TABLE 1. Cytokine-specific primer pair sequences used in PCR

Gene	Sense primer	Antisense primer	Size[a]	Reference
IL-1β	TCATGGGATGATGATGATAACCTGCT	CCCATACTTTAGGAAGACACGGATT	502	30
IL-2	TGATGGAGCTACAGGAGCTCCTGAG	GAGTCAAATCCAGAACATGCCGCAG	167	30
IL-4	CGAAGAACACCACAGAGAGTGAGCT	GACTCATTCATGGTGCAGCTTATCG	180	30
IL-5	GTGAAAGAGACCTTGACACAGCTG	CACACCAAGGAACTCTTGCAGGTA	290	10
IL-6	CTGGTGACAACCACGGCCTTCCCTA	ATGCTTAGGCATAACGCACTAGGTT	600	30
IL-10	ACCTGGTAGAAGTGATGCCCCAGGCA	CTATGCAGTTGATGAAGATGTCAAA	237	30
IL-12 p35	GCAAGAGACACAGTCCTGGG	TGCATCAGCTCATCGATGGC	618	30
IFN-γ	AGCGGCTGACTGAACTCAGATTGTAG	GTCACAGTTTTCAGCTGTATAGGG	243	30
TNF-α	GGCAGGTCTACTTTGGAGTCATTGC	ACATTCGAGGCTCCAGTGAATTCGG	307	30
TGF-β	TGGACCGCAACAACGCCATCTATGAG	TGGAGCTGAAGCAATAGTTGGTATCCAG	502	23
MIP-2	GCCAGTGAGCTGCGCTGTCAGTGC	GTTAGCCTTGCCTTTGTTCAGTATG	221	33
β-Actin	TGGAATCCTGTGGCATCCATGAAAC	TAAAACGCAGCTCAGTAACAGTCCG	348	30

[a] Predicted size of the PCR product (base).

Plasmid construction and transformation. For expression of murine IL-1β in *L. casei*, a plasmid was constructed from pIGM2, which was established in our previous study (13). Although the plasmid already included a promoter from *Lactobacillus brevis slpA* (*slpAp*) and a secretion signal of *L. casei prtP* (SS*prtP*) for heterologous expression and secretion, minor modification of the sequence was required in order to express the proper size of IL-1β. DNA fragments consisting of *slpAp*, SS*prtP*, and the IL-1β gene were generated by an overlap PCR technique. Total RNA was prepared from the spleen of a BALB/c mouse, followed by reverse transcription (RT) using Retroscript (Ambion). The IL-1β fragment was amplified from the total cDNA using primers IGM617 (GCG AAA TCC AAG CAA AGG CGG TTC CCA TTA GAC AAC TGC A) and IGM603 (CCC CCT CGA GCC GGC TTA GGA AGA CAC GGA TTC C). A DNA segment from *slpAp* to SS*prtP* was prepared by PCR with pIGM2 as the template and with primers IGM289 (CCC AAG CTT AGA TCT GAT TAC AAA GGC TTT AAG CAG G) and IGM618 (TGC AGT TGT CTA ATG GGA ACC GCC TTT GCT TGG ATT TCG C). The two resulting fragments were then connected by PCR with IGM289 and IGM603. The expression cassette of IL-1β was digested with BglII and XhoI and inserted into the same restriction site of pIGM2. Transformation of *L. casei* was performed by the method described previously (21).

Immunoblotting. Overnight cultures of recombinant lactobacilli were separated into bacterial cells and culture supernatants by centrifugation. The bacterial cells were washed with 50 mM Tris (pH 8.0) and treated with 5 mg/ml lysozyme and 20 U/ml of mutanolysin for 30 min. The spheroplasts were washed with 0.3 M sucrose in 50 mM Tris buffer, dissolved in Laemmli sample buffer, and boiled for 10 min. The culture supernatants were concentrated 50-fold using trichloroacetic acid, dissolved in Laemmli sample buffer, and boiled for 5 min. These samples were applied for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto a polyvinylidene fluoride filter. The blot was conjugated with anti-mouse IL-1β (Peprotech Inc.) and horseradish peroxidase (HRP)-labeled anti-rabbit IgG (Sigma). IL-1β-specific bands were visualized with ECL-plus (GE Healthcare) using a ChemiDoc system (Bio-Rad).

Quantitative determination of mIL-1β. Prewarmed MRS medium supplemented with erythromycin and bicarbonate buffer (MRSCE) (final pH, 7.0, 7.5, or 8.0) was inoculated with washed cells from 1/10 volume of overnight culture of recombinant lactobacilli. After 5 h of incubation, the culture supernatants were collected by centrifugation and sterilized using a 0.45-μm-pore filter (Millipore). The concentration of IL-1β in each culture supernatant was determined using an mIL-1β enzyme-linked immunosorbent assay (ELISA) kit (Biosource) in accordance with the manufacturer's instructions.

Caco-2 cell culture and stimulation with IL-1β. Caco-2 cells, purchased from the American Type Culture Collection, were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% fetal calf serum (FCS), 1% nonessential amino acids, 100 IU/ml of penicillin, and 100 μg/ml of streptomycin at 37°C in a 5% CO_2 incubator. A single-cell suspension was prepared by trypsin-EDTA treatment and seeded in a 98-well culture plate (2×10^4 cells/well). After overnight incubation, the culture supernatant of recombinant lactobacilli or purified recombinant murine IL-1β (Peprotech) was added to the Caco-2 culture, followed by an additional 16 h of incubation. The culture supernatants were then collected, and the amount of human IL-8 was determined using an OptEIA ELISA kit (BD Biosciences).

Mice. Female BALB/c mice and C3H/HeJ mice (8 to 10 weeks old) were purchased from Japan SLC (Shizuoka, Japan). The care and use of experimental animals complied with the Principal Law on Animal Experimentation of the National Institute of Health Sciences of Japan.

Ligated-intestinal-loop assay. BALB/c mice starved overnight were injected intraperitoneally (i.p.) with 0.1 ml of pentobarbital (80 mg/kg of body weight). The abdomen was incised, and the ileum was ligated with surgical thread approximately 0.5 cm each side of one Peyer's patch (PP). Recombinant lactobacilli (1×10^9 CFU/ml) suspended in MRSCE medium (pH 7.0) were injected into the ligated intestinal loop containing a single PP. The abdomen was then closed by sewing, and the mice were kept for 4 h before sacrifice. The loop was collected and washed extensively with phosphate-buffered saline (PBS). The single PP and a small piece of non-PP lamina propria (LP) in the loop were isolated and transferred to lysis buffer for RNA preparation. Total RNA was isolated using an SV Total RNA isolation system (Promega) in accordance with the manufacturer's instructions. The concentration of RNA was measured using a NanoDrop ND-1000.

RT-PCR. Total cDNA was prepared from 1 μg of RNA using Retroscript. PCR was then performed to detect IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 p35, gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), transforming growth factor beta (TGF-β), macrophage inflammatory protein 2 (MIP-2), and β-actin. The sequences of primers are shown in Table 1. The reaction conditions were 30 cycles of 94°C for 25 s, 50°C for 15 s, and 72°C for 40 s. The PCR products were applied to 2% agarose gel electrophoresis and visualized with ethidium bromide using a ChemiDoc gel documentation system.

Preparation and stimulation of PP cells. A single-cell suspension of PP was prepared from BALB/c mice in accordance with a protocol described previously (24). PP cells were prepared using collagenase type 1 (Sigma-Aldrich). The PP cells were then separated into $CD11c^+$ and $CD11c^-$ cells using a Magnetic Cell Separation (MACS) system (Miltenyi Biotec) in accordance with the manufacturer's instructions. The $CD11c^+$ and $CD11c^-$ cells were suspended in RPMI 1640 medium (Sigma) supplemented with 10% FCS and seeded in a 96-well microplate. The stimulants, which were recombinant *L. casei* cells and cell-free MRS culture supernatants, were added to the wells and incubated for 24 h. The culture supernatants of the PP cell cultures were collected and analyzed by ELISA. The quantity of IL-6 was measured using a mouse IL-6 ELISA kit (Biosource).

Immunization of mice. C3H/HeJ mice were previously optimized for immunization against SE (14). The mice were immunized i.g. with a mixture of 5×10^9 CFU/mouse of heat-killed SE (HKSE) and 5×10^9 CFU/mouse of viable recombinant *L. casei*. HKSE was prepared from an overnight culture. SE cells were collected, washed gently, and suspended in distilled water. The suspension was incubated at 60°C for 30 min and then freeze-dried. Complete inactivation of SE was confirmed by LB plate culture for a week. Recombinant *L. casei* for administration was prepared from overnight cultures. Prior to immunization, the lactobacilli were incubated in MRSCE (pH 8.0) for 1 h to enhance mIL-1β expression and then resuspended in fresh MRS supplemented with bicarbonate buffer (MRSC) (pH 8.0). A mixture of HKSE and recombinant lactobacilli was prepared by simple mixing of both suspensions in MRSC (2.5×10^{10} CFU/ml each; 0.2 ml/dose). Three consecutive daily doses were performed 3 times at 2-week intervals. The mice were sacrificed 2 weeks after the last immunization, and blood and feces were collected. Sera were prepared from blood samples by centrifugation. Fecal extracts were prepared as cleared supernatants of suspended feces in PBS (1:10). Both samples were stored at −20°C until they were used.

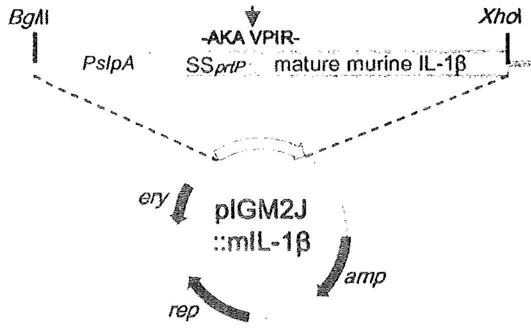

FIG. 1. Feature map of pIGM2J::mIL-1β. The structure, expression cassette, restriction sites, and cleavage point of the fusion protein (arrow) are shown. Genes: *amp*, ampicillin resistance; *rep*, replication protein; *ery*, erythromycin resistance.

Detection of SE-specific antibodies. SE-specific IgG in serum and IgA in fecal extracts were detected by ELISA. HKSE cells were homogenized in PBS using a FastPrep bead beater (Bio 101), and the cleared lysate was collected by centrifugation. The concentration of protein in the HKSE lysate was determined by a Bradford protein assay (Bio-Rad). A microtiter plate was coated overnight with 5 μg/ml (as a protein solution) of HKSE lysate. Properly diluted samples were then added to each well and incubated for 2 h. HRP-conjugated anti-mouse IgG (or IgA) was added and incubated for 1 h. A TMB Substrate Reagent Set (BD Biosciences) was used for color development, and 2 N H_2SO_4 was added to stop the reaction. The optical density at 450 nm was measured using a microplate reader.

Statistical analysis. Statistical significance ($P < 0.05$) was determined by Tukey's multiple-analysis test.

RESULTS

Secretion of IL-1β from recombinant *L. casei*. The ligated plasmid pIGM2J::m IL-1β, shown in Fig. 1, was introduced directly into *L. casei* by electroporation. The production of murine IL-1β by recombinant *L. casei* was confirmed by immunoblotting. As shown in Fig. 2, IL-1β-specific bands were detected in both cell extracts and the culture supernatants of recombinant *L. casei* (KJ725). A single band corresponding to the size of IL-1β fused to a signal peptide appeared in the lane of the cell extract of KJ725, while two bands were observed in the lane of the culture supernatant. The smaller band seemed to be the same size as purified IL-1β. No signal was detected from the control strain. The influence of the pH on IL-1β secretion was also determined. Approximately 1 μg/ml of IL-1β was secreted if the initial pH of the medium was between 7.0 and 8.0 (Table 2). However, only one-third of the IL-1β was detected without pH control.

