Generation and Characterization of Thymidine/D-Alanine Auxotrophic Recombinant *Lactococcus lactis* subsp. *lactis* IL1403 Expressing BmpB

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Abstract Genetic engineering of *Lactococcus lactis* to produce a heterologous protein may cause potential risks to the environment despite the industrial usefulness of engineered strains. To reduce the risks, we generated three auxotrophic recombinant *L. lactis* subsp. *lactis* IL1403 strains expressing a heterologous protein, BmpB, using *thyA*- and *alr*-targeting integration vectors: ITD ($thyA^-alr^+bmpB^+$), IAD ($thyA^+alr^-bmpB^+$), and ITDAD ($thyA^-alr^-bmpB^+$). After construction of integration vectors, each vector was introduced into IL1403 genome. Integration of BmpB expression cassette, deletion of *thyA*, and inactivation of *alr* were verified by using PCR reaction. All heterologous DNA fragments except *bmpB* were eliminated from those recombinants during double crossover events. By using five selective agar plates, we also showed thymidine auxotrophy of ITD and ITDAD and D-alanine auxotrophy of IAD and ITDAD. In M17G and skim milk (SYG) media, the growth of the three recombinants was limited. In MRS media, the growth of IAD and ITDAD was limited, but ITD showed a normal growth pattern as compared with the wild-type strain (WT). All the recombinants showed maximal BmpB expression at an early stationary phase when they were cultivated in M17G supplemented with thymidine and D-alanine. These results suggest that auxotrophic recombinant *L. lactis* expressing a heterologous protein could be generated to reduce the ecological risks of a recombinant *L. lactis*.

Introduction

Lactic acid bacteria (LAB) are food-grade microorganisms widely used in the food industry. For example, *Lactococcus lactis* is one species of LAB used for fermented dairy products such as cheese and fermented milk [16]. Genetic engineering of *L. lactis* has been used to enhance the probiotic potential of wild-type (WT) strains [18]. They have been genetically engineered to express enzymes or antigens, and to produce functional molecules.

Although the genetically engineered LAB (GE-LAB) are useful in the food industry, they may pose potential risks to the ecosystem including human [5]. The well-known problems with GE-LAB are the predominance of themselves in unwanted places and the dissemination of antibiotic resistance genes derived from them [15]. To overcome those problems, the survival of GE-LAB released into the environment can be limited by introducing a mechanism to kill them. Unnecessary DNA elements, including antibiotic resistance genes, should also be eliminated from them.

Two useful biological mechanisms to kill LAB cells have been reported. The first mechanism is the active containment based on cell lysis by cell wall-degrading enzymes such as enterolysin A [6] and autolysin [3]. However, the expression control of killing enzymes is challenging when GE-LAB are released into the environment, and the killing effect may be inactivated by spontaneous mutations in the killing genes or controlling promoters for the genes. The second mechanism is the passive containment, which is based on auxotrophy for specific nutrients such as thymidine [7, 14] and D-alanine [2, 13]. However, auxotrophic GE-LAB for a single specific nutrient can grow and survive where the nutrient is present. To overcome the shortcomings associated with the

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above strategies, dual lethal systems have been reported [11, 17].

In this study, we combined two kinds of auxotrophy, thymidine and ᴅ-alanine, to introduce a dual lethal system into recombinant *L. lactis* expressing a heterologous protein, BmpB, which has been reported to be a vaccine candidate against swine dysentery [9]. Growth in LAB media and BmpB expression of the recombinant *L. lactis* strain were evaluated and compared with those of WT and single auxotrophic strains.

Materials and Methods

Bacterial Strains and Plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* Top10F′ was used as a host bacterium for the construction of each integration vector targeting IL1403 *thyA* or *alr*.

