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Cloning and Characterization of a Novel *tuf* Promoter from *Lactococcus lactis* subsp. *lactis* IL1403

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Abstract Genetic engineering of lactic acid bacteria (LAB) requires a reliable gene expression system. Especially, a stable promoter is an important genetic element to induce gene expression in such a system. We report on a novel *tuf* promoter (P*tuf*) of *Lactococcus lactis* subsp. *lactis* IL1403 that was screened and selected through analysis of previously published microarray data. P*tuf* activity was examined and compared with three other known lactococcal promoters (P*dnaJ*, P*pfkA*, and P*usp45*) using different bacteria as expression hosts. Each promoter was, respectively, fused to the promoterless and modified *bmpB* gene as a reporter, and we estimated promoter activity through BmpB expression. All promoters were active in IL1403, and P*tuf* activity was strongest among them. The activity of each promoter differed by host bacteria (*Lactobacillus plantarum* Lb25, *Lactobacillus reuteri* ATCC23272, and *Escherichia coli* Top10F'). P*tuf* had the highest activity in IL1403 when growth reached late log phase. The activity of each promoter correlated with the expression of each cognate gene in the microarray data ($R^2 = 0.7186$, $P = 0.06968$). This study revealed that novel food-grade promoters such as IL1403 P*tuf* can be selected from microarray data for food-grade microorganisms and P*tuf* can be used to develop a reliable gene expression system in *L. lactis*.

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Introduction

Lactic acid bacteria (LAB) are food-grade microorganisms widely used in the food and animal industries. *Lactococcus lactis* is one species of LAB that has been used as a component of starter culture for fermented dairy products such as cheese and fermented milk [23]. Many studies have shown that *L. lactis* can be genetically engineered [13, 15, 26]. Recent researches in *L. lactis* have focused on the development of oral vaccines [2, 21, 26]. Generally, many genetic elements are required to develop a recombinant oral vaccine using LAB [12]. Among these elements, promoters are important and essential for foreign antigen expression. However, finding a promoter that fulfills all needed requirements is not easy. Therefore, screening and selecting of promoters is an important and on-going avenue of research.

Despite many efforts to screen promoters, the conventionally used strategies were very similar in terms of construction of promoter library and use of reporter genes such as *lacZ*, *lacG*, *gusA*, *cat*, *gfp*, and *luxAB* [3]. Construction of promoter library with high diversity requires a lot of labor, cost, and time. To avoid these problems and to screen and select novel promoters, we designed a new strategy using microarray data. In this study, we cloned and characterized a novel *tuf* promoter (P*tuf*) of *L. lactis* IL1403 which was selected through analysis of previously published microarray data.

Materials and Methods

Bacterial Strains and Plasmids

The bacterial strains and plasmids used in this work are listed in Table 1. *Escherichia coli* Top10F' was used as a

Table 1 Bacterial strains and plasmids

Strains and plasmids	Relevant characteristics	Origin
Strains		
E. coli Top10F'	Amp^r, Cloning/Expression host for pGEM-T easy	Invitrogen
L. lactis IL1403	His^- Iso^- Leu^- Val^-, plasmid-free	[19]
L. plantarum Lb25	Isolated from swine feces	[27]
L. reuteri ATCC 23272	Isolated from human feces	ATCC
Plasmids		
pGEM-T easy	Amp^r, M13*ori* pBR322*ori*, Linear T-overhangs vector	Promega
pET.MbmpB	Amp^r, pET-21b(+) derivative, Modified *bmpB* gene	[27]
pT.Ptuf.MbF	pGEM-T easy derivative, T7 and *tuf* promoter upstream of *bmpB*	This work
pT.Ppfk.MbF	pGEM-T easy derivative, T7 and *pfkA* promoter upstream of *bmpB*	This work
pT.Pusp.MbF	pGEM-T easy derivative, T7 and *usp45* promoter upstream of *bmpB*	This work
pIL252	*ermAM*, 4.6 kb, Low-copy number plasmid vector for LAB	[19]
pIL.CatT	pIL252 derivative, Promoterless *cat* gene	This work
pILPdnaJ.Mb	pIL.CatT derivative, *dnaJ* promoter upstream of *bmpB*	This work
pILPtuf.Mb	pIL.CatT derivative, *tuf* promoter upstream of *bmpB*	This work
pILPpfk.Mb	pIL.CatT derivative, *pfkA* promoter upstream of *bmpB*	This work
pILPusp.Mb	pIL.CatT derivative, *usp45* promoter upstream of *bmpB*	This work