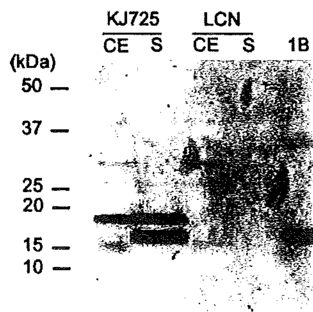

FIG. 2. Detection of IL-1β-specific bands by immunoblotting. Lysozyme-treated cell extracts (CE), concentrated culture supernatants (S), and 10 ng of purified IL-1β (1B) were applied to SDS-PAGE and immunoblotting. Anti-IL-1β antibody was used at 1:5,000, and HRP-conjugated anti-rabbit IgG was used at 1:50,000. Molecular masses are shown on the left.

TABLE 2. Yield of IL-1β in KJ725 culture with and without buffer

Culture condition	IL-1β yield (μg/ml)[a]
No buffer (pH 6.3)	0.326
Buffer (pH 7.0)	1.032
Buffer (pH 7.5)	0.966
Buffer (pH 8.0)	1.116

[a] Mean value of triplicate cultures.

Biological activity of the recombinant IL-1β. The biological activity of the recombinant IL-1β produced by KJ725 was determined by stimulation of the Caco-2 cells. Dose-dependent IL-8 release induced by IL-1β was observed in a Caco-2 cell culture stimulated with the supernatant of KJ725 (Fig. 3). Despite the same amount of IL-1β being used, the release of IL-8 by stimulation with KJ725 was less than that with purified IL-1β. No IL-8-inducing activity was detected by stimulation of Caco-2 cells with the culture supernatant of LCN.

Detection of cytokine expression by ligated-intestinal-loop assay. In order to evaluate the immunological impact of KJ725 *in vivo*, a ligated-intestinal-loop assay was performed. Preliminary experiments indicated that over 10^9 CFU/ml of bacterial suspension induced cytokine expression stably, and thus, it was applied in this study. The same experiment was repeated to normalize the results. Eleven kinds of cytokine expression in PP and LP were analyzed by RT-PCR. Specific bands of IL-1β, IL-6, IL-12, TNF-α, and TGF-β, with β-actin as an internal standard, were constantly detected in PP cells, while no bands, except for the band of β-actin, were detected in LP cells (Fig. 4).

Production of IL-6 by PP $CD11c^+$ cells. PP cells were separated into $CD11c^+$ cells and $CD11c^-$ cells using MACS. Each cell type was stimulated with the KJ725 cell or culture supernatant. As shown in Fig. 5, IL-6 was efficiently produced by

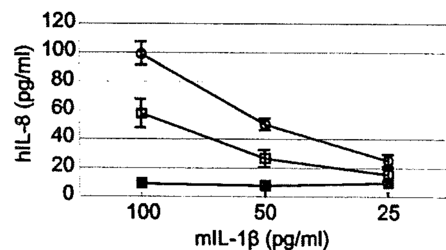

FIG. 3. Biological activity of recombinant murine IL-1β produced by KJ725. Human IL-8 released from Caco-2 cells was measured after 24 h of stimulation with culture supernatant of KJ725 (empty squares) or LCN (filled squares) or with purified murine IL-1β standard (empty circles). The concentration of total murine IL-1β in the culture supernatant of KJ725 (in MRSEC medium) was determined by ELISA and then adjusted to 100, 50, and 25 pg/ml by dilution in complete RPMI medium. The corresponding volume of culture supernatant of LCN was added to the Caco-2 cell culture as a negative control. The result shown is representative of three separate experiments; the values are the means ± standard deviations (SD) of duplicate samples.

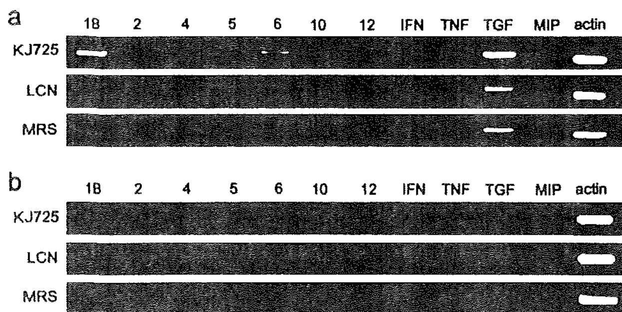

FIG. 4. Cytokine expression induced by ligated-intestinal-loop assay using RT-PCR. PCR products were applied to 2% agarose gels. Eleven kinds of cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, and IL-12p35 [lanes 1B to 12]; IFN-γ [IFN]; TNF-α [TNF]; TGF-β [TGF]; and MIP-2 [MIP]) expressed in PP cells (a) and LP cells (b) were detected by RT-PCR using specific primer pairs. As an internal control, a DNA fragment of β-actin (actin) was also amplified. The data represent at least three separate experiments.

CD11c$^+$ cells in response to both KJ725 cells and culture supernatant. Meanwhile, a low level of IL-6 was induced by stimulation with LCN cells and culture supernatant compared with KJ725 cultures (Fig. 6).

Enhancement of antibody production by IL-1β-producing *L. casei*. To evaluate the adjuvant effect of KJ725, i.g. immunization of mice was performed. HKSE was used as an immunogen and administered in combination with either KJ725 or LCN. As shown in Fig. 7, SE-specific antibodies were induced by i.g. immunization with HKSE. In the group that received HKSE and KJ725, the highest level of SE-specific IgG ($P < 0.05$ compared with the HKSE and LCN group or the MRS group) and IgA ($P < 0.05$ compared with the MRS group) was detected in their sera and feces.

DISCUSSION

The delivery of vaccine antigens by live bacterial carriers, both pathogenic and commensal bacteria, has been studied (17). Compared with vaccine delivery systems using attenuated pathogens, vaccines based on lactic acid bacteria require high doses, a high frequency of immunization, and highly immunogenic antigens for successful induction of protective immunity. For instance, *Lactobacillus plantarum* expressing *Borrelia burgdorferi* OspA was administered at 4 × 10^{10} cells in four consecutive daily doses with additional priming and two boosts (total, 16 days; 6.4 × 10^{11} cells), while an attenuated *Salmonella enterica* serovar Typhimurium producing OspA was inoculated at 10^8 organisms with a total of five weekly doses (total, 5 days; 5 × 10^8 cells) (5, 8). In this context, it is important to provide supplemental adjuvant effects for the development of vaccine using lactic acid bacteria. For this purpose, recombinant *L. casei* secreting mature murine IL-1β was constructed. Using the secretion system developed in our previous study, recombinant IL-1β conjugated with signal peptide was produced intracellularly, and a mature form of IL-1β was then secreted extracellularly. The results of immunoblotting indicated that the secretion system worked properly. At the same time, a considerable part of the extracellular IL-1β remained

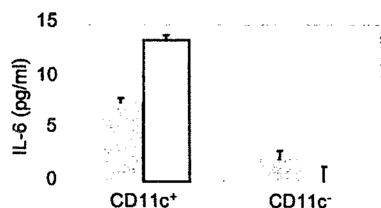

FIG. 5. IL-6 release by PP CD11c$^+$ or CD11c$^-$ cells. MACS-separated CD11c$^+$ or CD11c$^-$ cells (5 × 10^4 cells/well) were stimulated with KJ725 cells (solid bars; 5 × 10^5 CFU/well) or culture supernatant (open bars; 0.5%). After 24 h of incubation, samples were collected and released IL-6 was detected by ELISA. The results shown are representative of three separate experiments; the values are means and SD.

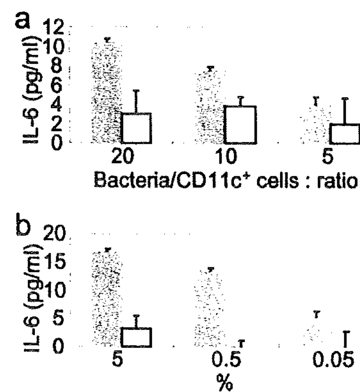

FIG. 6. IL-6 released from PP CD11c$^+$ cells. MACS-separated CD11c$^+$ cells (5 × 10^4 cells/well) were stimulated with recombinant *L. casei* cells (bacteria/CD11c$^+$ cell ratios, 20, 10, and 5) (a) or culture supernatant (5%, 0.5%, and 0.05% diluted in RPMI 1640 medium) (b). After 24 h of incubation, samples were collected and released IL-6 was detected by ELISA. The results are representative of three separate experiments; the values are means and SD. Solid bars, KJ725 cells/supernatants; open bars, LCN cells/supernatants.

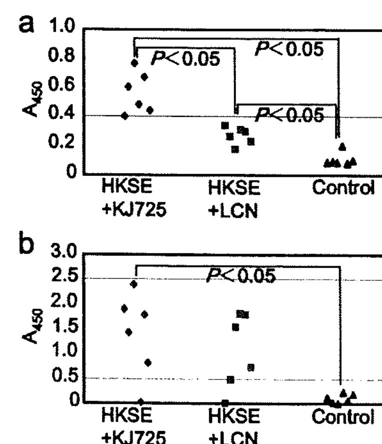

FIG. 7. SE-specific antibody production induced by HKSE with either KJ725 or LCN. Anti-SE IgG in 100-fold-diluted serum (a) and anti-SE IgA in 50-fold-diluted cecum lavage fluid (b) were detected by ELISA. Each value represents the absorbance at 450 nm (A_{450}). The immunization groups are shown at the bottom. Statistical significance was accepted at $P < 0.05$. The control was a group that received MRSC medium alone.

as a fusion protein. This protein seemed to be leaked or secreted without the involvement of signal peptidase. Further investigations are required to explain the phenomenon. The amounts of IL-1β production were measured under different pH conditions, because IL-10 production using the same system was found to be pH dependent in the previous study (13). The results showed that an approximately 3-fold-larger amount of the protein was produced when carbonate buffer was added to the culture. Considering this characteristic, MRS medium was supplemented with the buffer when the recombinant strains were applied to the *in vivo* assay. The biological activity of IL-1β was determined by the stimulation of Caco-2 cells as described previously (9, 25). In response to IL-1β produced by the recombinant *L. casei*, IL-8 was released from the cell culture. The activity of IL-1β from recombinant bacteria was approximately 60% of that of purified IL-1β. Because the amino acid sequence of the N terminus of IL-1β is critical for its biological activity, extra peptides at the N terminus drastically impaired the activity (12). In fact, the biological activity was lost by adding only 5 additional amino acids (DTNSD) at the N terminus of IL-1β (data not shown). As described above, part of IL-1β secreted by recombinant *L. casei* was conjugated with the signal peptide. Hence, contamination by inactive IL-1β resulted in lower biological activity.

The immunological properties of the recombinant *L. casei* producing IL-1β were analyzed *in vivo* using a ligated-intestinal-loop assay. In this assay, the expression of cytokine genes was detected only in the PP. The result of cytokine profiling by RT-PCR showed that the expression of IL-6 seemed to be upregulated. In order to confirm the induction of IL-6, PP cells were prepared and stimulated with IL-1β-secreting lactobacilli. The result showed that IL-6 was efficiently released from $CD11c^+$ cells by stimulation with both IL-1β-secreting bacteria and the culture supernatants in a dose-dependent manner. IL-6 is produced by monocytes, macrophages, dendritic cells, etc., and enhances antibody production from B cells (15, 20). Moreover, it was reported that $CD11c^+/B220^-/CD11b^+$ Peyer's patch DCs secrete IL-6 and induce antibody production from naïve B cells (24). These findings suggested that IL-1β-secreting lactobacilli could be applied in vaccination as an adjuvant that increases antibody production. In order to evaluate this effect, the immunization of mice was performed using HKSE in combination with the recombinant *L. casei* strains. The result showed that comparatively large amounts of SE-specific antibodies, both IgG and IgA, were achieved by immunization with HKSE plus IL-1β-secreting lactobacilli. This result suggested that the recombinant strain had adjuvant effects *in vivo*. If purified IL-1β was administered orally or intragastrically, it could be degraded by the digestive process in the gastrointestinal (GI) tract. Meanwhile, IL-1β released by the recombinant *L. casei* could be delivered directly to the GI tract with less exposure to gastric acid, bile, and digestive enzymes. This is an advantageous feature of adjuvant delivery using recombinant lactobacilli. Because IL-1β is a proinflammatory cytokine, there may be a risk of side effects. In this study, however, apparent side effects, such as weight loss and diarrhea, were not observed.