Culture Media and Cultivation

Escherichia coli and *L. lactis* were cultivated in Luria–Bertani broth and M17 broth containing 0.5% (w/v) glucose (M17G), respectively. The antibiotics used were ampicillin (100 μg/ml) for *E. coli* and erythromycin (5 μg/ml) for *L. lactis*. When indicated, 40 μM of thymidine or 200 μg/ml of ᴅ-alanine was added to the media. *E. coli* was cultivated at 37°C with agitation, and *L. lactis* was cultivated at 30°C without agitation.

Construction of Integration Vectors

As shown in Fig. 1a, integration vectors were constructed using the following DNA fragments; *thyA* flanking regions (*thyA* F1 and *thyA* F2), *alr* flanking regions (*alr* F1 and *alr* F3), *ermAM* with a promoter (Pro.*ermAM*), and modified *bmpB* with the IL1403 *pfkA* promoter (P*pfk.bmpB*). These fragments were amplified by PCR using the primers shown in Table 2 and cloned into the pGEM-T easy plasmid. IL1403 genomic DNA was used as a template to clone *thyA* F1, *thyA* F2, *alr* F1, *alr* F3, and the *pfkA* promoter (P*pfk*). The pILPpfk.Mb plasmid was used as a template to clone Pro.*ermAM* and modified *bmpB*, which encodes the partial cytosolic BmpB protein. Two *thyA* flanking regions were cut with *Xba*I, and they were ligated to each other by T4 DNA ligase. The ligated fragment (*thyA*F1.F2) was amplified by PCR and cloned into the pGEM-T easy plasmid to generate the pT.thyAF1.F2 plasmid. Pro.*ermAM* was cut with *Sal*I and *Sac*I, and inserted into the *Sal*I and *Sac*I site of pT.thyAF1.F2 to generate a *thyA*-targeting integration backbone pT.I2 plasmid. Similarly, the *alr*-targeting integration backbone pA.I2 plasmid was generated using the pGEM-T easy, alrF1.F3, and Pro.*ermAM*. After construction of the two integration backbone plasmids, P*pfk.bmpB* was cut with *Spe*I and *Xho*I, and inserted into the *Xba*I and *Xho*I site of pT.I2 and pA.I2, respectively, to generate the final integration vectors, pT.Ppfk.MbI2 and pA.Ppfk.MbI2.

Analysis of Nucleotide Sequences

The nucleotide sequences of all fragments were identified by sequencing based on Sanger's dideoxy sequencing

Table 1 Bacterial strains and plasmids

Strains/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	[12]
ITS	IL1403 derivative, pT.Ppfk.MbI2-integrated at *thyA* F1	This work
IAS	IL1403 derivative, pA.Ppfk.MbI2-integrated at *alr* F1	This work
ITD	ITS derivative, double crossover, *thyA* deleted, modified *bmpB*	This work
IAD	IAS derivative, double crossover, *alr* inactivated, modified *bmpB*	This work
ITDAS	ITD derivative, pA.I2-integrated at *alr* F1	This work
ITDAD	ITD derivative, double crossover, *alr* inactivated	This work
Plasmids		
pGEM-T easy	Amp^r, M13*ori* pBR322*ori*, linear T-overhangs vector	Promega
pIL252	*ermAM*, 4.6 kb, low-copy number plasmid vector for LAB	[12]
pILPpfk.Mb	pIL252 derivative, *pfkA* promoter, modified *bmpB*	[8]
pT.I2	pGEM-T easy derivative, IL1403 *thyA*-targeting	This work
pT.Ppfk.MbI2	pT.I2 derivative, P*pfk* upstream of modified *bmpB*	This work
pA.I2	pGEM-T easy derivative, IL1403 *alr*-targeting	This work
pA.Ppfk.MbI2	pA.I2 derivative, P*pfk* upstream of modified *bmpB*	This work

