Bioscience of Microbiota, Food and Health Vol. 41 (1), 20–29, 2022

Full Paper

Novel shuttle vector pGMβ1 for conjugative chromosomal manipulation of *Lactobacillus delbrueckii* subsp. *bulgaricus*

Daiki IWAMOTO[1], Megumi ISHIZAKI[1], Taiki MIURA[1] and Yasuko SASAKI[1*]

[1]*Graduate School of Agriculture, Meiji University, 1-1-1 Higashi-Mita, Tama-ku, Kawasaki, Kanagawa 214-8571, Japan*

Received February 16, 2021; Accepted September 22, 2021; Published online in J-STAGE October 18, 2021

***Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) is widely used as a starter for yogurt and cheese worldwide. Despite the economic importance of this bacterium in the dairy industry, there have been few genetic studies involving knockout or overexpression mutants to identify the functions of *L. bulgaricus* genes. One of the main reasons for this gap is the low transformation efficiency of available *L. bulgaricus* chromosome-integrating vectors upon performing conventional electroporation. We previously proposed the conjugal plasmid pAMβ1 as an integration vector for *L. bulgaricus*, as conjugation could avert the need for a restriction modification system; pAMβ1 does not replicate and integrate into the chromosome of *L. bulgaricus*. Here, we describe an effective chromosomal manipulation system involving a novel shuttle vector pGMβ1, which could improve the operability of the broad host-range conjugal plasmid pAMβ1. We further developed an enhanced filter-mating method for conjugation. To validate this system, the effectiveness of conversion of the lactate dehydrogenase gene *D-ldh* of *L. bulgaricus* to the *L-ldh* form of *Streptococcus thermophilus* was examined. As pGMβ1 and pAMβ1 are unable to replicate in *L. delbrueckii* subsp. *delbrueckii*, they were chromosomally integrated. However, these plasmids could replicate in *L. delbrueckii* subsp. *indicus* and *sunkii*. This integration system could unearth important gene functions in *L. bulgaricus* and thus improve its applications in the dairy industry. Moreover, this conjugation system could be used as a stable vector for the transformation of long cluster genes in several species of lactic acid bacteria.**

Key words: *Lactobacillus delbrueckii* subsp. *bulgaricus*, recalcitrant strains, chromosomal manipulation, conjugal shuttle vector, lactic acid bacteria

INTRODUCTION

Lactobacillus delbrueckii subsp. *bulgaricus* (*L. bulgaricus*) is used in the dairy industry worldwide as a starter to produce yogurt and cheese. This has led to high demand for in-depth genetic studies on *L. bulgaricus* to maximize its usefulness. Currently, complete genome sequence data are available for four strains of *L. bulgaricus* in the Kyoto Encyclopedia of Genes and Genomes database [1–4]. However, few genetic studies have used knockout or overexpression mutants; thus, the specific functions of this so-called "recalcitrant" bacterium remain poorly understood. The primary cause for the limited genetic work on *L. bulgaricus* is the lack of effective and reproducible methods for chromosome manipulation [5].

One of the difficulties in performing chromosome manipulations in *L. bulgaricus* is the lack of an appropriate vector with high transformation efficiency. The majority of *L. bulgaricus* strains do not harbor plasmids, with only four such plasmids reported to date: pDOJ1, 6.2 kb [6]; pLBB1, 6.1 kb [7]; pBUL1, 7.9 kb [8]; and LDBND_P, 6.2 kb [9]. It is also difficult to transform *L. bulgaricus* with plasmid DNA by conventional electroporation protocols as its elongated shape makes it highly sensitive to electrical pulses. Moreover, throughout its long history of use in the dairy industry, the industrial strains of *L. bulgaricus* have shown high restriction enzyme activity, primarily to confer protection against phages [10]. Therefore, targeting the genes coding for restriction enzymes could help overcome this limitation. Indeed, Sasaki's research group obtained transformants of pX3, derived from pBUL1, by electroporation into the T11 strain [5, 11, 12], which is a mutant deficient in restriction modification genes. Serror *et al.* [8] also succeeded in transforming several plasmids in *L. bulgaricus* VI104, with 10^4 transformants/μg DNA reported as the highest transformation efficiency obtained with the pLEM415 plasmid.

High transformation efficiencies are required to ensure chromosomal integration as it generally occurs at a low frequency. There are both replicative and non-replicative mechanisms for chromosomal integration. Using the non-replicative plasmid

*Corresponding author. Yasuko Sasaki (E-mail: y_sasaki@meiji.ac.jp)

doi: 10.12938/bmfh.2021-014

pJC4, Jang *et al.* [13] obtained the chromosomal integrant of *L. bulgaricus* ATCC11842^T with cellulase screening. Li *et al.* [4] reported *ccpA* disruption in *L. bulgaricus* ATCC11842^T by homologous recombination using a linear DNA fragment (CcpA-Tet-CcpA) and the pCT vector. Unfortunately, there have been no follow-up reports on disruption mutations using these methods.

With respect to the replicative approach, pG$^+$host [14] is an effective replicative and thermosensitive plasmid, which is derived from pWVO1 with a thermosensitive replicon (ts) and has been used in many lactococci [15, 16], streptococci [17, 18], and lactobacilli [19] to date. Sasaki and colleagues were only able to achieve successful integration in the chromosome of the T11 mutant strain (R$^-$M$^-$) using pSG$^+$E2, which contained a *ts* replicon of the pG$^+$host, whereas integration was not successful in the chromosomes of other industrial strains (personal communication). As the lower transfer frequency made it difficult to manipulate the chromosomal genes of *L. bulgaricus*, direct manipulation systems were explored. The plasmid pMC1, containing an *attP* site and the integrase (*int*) gene of the *L. bulgaricus* mv4 bacteriophage, was integrated in a site-specific manner into the tRNA^{Ser} gene of *L. bulgaricus* [8]. Subsequently, Serror *et al.* [5] developed a transposition mutagenesis system for *L. bulgaricus* using a pIP501 derivative as a delivery vector with IS1223 or IS1201. Additionally, Licandro-Seraut *et al.* [20] developed an *in vivo* random mutagenesis system (Pjunc-TpaseIS1223) in *Lacticaseibacillus casei* and *L. bulgaricus*, which was found to be particularly efficient in *L. casei*; however, more than 80% of the sequenced targets were located in intergenic regions in *L. bulgaricus.*