Several kinds of cytokines have been produced by lactic acid bacteria to complement vaccine efficacy (2, 28). The present study demonstrated adjuvant effects of recombinant *L. casei* secreting biologically active IL-1β. Although it is known that IL-1β can be used as a mucosal adjuvant, it has not been reported previously that intragastrically administered recombinant lactobacilli secreting cytokines exhibited an adjuvant effect. The recombinant strain developed in this study could be a powerful tool to improve the efficacy of vaccines based on lactic acid bacteria or other delivery agents that have relatively weak immunogenicity.

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Development of a Highly Efficient Protein-Secreting System in Recombinant *Lactobacillus casei*

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The available techniques for heterologous protein secretion in *Lactobacillus* strains are limited. The aim of the present study was to develop an efficient protein-secretion system using recombinant lactobacilli for various applications such as live delivery of biotherapeutics. For the construction of expression vectors, the *Lactobacillus brevis slpA* promoter, *Lactobacillus casei prtP* signal sequence, and mouse IL-10 sequences were used as a model system. Interestingly, the *slpA* promoter exhibited strong activity in *L. casei*, contrary to previous observations. In order to stabilize replication of the plasmid in *E. coli*, a removable terminator sequence was built into the promoter region. For the improvement of secretion efficiency, a DTNSD oligopeptide was added to the cleavage site of signal peptidase. The resulting plasmids provided remarkably efficient IL-10 secretion. Accumulation of the protein in the culture supernatant varied widely according to the pH conditions. By analysis of the secreted protein, formation of homodimers, and biological activity, IL-10 was confirmed to be functional. The presently constructed plasmids could be useful tools for heterologous protein secretion in *L. casei*.

Keywords: *Lactobacillus*, secretion, IL-10, *slpA*

Generally, lactic acid bacteria (LAB) are regarded as safe and useful commensal bacteria, which are known as probiotics and starters for food fermentation. Recently, several recombinant LAB secreting heterologous proteins have been developed as live delivery agents for biotheraputic applications using genetic modification techniques. For instance, *Lactococcus lactis* secreting biologically active interleukin 10 (IL-10) was established for the treatment of inflammatory bowel diseases in a murine model [21]. The successful study thereafter progressed to a clinical trial in humans [3]. Delivery of anti-infectives by LAB is also under investigation. For prevention of the transmission of human immunodeficiency virus type 1 (HIV-1), recombinant *L. lactis* and *Lactobacillus plantarum* that secrete microbiocidal cyanovirin-N were constructed and were capable of neutralizing the infectivity of HIV-1 *in vitro* [17]. Secretion of human CD4 in a strain of *Lactobacillus jensenii* has also been explored for the prevention of HIV-1 infection [4]. These studies suggested that protein-secreting systems in LAB could be useful and offer a promising strategy for medical applications in the future.

In methodological terms, the yield of secreted protein is preferred to be high for better effects. Hence, strong promoters should be used for the expression of heterologous genes. It is also known that specific amino acid sequences flanking signal peptides can enhance the efficiency of protein secretion [9, 10]. Using these strategies, several highly efficient secretion systems have been developed in LAB [17]. However, such systems are considered to be available in only a few specific strains because host–vector systems in LAB are limited and the activities of LAB promoters may be strain dependent. Moreover, the properties of LAB *in vivo*, such as immunomodulating activities and persistence in the gastrointestinal tract, are different among LAB strains [12, 13]. Therefore, a specific protein-secretion system has to be optimized for each LAB strain that is applied for a live delivery agent.

Lactobacillus casei IGM393, a subculture of ATCC 393 (pLZ15$^-$), is considered to be synonymous with *L. casei* BL23 [1]. IGM393 and its relatives have been used as a host for genetic modification and applied for live delivery agents. For example, the single chain variable fragment of an antibody that binds to a major adhesion molecule of *Streptococcus mutans* was expressed on the cell surface and showed protective effects against colonization with the pathogen [8]. Other studies investigated the efficacies of strains engineered to produce heterologous antigens for the prevention of an allergic disorder, an autoimmune disease,

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and an infectious disease [5, 7, 11]. Thus, it is clear that the development of a highly efficient protein-secreting system in this strain would be important and beneficial. For this purpose, the present study attempted to construct a plasmid with a expression cassette consisting of a strong promoter, a signal sequence, and a heterologous model protein. An *E. coli–Lactobacillus* shuttle vector plasmid, pLP402, established by Pouwels *et al.* [16], was used as the base plasmid. In a preliminary study, a strong promoter from the *slpA* gene (P_{slpA}) of *Lactobacillus brevis* was replaced with P_{amy} of pLP402; however, disruption of the plasmid was observed during cloning in *E. coli* (unpublished data). Such instabilities occasionally occur owing to promoters from LAB [2, 15, 23]. A terminator placed downstream of such a promoter could improve its stability in some cases but did not work in this case. In order to solve this problem, a removable terminator sequence was inserted into P_{slpA}. This modification drastically improved the stability of the plasmid in *E. coli* and retained the activity of the promoter after the terminator sequence was removed (unpublished data). Subsequently, an autologous signal sequence, *prtP*, which is a well-characterized extracellular protein, was connected to P_{slpA} to drive secretion of the protein. It was reported that a net negative charge followed by a signal peptidase cleavage site improved the secretion efficiency of proteins. In this study, a peptide sequence (DTNSD), which was used in a study by Pusch *et al.* [17], was inserted between the signal peptide of PrtP and the model protein, which was mouse IL-10. IL-10 is a regulatory cytokine, produced by lymphocytes and previously expressed in *L. lactis* as mentioned above. Considering its usefulness, the construction of recombinant *L. casei* producing and secreting a high yield of IL-10 seems to be beneficial. Moreover, *L. casei* might be a better live delivery agent because the optimal growth temperature of *L. casei* is the same as the body temperature of mammals, whereas that of *L. lactis* is 30°C. This paper shows the development of a highly efficient protein-secretion system in *L. casei*. The biological activity of IL-10 was tested, and formation of homodimers as well, as the influence of pH conditions in the bacterial culture, were also determined.

Materials and Methods

Bacterial Strains and Growth Conditions

L. casei IGM393 and recombinant strains were grown in Mann–Rogosa–Sharp (MRS) medium at 37°C. *L. brevis* JCM 1559 was purchased from the Japan Collection of Microorganisms (RIKEN BioResource Center, Saitama, Japan) and grown at 32°C. A recombinant *L. casei* strain harboring pLPEmpty, LCN, was used as a reference strain [7]. Erythromycin (5 μg/ml) was added to MRS (MRSE) for the selection of recombinant *L. casei* strains. For pH control, 50 mM carbonate buffer was supplemented into the medium at certain ratios ($NaHCO_3$:Na_2CO_3). The commonly used cloning host of *E. coli* strain JM109 (TaKaRa, Shiga, Japan) was grown in LB with or without 100 μg/ml ampicillin.

Preparation of Total cDNA from Mouse

A 9-week-old female BALB/c (Japan SLC, Shizuoka, Japan) mouse was sacrificed and the spleen was collected. Total RNA from the spleen was isolated using an RNeasy mini kit (Qiagen, Tokyo, Japan). Total cDNA was prepared by reverse transcription with RETROscript (Ambion, TX, U.S.A.) in accordance with the manufacturer's instructions. The care and use of experimental animals complied with local animal welfare laws and guidelines.

Construction of Plasmids and Transformation of *L. casei*

The sequences of all PCR primers used in this study are listed in Table 1. PCR products and plasmids were digested with restriction endonucleases followed by ligation and cloning in *E. coli* JM109. A high copy number plasmid for *E. coli*, pUC19, was used for cloning of *L. brevis PslpA*. An *E. coli–Lactobacillus* shuttle vector, pLP402, was the plasmid for expression. The detailed methodology for plasmid construction is combined with the Result section. The constructed plasmids were introduced into *L. casei* by electroporation. The method for the preparation of competent cells of *L. casei* and the pulse conditions for electroporation were as described previously [16].

Table 1. PCR primers used in this study.

Primers	Sequences	Restriction sites
IGM289	cccaagcttagatctgattacaaaggctttaagcagg	*Hin*dIII, *Bgl*II
IGM290	gggctcgaggcccggttgttcgcggccgcttttgttaagaattttatttcataacattagcgg	*Xho*I, *Not*I
IGM291	cccgtcgacgcccggttgttcgcggccgcttcggttatactattcttgcttgata	*Sal*I, *Not*I
IGM292	ggggaattcctgcagggatccaaacttgattgcataatctttcttcc	*Bam*HI, *Pst*I, *Eco*RI
IGM350	acatattttatgtttggagggtattggatg	
IGM351	catccaataccctccaaacataaaatatgt	
IGM468	ccccggatccgagcaggggccagtacagccg	*Bam*HI
IGM478	ccccctcgagttagcttttcattttgatcatcatgta	*Xho*I
IGM479	gcgaaatccaagcaaaggcgagcaggggccagtacagccg	
IGM480	cggctgtactggcccctgctcgcctttgcttggatttcgc	
IGM482	cggctgtactggcccctgctcggatccgagtttgtgtccgcctttgcttggatttcgc	*Bam*H I

Underlines indicate restriction sites.

Culture of Recombinant *L. casei* Under pH Control

Recombinant *L. casei* strains were grown in MRSE overnight. Bacterial cells were collected and washed with PBS, and the concentration of the cell suspensions was adjusted to 1×10^9 CFU/ml. Prewarmed MRSE and MRSE, the pH of which was adjusted to 6.5, 7.0, 7.5, 8.0, and 8.5, were inoculated with overnight culture (2×10^8 CFU/ml final concentration) and incubated for 5 h. The cultures were chilled on ice immediately after the incubation. The CFU count after the incubation was determined by a general plate-culture method. Cleared culture supernatants were collected by centrifugation followed by 0.22-µm-pore filter sterilization.

SDS–PAGE and Immunoblotting

The cleared supernatants were concentrated 10-fold using trichloroacetic acid or ultrafiltration (Microcon 10; Millipore) and dissolved in Laemmli buffer. Proteins were separated by 10–20% gradient SDS–PAGE and electrically blotted onto a PVDF membrane. Specific signals were detected with anti-mouse IL-10 (PeproTech, London, U.K.) and Alexa Fluor 488 anti-rabbit IgG (Molecular Probes) antibodies. The specific band of IL-10 was visualized and analyzed using a Molecular Imager FX and Quantity One (BIO-RAD, Tokyo, Japan).

Biological Activity of mIL-10

The biological activity of mIL-10 was assessed by the stimulation of mouse MC/9 mast cells as described previously [25]. MC/9 cells were purchased from the American Type Culture Collection (ATCC) and maintained in high glucose (4.5 g/l) Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich Japan, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS), 10% rat T-STIM (Japan BD, Tokyo, Japan), 0.05 mM 2-mercaptoethanol, and penicillin/streptomycin (complete DMEM) at 37°C in 5% CO_2. For the proliferation assay, MC/9 cells were washed with complete DMEM without rat T-STIM (incomplete medium) and suspended in incomplete medium supplemented with 5 ng/ml of recombinant mouse IL-4 (PeproTech EC, London, U.K.). The cell suspension was seeded into a 96-well flat-bottom microplate at 5×10^3 cells/well.