Fig. 1 Schematic diagram of integration vectors and verification of integration. **a** Gene replacement via homologous recombination. *Arrows* indicate primers used to detect DNA fragments in panel **b**. **b** Verification of crossover and detection of unnecessary DNA fragments by PCR
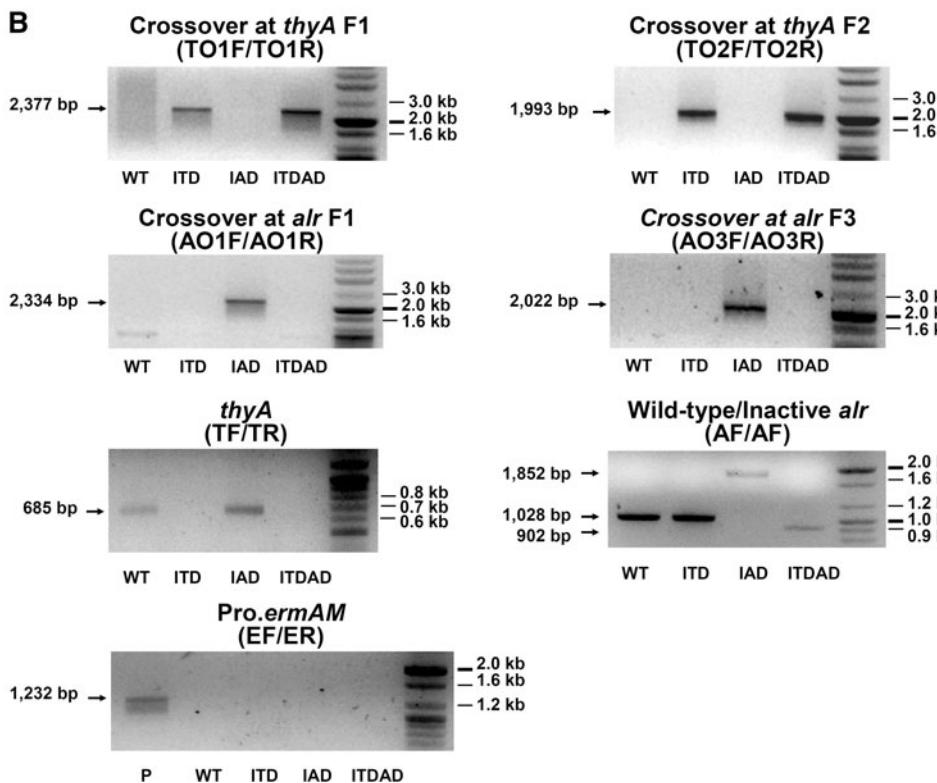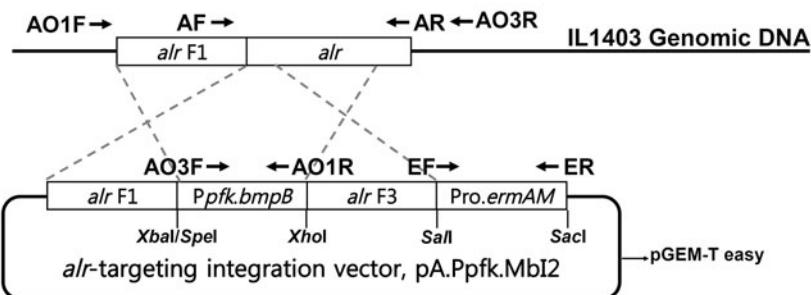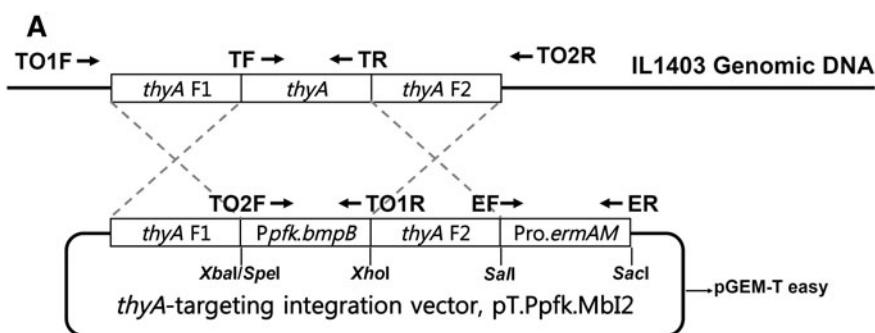
method and the absence of mutations was confirmed. DNA sequencing was carried out by using an Applied Biosystems 3730xl sequencer of the National Instrumental Center for Environmental Management (NICEM, Seoul National University, South Korea).