host bacterium for construction of pGEM-T easy-derived vectors and BmpB expression. *L. lactis* IL1403 was used as a host bacterium for construction of pIL252-derived vectors and BmpB expression.

Culture Media, Bacterial Transformation, and DNA Isolations

E. coli, *L. lactis*, and *Lactobacillus* strains were cultivated in Luria-Bertani broth, M17 broth containing 0.5% (w/v) glucose (called M17G), and MRS broth, respectively. The used antibiotics were ampicillin (100 μg/ml) for *E. coli* and erythromycin (5 μg/ml) for LAB. All bacterial species were transformed by electroporation as described previously [1] and according to manufacturer's instructions. Plasmid DNA was isolated from *E. coli* with the Plasmid Purification Mini Kit (Nucleogen, South Korea) according to manufacturer's instructions. Plasmid DNA from *L. lactis* and *Lactobacillus* strains was isolated as described previously [16].

Analysis of Microarray Data for IL1403 and Statistical Analysis

We collected two sets of microarray data on IL1403 from the Gene Expression Omnibus (GEO) database of National Center for Biotechnology Information (NCBI) website (Series GSE5761 and GSE4872). These sets contain exponential phase-specific gene expression profiles of IL1403 cultivated in either a chemically defined medium (CDM) (Sample GSM134562, GSM134563 and GSM134564 within Series GSE5761) [17] or a CDM with ten-fold reduced concentrations of isoleucine, leucine, and valine (Sample GSM109311, GSM109314, GSM109315 within Series GSE,4872). Sorting of microarray data and linear regression was carried out using Microsoft Excel or R [22].

Construction of Plasmid Vectors

pIL.CatT was constructed by subcloning a 971-bp fragment containing the RBS region, *cat*, the transcriptional terminator for *pepN* of IL1403, an N-terminal *Sal*I site, and a C-terminal *Eco*RI site into the *Sal*I and *Eco*RI sites of pIL252. A schematic diagram for the construction of expression vectors is shown in Fig. 1. Four promoters were cloned by PCR-amplification (Table 2) with IL1403 genomic DNA as a template using the i-MAX™ II DNA polymerase (iNtRON BIOTECHNOLOGY, South Korea). The amplicons and pET.MbmpB [9] were cut with *Nde*I, and the resulting fragments were ligated. The ligated DNA was used as a template for PCR-amplification using four sets of primers (Table 2 and Fig. 1). Each amplicon was, respectively, subcloned into pGEM-T easy and pIL.CatT to generate *E. coli* expression vectors (pT.Ptuf.MbF, pT.Ppfk.MbF, and pT.Pusp.MbF) and LAB expression vectors (pILPdnaJ.Mb, pILPtuf.Mb, pILPpfk.Mb, and pILPusp.Mb).

Analysis of Nucleotide Sequences

DNA sequencing was carried out using Applied Biosystems 3730xl by the National Instrumental Center for