The recombinant lactobacilli were incubated for 5 h in DMEM supplemented with erythromycin. The culture supernatant was collected by centrifugation and sterilized using a 0.22-µm-pore filter. The concentration of murine IL-10 in the cleared supernatant was determined by ELISA. The culture supernatants, supplemented with 10% FBS, were added to the 96-well plate containing MC/9 cells. After incubation for 48 h, the viability of the MC/9 cells was determined using a CellTiter-Blue Cell Viability Assay (Promega KK, Tokyo, Japan) in accordance with the manufacturer's instructions. Resazurin solution was added to the culture and incubated at 37°C for 4 h. The level of resorufin, which is derived from resazurin, was measured using a fluorescence microplate reader (excitation: 530 nm; emission: 620 nm). Units of IL-10 activity were calculated by comparing with MC/9 culture stimulated with purified IL-10 standards (BioLegend, CA, U.S.A.). The ED_{50} of purified IL-10 was approximately 10 pg/ml in these assay conditions, which corresponded to 10^8 units/mg.

Cytokine Quantification by Enzyme-Linked Immunosorbent Assay (ELISA)

Mouse IL-10 secreted from recombinant lactobacilli was quantified using mouse IL-10 Instant ELISA (Bender MedSystems, CA, U.S.A.), and mouse TNF-α released from RAW264.7 cells was detected using OptEIA (BD Pharmingen, CA, U.S.A.) in accordance with the manufacturer's instructions. Cleared supernatants from recombinant *L. casei* culture and medium from RAW264.7 cells were properly diluted and applied to the assay. The concentration of cytokine was calculated from the standard curve.

RESULTS

Construction of Plasmids

A construction-flow diagram of the plasmid vectors is shown in Fig. 1. The amplicons, primer pairs, and template DNA for amplification by PCR are shown in Table 2. The DNA fragment encoding murine IL-10 was amplified from total cDNA of mouse spleen and inserted into the *Bam*HI–*Xho*I sites of pLP402 (pLP402::mIL-10). In order to build a terminator inside of P_{slpA}, the promoter was divided into

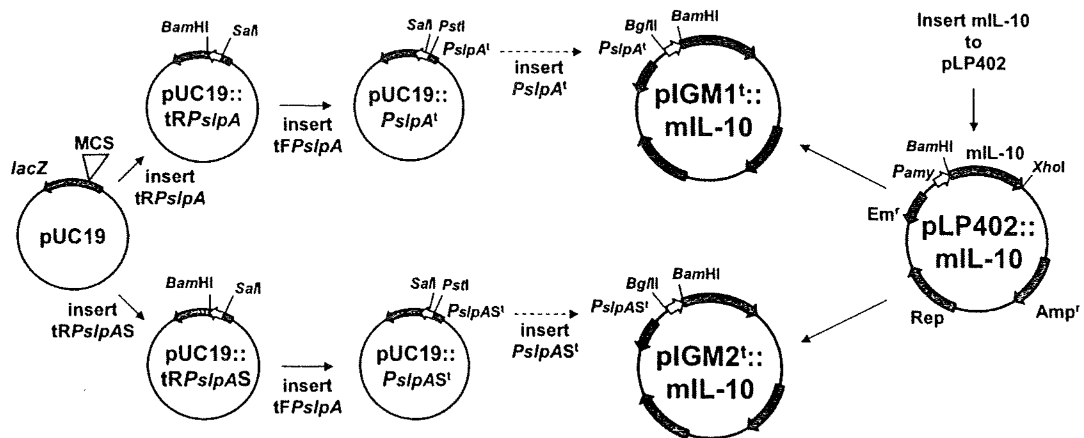

Fig. 1. Construction-flow diagram of expression vectors.

MCS: multiple cloning site; Rep: replication protein; Em^r: erythromycin resistance; Amp^r: ampicillin resistance.

Table 2. PCR amplicons generated in this study.

Amplicons	Primer pairs	Template DNA
mIL-10	IGM468, IGM478	Total cDNA of mouse spleen (BALB/c)
tR*PslpA*	IGM291, IGM292	*L. brevis* JCM 1559 chromosomal DNA
tR*PslpA*SF	IGM291, IGM351	*L. brevis* JCM 1559 chromosomal DNA
tR*PslpA*SR	IGM350, IGM482	*L. casei* IGM393 chromosomal DNA
tR*PslpA*S	IGM291, IGM482	tR*PslpA*SF and tR*PslpA*SR
tF*PslpA*	IGM289, IGM290	*L. brevis* JCM 1559 chromosomal DNA
*PslpA*SF	IGM289, IGM480	pUC19::*PslpA*St
mIL-10RE	IGM479, IGM478	Total cDNA of mouse spleen (BALB/c)
PslpA-mIL-10	IGM289, IGM478	*PslpA*SF and mIL-10RE

two fragments and amplified separately by PCR using primers IGM290 and IGM291 that included artificial sequences designed to generate the terminator. The terminator sequence was based on that of the *ldh* gene (T_{ldh}) of pLP402 and modified to eliminate mismatches. The downstream fragment of P_{slpA} was connected to the signal sequence of *prtP* (tR*PslpA*S), which was generated by overlap PCR using tR*PslpA*SF and tR*PslpA*SR as the template, and inserted into the *Bam*HI–*Sal*I sites of pUC19 (pUC19::tR*PslpA*S). The primer for tR*PslpA*SR amplification (IGM482) included a sequence encoding the DTNSD oligopeptide. For intracellular expression, P_{slpA} without a signal sequence (tR*PslpA*) was also cloned into the plasmid (pUC19::tR*PslpA*). The remaining upstream region of *PslpA* (tF*PslpA*) was generated and digested with *Hin*dIII–*Xho*I and inserted into the *Hin*dIII–*Sal*I sites of pUC19::tR*PslpA* and pUC19::tR*PslpA*S. The resulting plasmids (pUC19::*PslpA*ᵗ and pUC19::*PslpA*Sᵗ) including the terminator inside of the promoter were constructed. The modified P_{slpA} region was then collected by digestion with *Bam*HI and *Bgl*II, followed by insertion into the same sites of the pLP402::mIL-10 (pIGM1ᵗ::mIL-10 and pIGM2ᵗ::mIL-10). The expression cassette to secrete the exact mIL-10 product was generated by overlap PCR from two DNA templates, *PslpA*SF and mIL-10RE. The amplified expression cassette was then inserted into the *Bgl*II–*Xho*I sites of pLP402 (pIGM2ᵗJust::mIL-10). A map of the expression cassette and the differences between pIGM2ᵗ::mIL-10 and pIGM2ᵗJust::mIL-10 is shown in Fig. 2.

Expression of IL-10 in Recombinant *L. casei*

The plasmids, pIGM2ᵗJust::mIL-10 and pIGM2ᵗ::mIL-10, were digested with *Not*I and then self-ligated in order to remove the terminator. By electroporation, two recombinant *L. casei* strains, EK3 (harboring pIGM2Just::mIL-10) and EK7 (harboring pIGM2::mIL-10), were obtained. Under normal or pH-control conditions, the expression and secretion levels of mIL-10 by EK3 and EK7 were determined. IL-10-specific bands were detected from the supernatants of EK3 and EK7, and thus expression and secretion of mIL-10 were confirmed (Fig. 3A). Interestingly, the intensity of the specific signals depended on the pH conditions, which indicated that the efficiency of production or secretion was affected by pH. Relatively high secretion levels were achieved at pH 7.0–8.0 in EK3 and pH 6.5–8.5 in EK7, whereas weak signals were detected under normal (pH 5.5) conditions. In order to investigate whether mIL-10 secreted from recombinant strains can form a homodimer, culture supernatants concentrated slightly by ultrafiltration were analyzed by nonreducing SDS–PAGE and immunoblotting. As shown in Fig. 3B, dual bands were detected from the lanes of EK3, EK7, and purified IL-10. The larger band

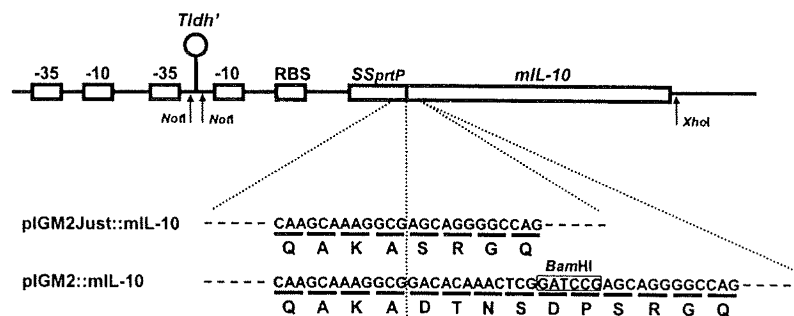

Fig. 2. Expression cassette of pIGM2Just::mIL-10 and pIGM2::mIL-10.
Features of the expression cassette are shown. Nucleotides and amino acid sequences of *SSprtP*–mIL-10 junctions are also shown. RBS: ribosome binding site; *SSprtP*: signal sequence of *prtP*.

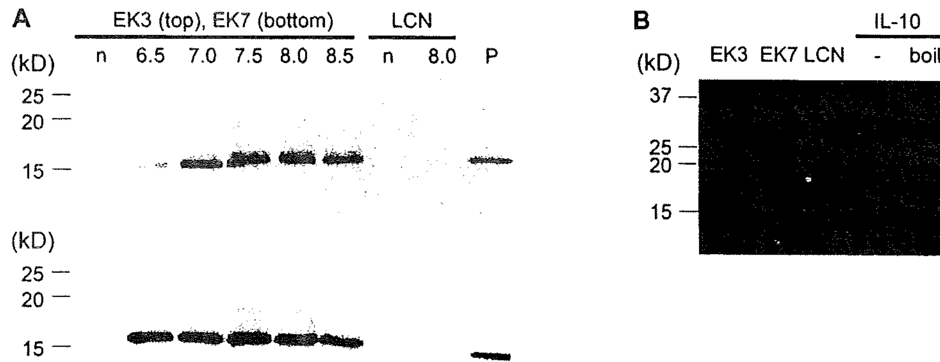

Fig. 3. Analysis of mIL-10 secreted by recombinant *L. casei*.
A. mIL-10 secretion under pH control. Proteins were concentrated by TCA, dissolved in Laemmli buffer, and boiled for 5 min. Molecular mass (left margin) and pH (top margin) are shown. n: normal conditions (no pH control); P: purified mouse IL-10, 10 ng/lane. **B.** Detection of mIL-10 homodimer. Proteins were concentrated by ultrafiltration and mixed with Laemmli buffer without 2-mercaptoethanol (2-ME). Boiled (with 2-ME) purified IL-10 is also shown for reference.

was approximately double the size of the smaller band; thus, mIL-10 produced by EK3 and EK7 could form homodimers.

Quantification of mIL-10 Secreted by Recombinant Strains

The amount of mIL-10 in the culture supernatants was measured by ELISA. The values of each sample are listed in Table 3. Under normal culture conditions (without buffer), mIL-10 was barely detected from EK3 and EK7 cells. However, pH control of the culture increased mIL-10 secretion levels, which varied drastically depending on the pH. The highest amount of secretion was achieved at pH 8.0 in EK3 and at pH 7.5 in EK7. On the other hand, the mIL-10 content in the culture supernatant was relatively low at pH values under 6.5 or over 8.5. Because growth rates differed among pH conditions, the protein-secreting efficiency was also calculated. As a result, the maximum efficiency was achieved at pH 8.0 in both EK3 and EK7 cells. In each culture condition, the secretion efficiency of EK7 was much higher than that of EK3.

Table 3. Quantification of secreted mIL-10.