Integration of Foreign DNA

Each integration vector was introduced into IL1403 competent cells by electroporation [1]. Transformants were then incubated at 30°C for 8 h in 50 ml of M17G

Table 2 Primers for cloning, sequencing, and detecting

Primer name	Sequences of primer	Amplicons (size)
ILthyAf1NEcoRI	TGAATTCGCGCCTCTGGTAATGGTAGT	*thyA* F1 (1,365 bp)
ILthyAf1CXbaI	TTCTAGAGAATCTTCCTATCTTTTTATT	
ILthyAf2NXbaIXhoI	TTCTAGACTCGAGATTAACCAAAAAGTTACTGACAGAA	*thyA* F2 (1,159 bp)
ILthyAf2CSalI	TGTCGACTTCTCAATAGCCCAAGCTCC	
ILalrF1EcoRI	TGAATTCGAAGTTGTTCTTCACAGC	*alr* F1 (1,315 bp)
ILalrF1XbaI	TTCTAGACTTTTAAACCTTCCTCTAGT	
ILalrF3NXbaXho	TTCTAGACTCGAGGGCTCGGTTGAGGTTTCTAAAGCGG	*alr* F3 (692 bp)
ILalrF3CSal	TGTCGACACTTGTTTCATAAGTTGCACCGTAACC	
EF (ErmAMN2SalI)	TGAACTTGTCGACCAAGCAAATACAATCATTGTC	Pro.*ermAM* (1,232 bp)
ER (ErmAMC2SacI)	GCGACTCGAGCTCTTATTTCCTCCCGTT	
pfkN1BglSpe	AGATCTGAAACTAGTAAAACGTTTCATACAGT	P*pfk.bmpB* (972 bp)
pkfC1Nde	GCGTTTCATATGAATCTGTCCTCCG	
Bmp_C_XhoI	TCTCGAGTTATTTCCAAGTAGGAAGATAAG	
TO1F (ILthyF1out)	TATGATGCTTATCTGTTCAAT	Crossover at *thyA* F1 (2,377 bp)
TO1R (Bmp_C_Xho)	TCTCGAGTTATTTCCAAGTAGGAAGATAAG	
TO2R (ILthyF2out)	AATCAAATTTGCCTGGACG	Crossover at *thyA* F2 (1,993 bp)
TO2F (MbmpN_SpeI)	ACTAGTATGGCTTGTAAATCAACACAT	
AO1F (AF1_1)	GAACCAGAAATTCGTAATGG	Crossover at *alr* F1 (2,334 bp)
AO1R (Mb_C2)	TTGATAACAGCACAAGCAAC	
AO3R (AF3_1)	TCCTTACAAATTCGACTCGT	Crossover at *alr* F3 (2,022 bp)
AO3F (MbmpN_SpeI)	ACTAGTATGGCTTGTAAATCAACACAT	
TF (ILthyN1)	GTAACAGGCTCTTTCGTTAC	*thyA* (685 bp)
TR (ILthyC1)	GTTCATAGTCCACAAGCTC	
AF (ILAlrOutN2)	AACATCCTTTTGATGGACAG	WT/Inactive *alr* (1,028/1,852, 902 bp)
AR (ILAlrC2)	CTCCATTTACCAGTACTTTG	

supplemented with thymidine and D-alanine (M17G.TD). After incubation, cells, harvested from 4 ml of the culture, were spread on M17G.TD plates supplemented with erythromycin (M17G.TD.Em5) and the cells were incubated at 30°C for 48 h. Colonies formed after incubation were assumed to be single crossover integrant candidates. To induce re-crossover at a different flanking region, single crossover cells were continuously sub-cultivated using M17G.TD without erythromycin. After approximately 100 generations, erythromycin-sensitive colonies were isolated and assumed to be WT revertants or double crossover integrant strains.