We previously proposed a gene-targeting chromosomal integration method involving the conjugation of pAMβ1 [21] for targeted gene disruption and gene replacement in *L. bulgaricus* [22, 23]. Conjugation [22] is a specific system that mediates the transfer of DNA in a wide range of bacterial genera. Intergeneric transfer has also been reported in some cases. pAMβ1 is a conjugative, broad-host-range plasmid that is isolated from *Enterococcus faecalis* and is capable of autonomously replicating in many lactic acid bacteria, including *Lactococcus lactis* (*Lc. lactis*) [24], streptococci [25], and several genera of the order Lactobacillales such as *L. casei* [26], *Lactiplantibacillus plantarum, Lactobacillus acidophilus,* and *Ligilactobacillus salivarius* [27], as well as in other gram-positive bacteria such as *Bacillus thuringiensis* [28] and *Clostridium acetobutylicum* [29]. Although pAMβ1 can replicate in many gram-positive bacteria, our previous study showed that it was unable to do so in *L. bulgaricus* and that it integrated into the chromosome. Intergeneric transfer of pAMβ1 was achieved using an improved filter-mating method under erythromycin (Em) selective pressure; however, the transfer frequency was markedly reduced, reaching only approximately 10^{-9} per recipient compared with a transfer frequency of 10^{-4} per recipient from *E. faecalis* to *L. plantarum* [30]. This chromosomal integration of pAMβ1 was observed not only in *L. bulgaricus* but also in two other subspecies of *L. delbrueckii* subsp. *delbrueckii* and *L. delbrueckii* subsp. *lactis*. However, insertion of a 1.0 kb homologous fragment of the *L. bulgaricus* chromosome into pAMβ1 increased the conjugal transfer frequency by more than 100-fold, resulting in successful gene conversion in *L. bulgaricus.*

Although the use of conjugation as a gene-transforming tool is a classical method, gene transfer via a single strand can avert the use of restriction enzymes that generally break double-stranded DNA [31]. However, a significant issue arises when preparing the theta-type plasmid pAMβ1 in *Lc. lactis* or *Streptococcus thermophilus*, due to its large size (27,815 bp; National Center for Biotechnology Information accession no. NC_013514) and varying copy number (one or two) [21]. To overcome these limitations, we developed a new method using the shuttle vector pGMβ1 to manipulate chromosomal genes. We then used this system to examine the replication of pAMβ1 in two other subspecies of *Lactobacillus delbrueckii*: subsp. *indicus* [32] and subsp. *sunkii* [33].

Notably, the shuttle vector pGMβ1 enabled the efficient and convenient construction of an integration plasmid in *Escherichia coli* DH5a. We could not determine the adequate conditions required for selection of the plasmid transfer conjugant of *L. bulgaricus* when *E. coli* was used as the donor, although *E. coli* showed higher tolerance than *L. bulgaricus* under all tested conditions (temperature, salt, antibiotic resistance, and nutrition). *Lc. lactis* IL1403 was selected as the donor for two reasons: (1) the transfer conjugant of *L. bulgaricus* could be differentiated from *Lc. lactis* at a specific temperature (45°C) and by resistance to erythromycin (Em^R), and (2) IL1403 showed a high transfer frequency and low restriction ability when the plasmid constructed in *E. coli* was electroporated into it. The combination of the improved filter-mating method [30] and gene targeting using a homologous region could help to overcome the extremely low chromosomal integration frequency. To further validate this conjugal intergenic integration method with pGMβ1, in the present study, we tested the conversion of the lactate dehydrogenase gene *D-ldh* of *L. bulgaricus* to the *L-ldh* form of *S. thermophilus.* This method of integration could facilitate the discovery of important gene functions in *L. bulgaricus* and thus improve its applications in dairy production.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Table 1 shows the strains and plasmids used in this study. *Lc. lactis* [34] and *S. thermophilus* [35] were cultured on GM17 and LM17 media, which were comprised of M17 (Becton, Dickinson and Company) supplemented with 5 g/L glucose or lactose for 16 to 18 hr at 32°C or 37°C, respectively, without shaking. SMY medium contained 10% skim milk with 0.1% yeast extract (Thermo Scientific™ Oxoid™ Yeast Extract Powder).

Lactobacillus strains (Table 1) were inoculated at 1% (vol/vol) on de Man, Rogosa and Sharpe (MRS) medium (Becton, Dickinson and Company) and cultured at 42°C without shaking. Before the main culture, the precultures were performed at 37°C for 16 to 18 hr. The *E. coli* strain was grown on Luria–Bertani (LB) medium at 37°C with shaking.

Construction of pGMβ1

The schematic for construction of the shuttle vector pGMβ1 is shown in Fig. 1A. Table 2 shows the primers used in this study. The fragment containing the whole length of the pGEM-T Easy vector (the replication origin, f1 ori and ampicillin-resistance gene) was amplified with the primers LAB184 and LAB185C, which contained the AvaI (BmeT110I, Takara Bio) site (underlined in Table 2), using the pGEM-T Easy vector as a template for polymerase chain reaction (PCR; Gflex DNA

Table 1. Strains and plasmids

Strain		Reference
Escherichia coli	DH5-alpha	Takara Bio Inc.
Lactococcus lactis	IL1403	[12]
Streptococcus thermophilus	ST1131	[18]
Lactobacillus delbrueckii		
subsp. *bulgaricus*	LB2038	[18]
subsp. *bulgaricus*	LB600	[35]
subsp. *indicus*	JCM15610^T	Japan Collection of Microorganisms
subsp. *indicus*	SAK	This study; Laboratory collection
subsp. *sunkii*	JCM17838^T	Japan Collection of Microorganisms
subsp. *lactis*	JCM1248^T	Japan Collection of Microorganisms
subsp. *delbrueckii*	JCM1012^T	Japan Collection of Microorganisms
Plasmid		**Reference**
pGEM-T easy	3,015 bp	Promega Co.
pAMβ1	27,815 bp	[30]
pGMβ1	30,831 bp	This study
pβL−Int1	30,943 bp	[37]
pGMβ$_{int}$1	32,778 bp	This study
pGMβ$_{int}$2	32,708 bp	This study