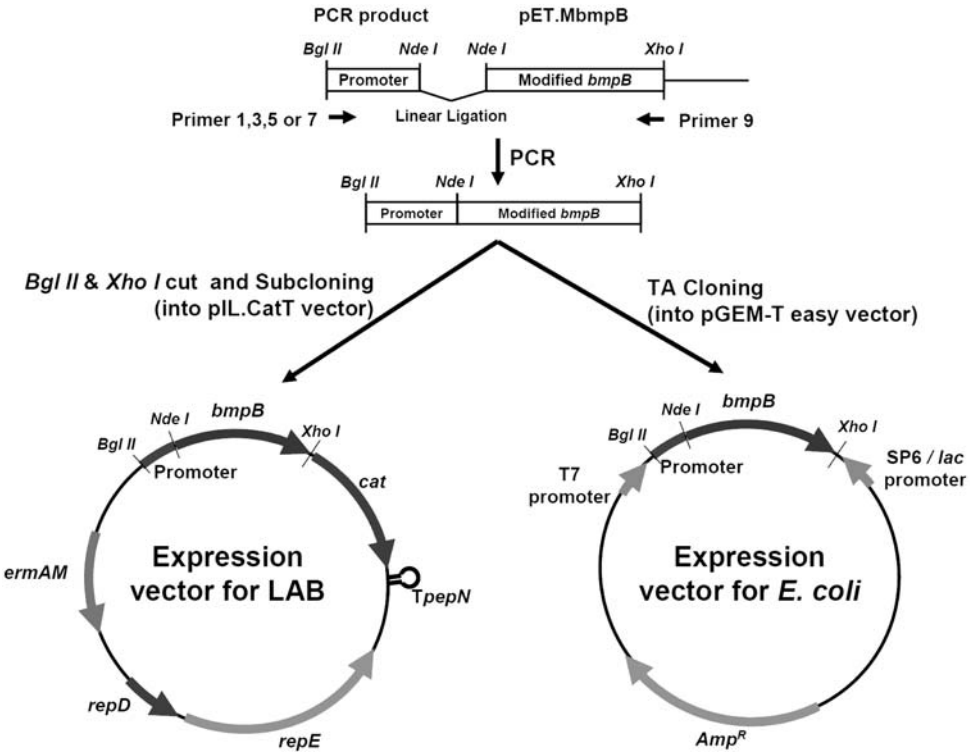

Fig. 1 Schematic diagram for the construction of BmpB expression vectors. T*pepN* transcription terminator of the IL1403 *pepN* gene; *ermAM* erythromycin resistance gene; Amp^R ampicillin resistance gene; *cat* chloramphenicol resistance gene; *repD* replication protein D; *repE* replication protein E

Environmental Management (NICEM, Seoul National University, South Korea).

Bacterial Cell Harvest for Western Blot Assay

A single bacterial colony was inoculated into 3 ml of fresh broth medium and incubated at 30°C without agitation or at 37°C with agitation for 17 h. The resulting culture (30 µl) was subsequently inoculated into 3 ml of fresh broth medium and incubated until the final optical density at 600 nm (OD_{600}) was about 2.3 (*E. coli*), 2.3 (*L. lactis*), 3.6 (*L. plantarum*), or 6.0 (*L. reuteri*). All cultures were centrifuged at 5,000*g* for 10 min at 4°C to harvest cells, and the cells were stored at −80°C until analysis.

Protein Extraction, SDS-PAGE, and Western Blot Assay

In order to extract protein from *E. coli*, the harvested cells were washed twice with PBS and resuspended in 200 µl of PBS containing 1% (w/v) SDS. After briefly vortexing, resuspended cells were incubated at 95°C for 5 min. Cell debris was removed from the cell extract by centrifugation at 14,000*g* for 10 min. The protein-containing supernatant was stored at −80°C until analysis. In order to extract protein from LAB, the harvested cells were washed twice with PBS and resuspended in 200 µl of PBS. Glass beads (212–300 µm, Sigma) were supplemented, and the cells were incubated on ice and shaken intermittently for 1 h by vortex. Cell debris was removed from the cell extract by centrifugation at 14,000*g* for 10 min. The protein-containing supernatant was stored at −80°C until analysis. SDS-PAGE was carried out with a 12% poly-acrylamide gel. Each well was loaded with 20 µg of *E. coli* protein or 40 µg of LAB protein in the same volume. The expressed BmpB was detected with polyclonal anti-BmpB mouse serum (1:4,000, Aprogen, South Korea) and anti-mouse IgG, HRP-linked antibody (1:20,000, Cell Signaling Technology, USA) as described previously [9].