Initial pH	Concentration of IL-10, ng/ml (ng/10^8 CFU)	
	EK3	EK7
5.5 (without buffer)	6 (1.2)	8 (1.8)
6.5 (with buffer)	24 (4.8)	354 (77.4)
7.0 (with buffer)	79 (16.5)	543 (132.3)
7.5 (with buffer)	97 (27.9)	615 (167.1)
8.0 (with buffer)	144 (60.6)	501 (231.0)
8.5 (with buffer)	46 (32.1)	189 (132.6)

Values represent the mean of duplicate assays. Results are representative of three separate experiments.

Biological Activity of Recombinant IL-10

MC/9 cells were stimulated with mIL-10 produced by recombinant lactobacilli and the proliferation of mast cells was determined. As shown in Fig. 4, the supernatants of both EK3 and EK7 provided specific activity for MC/9 proliferation. The concentration of mIL-10 in each culture was 1.1 ng/ml for EK3, 1.6 ng/ml for EK7, and not detected from LCN. The titer of biological activity was calculated compared with the activity of purified IL-10 (1×10^8 units/mg). As a result, the titers of active IL-10 in EK3 and EK7 cultures were defined as 6×10^6 units/mg.

DISCUSSION

Live delivery systems using LAB appear to be promising for medical applications. The present study attempted to

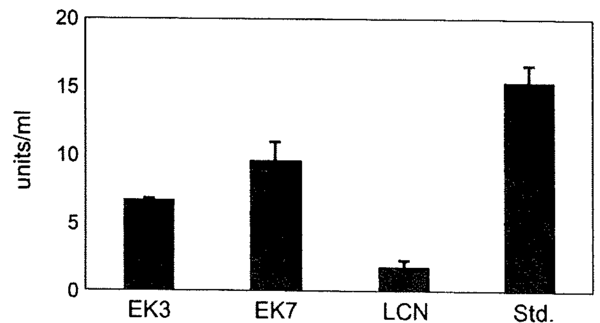

Fig. 4. Biological activity of mIL-10 produced by recombinant lactobacilli (n=4).
MC/9 cells were stimulated with culture supernatants of recombinant lactobacilli (50% volume). Each bar represents the mean value plus standard deviation. The concentration (units/ml) of each sample was calculated using a standard curve. Std. (standard): 125 pg/ml of purified IL-10.

develop a highly efficient protein-secretion system in *L. casei* using the *slpA* promoter, a *prtP* signal peptide, and a DTNSD motif. The base plasmid pLP402 contains removable T_{ldh}, flanking *Not*I sites on both sides, to stabilize the plasmid during cloning in *E. coli.* The terminator sequence was thus preserved during cloning; however, the plasmid, the promoter of which was replaced with P_{slpA}, was extremely unstable. This result suggested that cloning of P_{slpA} in this plasmid required stricter repression of the promoter activity. It was hypothesized that a strong terminator set inside of P_{slpA} could improve the stability of the plasmid. It is known that P_{slpA} of *L. brevis* consists of dual tandem −35 and −10 regions [26]. A terminator was built between the downstream −35 and −10 sites, and hence the downstream −35 and −10 region was inactivated and the upstream −35 and −10 region was still active, but transcription could be blocked by the downstream terminator. Because this artificial terminator was also flanked with *Not*I sites on both sides, the terminator could be removed easily and the same distance between the −35 and −10 site as that of the original P_{slpA} could be recovered. Plasmids constructed in this study, pIGM2'Just::mIL-10 and pIGM2'::mIL-10, showed good stablity. Therefore, this strategy could be applicable for the cloning of other LAB promoters that are unstable in *E. coli.*

In a previous study, the *L. brevis slpA* promoter was applied for heterologous protein expression but showed poor functionality in *L. casei* ATCC393 [18]. Contrary to the previous report, the present study showed that P_{slpA} exhibited high expression levels in *L. casei* IGM393. This opposing result is surprising because *L. casei* IGM393 is considered to be synonymous with ATCC393 as well as BL23 [1]. Moreover, *L. brevis* JCM 1559 is the same strain as ATCC 8287, which means the same P_{slpA} was used in both studies. The P_{slpA} in the present study was modified; however, it was observed that P_{slpA} worked equally well in *L. casei* even if the promoter was not modified (unpublished data). Thus, the genes encoding the heterologous proteins and the vector plasmids are the only major differences between the previous (*bla* with pKTH2121, pWV01 replicon) and the present (*IL-10* with pIGM2, p353-2 replicon) studies. Although it is difficult to define which factor is attributable to this difference in expression at present, *L. brevis* P_{slpA} can be applied for a highly efficient expression system in *L. casei.*

The S-layer protein originally had a signal peptide, and thus it was firstly attempted to use this sequence; however, heterologous protein production, but no secretion, was observed (unpublished data). This result suggested that the secretion signal of *slpA* may not be functional in *L. casei.* In order to solve this problem, the sequence encoding the secretion signal of *L. casei prtP* was applied for vector construction. The DNA sequence encoding the signal peptide and RBS of *prtP* was connected to the modified promoter. By this strategy, expression and secretion of mouse IL-10 were finally achieved using both pIGM2Just::mIL-10 and pIGM2::mIL-10. As expected, the protein conjugated to the DTNSD motif appeared in culture supernatant much more than IL-10 without the oligopeptides. This peptide sequence was originally from lactococcal secreted protein Usp45 [17]. The result of this study indicated that the DTNSD motif is functional in *L. casei* as well. Another oligopeptide providing a global net charge of −2 is also known to increase secretion efficiency [10]. As predicted in this paper, these oligopeptides are probably available for most LAB strains, which share the same protein-secreting mechanism.

In nature, IL-10 fulfills its physiological function as a homodimer [24, 27]. Thus, IL-10 produced by recombinant *L. casei* was analyzed to determine whether the dimeric protein can be formed. Immunoblotting analysis under nonreducing conditions showed that IL-10-specific bands were detected at both monomeric and dimeric sizes. This result indicated that the protein expressed by the recombinant strain could form IL-10 homodimers. The result also demonstrated that the DTNSD motif, the extra peptide connected to the N-terminus of recombinant IL-10, did not interfere with the formation of dimeric proteins.

Schotte *et al.* [19] reported that the secretion level of IL-10 depended on the pH conditions of the medium. In the present study, a similar effect was observed even though the expression system and host strain were different. This result suggested that the absence of IL-10 at low pH may be attributed to a characteristic of IL-10, although its mechanism is still not clear. It is known that lower pH affects the conformation of IL-10 and dissociates the homodimeric protein into a monomeric form [22], which might be subjected to breakdown in bacterial culture. Lactic acid bacteria produce proteases, the activity of which is high in acidic conditions [14, 20]. Hence, the recombinant IL-10 might be degradated by such acid proteases produced by *L. casei.* The P_{slpA} promoter could be pH sensitive as well. The amount of mRNA of *L. brevis slpA* is high at the exponential phase, whereas it is low at the stationary phase [6]. The pH at the stationary phase was lower than that at the exponential phase, and thus the activity of the *L. brevis slpA* promoter may be pH dependent. In this study, the maximum IL-10 yield was achieved at pH 7.5−8.0 by the secretion system with the DTNSD motif. Under optimal conditions, the efficiency of secretion during 5 h was estimated at >2 μg/10^9 CFU. This yield was much higher than that of recombinant *L. lactis* constructed in a previous study, which was approximately 0.6 μg/10^9 CFU [21]. The secretion system developed in this study exhibits remarkably high efficiency. In order to detect the biological activity of IL-10, MC/9 cells were stimulated with the culture supernatants of recombinant lactobacilli. As a result, the proliferation of MC/9 cells was

induced by the released mIL-10, which indicated that the secreted protein was biologically active. Both mIL-10 cultures showed similar activity levels regardless of the DTNSD motif, although the titers of the biological activity were less than that of the reference IL-10 standard.

In summary, the presently constructed plasmids (1) are expression vectors for *L. casei* based on pLP402, (2) multiply stably in *E. coli* owing to a removable terminator, (3) provide a high level of heterologous protein expression under the control of P_{slpA}, (4) induce secretion of the protein by means of the signal peptide of PrtP, and (5) accelerate secretion using the DTNSD motif.

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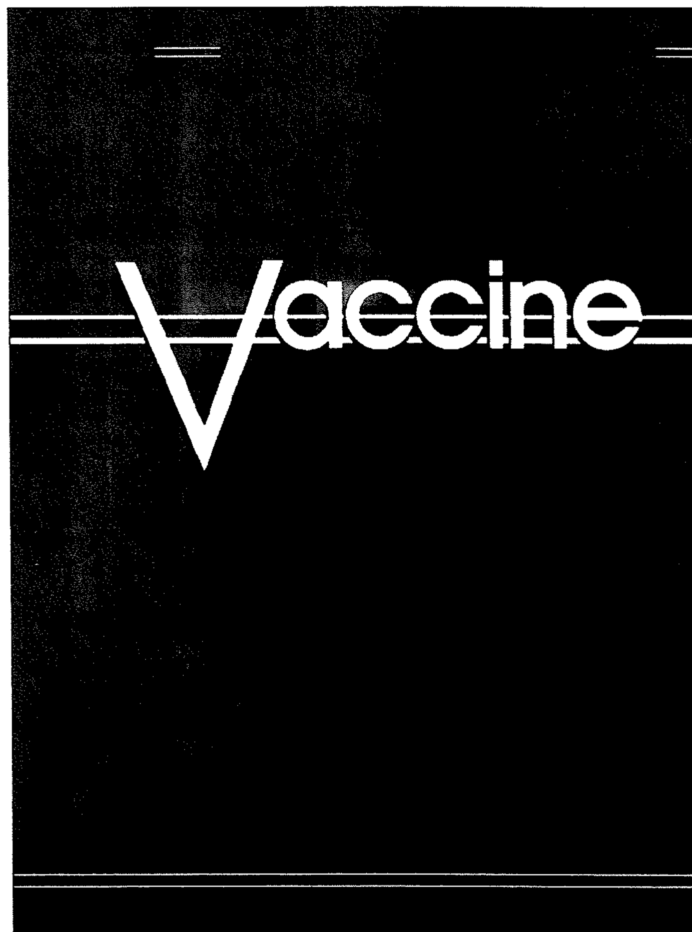

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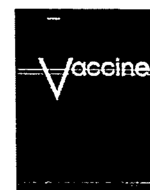

Innate and acquired immune responses induced by recombinant *Lactobacillus casei* displaying flagellin-fusion antigen on the cell-surface

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ABSTRACT

Bacterial flagellins are known as antigens that induce innate immune responses through TLR5 and boost immune responses in combination with other antigens. The aim of the present study was to determine the immunological properties of recombinant *Lactobacillus casei* producing flagellin and flagellin-fusion antigens *in vitro* and *in vivo*. Recombinant lactobacilli expressing Salmonella FliC and FliC fused to truncated SipC on the cell-surface were constructed. Fusion and non-fusion flagellin associated with *L. casei* retained the ability to induce IL-8 production by Caco-2 cells. Immunization of mice with these recombinant strains induced antigen-specific antibodies and cytokine production. The results showed that the outside epitope of the heterologous antigen was recognized more easily by the immune system than the inside epitope. The immune responses elicited by the Lactobacillus-associated antigens were mainly Th1 while that by the soluble antigen was Th2, although some of the responses were mixed.
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1. Introduction

Lactic acid bacteria (LAB) have been considered for use as a vaccine delivery vehicle over the past decade because these bacteria are generally regarded as safe. So far, a number of genetically modified LAB producing pathogenic antigens have been established and their efficacies for vaccination demonstrated [1–4]. Previously, it was reported that vaccination with recombinant *Lactobacillus casei* that exhibited flagellin on the cell-surface conferred protective immunity against infection by *Salmonella enterica* serovar Enteritidis (SE) [5].