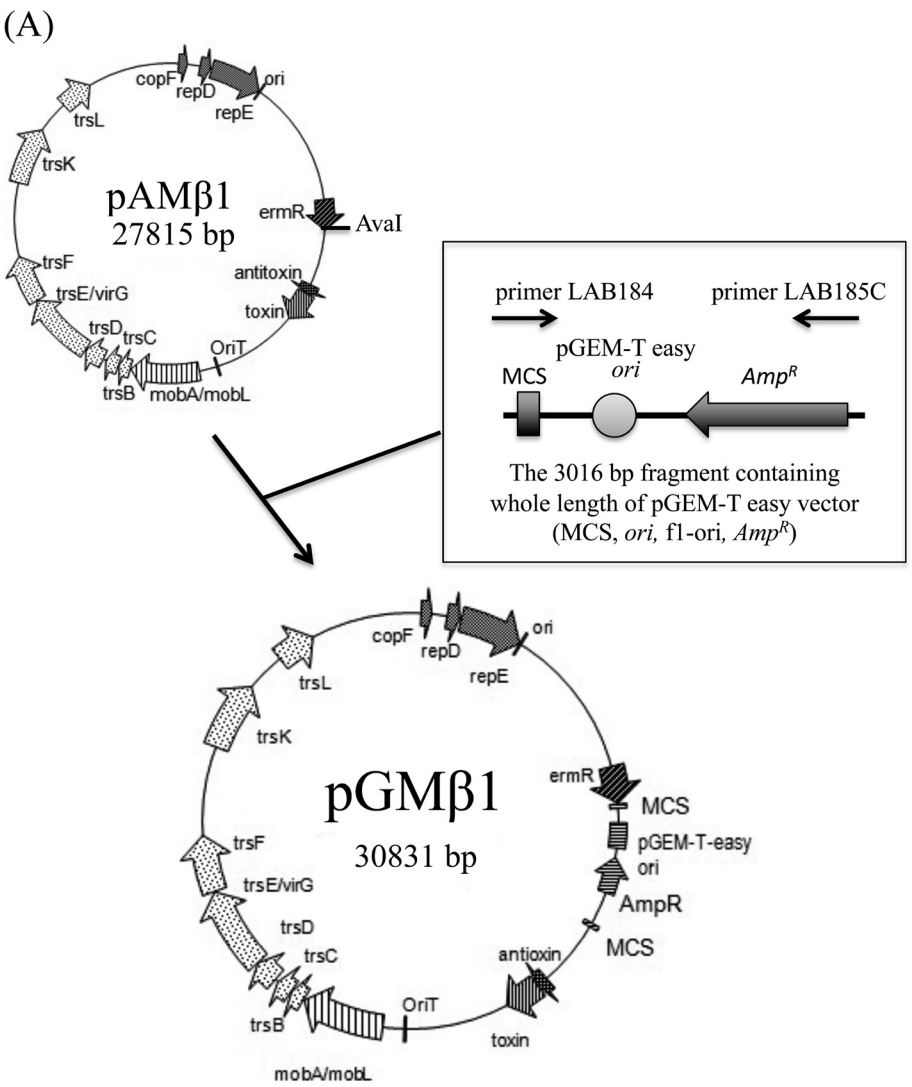


Fig. 1. (A) Construction of the conjugative shuttle plasmid pGMβ1. (A) The 3016 bp fragment containing the replication origin and ampicillin resistance gene of the pGEM-T Easy vector was inserted at the AvaI site of pAMβ1.

doi: 10.12938/bmfh.2021-014

Table 2. Primer list

Primer	Sequence (5′–3′)	Template (Plasmid or chromosome)	Length (bp)
LAB011	GGGCATTTAACGACGAAACT	erythromycin resistance gene of pAMβ1	LAB011/LAB012C
LAB012C	GGCGTGTTTCATTGCTTGAT	erythromycin resistance gene of pAMβ1	509 bp
LAB216	GGGTGGAACCATCTCTGGCGAAC	5' upper region of *D-lactate dehydrogenase* of *L. bulgaricus*	LAB216/LAB011
	:440 bp	upper from the initiation codon of *D-ldh (L-ldh)* of *L. bulgaricus*	2,822 bp (2,837 bp)
LAB084	GGATGACTGCAACTAAACTA	from 1–18 nt of *L-lactate dehydrogenase* of *S. thermophilus*	LAB084/LAB085C
LAB085C	CCTTAGTTTTTTGAAGCTTCTTGGA	from 968–987 nt of *L-lactate dehydrogenase* of *S. thermophilus*	987 bp
LAB105	GCGCTTAGAATCGCTTTAGGAAAC	fragment (1) Forward: 5–3076 nt of pGMβ1	LAB105/LAB106C
LAB106C	CGGGTTCTTCAAATATTTCTCCAAG	fragment (1) Reverse: 5–3076 nt of pGMβ1	3,072 bp
LAB107	CCCGATTACATGGATTGGATTAGTTC	fragment (2) Forward: 3073–6322 nt of pGMβ1	LAB107/LAB108C
LAB108C	GCACTATCAACACACTCTTAAGTTTG	fragment (2) Forward: 3073–6322 nt of pGMβ1	3,250 bp
LAB109	CTTAGAAGCAAACTTAAGAGTGTGTTG	fragment (3) Forward: 6299–9395 nt of pGMβ1	LAB109/LAB110C
LAB110C	GGGTGCTGTTGTTTAAAGGTATC	fragment (3) Reverse: 6299–9395 nt of pGMβ1	3,097 bp
LAB111	CCCTAATTTTGATGAACTAGCGAAAC	fragment (4) Forward: 9393–12377 nt of pGMβ1	LAB111/LAB112C
LAB112C	CGAGCGAATAGCGAGCAAAATATTAAC	fragment (4) Reverse: 9393–12377 nt of pGMβ1	2,985 bp
LAB113	GCACCTTTTTCAATTAGACGCTTTG	fragment (5) Forward: 12313–15426 nt of pGMβ1	LAB113/LAB114C
LAB114C	GGCTTGTTTCACTTGATCGCTATTC	fragment (5) Reverse: 12313–15426 nt of pGMβ1	3,114 bp
LAB115	GGGAGTTAGTTATGAATAGCGATCAAG	fragment (6) Forward: 15389–18359 nt of pGMβ1	LAB115/LAB116C
LAB116C	CGGACTAACGCCGTAAATATCTTC	fragment (6) Reverse: 15389–18359 nt of pGMβ1	2,970 bp
LAB117	CGGCGTTAGTCCGAAGAAAG	fragment (7) Forward: 18347–21446 nt of pGMβ1	LAB117/LAB118C
LAB118C	GCTTCTACTCCTCTCCTAATTGAATG	fragment (7) Reverse: 18347–21446 nt of pGMβ1	3,099 bp
LAB119	CCCAAAGAACGACCATTCAATTAG	fragment (8) Forward: 21428–24573 ntof pGMβ1	LAB119/LAB120C
LAB120C	CGCCCTCAAAGACATTAGAGATAG	fragment (8) Reverse: 21428–24573 nt of pGMβ1	3,145 bp
LAB121	GCTGGTGAGGCTATCTCTAATG	fragment (9) Forward: 24540–40 nt of pGMβ1	LAB121LAB122C
LAB122C	GGACTGGATCGTGTTTCCTAAAG	fragment (9) Reverse: 24540–40 nt of pGMβ1	3,316 bp
LAB184	CAACTCGGGCACTAGTGAATTCGCGGCCGCCTG	whole nucleotides of pGEM-T-Easy vector (+AvaI)	LAB184/LAB185C
LAB185C	GTTCCCGAGGAATTCCCGCGGCCGCCATGGC	whole nucleotides of pGEM-T-Easy vector (+AvaI)	3,016 bp
LAB270	CCAGAGCTCGGATCCAACACCAGATCAAGAG	insertion fragment for *L-ldh* gene conversion with *D-ldh* of *L. bulgaricus* (+SacI)	LAB270/LAB271C
LAB271C	GGTGAGCTCGTCTATCACAACCGACAACGG	insertion fragment for *L-ldh* gene conversion with *D-ldh* of *L. bulgaricus* (+SacI)	1,950 bp
LAB345	CTTATCCATTAAAAGCTAAAACGAAAACCCGCGG	3' down region of *D-lactate dehydrogenase* of *L. delbrueckii* JCM1012	LAB345/LAB346C
LAB346C	GGCGAGCTCGAAATGAAGGAATTCATCCTGCC	3' down region of *D-lactate dehydrogenase* of *L. delbrueckii* JCM1012	516 bp

polymerase, Takara Bio). The fragment was purified with a QIAquick PCR purification and gel extraction kit (Qiagen). The PCR product, a 3016 bp fragment, was ligated at the AvaI site of pAMβ1 (DNA ligation kit ver.1, Takara Bio). The ligation mixture was electroporated into *E. coli* DH5a using a Gene Pulser (Bio-Rad; 2.5 kV, 25 μF, 300 Ω). Transformants were selected in the presence of 50 μg/mL ampicillin (Sigma-Aldrich) and 250 μg/mL erythromycin (Sigma-Aldrich) in LB broth, and the plasmids were prepared using the alkaline sodium dodecyl sulfate (SDS) extraction procedure [36].

