of the recombinant peptide induced with 10 ng/mL nisin was achieved at an initial OD_{600} of 0.2, and the expression remarkably decreased as the OD_{600} increased (Fig. 6b). However, in the presence of 40 ng/mL nisin, the expression of the peptide was stable and observed over a wide range of OD_{600} values from 0.2 to 1.2 (Fig. 6c), although low expression was observed at an OD_{600} of 1.8. Therefore, compared with 10 ng/mL nisin, 40 ng/mL nisin was suitable for stable expression of the peptide over a wide range of OD_{600} values.

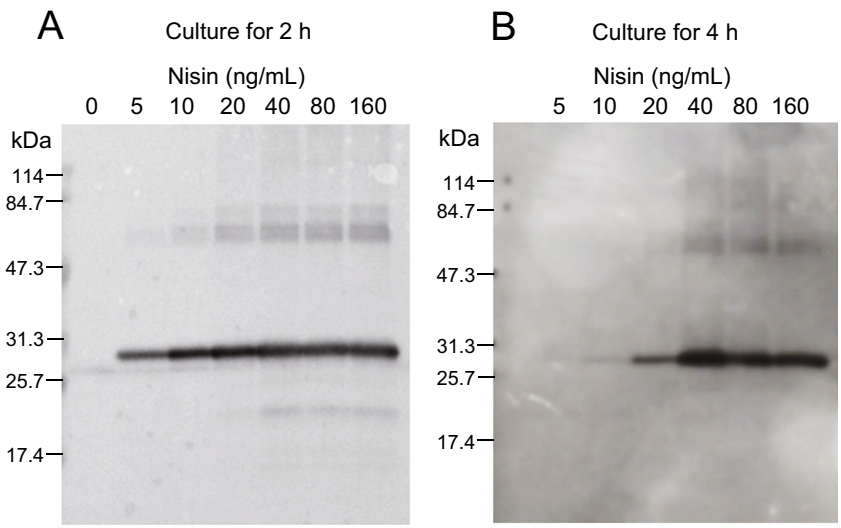

Fig. 4 Effects of nisin concentration on the expression of the epitope peptide in *L. lactis*. The indicated concentrations of nisin were added to the pre-cultured recombinant *L. lactis* at an OD_{600} of ~ 0.4. Culturing was continued for 2 or 4 h at 30 °C to allow for expression, and harvested cells were subjected to SDS-PAGE. The recombinant peptide was detected by western blotting with an anti-FLAG antibody. **a** Culture incubated with nisin for 2 h. **b** Culture incubated with nisin for 4 h

Estimation of the amount of expressed epitope peptide

The amount of expressed T cell epitope peptide was estimated by western blotting followed by quantification using a chemiluminescence imaging device. Affinity-purified epitope peptide from the recombinant *E. coli* was used as a standard. Figure 7 shows the purified *E. coli* recombinant peptide and the peptide expressed by *L. lactis* in this study. By measuring the strength of chemiluminescence from the recombinant peptides in a chemical luminescence imaging device, the ratio of chemiluminescence for 0.65 μg of *E. coli* peptide, 0.32 μg of *E. coli* peptide, and the expressed amount of *L. lactis* peptide was determined to be 1:051:082. Based on these results, the peptide concentration in the culture of *L. lactis* was estimated to be ~ 22 mg/L.

Discussion

In the present study, we successfully constructed recombinant *L. lactis* that expresses the T cell epitope peptide of Japanese cedar pollen allergens upon exposure to nisin. Lactic acid bacteria (LAB) are GRAS organisms and have therefore attracted attention as potential delivery vehicles for various vaccines (Pontes et al. 2011; Tarahomjoo 2012). In particular, LAB are useful for the induction of mucosal immunity against pathogens that infect through the mucosal membranes of the host (Wells 2011; Bermúdes-Humarán et al. 2013; Wang et al. 2016). Therefore, vaccination using recombinant LAB is usually conducted by oral or nasal administration of the recombinant bacteria.