Verification of Integration

Integration of the vectors into the IL1403 genome was verified by PCR using i-MAX II DNA polymerase (iNtRON Biotechnology, South Korea). Genomic DNA extracted from IL1403-derived integrant strains was used as a template. The primers used and their locations are indicated in Fig. 1a and b and Table 2.

Test of Auxotrophy and Erythromycin-Sensitivity Using Selective Agar Plates

Each test strain was cultivated overnight with M17G.TD broth. Each overnight culture was rubbed on five selective agar plates and the plates were incubated for 36 h. After incubation, the plates were scanned.

Growth Analysis in Growth Media

For growth analysis of the three recombinant strains, M17G, MRS, and SYG [10% (w/v) skim milk, 0.5% (w/v) yeast extract, and 0.5% (w/v) glucose] were used as growth media. An overnight culture of each strain was inoculated into 10 ml of growth media and the cells were incubated at 30°C until specified time points. During incubation, the optical density of the culture at 600 nm (OD600) or the number of colony-forming units per ml (CFU/ml) was measured. In the SYG medium, we could not detect the OD600 value due to the high turbidity of the medium.

Bacterial Cell Harvest for Protein Extraction

Single colony of each strain was inoculated into 10 ml of fresh M17G.TD broth, and the cells were incubated overnight at 30°C without agitation. The overnight culture (500 µl) was subsequently inoculated into 50 ml of fresh M17G.TD broth, and the cells were incubated at 30°C without agitation. The OD600 was measured to draw a growth curve. At specified time points, 5 ml of culture was collected and centrifuged at 5,000g for 10 min at 4°C to harvest cells. The harvested cells were stored at −80°C until protein extraction.

BmpB Detection by SDS-PAGE and Western Blot Assay

To extract proteins from IL1403-derived recombinant cells, the harvested cells were washed twice with phosphate buffered saline (PBS) and resuspended in 200 µl of PBS. Glass beads (212–300 µm, Sigma) were added, 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 14,000g for 10 min. The protein-containing supernatant was stored at −80°C until analysis. Quantitative analysis of protein was carried out with Protein Assay (Bio-Rad, USA, #500-0006) and bovine serum albumin (BSA) according to manufacturer's instructions. SDS-PAGE was carried out with a 12% polyacrylamide gel. Each well was loaded with 40 µg protein. 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) [8].

Results

Generation of Three Double Crossover Integrant Strains

We constructed thyA- and alr-targeting integration vectors (pT.Ppfk.MbI2 and pA.Ppfk.MbI2) using the pGEM-T easy plasmid, which does not replicate in LAB. Each vector was introduced into L. lactis IL1403 by electrotransformation and expected to be inserted into the cognate target locus of chromosome via homologous recombination. After transformation erythromycin-resistant colonies were isolated and identified as single crossover integrant strains (ITS and IAS) by PCR (data not shown). These single crossover strains are still having the full ORF sequences of thyA and alr within their genomes. The single crossover thyA-integrant, ITS, did not show thymidine

auxotrophy in M17G, but the single crossover alr-integrant, IAS, showed D-alanine auxotrophy (data not shown). During continuous sub-cultivation of these strains using M17G.TD, double crossover strains (ITD and IAD) were isolated. In a similar way, a thymidine/D-alanine double auxotrophic strain (ITDAD) was isolated from sub-cultivation of an ITD-derived alr-integrant (ITDAS).