The non-deletion 30.8-kb plasmid was selected and analyzed with several restriction enzymes (NotI, HpaI, and PvuII, Takara Bio). The resulting plasmid was designated pGMβ1 and was electroporated into *Lc. lactis* IL1403 according to a previous protocol [35], followed by selection with 25 μg/mL Em in GM17 medium.

Construction of pGMβ$_{ldh}$1 and pGMβ$_{ldh}$2 plasmids for gene conversion

As the conversion occurred through double homologous recombination, pGMβ$_{ldh}$1 was constructed so as to contain an *L-ldh* gene and two homologous regions, the 5′ upstream and the 3′ downstream region of *D-ldh*. The insertion fragment was amplified with the primers LAB270 and LAB271C, which contained a SacI site (Takara Bio), using the pβL-Int1 vector [37] as a template. The pβL-Int1 vector contained a 359 bp fragment of the 5′ upstream region and a 586 bp fragment of the 3′ downstream region of *D-ldh* (LBU_ 0066: according to KEGG) derived from the *L. bulgaricus* LB2038 chromosome and situated at either end of the 987 bp region of the *L-ldh* gene of strain ST1131 (Fig. 1B).

The resulting 1950 bp fragment (1932 bp + primer) was ligated into pGMβ1 at the SacI site to generate the plasmid pGMβ$_{ldh}$1 (Fig. 1B). The 359 bp fragment at the 5′ upstream region of *D-ldh* is highly conserved among the five subspecies of *L. delbrueckii*. However, the sequence of the 3′ region could be classified into two groups: one group contained subsp. *bulgaricus* and subsp.

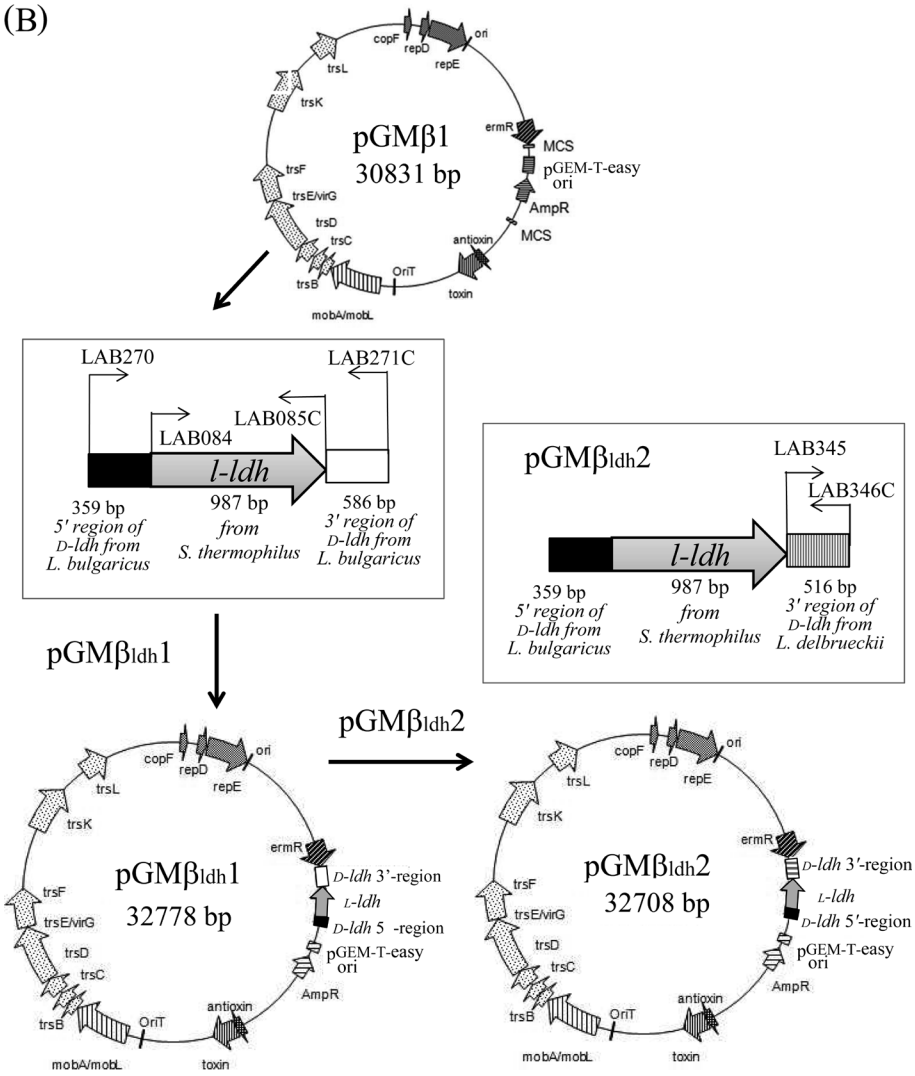


Fig. 1. (B) pGMβ$_{ldh}$1 and pGMβ$_{ldh}$2 were constructed for conversion of the *L-ldh* gene of *S. thermophilus* and *D-ldh* gene of five subspecies of *L. delbrueckii* with double-crossover events. There was a difference in the 3′ downstream sequence of *D-ldh* between pGMβ$_{ldh}$1 and pGMβ$_{ldh}$2. pGMβ$_{ldh}$1 was used for *L. bulgaricus* and *L. indicus*, and pGMβ$_{ldh}$2 was used for *L. delbrueckii* and *L. sunkii.*

indicus, and the other contained subsp. *delbrueckii*, subsp. *lactis*, and subsp. *sunkii.* Then plasmid pGMβ$_{ldh}$2 was then constructed, which contained a different 516 bp fragment containing the 3′ downstream region of *D-ldh* amplified using the primers LAB345 and LAB346C and the *L. delbrueckii* subsp. *delbrueckii* JCM1012^T chromosome as the template.

We used pGMβ$_{ldh}$1 for *L. bulgaricus* and *L. indicus*, and pGMβ$_{ldh}$2 for *L. delbrueckii*, *Lactobacillus delbrueckii* subsp. *lactis* (*Lb. lactis*) and *L. sunkii* to achieve the gene conversion from *D-ldh* to *L-ldh*.

Conjugation experiment

Conjugation by filter mating was performed between the donor, *Lc. lactis* IL1403 (pGMβ$_{ldh}$1, pGMβ$_{ldh}$2, or pAMβ1), and the recipients, *S. thermophilus* and five subspecies of *L. delbrueckii*, according to our proposed method. The detailed workflow of the conjugation method is outlined in Fig. 2. The donor and recipient mixtures were washed twice in 20 mM phosphate buffer (pH 7.5), trapped on a filter membrane (0.45 mm), and passed through 60–80 mL of sterilized water on the filter membrane using a vacuum pump. After the integration was confirmed by checking the chromosomal construction check using PCR, successive culturing without Em was performed continuously until a double-crossover event occurred. The number of times these steps were repeated to obtain an Em-sensitive clone depended on the target genes.