Results

Screening and Selecting of a Promoter Through Analysis of Microarray Data

We collected and analyzed two sets of previously published microarray data on IL1403 (Series GSE5761 and GSE4872) to identify highly expressed genes in IL1403. All data were sorted by normalized signals (Table 3). The first set (Series GSE5761) of data was derived from IL1403 cells cultivated in CDM. The second data set (Series GSE4872) was derived from IL1403 cells cultivated in CDM with ten-fold reduced concentrations of isoleucine, leucine, and valine, as compared to CDM. The spot having the highest expression signal in each set was identified as the same gene, *tuf* (Table 3), which encodes the translation elongation factor Tu (EF-Tu). According to the annotation

Table 2 Primers for cloning and sequencing

Primer #	Primer name	Sequences of primer	PCR product
1	dnaJN1BglSpe	CGC**AGATCTACTAGT**GTGGGTGACGATTGATTTT	P*dnaJ*
2	dnaJC1NdeI	CAT**CATATG**TTTCTAAATTACTCACTTCCTTTAC	
3	tufN1SpeBgl	GC**ACTAGTAGATCT**ATTTTACTACTTC	P*tuf*
4	tufC1Nde	CTTCTTTAGC**CATATG**AAAAATGTCTCCT	
5	pfkN1BglSpe	**AGATCT**GAA**ACTAGT**AAAACGTTTCATACAGT	P*pfkA*
6	pkfC1Nde	GCGTTT**CATATG**AATCTGTCCTCCG	
7	PuspN2BglSpe	GTTTACC**AGATCT**CG**ACTAGT**CTTTTGCTT	P*usp45*
8	PuspC1NdeI	TT**CATATG**TGTAATTTTTTAATTTTTCCTC	
9	Bmp_C_XhoI	T**CTCGAG**TTATTTCCAAGTAGGAAGATAAG	Promoter + *bmpB*

data for the IL1403 genome (NC_002662), we could infer that the *tuf* gene has its own promoter and does not share that promoter with any other genes. Therefore, in this study, we examined the novel P*tuf*.

Sequence Analysis and Cloning of a Putative Promoter

To compare the activity of the P*tuf* with that of other promoters, we selected three other known lactococcal promoters for the *dnaJ*, *pfkA*, and *usp45* genes (P*dnaJ*, P*pfkA*, and P*usp45*) of *L. lactis* subsp. *cremoris* MG1363 [11, 20, 24]. They were reported to have activity in MG1363, and their cognate IL1403 promoters were also expected to have activity in IL1403 but their activity was not examined in IL1403. Therefore, we used them not only as positive controls but also as samples in this study. The 180-bp region of putative P*tuf* containing the −35 and −10 hexamers was cloned (Fig. 2). The putative promoters for the other three genes were also cloned according to previous reports. Sequences for each cognate MG1363 promoter are similar to those of IL1403, with similarities of 95% (P*tuf*, 180 bp), 86% (P*dnaJ*, 333 bp), 99% (P*pfkA*, 140 bp), and 92% (P*usp45*, 193 bp). Each cloned promoter was, respectively, fused to the promoterless and modified *bmpB* gene (Fig. 1), which was derived from *Brachyspira hyodysenteriae*, a major pathogen of swine dysentery. Since administration of BmpB was reported to protect pigs from this disease [10], we selected the *bmpB* for potential use of *L. lactis* expressing BmpB as an oral vaccine.

Construction of BmpB Expression Vectors Using Lactococcal Promoters

We constructed BmpB expression vectors to examine promoter activity in *E. coli* and LAB. Four promoters were fused to modified *bmpB*, respectively. Each fused fragment was subcloned into pGEM-T easy and pIL.CatT to generate two expression vectors (Fig. 1). For no apparent reason, we failed to construct an *E. coli* expression vector containing P*dnaJ*. All LAB expression vectors were transformed into *L. lactis* IL1403, *L. plantarum* Lb25, and *L. reuteri* ATCC23272.