The genetic structure of the recombinant strains was confirmed by PCR (Fig. 1b, Table 2). In ITD and ITDAD, crossover at thyA F1 and F2 was identified by 2,377-bp and 1,993-bp bands, respectively. In IAD and ITDAD, crossover at alr F1 and F3 was identified by 2,334-bp and 2,022-bp bands, respectively. These four bands were made between IL1403 genomic DNA and an integration vector, which are separate DNA molecules if the crossover did not occur. In ITD and ITDAD, the absence of thyA was identified by the absence of the 685-bp band shown in WT and IAD. In IAD, insertion of bmpB and truncation of alr were identified by the 1,852-bp band, which is longer than the 1,028-bp band shown in WT and ITD. In ITDAD, truncation of alr was identified by the 902-bp band, which is shorter than the 1,028-bp band of WT and ITD. In all the four strains, the absence of Pro.ermAM was identified by the absence of the 1,232-bp band, which is shown in a Pro.ermAM-containing strain (P). All recombinant and wild-type strains could not grow when erythromycin was supplemented, which means that they have no active ermAM gene (Fig. 2). These results suggest that all three recombinant strains are in double crossover at thyA or alr locus and they did not have any heterologous DNA elements except the BmpB expression cassette.
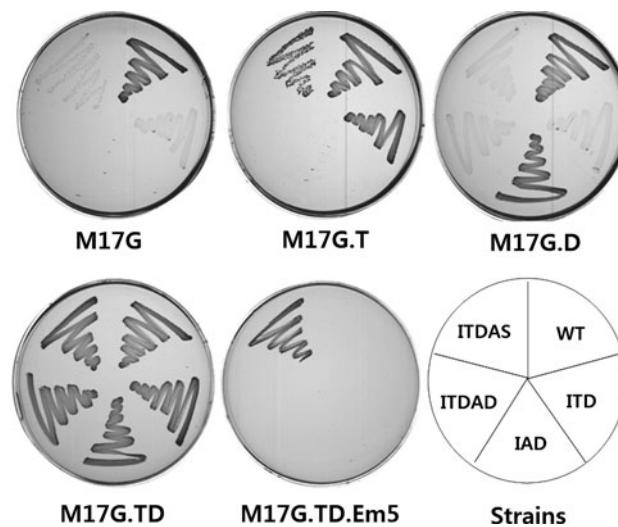

Fig. 2 Test of auxotrophy and erythromycin-sensitivity of recombinant strains. T supplemented with thymidine; D supplemented with D-alanine

Growth of Recombinant *L. lactis* Strains in M17G, MRS, and SYG

The growth of *thyA*-deficient LAB strains is dependent on the presence of thymidine or thymine, because these strains cannot produce dTTP without supplementation [7, 14]. Likewise, the growth of *alr*-deficient LAB strains is dependent on the presence of ᴅ-alanine, which is required to synthesize normal cell walls [2, 13]. To show the thymidine or/and ᴅ-alanine dependency of the three recombinant strains (ITD, IAD, and ITDAD), we checked auxotrophy of each recombinant using five different M17G-derived selective agar plates (Fig. 2). ITD or IAD could grow normally only when thymidine or ᴅ-alanine was supplemented, respectively. ITDAD could grow normally only when both thymidine and ᴅ-alanine were supplemented.