Flageller antigens have been investigated as a protective antigen for vaccination against *Salmonella* [6,7]. At the same time, flagellins are also known as agonists of Toll-like receptor 5 (TLR5) and are required for Ipaf activation, which is involved in innate immune responses during *Salmonella* infection [8–10]. Moreover, adjuvant activities of flagellins were reported in previous studies. Cuadros et al. demonstrated that a flagellin-EGFP fusion protein could evoke EGFP-specific immune responses while EGFP only was not able to induce antigen-specific immunity [11]. Huleatt et al. found that a recombinant flagellin-ovalbumin fusion protein induced rapid and potent antigen-specific immune responses in the absence of supplemental adjuvant [12]. These findings indicate that flagellins can elicit both innate and acquired immunity. In other words, flageller antigens are applicable for vaccination as a protective antigen and as an adjuvant.

Because our previous study focused on SE flagellin (FliC) as a single protective antigen, innate immune responses and adjuvant activities induced by FliC-producing *L. casei* remain to be investigated. In the present study, recombinant *L. casei* expressing FliC-fusion antigen on the cell-surface was constructed. As a fusion partner, SipC protein of SE was selected. SipC is a member of the proteins involved in type III secretion systems (TTSSs) and possesses dual functions, including translocation of effectors and actin modulation [13,14]. A specific immune response to SipC is induced during infection by *Salmonella*, and the CD4$^+$ T cell epitope I-A^d/SipC 381-94 has been defined already [15]. In addition, a SipC homolog, IpaC, was used as a component of a vaccine to confer protection against *Shigella flexneri* infection [16]. In consideration of these findings, SipC seems to be a promising candidate as a protective antigen. Because the N-terminal region of SipC may cause the insolubility of recombinant proteins and does not include the T cell epitope, the amino acid residues from 201 to 409, corresponding to the C-terminus of SipC (cSipC), were used in this study. Two types of cSipC fusion proteins, conjugated to either the N-terminus or C-terminus of FliC, were constructed in order to determine any differences in their immunogenicity.

The present study attempted to evaluate the immunological properties of recombinant *L. casei* producing fusion antigens composed of FliC and cSipC *in vitro* and *in vivo*. An innate immune response through TLR5 was determined using human intestinal Caco-2 epithelial cells. Caco-2 cells express TLR5 and are responsive to flagellin [17] but are not responsive to TLR2 or TLR4 agonists

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due to the absence of TLR4 expression and the low expression level of TLR2, TLR1, and TLR6 [18–20]. TLR5-stimulating activity was detected by the release of interleukin 8 (IL-8) from a Caco-2 cell culture [21]. Induction of acquired immunity was determined by parenteral immunization of mice followed by detection of antigen-specific antibodies and cytokines.

2. Materials and methods

2.1. Bacterial strains and growth conditions

A list of recombinant strains used in the present study is shown in Table 1. A plasmid-free strain of *L. casei* IGM393 and recombinant strains including a FliC-expressing strain (LCF) and a non-expressing control strain carrying pLPEmpty (LCN), which were constructed in the previous study, were grown in de Mann Rogosa and Sharpe (MRS) broth (Difco). Erythromycin (5 μg/ml) was added to MRS only for recombinant strains. As described previously, *Lactobacillus*-carrying medium (LCM) supplemented with 1% mannitol and 5 μg/ml erythromycin was used for induction of the expression of heterologous antigens [5]. A human clinical isolate of *Salmonella enterica* serovar Enteritidis (SE) #40 [22] was cultured in Luria–Bertani (LB) broth (Difco). For the cloning of plasmids, *Escherichia coli* JM109, grown in LB medium containing 100 μg/ml ampicillin, was used in this study.

2.2. Preparation of proteins and antisera

Preparation of the SE antigen, the truncated C-terminus of SipC (cSipC), was performed using a histidine-tagged system in accordance with the manufacturer's instructions (Qiagen). Briefly, the partial *sipC* gene encoding cSipC (amino acid residues 201–409) was amplified from SE chromosomal DNA by PCR with a set of primers, IGM389 (ccc cgg atc cga atg aaa gag gcg cgc tta aa) and IGM390 (ggg gct cga gag cgc gaa tat tgc ctg cga). The amplified DNA fragment was digested with BamHI and XhoI and inserted into the BamHI–SalI sites of pQE31. *E. coli* M15 was then transformed with the ligated plasmid. The expression and purification of His-tagged protein (His-cSipC) were carried out under denaturing conditions. The protein was renatured by dialysis against PBS. The flagellin from SE was prepared in accordance with the method by Ibrahim et al. [23]. The purity of His-cSipC and flagellin (FliC) was verified by 10% SDS-PAGE followed by CBB staining, and the concentration of proteins was quantified by Bradford's method (Bio-Rad).

In order to prepare anti-cSipC serum, 8–10-week-old female BALB/c mice were immunized intraperitoneally (i.p.) with the purified protein. Ten micrograms of protein with Freund's complete adjuvant (FCA) was injected into a mouse 3–4 times at 3-week intervals between each administration. The care and use of experimental animals complied with local Animal Welfare Laws and Guidelines. Total blood was collected two weeks after the last booster and serum was prepared by centrifugation. The antibody's specificity was checked by western blotting analysis. The anti-flagellin antibody used in this study was the same as that prepared previously [24].

Table 1
Bacterial strains used in this study.

Strains	Characteristics	References
L. casei		
IGM393	Plasmid-free host, subculture of ATCC 393 (pLZ15⁻)	[5]
LCF	Carrying pLP401::FliC, surface-display, Em^r	[5]
LCS	Carrying pLP401::cSipC, surface-display, Em^r	This study
LCFS	Carrying pLP401::FliC = cSipC, surface-display, Em^r	This study
LCSF	Carrying pLP401::cSipC = FNC, surface-display, Em^r	This study
LCN	Carrying pLPEmpty, non-expressing control strain, Em^r	[5]
S. Enteritidis		
#40	Clinical isolate, source of genes and proteins	[24]
E. coli		
JM109	Cloning host	Takara Bio Inc.

Em^r: erythromycin resistance.

2.3. Transformation of *L. casei*

As the expression vector for cell-surface anchoring of the heterologous antigens, the plasmids pLP401::cSipC, pLP401::cSipC = FliC, and pLP401::FliC = cSipC were constructed from pLP401 by the same technique as described previously [5]. In brief, DNA fragments encoding these antigens were amplified from SE #40 chromosomal DNA by PCR with primers IGM389 and IGM390 for cSipC. In order to construct the fusion protein, FliC = cSipC, overlap PCR was performed. As a first step, DNA fragments encoding FliC and cSipC were synthesized using chimeric primers that included both sequences of *fliC* and truncated *sipC*; IGM200 (gaa aag gat ccg gca caa gtc att aat aca aac agc ct) and IGM423 (ttt aag cgc gcc tct ttc att acg cag taa aga gag gac gt) for the front segment (FliC-) and IGM422 (acg tcc tct ctt tac tgc gta atg aaa gag gcg cgc tta aa) and IGM390 for the rear segment (-cSipC). As a second step, the two segments were connected and amplified by PCR using primers IGM200 and IGM390. Another chimeric gene encoding cSipC = FliC was prepared by the same technique but using different primers, IGM389 and IGM421 (gta tta atg act tgt gcc ata gcg cga ata ttg cct gcg a) for the front segment (cSipC-), IGM420 (tcg cag gca ata ttc gcg cta tgg cac aag tca tta ata c) and IGM201 (tcg ccg tcg aca cgc agt aaa gag agg acg tt) for the rear segment (-FliC), and IGM389 and IGM201 for the connection. These PCR products were digested with BamHI and XhoI, and inserted into the same restriction sites of pLP401. The ligated plasmid was then introduced into *E. coli* JM109 for cloning. In order to convert it into a mature plasmid, the constructed plasmid was treated with NotI followed by self-ligation. The preparation of competent cells and electroporation of *L. casei* were carried out in accordance with the method of Pouwels et al. [25].

2.4. Immunoblotting and flow cytometry

The procedure to confirm the expression and surface presentation of heterologous proteins was described previously [5]. Briefly, transformed bacteria were grown, collected, and disrupted in SDS-PAGE sample buffer. Culture supernatants were also collected and concentrated 10-fold using 10% trichloroacetic acid. Proteins were separated by SDS-PAGE and transferred to a PVDF membrane (Immobilon™-P, Millipore) by electroblotting. The blot was then conjugated with appropriate primary antibodies (anti-FliC rabbit Ab or anti-cSipC mouse Ab) and Alexa Fluor™ 488 goat anti-rabbit (or anti-mouse) IgG (Molecular Probes) and analyzed using a Molecular Imager FX (Bio-Rad).

For FACS analysis, intact bacterial cells were stained with a rabbit anti-FliC (or anti-cSipC) antibody and Alexa Fluor™ 488 goat anti-rabbit (or anti-mouse) IgG in PBS supplemented with 1% BSA and 0.05% Tween-20. The labeled bacterial cells were then analyzed using a FACSCalibur flow cytometer and CELLQuest software (BD).

2.5. Stimulation of Caco-2 cells using recombinant *L. casei*

Bacterial cells for stimulation were prepared as follows. Pre-warmed LCM supplemented with erythromycin was inoculated with a 5% volume of overnight culture of the respective bacterial strains and incubated for 5 h. The bacterial cells were

collected and washed twice with PBS and once with distilled water. The bacterial suspensions in distilled water were then lyophilized.

Caco-2 cells, established from epithelial cells of human colon adenocarcinoma, were purchased from American Type Culture Collection (ATCC) and maintained in a complete medium of Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.1% (v/v) non-essential amino acid, 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin. Every culture of Caco-2 cells was incubated at 37 °C in 5% CO_2. Semi-confluent cultures of Caco-2 cells were collected and suspended in complete medium and seeded into a 96-well flat-bottom microplate (1×10^4 cells/0.2 ml/well). After 24 h incubation, the medium was replaced with fresh medium including bacteria or purified proteins. The culture supernatant was collected after 4 h and stored at −20 °C until analysis.

2.6. Immunization of mice

Female 8-week-old C3H/HeJ mice (Japan SLC) were immunized i.p. with recombinant lactobacilli, purified cSipC, and/or flagellin (5 mice/group). On the days of immunization, prewarmed LCM supplemented with erythromycin was inoculated with a 5% volume of overnight culture of the respective bacterial strains and incubated for 5 h. The bacterial cells were then collected and washed with PBS. The bacterial cell suspensions for administration were adjusted to 1×10^7 cfu in 0.1 ml PBS per dose. The mice received three injections with 2-week intervals between each dose. Two weeks after the last booster, blood and the spleen were collected. Sera were prepared from the blood samples by centrifugation and stored at −20 °C until use. The care and use of experimental animals complied with local Animal Welfare Laws and Guidelines.

2.7. Enzyme-linked immunosorbent assay (ELISA)

Human interleukin 8 (IL-8) released into the culture supernatants was detected using IL-8 OptEIA ELISA sets (BD Biosciences, San Diego, CA, USA). Appropriately diluted culture supernatants were assayed in accordance with the manufacturer's instructions. Concentrations of the cytokines were calculated using a standard curve.

In order to determine the titers of antigen-specific antibodies, a standard ELISA was performed in accordance with the protocol described previously [5]. Briefly, 96-well microplates were coated with 5 μg/ml of protein (FliC or cSipC), blocked with 1% BSA, and incubated with serially diluted serum. Antigen-specific antibodies were conjugated with alkaline phosphatase (AP)-labeled anti-mouse IgG (Sigma), IgG1, and IgG2a (Southern Biotechnology Associates Inc., AL, USA). For color development, 4-nitrophenylphosphate (SIGMA) was used. The absorbance was read after 1 h at 405 nm. Endpoint titers were defined as the maximum dilution that gave an absorbance above the cut-off value (0.1), which was calculated based on the mean optical density of normal mouse sera.