Table 3 List of spots with high level of expression signal in microarray data for IL1403[a]

Spot ID[b]	ORF[c]	Normalized values[d]	
		CDM[e]	ILV[f]
1980	*tuf*	11.023	17.160
1979	*tuf*	10.497	17.113
1755	*rpmE*	7.130	6.930
1772	*rpoA*	6.653	7.847
1756	*rpmE*	6.550	7.380
1771	*rpoA*	6.513	7.890
50	*als*	5.693	6.770
49	*als*	5.613	6.523
1723	*rplJ*	5.503	5.763
1499	*ptnAB*	5.423	5.533
1724	*rplJ*	5.333	6.220

[a] This table was regenerated by sorting of the microarray data

[b] Spot ID used in microarray data

[c] Gene names for open reading frames

[d] Normalized values of expression signal, not log-transformed, average values for triplicate results

[e] Chemically defined medium

[f] CDM with ten-fold reduced concentrations of isoleucine, leucine and valine

BmpB Expression by Lactococcal Promoters and Host-Range of Promoters

In order to examine the activity of the four lactococcal promoters, cells of all wild-type and recombinant strains were cultivated and harvested. Protein was extracted, and BmpB expression induced by these promoters was detected by western blot assay (Fig. 3). As expected, BmpB expression was induced by all promoters when *L. lactis* IL1403 was used as the expression host. The promoterless

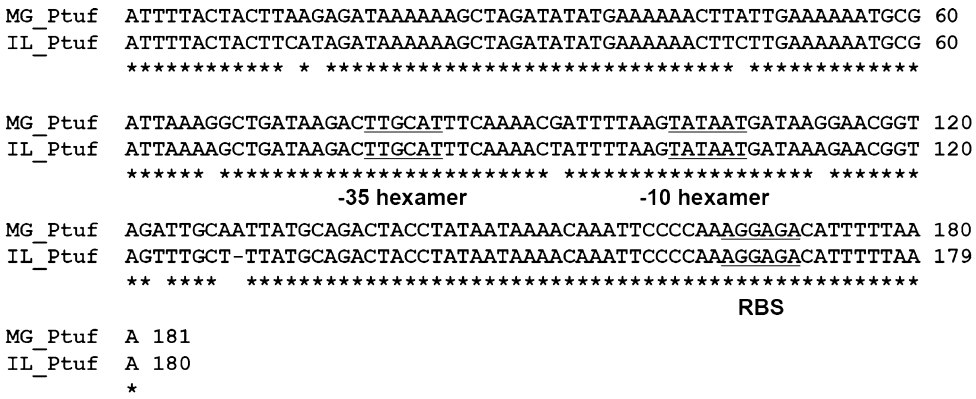

Fig. 2 Nucleotide sequence of IL1403 P*tuf*. Two P*tuf* sequences derived from IL1403 and MG1363 were aligned using ClustalW. Putative −35/−10 hexamers and RBS were indicated

vector did not induce BmpB expression (data not shown). P*tuf* activity was strongest among the promoters examined in IL1403. In IL1403, activity of P*pfkA* was stronger than that of P*dnaJ* or P*usp45*. This result was restricted to IL1403, and we could not expect the same result in other bacteria. Therefore, we examined these promoters using other LAB species and unrelated bacteria (*E. coli*). As shown in Fig. 3, these promoters have different host-range. Only P*usp45* could weakly induce BmpB expression in *L. plantarum* Lb25, the others were weakly active in *L. reuteri* ATCC23272. In *E. coli* Top10F', P*tuf* and P*pfkA* were strongly active, but P*usp45* was not active.

Promoter Activity was Dependent on Growth Phase

We showed that P*tuf* and P*pfkA* were strong in IL1403. Since expression of foreign genes by strong promoter can sometimes cause problems, such as growth inhibition of host cells [4], we examined the growth pattern of wild-type and two recombinant IL1403 strains (Fig. 4a), but there was no difference in the growth pattern between them. Some genes are expressed in a growth phase-dependent manner in LAB [5]. Therefore, we investigated the growth phase-dependent activity of the two promoters (Fig. 4b). We sampled LAB cells at indicated times during cultivation (Fig. 4a), and promoter activity was estimated by BmpB expression at each time. Maximal activity was observed at late log phase for P*tuf* and at early stationary phase for P*pfkA*, and the activity was maintained at early stationary phase.