A growth curve of each recombinant strain was compared with that of WT IL1403 using conventional LAB media such as M17G and MRS. In addition, pH changes were also measured because LAB produce lactic acid, thereby lowering the pH of the medium. When M17G was used (Fig. 3a), the growth of the three recombinants was
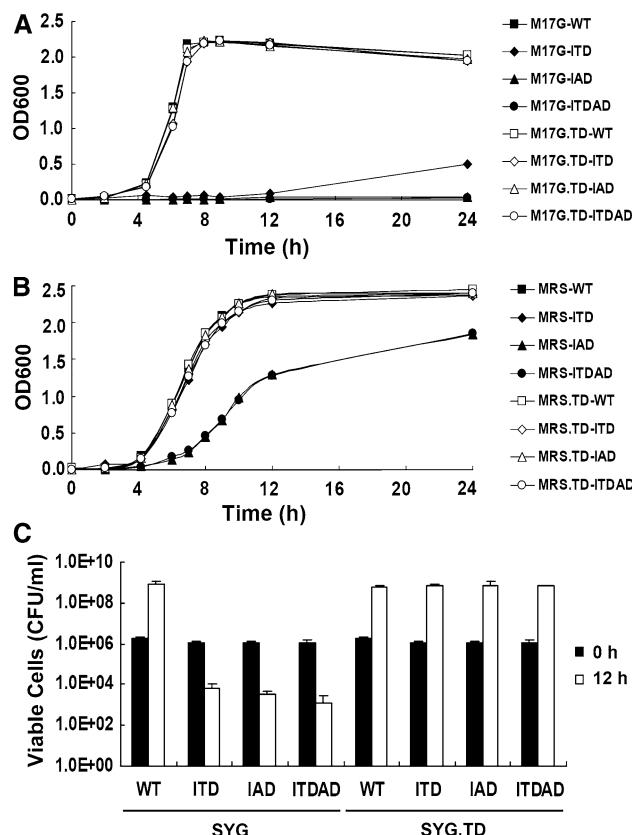

limited as compared with the WT strain. The growth limitation of two *alr*-integrants (IAD and ITDAD) was affected to a greater extent than that of ITD. The pH value of M17G changed from 6.90 at 0 h to 5.85 (WT), 6.34 (ITD), and 6.85 (IAD and ITDAD) after 24 h of incubation. When MRS was used (Fig. 3b), the growth of only the two *alr*-integrants was limited, while the ITD strain showed a normal growth pattern identical to that of the WT strain. The pH value of MRS changed from 6.73 at 0 h to 4.58 (WT), 4.60 (ITD), and 5.28 (IAD and ITDAD) after 24 h of incubation. In contrast, when the two media were supplemented with thymidine and ᴅ-alanine, all recombinants were able to grow and change the pH of media in a manner identical to that of the WT strain, even though the growth of ITD was slightly retarded in M17G.TD.

Milk has been recognized as a nutrient-rich niches for LAB [10], but it has insufficient pyrimidines to support the growth of some LAB strains. The pyrimidine source present in milk is orotate, an intermediate compound in pyrimidine biosynthesis. However, only some LAB strains can utilize orotate to synthesize pyrimidines and grow in milk [7]. To examine the growth and thymidine or/and ᴅ-alanine dependency of the auxotrophic recombinants in milk, a skim milk-based SYG medium was used. When SYG was used (Fig. 3c), all the recombinants showed similar levels of growth limitation and cell death, and only the WT strain could grow normally and lower the pH of SYG thereby inducing curd formation (data not shown). The pH value of SYG changed from 6.54 at 0 h to 4.72 (WT), 6.15 (ITD), and 6.56 (IAD and ITDAD) after 24 h of incubation. On the contrary, when thymidine and ᴅ-alanine were added to SYG, normal growth and curd formation were observed in all strains (data not shown).

BmpB Expression in Recombinant *L. lactis* Strains

A heterologous gene, modified *bmpB*, was inserted into the genome of each recombinant. When they are expected to be used as LAB-based oral vaccines against the swine dysentery, the BmpB expression is an important issue because expressed BmpB antigen should be delivered to the intestinal immune system. Therefore, in this study, the BmpB expression in the recombinants was examined. All the recombinant strains were able to express BmpB and the expression was growth phase-dependent. The maximal BmpB expression was observed at an early stationary phase regardless of the strain type (Fig. 4a, b).