As the frequency of chromosomal integration is remarkably low in *L. bulgaricus*, the transfer conjugant cannot be obtained by the conventional filter-mating method. To increase the transfer frequency, sterilized water was passed through the filter membrane under reduced pressure after donor and recipient cells were trapped on the filter membrane, as this enables tight contact between the donor and recipient [30].

To validate whether pAMβ1 could replicate in *L. indicus* and *L. sunkii*, the conjugation experiment was performed with *Lc. lactis* IL1403 (pAMβ1) as the donor and *L. indicus* JCM15610^T,

L. indicus SAK and *L. sunkii* JCM17838^T as the recipients. The next conjugation experiment was performed with *L. indicus* JCM15610^T (pAMβ1) and *L. sunkii* JCM17838^T (pAMβ1) as the donor and *Lc. lactis* IL1403 as the recipient. In this case, transconjugants of *Lc. lactis* IL1403 (pAMβ1) were selected on Em resistance and growth in the presence of 4% NaCl supplemented on GM17. Field-inversion gel electrophoresis (FIGE) was used to detect the pAMβ1 plasmid for confirmation of intergenic conjugation.

Plasmid preparation and detection

Plasmids were extracted by the alkaline SDS extraction procedure [37]. Only 200 µL/mL lysozyme (Sigma-Aldrich) was used for the lysis of stationary phased cells of *S. thermophilus* and *Lc. lactis*. For the lysis of *L. delbrueckii* cells, 2 µL/mL mutanolysin (Sigma-Aldrich) was added along with the lysozyme. Detection of all plasmids over 27 kb in size was performed by FIGE [38], which was performed using a Bio-Rad Sub-Cell GT system. The electrophoresis conditions were 400 V for 2 hr and 50 min with 12-sec switchable conductivity. As plasmid extraction was challenging, particularly from steady state cells of *L. sunkii* and *L. indicus*, only log phase cells (OD_{660}= 0.4) could be used. Although the plasmid yield was extremely low, 10 ng plasmid was obtained from 400 mL of MRS culture medium (OD_{660}=0.4) containing *L. sunkii* or *L. indicus* cells.

Southern hybridization was performed to confirm that pAMβ1 replicated in *L. sunkii.* The Em resistance gene of pAMβ1, which was used as a probe, was amplified with the primers LAB011 and LAB012C and labelled using DIG-High Prime DNA Labeling and Detection Starter Kit 1 (Sigma-Aldrich).

Preparation of donor and recipient cells for conjugation

The donor, *Lc. lactis* IL1403 (pGMβ$_{ldh}$1, pGMβ$_{ldh}$2), was cultured at 30°C in GM17 medium supplemented with 25 µg/mL Em. Fresh cells provided a higher conjugation frequency and were more efficient donors than frozen cells. In contrast, frozen cells (*Lactobacillus* and *Streptococcus* strains) exhibited a high conjugation frequency as recipients. The optimal donor to recipient cell ratio was determined to be 1:10 or 1:1.

Selection of chromosomal integrants (transfer conjugants)

During conjugation in the filter membrane, the donor and recipient were cultured together, and only the transfer conjugants were selected. When *E. coli* was used as the donor, it was difficult to select only the transfer conjugants of *L. bulgaricus*. However, when *Lc. lactis* was used as the donor, the transfer conjugants of *L. bulgaricus* could be selected with a high temperature (45°C) and 25 µg/mL Em in MRS, because *Lc. lactis* was unable to grow at 45°C. Two variations of chromosomal integrations were expected for each integrated vector (pGMß$_{ldh}$1 or pGMß$_{ldh}$2), as they contained two homologous fragments of the 5′ region and 3′ region of the *D-ldh* gene on the chromosome (<1> and <2> in Fig. 1C). Chromosomal integration was confirmed by PCR with the primers LAB011 and LAB216, or the primers LAB085C and LAB216 as shown in <2> in Fig. 1C.

Acquisition of mutants and revertants after double-crossover recombination

Successive culturing in Em-free MRS was performed at 42°C to acquire the double-crossover products, after which Em-sensitive colonies were selected. Identification of the mutants or revertants was performed using PCR with the primers LAB085C and LAB216, and high-performance liquid chromatography (HPLC) was performed for D/L-lactate determination.

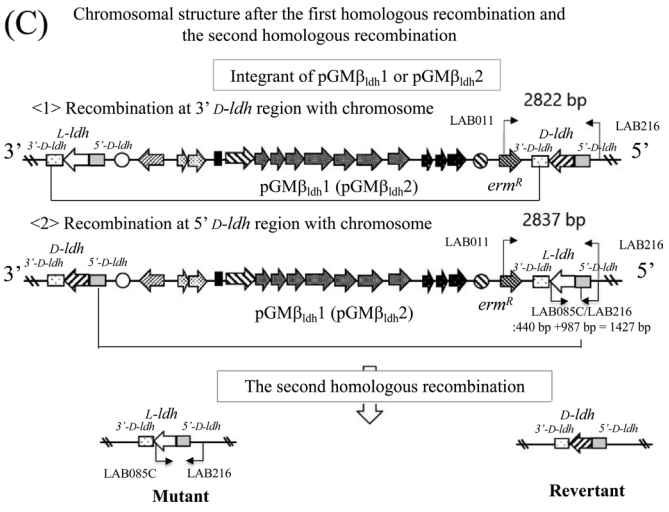


Fig. 1. (C) The chromosomal structures of the integrants after first recombination: in the case of <1>, the first homologous recombination between the chromosome and pGMb$_{ldh}$1 (pGMb$_{ldh}$2) occurred at the 3′ downstream region of *D-ld*h, and in the case of <2>, the first homologous recombination occurred at the 5′ upstream region of *D-ldh*.

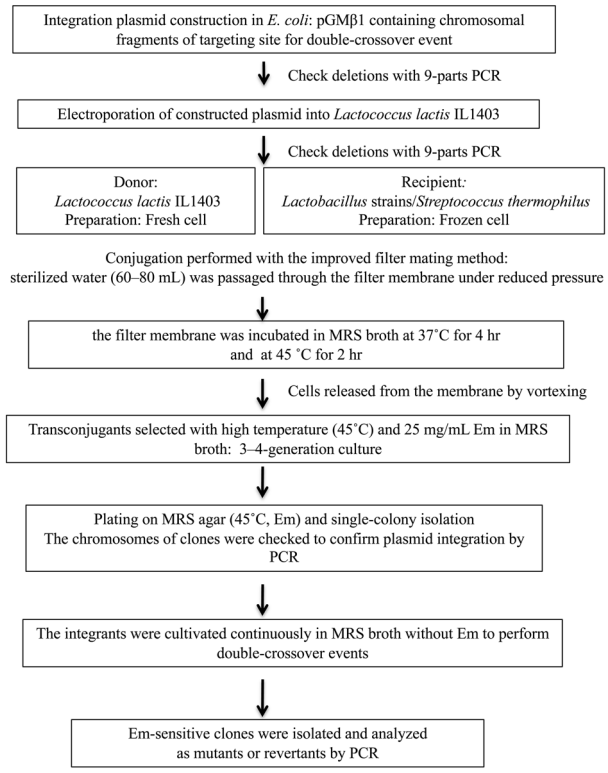


Fig. 2. Flowchart of the conjugation experiment. The detailed workflow of the conjugation method is outlined.