Correlation Between Promoter Activity and Microarray Data

Since the promoters used in this study were derived and cloned from IL1403, we wondered whether the level of BmpB expression induced by each promoter correlates with the expression level in the microarray data. The microarray data (Series GSE5761) were sorted by the normalized signal, and then regression of the normalized signal (Y) on the rank (X) was analyzed ($\hat{Y} = 7.9752 - 0.9398 \ln(X)$, $R^2 = 0.9809$, $P < 0.0001$). In order to linearize the curvilinear relation, we transformed the value of the normalized signal, and then regression of the transformed signal (Y) on the rank (X) was analyzed ($\hat{Y} = 168.33 + 0.8894\ X$, $R^2 = 0.9963$, $P < 0.0001$). Finally, we analyzed the correlation between the level of BmpB expression (Y) by each promoter and the transformed signal (X) of each cognate gene ($\hat{Y} = 2.2964 - 0.0021\ X$, $R^2 = 0.7186$, $P = 0.06968$, Fig. 5). Since there was no value of *pfkA* in the microarray data, we substituted values of *ldh* and *pyk* genes for a value of *pfkA*. This was because the two genes lie downstream of *pfkA* and are under the control of P*pfkA*. Although the sample number is low, we observed a correlation tendency. This result suggests that a gene with high microarray signal may have a strong promoter and that a gene with low signal may have a weak one.

Discussion

Previously, bacterial P*tuf* had been investigated only in *Chlamydia trachomatis* [18] and *Lactobacillus johnsonii* [25]. There are no reports of P*tuf* in *L. lactis*. We have now identified and cloned a novel P*tuf* of *L. lactis* IL1403.

Based on our present results regarding IL1403 P*tuf*, we do not know whether they need trans-elements. However, they worked without any additional trans-elements in IL1403. On the contrary, the nisin-inducible promoter needs additional trans-elements such as NisK and NisR proteins [6]. Therefore, the short IL1403 P*tuf* can be easily and simply used in IL1403.

Generally, promoters have their own host-range. As shown in Fig. 3, P*tuf* and P*pfkA* did not work in *L. plantarum*, but they worked in *L. lactis, L. reuteri*, and *E. coli*. Therefore, these promoters also have their own host-range. One previous study showed that LAB promoters working in *L. lactis* have similar activity in *E. coli* [8]. We also showed a similar result using P*tuf* and P*pfkA*.

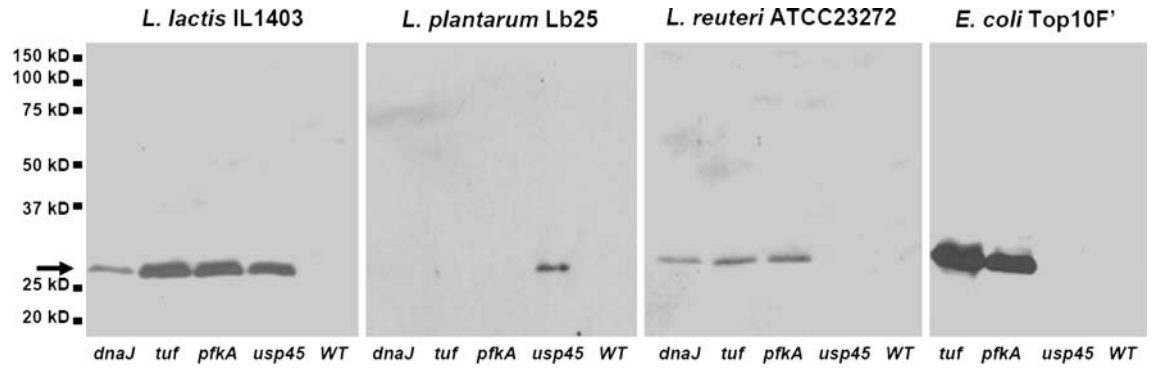

Fig. 3 Detection of BmpB induced by lactococcal promoters. BmpB expression in different bacteria harboring different plasmids was detected. *Arrow* indicates BmpB. This figure is a representative result from three independent experiments. *dnaJ*, pILPdnaJ.Mb; *tuf*, pILPtuf.Mb or pT.Ptuf.MbF; *pfkA*, pILPpfk.Mb or pT.Ppfk.MbF; *usp45*, pILPusp.Mb or pT.Pusp.MbF; WT, no plasmid