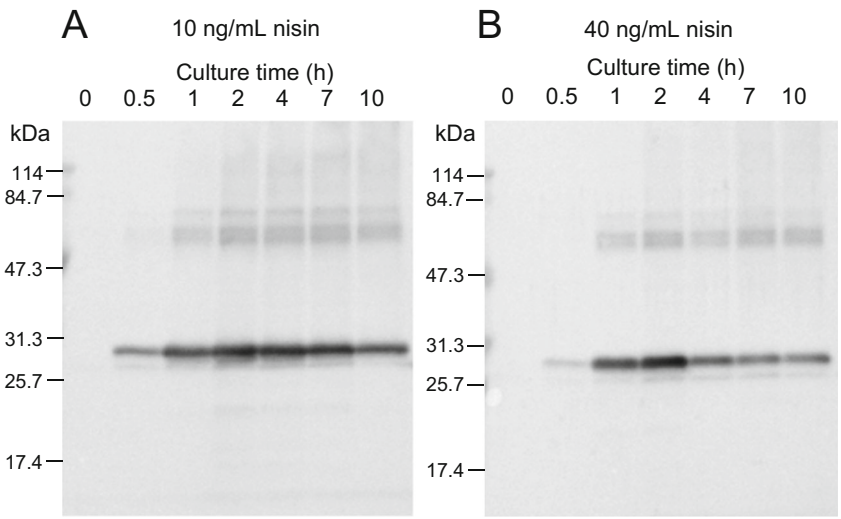

Fig. 5 Time course of nisin-induced expression of the epitope peptide. Nisin was added to the pre-cultured recombinant *L. lactis* at an OD_{600} of ~ 0.4, and the culture was continued up to 10 h at 30 °C. Harvested cells were subjected to SDS-PAGE, and the recombinant peptide was detected by western blotting with an anti-FLAG antibody. **a** 10 ng/mL nisin. **b** 40 ng/mL nisin

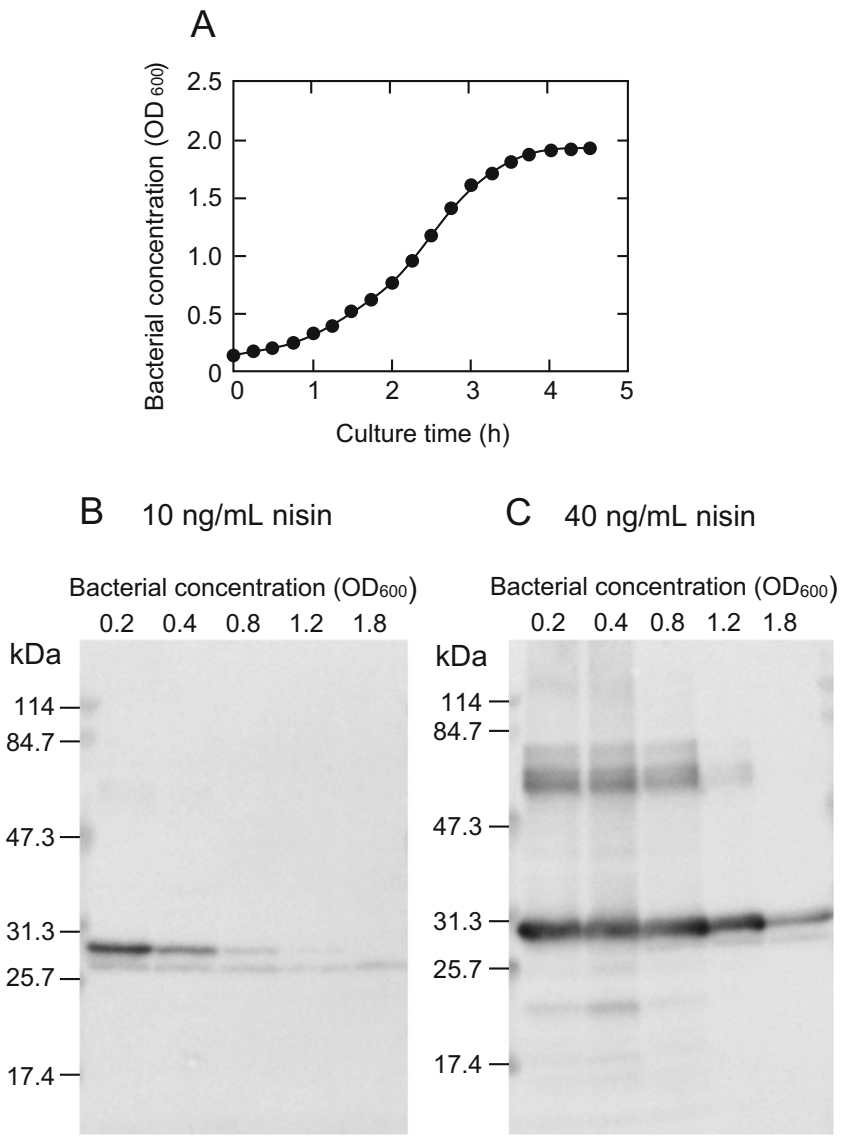

Fig. 6 Effects of the bacterial concentration at the time of nisin addition on the expression of the epitope peptide. Harvested cells were subjected to SDS-PAGE, and the recombinant peptide was detected by western blotting with an anti-FLAG antibody. **a** Time course of *L. lactis* growth monitored at OD_{600}. **b** Expression of the epitope peptide induced by the addition of 10 ng/mL nisin followed by 4-h culture at 30 °C. **c** Expression of the epitope peptide induced by the addition of 40 ng/mL nisin followed by 2-h culture at 30 °C. Bacterial concentrations (OD_{600}) at the time of nisin addition are indicated