Determination of D-lactate and L-lactate production

The wild-type, chromosomal integrant, and *ldh*-converted mutant were cultured overnight in SMY medium. After centrifugation, the supernatant was purified with (Carrez I and II), potassium hexacyanoferrate(II) trihydrate and zinc sulfate heptahydrate (Wako) as described previously [35]. HPLC (Shimadzu Corporation) was performed using SUMICHIRAL OA-5000 and OA-5000L columns to separate and measure L-lactate and D-lactate under the following conditions: mobile phase, 2 mM copper (II) sulfate pentahydrate (Wako); flow rate, 1.0 mL/min; oven temperature, 40°C; UV detection, 254 nm.

RESULTS

Construction of the shuttle vector pGMβ1

Among the 30 *E. coli* transformants examined for size and restriction sites after electroporation of the ligation mixture, 10 clones produced fragments of approximately 30.8 kb, whereas the other 20 clones showed various deletions in the pAMβ1 region.

To detect deletions over 100 bp in size, PCR amplification of the fragments in which pGMβ1 was divided into nine parts (Table 2) was performed, and no deletions were detected in the nine parts of the PCR products of pGMβ1 (Fig. 1A).

Ldh gene conversion using pGMβ$_{ldh}$1

To validate the utility of pGMβ1 for conjugation, the conversion of the *ldh* gene in *L. bulgaricus* and the alteration of the stereochemistry of lactic acid from the D- to L-isomer using a double-crossover event were examined. In addition, the conjugation to other subspecies of *L. delbrueckii* was assessed.

The five subspecies of *L. delbrueckii* wild-type strains produced only D-lactate, whereas *S. thermophilus* produced only L-lactate. pGMβ$_{ldh}$1 (Fig. 1B) constructed in *E. coli* was electroporated into *Lc. lactis* IL1403 and conjugation was performed from IL1403 (pGMβ$_{ldh}$1) to *L. bulgaricus* LB2038 or LB600 as the recipient. After filter-mating conjugation (Fig. 2), transfer conjugants (integrants) were selected on the basis of Em resistance at 45°C, demonstrating the production of both the D- and L-isomers of lactic acid (Fig. 3). The integration of pGMβ$_{ldh}$1 into the *L. bulgaricus* chromosome could be determined by PCR with the primers LAB011 (*erm^R*) and LAB216 or the primers LAB085C (*L-ldh*) and LAB216, as shown in Fig. 1C.

Transconjugants were then inoculated into Em-free MRS medium to induce a double-crossover event, and the isolated Em-sensitive clones were found to produce either L- or D-lactic acid only (Fig. 3). A slight peak of D-lactic acid was observed (shown in Fig. 3C) that suggested the possibility of D-lactic acid production from one other *D-ldh* gene (LBU_1637) in the genome. Approximately 200 generations were required to obtain the first Em-sensitive clone. After 10–15 successive cultures, the Em-sensitive clones constituted more than half of the colony.

The converted mutants and revertants were both Em sensitive (Fig. 1C). The mutants harbored the *L-ldh* gene, which was detected by PCR using the primers LAB085C (*L-ldh*) and LAB216, and L/D-lactate was measured by HPLC (Fig. 3). The acidification rate in the SMY medium, in which the mutants were cultured, was the same as for the wild-type strain of *L. bulgaricus* LB2038 or LB600.

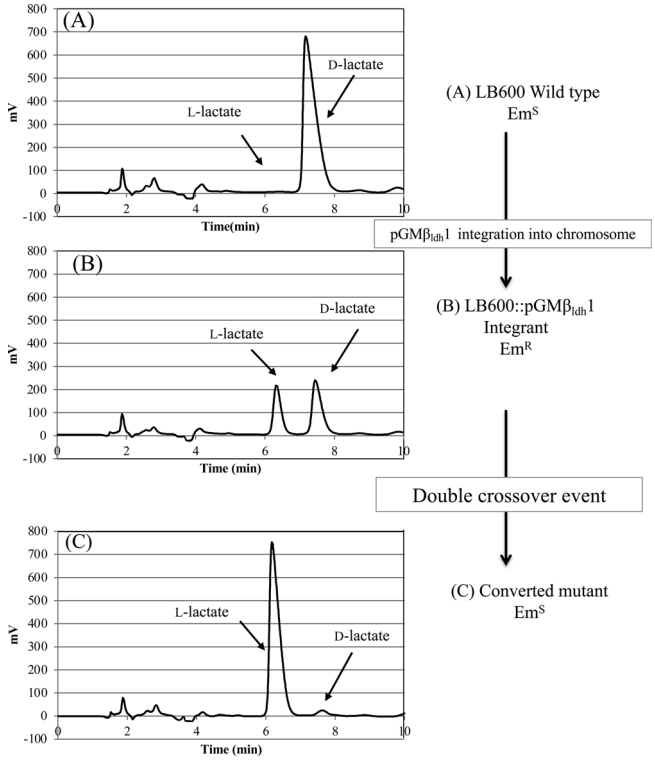


Fig. 3. Analysis of the D- and L-lactate of *L. bulgaricus* by high-performance liquid chromatography. (A) Conversion of the *ldh* gene in *L. bulgaricus* LB600 to change the stereochemistry of lactic acid from the D- to the L-isomer using a double-crossover event. Wild-type LB600 produced only D-lactate and was sensitive to Em. (B) The transconjugant strain with chromosomal integration of pGMβ$_{ldh}$1 produced D- and L-lactate and was resistant to Em. Both the mutant and revertant after the double-crossover event were sensitive to Em. (C) The revertant (wild-type) produced only D-lactate and the conversion mutant produced only L-lactate.

Replication ability of pGMβ1 in other subspecies of L. delbrueckii

We further examined whether pGMβ1 could be functioning as a chromosomal integrating vector during the transfer of pGMβ$_{ldh}$2 to *L. delbrueckii* JCM1012^T, *Lb. lactis* JCM1248^T, and *L. sunkii* JCM17838^T, and during the transfer of pGMβ$_{ldh}$1 to *L. indicus* JCM15610^T. Figure 4A shows there were two groups, one group showing high conjugation frequencies (*L. indicus*, pGMβ$_{ldh}$1; *L. sunkii*, pGMβ$_{ldh}$2) and one group showing low conjugation frequencies (*L. bulgaricus,* pGMβ$_{ldh}$1; *L. delbrueckii*, pGMβ$_{ldh}$2). In the case of *S. thermophilus,* the positive control showed a high conjugation frequency (10^{-3}/recipient), wherein pAMβ1 could be replicated. After double crossover, *ldh* gene conversion was only successful in *L. bulgaricus* and *L. delbrueckii,* and their conjugal transfer frequencies were low (under 10^{-5}/recipient) owing to chromosomal integration. In contrast, the transfer frequencies of *L. indicus* JCM15610^T and *L. indicus* SAK, or *L. sunkii* JCM17838^T were high, reaching over 10^{-3}/recipient, which was the same level as observed for *S. thermophilus*. As transfer conjugants could not be obtained in *Lb. lactis* JCM1248^T, the strains of the other four subspecies showed Em resistance, and the production of D-lactate and L-lactate after conjugation. After successive cultures, although the *ldh*-converted mutants

doi: 10.12938/bmfh.2021-014

(Fig. 1C) were detected in *L. delbrueckii,* as in *L. bulgaricus*, they were not detected in *L. indicus* and *L. sunkii*. The higher conjugal transfer frequencies were similar to that of *S. thermophilus*, suggesting that pGMβ$_{ldh}$1 or pGMβ$_{ldh}$2 could be replicated in these strains (Fig. 4A). The *ldh*-converted mutants were detected in *L. delbrueckii*, which showed a low transfer frequency that was similar to that observed in *L. bulgaricus*.