2.8. Ex vivo re-stimulation of spleen cells with SE antigens

The procedure for the stimulation of spleen cells was described previously [5]. The spleen was removed from the immunized mouse, and erythrocyte-free cells were prepared in complete RPMI-1640 medium (+10% fetal calf serum and penicillin/streptomycin). The cells were seeded into a 96-well microplate (1×10^6 cells/well) and supplemented with flagellin (10 μg/ml), cSipC (50 μg/ml), concanavalin A (5 μg/ml), or PBS. Each culture was incubated at 37 °C in a CO_2 incubator. After 72 h incubation, cleared culture supernatants were obtained by centrifugation and stored at −80 °C until analysis.

2.9. Cytokine profiling

Eight kinds of cytokines, interleukin-2 (IL-2), IL-4, IL-5, IL-10, IL-12 (p70), granulocyte/macrophage-colony stimulating factor (GM-CSF), gamma interferon (IFN-γ), and tumor necrosis factor alpha (TNF-α), were measured using a Bio-Plex suspension array system with a mouse Th1/Th2 cytokine panel (Bio-Rad). Appropriately diluted supernatants from spleen cell cultures were analyzed in accordance with the manufacturer's instructions. The samples were assayed in duplicate.

2.10. Statistical analysis

Statistical significance was determined using Tukey's multiple comparison test.

3. Results

3.1. Construction of recombinant strains expressing SE antigens

Three types of constructed strains carrying pLP401::cSipC,::FliC = cSipC, and ::cSipC = FliC were analyzed by immunoblotting in the present study. By detection of antigens with an anti-flagellin antibody, specific bands were detected in the lanes for *L. casei* expressing FliC (LCF), FliC = cSipC (LCFS), and cSipC = FliC (LCSF) (Fig. 1a). Flagellin-specific signals were detected in both the cell extract and the supernatant of the SE culture. As shown in Fig. 1b, specific signals were observed from strains producing cSipC (LCS), LCFS, or LCSF by conjugation with anti-cSipC antibody. In this case, SipC-specific signals were detected in the supernatant of SE cultures. The molecular masses of FliC and cSipC produced by recombinant lactobacilli were higher than the corresponding purified antigens because these antigens of lactobacilli were fused to the anchor peptide from the pLP401 vector. No specific signal was detected in the LCN lane.

The surface-associated antigens on the bacterial cells were detected by flow cytometry. As shown in Fig. 1c, the peak of the cell count shifted to a higher fluorescence position by labeling with the corresponding antibody, and the fluorescence means that all these recombinant strains displayed the antigens on the cell-surface.

3.2. The activity of recombinant strains for induction of IL-8 released from Caco-2 cells

Stimulation of Caco-2 cells with recombinant lactobacilli or purified flagellin induced the release of IL-8 in a dose-dependent manner (Fig. 2). Because bacterial cells were not inactivated but lyophilized once, antibiotics were included in the culture, and the incubation time was relatively short, and growth of bacterial cells was not observed during this assay. The relatively high levels of IL-8 were detected only in the culture exposed to agents including FliC. Despite Caco-2 cells being stimulated with the same amount of bacterial cells, LCF induced much less IL-8 production than LCFS or LCSF. In particular, the amount of IL-8 evoked by 1000 μg/ml LCF was almost same as that by 100 μg/ml LCFS or LCSF. These concentrations of LCF, LCFS, and LCSF, exhibited nearly equal activity in IL-8 induction as 10 ng/ml of purified flagellin.

3.3. Analysis of SE antigen-specific antibodies

The specific IgG titers against cSipC and FliC were measured by ELISA, as shown in Fig. 3. cSipC-specific IgG was produced by mice immunized with LCS, LCSF, LCFS, purified cSipC, and a

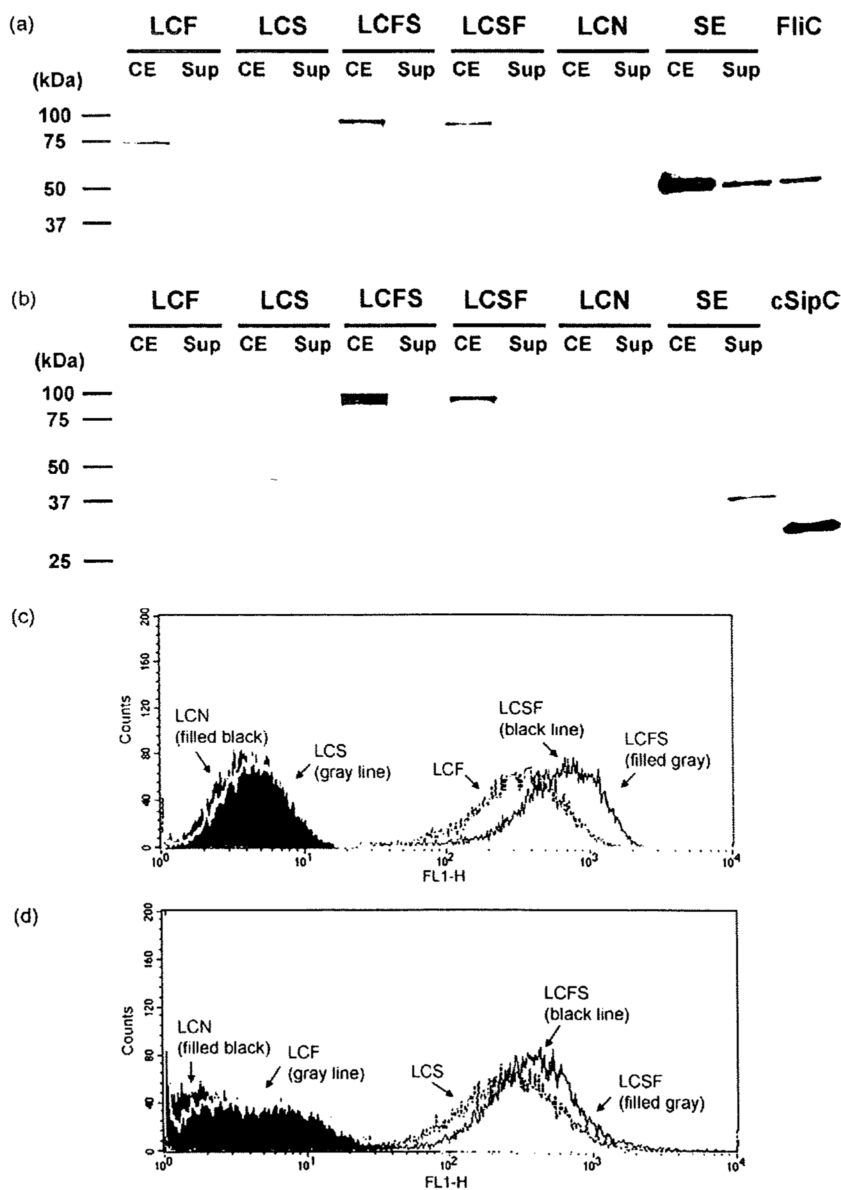

Fig. 1. Expression and surface-location of FliC-fusion antigens. Expression of FliC (a) and/or cSipC (b) was detected by immunoblotting analysis. Approximate 10^8 cfu of cell extract (CE), 50-fold concentrated culture supernatant (Sup), 10 ng of purified FliC, or 25 ng of purified cSipC were loaded onto an acrylamide gel. Molecular sizes are shown on the left. Surface-located antigens were detected using FACS analysis. Five kinds of recombinant lactobacilli, LCF, LCS, LCFS, LCSF, and LCN, were conjugated with anti-FliC (c) or anti-cSipC (d) antibodies and Alexa 488-labeled anti-rabbit IgG or anti-mouse IgG. Ten thousand events were analyzed. The horizontal axis (FL1) represents the intensity of fluorescence, and the vertical axis represents the number of bacterial particles.

mixture of purified cSipC and flagellin. The flagellin-specific IgG was detected in sera from mice that received LCF, LCSF, LCFS, or the mixture of purified cSipC and flagellin. No significant difference was shown for cSipC-specific IgG titer between the groups immunized with cSipC-producing lactobacilli (LCS, LCSF, and LCFS). On the other hand, the flagellin-specific IgG titers of the LCF- or LCFS-immunization groups were significantly higher than that of the LCSF-immunization group. Immunization with purified soluble antigens without adjuvant also evoked specific IgG. In addition, the titer of cSipC-specific IgG induced by inoculation with a mixture of cSipC and flagellin was higher than that of cSipC only. SE antigen-specific IgG was not detected from the immunized groups of LCN and PBS.

In order to determine the IgG1/2a ratio, which represents the Th2/Th1 response, the same ELISA but using anti-IgG1 and anti-IgG2a antibodies for detection was performed. For both anti-cSipC and anti-FliC IgG1/2a ratios, the groups immunized with soluble antigens showed greater values than the groups that received antigens exposed on the bacterial surface (Table 2). No significant difference was observed between the groups immunized with soluble antigens or between groups that received recombinant lactobacilli expressing SE antigens.

3.4. Th1/Th2 cytokines in cultures of antigen-stimulated spleen cells

Eight kinds of cytokine in spleen cell cultures, which were stimulated with SE antigens, were measured using a Bio-Plex suspension array system. Stimulation with ConA induced non-specific proliferation of splenocytes and the production of high levels of various cytokine, while poor cell-proliferation and cytokine production were observed in spleen cells incubated with PBS (data

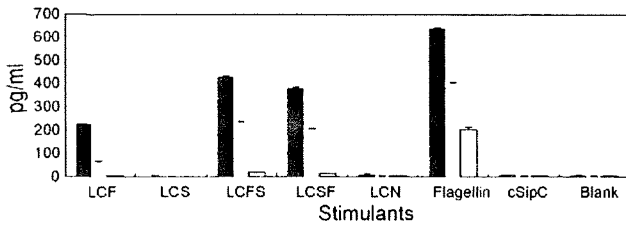

Fig. 2. Quantification of human IL-8 secreted by Caco-2 cells. The culture of Caco-2 cells was stimulated with recombinant lactobacilli (1000, 100 and 10 μg/ml) or purified proteins (100, 10, 1 ng/ml). Values represent mean + SEM ($n = 3$). Results are representative of at least three independent experiments. Black bar: 1000 μg/ml or 100 ng/ml. Gray bar: 100 μg/ml or 10 ng/ml; open bar: 10 μg/ml or 1 ng/ml.

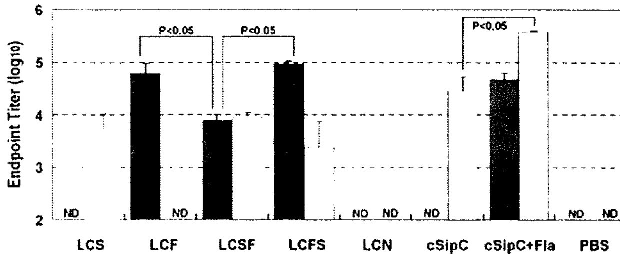

Fig. 3. IgG titer in serum of immunized mice. Endpoint titers of antigen-specific IgG are given as $-\log_{10}$(dilution factor) + standard deviation (SD). The types of immunization are shown at the bottom. Open bars represent cSipC-specific IgG titer and gray bars designate the titer of FliC-specific IgG. Significant differences ($P < 0.05$) were analyzed between groups LCS, LCF, LCSF, LCFS, LCN, and PBS, or between cSipC, cSipC + Fla, and PBS. ND: not detected

Table 2
Antigen-specific IgG1/2a ratio.