Among LAB, *L. lactis* has been most widely used because introduction of the NICE system into *L. lactis* allowed for efficient control of target gene expression by nisin. Many studies on nisin-induced expression of vaccine antigens have been reported, including antigens against *Helicobacter pylori* (Zhang et al. 2009; Chen et al. 2011; Zhang et al. 2015; Zhang et al. 2016), influenza virus (Joan et al. 2016), *E. coli* O157:H7 (Ahmed et al. 2014), hepatitis A virus (Zhang et al. 2011; Berlec et al. 2013), *Brucella abortus* (Ribeiro et al. 2002), and *Streptococcus pneumoniae* (Medina et al. 2008). However, to the best of our knowledge, there has only been one previous report on the expression of Japanese cedar pollen allergens in LAB (Ohkouchi et al. 2012), in which Cry j 1 was expressed in *Lactobacillus plantarum*.

In the present study, the recombinant T cell epitope peptide expressed in *L. lactis* was confirmed to be immunogenic because it was recognized by anti-FLAG antibody and an antibody raised against a sequence present in the epitope peptide. The amount of epitope peptide expressed was dependent on the nisin concentration, culture time in the presence of nisin, and the bacterial concentration at the time of nisin addition. Similar properties of nisin-induced expression of antigens have been reported by Zhang et al. (2009). The optimal peptide expression conditions determined by this study are a culture time of 2 h in the presence of 40 ng/mL nisin, added at bacterial concentrations of OD_{600} 0.2–1.2.

When the recombinant T cell epitope peptide expression was induced for 4 h in the presence of higher nisin concentrations (80–160 ng/mL), the expression was slightly inhibited (Fig. 4b). The inhibitory effect of nisin was clearly apparent when the culture was continued for 10 h in the presence of 40 ng/mL nisin (Fig. 5b), although the inhibition was only slight at a nisin concentration of 10 ng/mL (Fig. 5a). These results indicate that exposure to high concentrations of nisin

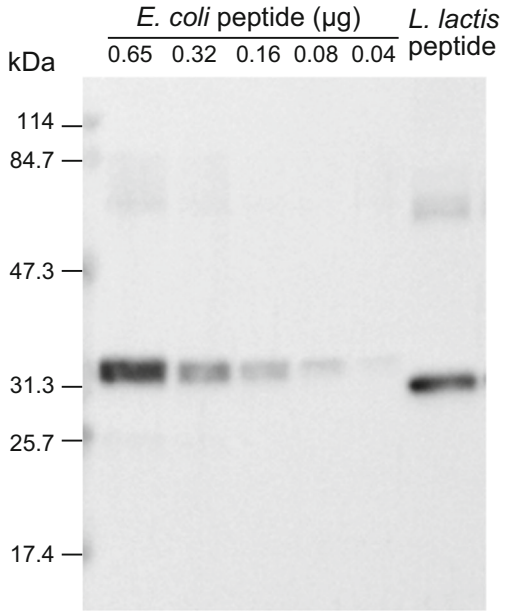

Fig. 7 Estimation of the epitope peptide concentration by western blotting. The expression of the epitope peptide by *L. lactis* was induced by 40 ng/mL nisin, and the peptide was detected with an anti-FLAG antibody. The purified epitope peptide from recombinant *E. coli* (0.65, 0.32, 0.16, 0.08, and 0.04 μg/lane, respectively) and the epitope peptide from *L. lactis* culture were used for western blotting

induces metabolic inhibition in *L. lactis*. This is not surprising because nisin is known to have antibacterial activity; to prevent this issue, amounts of nisin in the nanograms per milliliter range, which is subtoxic, are used for nisin-induced expression (Mierau et al. 2005).

Anti-FLAG antibody used as a primary antibody did not cross-react with *L. lactis* proteins in the absence of nisin (Fig. 4a). However, after peptide expression was induced by nisin, faint bands were detected by the anti-FLAG antibody in the upper and lower regions of the bands corresponding to the expressed peptide. Given that the density of these faint bands increased as the peptide expression progressed, these faint bands are likely to be products related to the expressed antigen peptide. Although the origin of these bands was not definitively confirmed, we speculate that the bands in the upper region are a dimer or an aggregate of the expressed peptide and the bands in the lower region are decomposed fragments of the peptide.

The yield of the epitope peptide in the present study was ~ 22 mg/L of culture medium, which was comparable with previously reported antigen yields of 27.26 mg/L (Zhang et al. 2009), 12.9 mg/L (Chen et al. 2011), and 8 mg/L (Ribeiro et al. 2002) in *L. lactis*. However, the epitope peptide yield in *L. lactis* was much smaller compared with the previously reported yield of ~ 120 mg/mL in *E. coli* (Hoang et al. 2015). Based on these results, we propose that the recombinant *L. lactis* constructed here is a potential candidate for vaccination against pollinosis. The efficacy of the epitope peptide will be elucidated in future clinical studies.

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Compliance with ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

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