To determine replication ability, we assessed the conjugal transfer of pAMβ1, which does not have a homologous region for chromosomal integration. Em-resistant strains of *L. indicus* JCM15610^T and *L. sunkii* JCM17838^T were obtained at high transfer frequencies (Fig. 4B). It was difficult to separate an open circular (OC) form of plasmid that was over 20 kb in size from a linear form of plasmid or chromosome by the usual electrophoresis method. Both an OC and linear form of pAMβ1 were observed in the transconjugant of *L. indicus* (pAMβ1) using field inversion gel electrophoresis (FIGE) (Fig. 5, lane 2), as the applied DNA was about 20 ng in all lanes. Though it was difficult to detect a covalently closed circular (CCC) form in lanes 2 and 3, the applied plasmid DNA was increased from 20 ng to 50 ng, and the pAMβ1 could not be moved from the agarose well. A CCC, OC, and linear form of pAMβ1 were observed in the transconjugant of *L. sunkii* (pAMβ1) using FIGE (Fig. 5, lane 5) with the same applied DNA (20 ng). Southern hybridization using a fluorescently labeled nucleic acid probe (*erm^R*) of pAMβ1 confirmed that pAMβ1 could replicate in *L. sunkii* JCM17838^T, and the results suggested that pAMβ1 was present in the CCC, OC, and linear forms (Fig. 5, lane 8).

The next conjugation was performed using *L. indicus* and *L. sunkii* as the donors, and *Lc. lactis* IL1403 as the recipient to evaluate the transfer ability of the pAMβ1 in *L. indicus* and *L. sunkii*.

The pAMβ1 plasmids could be detected in the transfer conjugants of IL1403 based on Em resistance and growth in the presence of 4% NaCl, although the transfer frequency was 10^{-8} for *L. indicus* and 10^{-6} for *L. sunkii*, both of which were extremely low compared with the results of prior conjugation with *Lc. lactis* as the donor (Fig. 4B).

DISCUSSION

We constructed a new conjugative shuttle vector, pGMβ1, that enabled manipulation of the chromosomal genes of *L. bulgaricus*, which is otherwise considered to be a "recalcitrant" bacterium, using an improved filter-mating method. The new vector contains a high copy number of *ori* from *E. coli*, which helped to overcomes the limitations of pAMβ1 as a low-copy and large-sized theta-type plasmid. We were further able to enhance the low conjugation frequency caused by the chromosomal integration of pAMβ1 with the improved filter-mating method and the application of double homologous recombination. Moreover, we

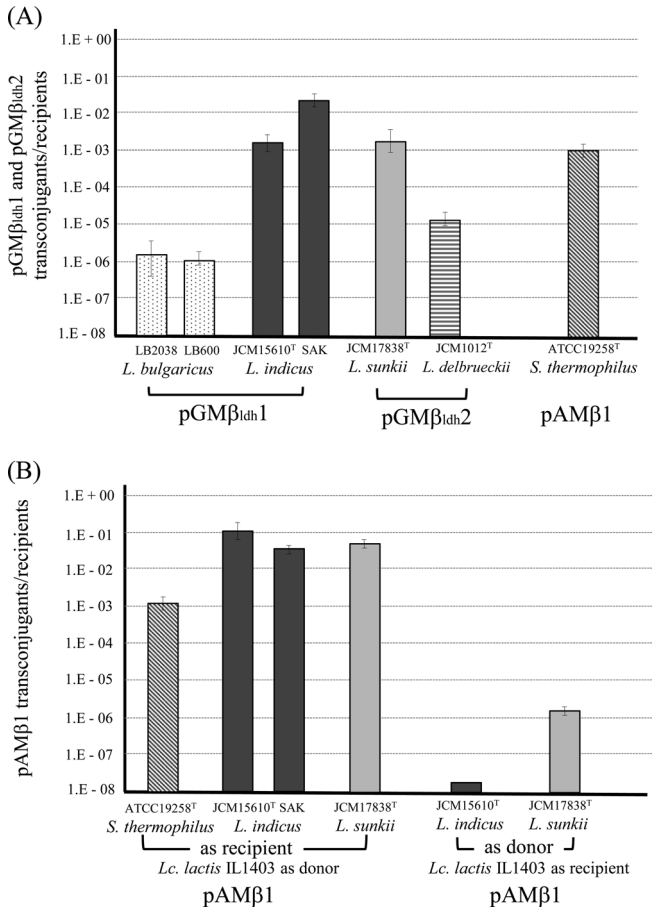
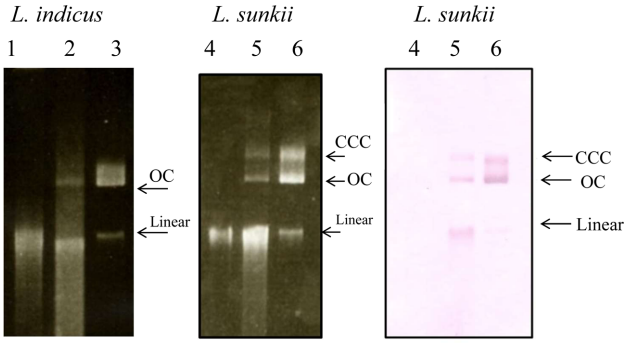


Fig. 4. Conjugal frequencies of pGMβ$_{ldh}$1, pGMβ$_{ldh}$2(A), and pAMβ1(B) per recipient. Conjugal experiments: n=3. The positive control was pAMβ1 transferred to *S. thermophilus*, wherein pAMβ1 could be replicated, and the conjugal frequency was high (10^{-3}/recipient).