(a)

Immunization group	IgG1/2a ratio	
LCS	0.56 ± 0.16	
LCFS	0.58 ± 0.15	
LCSF	0.63 ± 0.26	$P<0.05$
cSipC	1.28 ± 0.17	
cSipC+FliC	1.06 ± 0.02	

(b)

Immunization group	IgG1/2a ratio	
LCF	1.07 ± 0.04	
LCFS	0.95 ± 0.04	
LCSF	0.79 ± 0.17	$P<0.05$
cSipC+FliC	1.11 ± 0.05	

The ratios of cSipC-specific (a) or FliC-specific (b) IgG1/2a were calculated from endpoint titers ($\log_{10}$) of the specific IgG1 and IgG2a titers. Values represent mean ± SD.

not shown). Thus, the viability and vitality of the cells were confirmed and cytokine production was dependent on stimulation. Significantly higher levels of IL-2, IL-5, GM-CSF, and IFN-γ were released by flagellin-stimulated cells from LCFS-immunized mice (Table 3). By immunization with the cSipC + FliC mixture, the flagellin-stimulated cells produced significant levels of IL-4, IL-5, and IL-12, and cSipC-stimulated cells released relatively large amounts of IL-4, IL-10, IL-12, and TNF-α. The cSipC-stimulated cells from the cSipC-primed group released higher levels of IL-5 than the control group. The rest of the values were not significantly different.

4. Discussion

Genetically modified *L. casei* strains that produced a SE antigen with or without FliC-fusion were constructed. Flow cytometric analysis showed that these recombinant strains exhibited antigens on their cell surfaces. In order to investigate whether these recombinant lactobacilli have TLR5-stimulating activity, IL-8 release from stimulated Caco-2 cells was determined. The results showed that remarkable amounts of IL-8 were detected from each culture stimulated with recombinant *L. casei* producing either FliC or FliC-fusion antigens. Thus, the induction of an immune response through TLR-5 was suggested. Unexpectedly, the IL-8 accumulation evoked by the strains expressing FliC-fusion proteins was greater than that with the strain expressing FliC alone. Because the TLR5-stimulating activity was dose dependent, this result indicated that the contact between FliC-fusion proteins of recombinant bacteria and TLR5 of Caco-2 cells was more frequent than that between cell-anchored FliC and TLR5.

According to the result of flow cytometric analysis, the two recombinant strains expressing FliC-fusion proteins displayed FliC more efficiently than the FliC-expressing strain. This data seemed to correlate with the result of the IL-8 release assay. Thus, the difference in TLR5-stimulating activity could be explained by the unequal presence of FliC on the bacterial surface. There are other possibilities such as FliC-fusion proteins having higher TLR5-stimulating activity than FliC, or FliC-fusion proteins are more stable than FliC; however, there is no evidence to support these characteristics.

In order to investigate antigen-specific acquired immune responses, C3H/HeJ mice were immunized with recombinant *L. casei* and purified SE antigens by i.p. injection. The production of antigen-specific antibodies was induced without additional adjuvants. Soluble cSipC showed immunogenicity to produce antigen-specific IgG. In combination with purified flagellin, soluble cSipC induced higher IgG production. McSorley et al. reported that bacterial flagellin provides an adjuvant effect on CD4+ T cells [26]. Thus, it is probably the same reason why cSipC-specific antibody production was enhanced in combination with flagellin. Although immunization with all lactobacilli producing FliC or FliC-fusion proteins evoked anti-FliC IgG, the titer of the group immunized with LCSF was significantly lower than that with either LCF or LCFS. Meanwhile, the anti-cSipC IgG titer in the LCFS-immunized group was less than that in the LCSF-immunized group, although the difference was not statistically significant. Taken together, epitopes that were present on the outside part of the epitope on the bacterial cell could be easily recognized by immune cells and elicit IgG production.

It is generally known that analysis of the IgG subclass helps to determine the tendency of Th1- and Th2-type responses. In particular, induction of IgG1 represent a Th2-type response while the production of IgG2a indicates Th1-type. In this study, the IgG1/2a ratios of anti-FliC and anti-cSipC IgG were determined. The analysis of antibodies, especially anti-cSipC IgG, showed that immunization with soluble antigens resulted in a relatively higher IgG1/2a ratio, while immunization with antigens exposed on the surfaces of *L. casei* exhibited a relatively lower IgG1/2a ratio. This evidence suggested that the immune responses evoked by soluble antigens were Th2 dominant but *L. casei* associated antigens tended to induce Th1. Cunningham *et al.* reported previously that the responses to soluble FliC are Th2, while those to FliC on *Salmonella* are Th1 [27]. Although the host bacteria and the structure of the flagellar antigen are different, the present data may support their result. The Th1 shift might be provided by the nature of *Lactobacillus* strains because there is a large body of evidence that indicates their property of inducing Th1-type responses [28–31]. In contrast, previous studies reported different types of immune responses induced by commensal bacteria expressing tetanus toxin fragment C (TTFC). Medaglini

Table 3
Profiling of Th1/Th2 cytokines in antigen-stimulated culture of spleen cells ($n = 5$).

Immunization group	Th1-type cytokines		Immunization group	Th2-type cytokines and TNF-α	
	Flagellin-stimulated	cSipC-stimulated		Flagellin-stimulated	cSipC-stimulated
IL-2			IL-4		
LCS	51.2 ± 15.9	31.3 ± 22.4	LCS	1.2 ± 0.4	0.5 ± 0.6
LCF	141.8 ± 95.5	36.2 ± 18.0	LCF	7.8 ± 4.8	1.0 ± 1.7
LCSF	123.3 ± 74.7	52.1 ± 27.3	LCSF	4.1 ± 3.5	0.6 ± 0.3
LCFS	229.2 ± 62.7*	56.0 ± 26.1	LCFS	11.1 ± 5.6	0.4 ± 0.2
LCN	53.8 ± 21.9	38.2 ± 22.1	LCN	0.8 ± 0.2	0.3 ± 0.3
cSipC	27.1 ± 13.0	37.5 ± 17.3	cSipC	2.6 ± 1.9	6.9 ± 2.2
cSipC + FliC	89.0 ± 26.1	68.7 ± 34.1	cSipC + FliC	34.2 ± 22.3*	99.4 ± 67.0*
Saline	30.2 ± 24.5	25.5 ± 19.3	Saline	1.4 ± 0.9	0.2 ± 0.1
IL-12 p70			IL-5		
LCS	4.9 ± 2.2	1.8 ± 0.2	LCS	0.9 ± 1.3	0.6 ± 0.8
LCF	7.0 ± 1.6	1.7 ± 0.8	LCF	5.1 ± 6.1	0.8 ± 0.6
LCSF	7.1 ± 2.8	2.2 ± 0.7	LCSF	3.8 ± 3.9	0.4 ± 0.1
LCFS	9.5 ± 1.7	2.3 ± 0.5	LCFS	16.2 ± 11.6*	1.4 ± 1.6
LCN	4.9 ± 1.6	2.3 ± 1.4	LCN	0.6 ± 0.8	0.5 ± 0.3
cSipC	4.0 ± 1.1	3.6 ± 1.6	cSipC	1.1 ± 0.5	34.9 ± 26.5*
cSipC + FliC	12.1 ± 4.9*	20.5 ± 11.9*	cSipC + FliC	13.7 ± 7.8*	25.9 ± 24.4
Saline	4.6 ± 1.5	1.4 ± 0.6	Saline	0.6 ± 0.1	0.1 ± 0.1
GM-CSF			IL-10		
LCS	24.0 ± 10.1	15.9 ± 8.4	LCS	699.9 ± 403.3	34.1 ± 19.0
LCF	94.1 ± 43.3	11.1 ± 6.5	LCF	914.9 ± 259.4	36.8 ± 36.2
LCSF	97.5 ± 113.0	8.4 ± 3.7	LCSF	1014.4 ± 171.8	38.3 ± 10.1
LCFS	180.9 ± 113.3*	14.0 ± 13.2	LCFS	1113.7 ± 415.8	45.1 ± 18.8
LCN	29.2 ± 22.0	9.4 ± 6.0	LCN	760.3 ± 250.2	32.2 ± 9.5
cSipC	18.4 ± 8.5	10.2 ± 4.9	cSipC	503.5 ± 212.8	80.2 ± 52.5
cSipC + FliC	64.2 ± 33.9	17.9 ± 9.0	cSipC + FliC	1015.5 ± 415.8	569.3 ± 376.5*
Saline	27.6 ± 15.6	5.4 ± 0.6	Saline	548.7 ± 131.1	17.4 ± 9.9
IFN-γ			TNF-α		
LCS	65.7 ± 80.1	79.4 ± 142.1	LCS	11.3 ± 1.5	7.9 ± 4.0
LCF	468.8 ± 325.4	30.1 ± 32.7	LCF	12.7 ± 1.9	9.5 ± 2.2
LCSF	596.0 ± 859.1	39.7 ± 40.1	LCSF	15.1 ± 3.3	8.3 ± 1.3
LCFS	1149.6 ± 760.4*	93.9 ± 104.5	LCFS	16.7 ± 3.2	10.6 ± 1.7
LCN	187.1 ± 320.2	78.3 ± 54.7	LCN	11.6 ± 2.6	8.4 ± 1.3
cSipC	14.3 ± 11.8	10.9 ± 8.3	cSipC	10.6 ± 2.6	11.5 ± 2.3
cSipC + FliC	251.2 ± 164.6	144.0 ± 117.3	cSipC + FliC	14.3 ± 2.2	14.6 ± 4.5*
Saline	47.7 ± 48.3	8.3 ± 7.2	Saline	12.9 ± 9.0	7.6 ± 1.7

Eight kinds of cytokines were measured using a multiplex suspension beads assay (Th1/Th2 panel). Values represent mean ± SD.
* $P < 0.05$ (vs LCN and saline).

et al. demonstrated that the IgG1 subclass was predominant after parenteral immunization with recombinant *Streptococcus gordonii* with TTFC exposed on the cell-surface [32]; a similar result was shown by Grangette et al. using *Lactobacillus plantarum* producing TTFC intracellularly [33]. In the present study, unlike anti-cSipC IgG, the IgG1/2a ratio of anti-FliC induced by recombinant *L. casei* did not always show a clear Th1 shift. This evidence suggested that the antigens expressed by recombinant bacteria could have a significant influence on Th1/Th2 dominance as well. Controlling the Th1/Th2 balance is important to confer proper immunity, although it is rarely understood how recombinant lactobacilli expressing heterologous antigens induce immune responses. Hence, elaborate studies are required to develop vaccines based on *Lactobacillus* strains.

The profiling of cytokine production by *ex vivo* re-stimulation of spleen cells showed significant differences with the group immunized with LCFS. By stimulation with FliC, the spleen cells released greater amounts of Th1-type cytokines, such as IL-2, GM-CSF, and IFN-γ. Interestingly, IL-5 production, which represents a Th2-type immune response, was also high, although the production of IL-4 and IL-10 was not different from the non-immunized group. These mixed Th1/Th2 responses might explain the unbiased IgG1/2a ratio of anti-FliC induced by LCFS-immunization. In contrast to this reaction, the cells from mice immunized with FliC plus cSipC exhibited mainly Th2-type cytokine production. Greater amounts of IL-4 and IL-5 were produced by FliC-stimulation, and IL-4 and IL-10 were also induced by cSipC-stimulation. Notably, IL-12 was also released by stimulation with both FliC and cSipC. Therefore, these immune responses were mixed Th1/Th2-type although they were different from the immune responses by LCFS-immunization.

The present study demonstrated that FliC and FliC-fused antigens displayed on the cell-surface of *L. casei* elicit innate immune responses *in vitro* and showed that immunogenicities of these recombinant lactobacilli were affected by the species and the physical position of the antigens. It was also suggested that the adoptive immunity induced by the recombinant lactobacilli was mixed but mainly Th1-type. Because flagellin is considered to be a potential adjuvant, information provided in this study could be useful for designing of vaccines using lactobacilli as delivery agents.

Acknowledgements

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