Discussion

We generated three recombinant *L. lactis* strains expressing BmpB. They do not have any antibiotic resistance gene and

Fig. 3 Growth of recombinant auxotrophic strains. Growth curves in M17G (**a**) and in MRS (**b**) were made from two independent experiments. Growth in SYG (**c**) was monitored and this figure is a representative result from two independent experiments

Fig. 4 Growth phase-dependent BmpB expression. **a** Growth curve of the four strains in M17G.TD. **b** Growth phase-dependent BmpB expression. The *arrows* indicate BmpB expression. Sampling time points are indicated in panel **a**
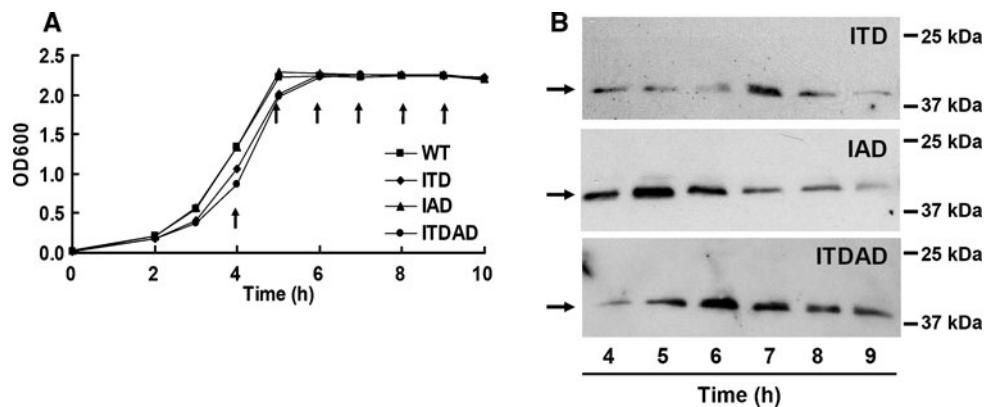
vector-derived DNA fragments, and the only heterologous genetic element inserted into their chromosome is the gene of interest (modified *bmpB*). The methods we used to engineer *L. lactis* seem to be safer than conventional methods using plasmid-based expression vectors, because expression vectors generally have selection markers such as antibiotic resistance genes and contain DNA elements to replicate and maintain themselves in host bacteria. These antibiotic resistance genes can be transferred to other bacteria, including pathogens. The transfer is one of the major problems of genetically modified organisms [15].

The growth of auxotrophic strains in M17G, MRS, and SYG was limited. In particular, the two D-alanine auxotrophs (IAD and ITDAD) experienced not only growth limitation but also cell death (Fig. 3c). But the thymidine auxotroph, ITD, showed a normal growth pattern in MRS medium without additional thymidine. MRS is a representative LAB growth medium and widely used in LAB cultivation. Due to both normal growth of ITD in MRS and absence of antibiotic resistance gene, we may misjudge that the recombinant strain is a WT strain. However, the defect of a single auxotrophy of ITD could be overcome by a dual lethal system based on thymidine and D-alanine auxotrophy (ITDAD). Nevertheless, the mortality of IT-DAD was not greater than that of the single lethal system based on D-alanine auxotrophy (IAD).

Gene expression in LAB has been reported to be affected by the growth phase [4]. In this study, BmpB expression was also growth phase-dependent. The same BmpB expression pattern was observed when the BmpB expression cassette (P*pfk.bmpB*) was used in a plasmid expression vector [8]. Therefore, the growth phase-dependency of BmpB expression is likely to be a result of P*pfk*. Maximal BmpB expression was observed in the early stationary phase. This time point is optimal for cell harvesting if the recombinants are to be used as oral vaccines, because both cell yield and BmpB expression are maximal at this time point.

In conclusion, we generated recombinant *L. lactis* strains using genetic engineering based on gene replacement and auxotrophy. The resulting recombinant strains were able to express a heterologous protein, BmpB. In addition, the recombinants showed growth limitation and cell death in growth media. The approach we took can be used to generate safe GE-LAB. However, further study is needed to examine their in vivo mortality and effects as LAB-based oral vaccines.

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