1. Chromosomal DNA of *L. indicus* (wild-type)
2. Plasmid pAMβ1 from transconjugant of *L. indicus*
3. *Lc. lactis* IL1403 (pAMβ1)
4. Chromosomal DNA of *L. sunkii* (wild-type)
5. Plasmid pAMβ1 from transconjugant of *L. sunkii*
6. *Lc. lactis* IL1403 (pAMβ1)

<Southern hybridization> probe:Em^R gene
7. Chromosomal DNA of *L. sunkii* (wild-type)
8. Plasmid pAMβ1 from transconjugant of *L. sunkii*
9. *Lc. lactis* IL1403 (pAMβ1)

Fig. 5. Detection of pAMβ1 plasmid by field-inversion gel electrophoresis (FIGE). As the size of pAMβ1 was approximately 28 kb, it was difficult to discriminate closed circular (CC, supercoiled), open circular (OC), and linear forms. FIGE could separate the CC, OC, and linear forms of pAMβ1 under the following conditions: 400 V for 2 hr and 50 min and 12-sec switchable conductivity. The forms of the plasmid pAMβ1 present in the of *L. indicus* (lane 2) and *L. sunkii* (lane 5) transconjugants and the results of Southern hybridization blot obtained using the *erm^R* probe with the plasmid pAMβ1 (lanes 4, 5, and 6) are shown.

doi: 10.12938/bmfh.2021-014

confirmed the utility of this method by demonstrating successful gene conversion from *D-ldh* to *L-ldh* on the *L. bulgaricus* and *L. delbrueckii* chromosomes.

The proposed conjugation method (outlined in Fig. 2) can be expanded to other research applications. In this method, first, the targeting fragment containing the double homologous recombination region was ligated into pGMβ1 and electroporated in *E. coli.* The length of the homologous region for recombination is recommended to be over approximately 400 bp. Second, the constructed plasmid was electroporated to *Lc. lactis* IL1403, which lacks an intrinsic restriction modification system. Third, improved filter-mating conjugation was performed between *Lc. lactis* IL1403 and *L. bulgaricus*; transfer conjugants were selected at 45°C in the presence of 25 mg/mL Em. Finally, the Em-sensitive clones (revertants or converted mutants) were obtained by successive culturing in Em-free medium.

Conjugation [22] is a strong and specific process that mediates the transfer of DNA between a wide range of bacteria, including possibly intergeneric transfer [23]. pAMβ1 is described as a "promiscuous" plasmid because of its broad host range and the *tra* gene that confers conjugal transfer abilities to fragments over 20 kb in length. Although pAMβ1 could replicate in many lactobacilli, it was unable to do so in *L. delbrueckii* subsp. *bulgaricus* and subsp. *delbrueckii* and was subsequently integrated into their chromosomes. Although we did not obtain the integrant of *Lb. lactis* in this study, the integration of pAMβ1 was observed using Southern hybridization in our prior work. When pAMβ1 recombined with the chromosomes of *L. bulgaricus* and *L. delbrueckii,* it was cleaved and recombined at a specific site. Southern blot analysis showed that recombination occurred within a specific 1.0 kb region, from nucleotide 2216 to 3194, of pAMβ1. This region contains the Rep origin (nucleotides 2705–2748) and a resolution site (*res*; nucleotides 2951–3039), where it overlaps with an unusual resolvase; thus, this enzyme interacts with DNA (*res* site) and promotes recombination of the resolution system in pAMβ1 [39–41]. However, the specific role of this resolvase in chromosomal recombination in this pGMβ1 system, which contains a double homologous region for recombination, is unknown.

In contrast to our expectation, pAMβ1 could replicate in both *L. indicus* and *L. sunkii*, which are newly identified *L. delbrueckii* subspecies. As high transfer frequencies of pAMβ1 were observed in these two subspecies, pGMβ1 was expected to be an ordinal plasmid vector for the introduction of foreign genes.

The conjugal transfer of pAMβ1 from *L. indicus* JCM15610^T (pAMβ1) and *L. sunkii* JCM17838^T (pAMβ1) to *Lc. lactis* IL1403 showed that pAMβ1 could be replicated, and its ability to be transferred was maintained in *L. indicus* and *L. sunkii*. The higher ratio of the linear form of pAMβ1 in *L. sunkii* (pAMβ1) and *L. indicus* (pAMβ1) compared with and that of *Lc. lactis* (pAMβ1) (Fig. 5) might explain the low conjugal transfer frequencies of *L. sunkii* (pAMβ1) and *L. indicus* (pAMβ1) to *Lc. lactis* (pAMβ1) (Fig. 4B). In particular, the CCC form could not be detected in *L. indicus* (Fig. 5), and it might be related to the lower transfer frequency of *L. indicus* to *L. sunkii* (Fig. 4B). In general transformation using electroporation, the CCC plasmid DNA shows a 10-10^3 -fold higher transfer frequency than linear plasmids [42], and the same tendency, that a low ratio of CCC caused reduced conjugal transfer frequencies, has been suggested.

As another cause for the low transfer frequency, the possibility that pAMβ1 could exist in both a plasmid form and a chromosomal integration form. Although we examined the PCR analysis to identify the integrated construction of pAMβ1 in the chromosomes of Em^R strains of *L. indicus* JCM15610^T and *L. sunkii* JCM17838^T, we were unable to identify results showing integrated construction. However, we observed the coexistence of integration and plasmid forms in the *L. indicus* SAK strain, suggesting the possibility of coexistence in other strains.

pAMβ1 belongs to "Θ class D" [43], and the host RNA polymerase I and replisome have been reported as host factors that contribute to pAMβ1 replication [44, 45]. The detailed factors contributing to the difference in the pAMβ1 replication mechanism among the hosts *L. bulgaricus/L. delbrueckii* and *L. indicus/L. sunkii* remain to be clarified.

Although we propose pGMβ1 as a novel chromosomal integration system for *L. bulgaricus*, this vector also has potential value for other applications in replicable hosts, especially bacteria in which transformation is challenging. For instance, as pGMβ1 is a large theta-type plasmid, it is expected to serve as a stable vector for the insertion of long cluster genes. Conjugation can avert the limitations of a restriction modification system and maintain a high transfer ability for pGMβ1; thus, this method is expected to offer a general and reproducible transformation tool for almost all lactobacilli, including *L. indicus* and *L. sunkii*, especially for industrial strains that do not have high transformation efficiency by electroporation.

L. bulgaricus is regarded as a natural genetically modified organism [10]; however, it has evolutionarily adapted and been optimized for utilization over its long history, beginning in 3200 B.C. In contrast to many other lactic acid bacteria, *L. bulgaricus* does not contain a prophage [2]. Hence, virulent phages against *L. bulgaricus* are extremely rare in the food industry, and it is also very hard to transform this organism with foreign DNA [46]. This provides a possible explanation for why there are so few reproducible reports addressing the chromosomal manipulation of *L. bulgaricus.*

This conjugal chromosomal manipulation method enables gene-targeting conversion, deletion, and insertion in a reproducible manner, making it possible to evaluate the biological functions of genes in *L. bulgaricus.*

In conclusion, the proposed conjugation method can conquer several limitations of electroporation-based transformation observed in bacteria and represents a reproducible and reliable chromosomal manipulation system for industrial strains of *L. bulgaricus*.

ACKNOWLEDGEMENTS

We are very grateful to Prof. D. Clewell for providing the pAMβ1 plasmid and to Prof. S. Ikematsu (National Institute of Technology, Okinawa College) for the sequencing of pGMβ1. We thank Dr. T. Sasaki and Dr. Y. Ito for numerous critical comments regarding pAMβ1 integration. We would like to thank Editage (www.editage.com) for English language editing.

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