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

Kajikawa, Akinobu[1], Eiko Ichikawa[2], and Shizunobu Igimi[2]*

[1]*Department of Food, Bioprocessing, and Nutrition Sciences, North Carolina State University, Box 7624, Raleigh, NC 27695, U.S.A.*
[2]*Division of Biomedical Food Research, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, Japan*

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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,

*Corresponding author
Phone: +81-3-3700-9164; Fax: +81-3-3700-9246;
E-mail: igimi@nihs.go.jp

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	gggctcgaggcccggttgttcgcggccgctttgttaagaattttatttcataacattagcgg	*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

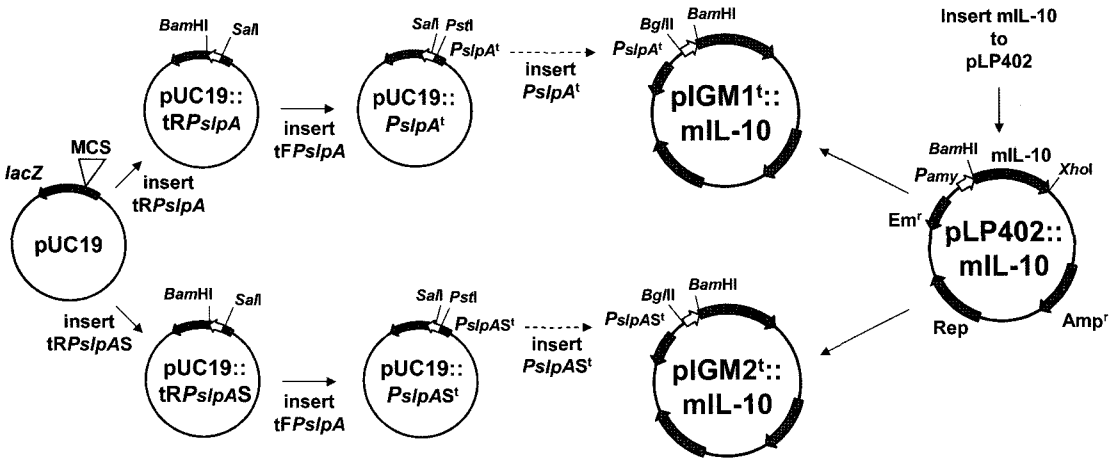

Fig. 1. Construction-flow diagram of expression vectors.
MCS: multiple cloning site; Rep: replication protein; Em^r: erythromycin resistance; Amp^r: ampicillin resistance.

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*S^t) 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 (pIGM1^t::mIL-10 and pIGM2^t::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 (pIGM2^{t}Just::mIL-10). A map of the expression cassette and the differences between pIGM2^t::mIL-10 and pIGM2^{t}Just::mIL-10 is shown in Fig. 2.

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

The plasmids, pIGM2^{t}Just::mIL-10 and pIGM2^t::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

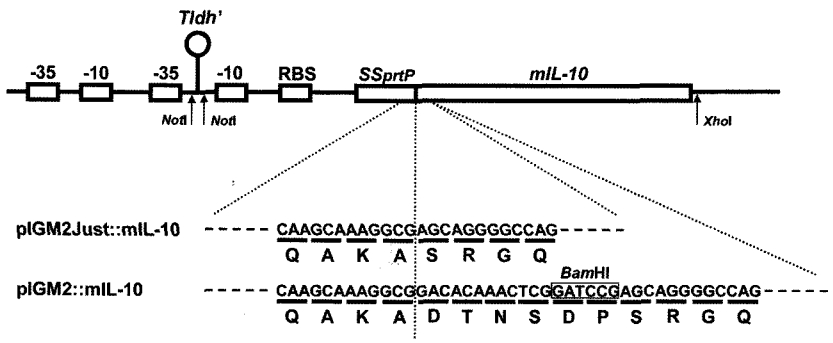

Fig. 2. Expression cassette of pIGM2Just::mIL-10 and pIGM2::mIL-10.
Features of the expression cassette are shown. Nucleotides and amino acid sequences of *SSprtP*–mIL-10 junctions are also shown. RBS: ribosome binding site; *SSprtP*: signal sequence of *prtP*.

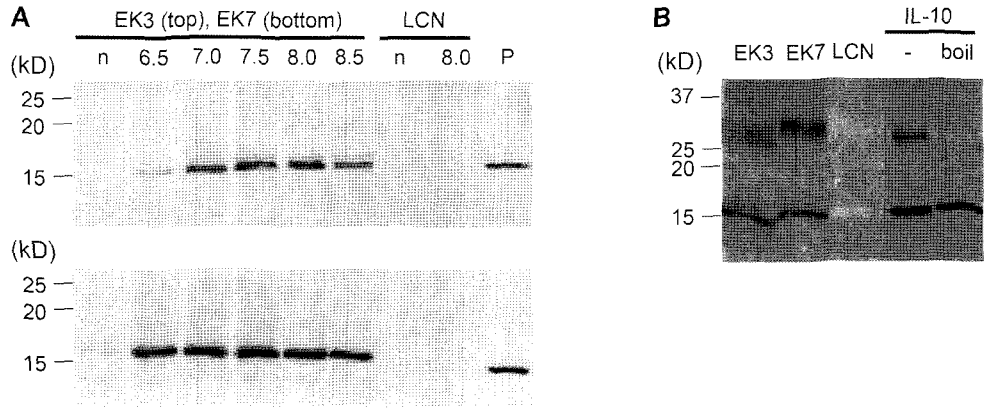

Fig. 3. Analysis of mIL-10 secreted by recombinant *L. casei*.
A. mIL-10 secretion under pH control. Proteins were concentrated by TCA, dissolved in Laemmli buffer, and boiled for 5 min. Molecular mass (left margin) and pH (top margin) are shown. n: normal conditions (no pH control); P: purified mouse 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

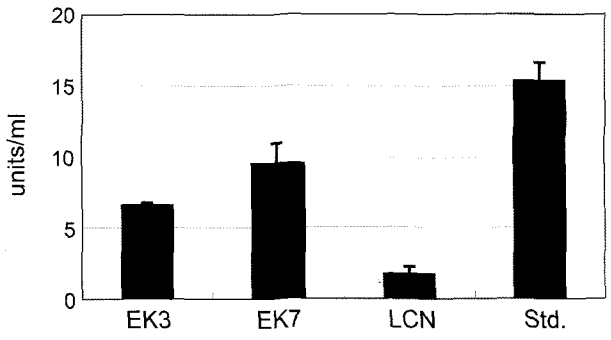

Fig. 4. Biological activity of mIL-10 produced by recombinant lactobacilli (n=4).
MC/9 cells were stimulated with culture supernatants of recombinant lactobacilli (50% volume). Each bar represents the mean value plus standard deviation. The concentration (units/ml) of each sample was calculated using 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^{t}Just::mIL-10 and pIGM2^t::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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