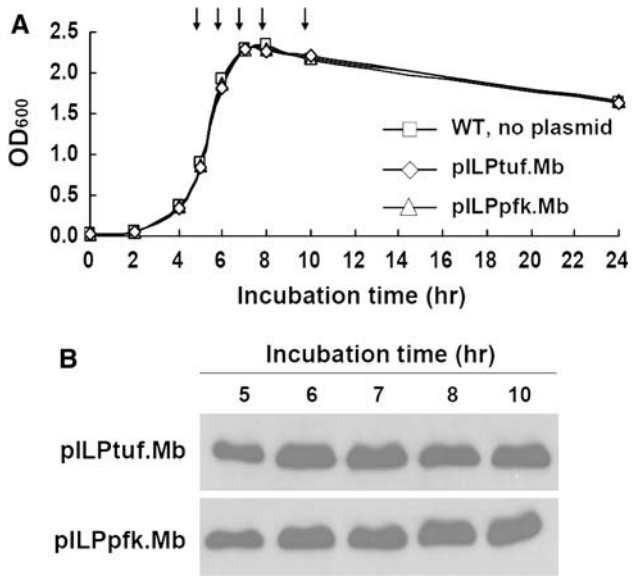

Fig. 4 Growth curve and growth phase-dependent BmpB expression. **a** Growth curve of three strains; wild-type IL1403 and its recombinants harboring pILPtuf.Mb or pILPpfk.Mb. *Arrows* indicate sample-collecting times. **b** Growth phase-dependent BmpB expression induced by two promoters. These figures are representative results from three (**a**) or two (**b**) independent experiments

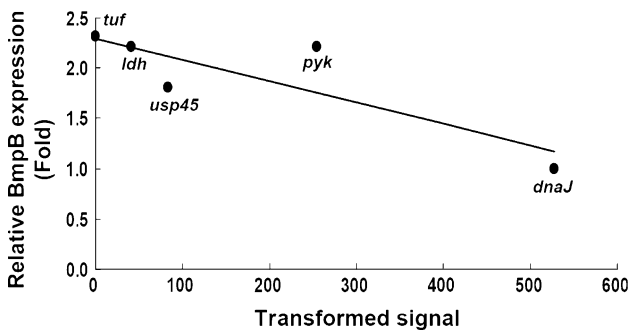

Fig. 5 Correlation between the level of BmpB expression and the value of transformed signal. Relative BmpB expression indicates intensity of each BmpB band divided by the intensity of the BmpB band induced by P*dnaJ*

Several proteomic studies of *L. lactis* reported that the product of IL1403 or MG1363 *tuf* gene was detected at high level by two-dimensional gel electrophoresis (2-DE) [7, 14]. These previous reports are coincident with the strong activity of P*tuf* observed in this study. The *las* operon of *L. lactis* consists of *pfkA*, *ldh* and *pyk* and is involved in glycolysis. This operon has a strong promoter in *L. lactis* MG1363 [20], and products of these IL1403 genes were also detected at high levels by 2-DE [7]. These previous reports are coincident with the strong P*pfkA* activity observed in this study.

Since genetically engineered microorganisms may have potential risks and bio-hazards, many studies have focused on food-grade gene expression systems which consist of genetic elements derived from food-grade microorganisms [4, 8, 11]. LAB are representative food-grade microorganisms. Therefore, the promoters examined in this study are all food-grade genetic elements, and we can use them as food-grade promoters to develop LAB-based applications and as genetic tools to investigate physiology of LAB.

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