Novel shuttle vector pGMß1 for conjugative chromosomal manipulation of Lactobacillus delbrueckii subsp. bulgaricus

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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 pAMß1 as an integration vector for L. bulgaricus, as conjugation could avert the need for a restriction modification system; pAMß1 does not replicate and integrate into the chromosome of L. bulgaricus. Here, we describe an effective chromosomal manipulation system involving a novel shuttle vector pGMß1, which could improve the operability of the broad host-range conjugal plasmid pAMß1. 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 pGMß1 and pAMß1 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 V1104, 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...
pJC4, Jang et al. [13] obtained the chromosomal integrant of *L. bulgaricus* ATCC11842 with cellulase screening. Li et al. [4] reported *cepA* disruption in *L. bulgaricus* ATCC11842 by homologous recombination using a linear DNA fragment (*CepA-Tet-CepA*) 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 effectivel 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+ [30]. This chromosomal integration of pAMβ1 was observed not only in *L. bulgaricus* but also 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* DH5α. We could not determine the adequate conditions required for selection of the plasmid transfer conjugal 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 conjugal of *L. bulgaricus* could be differentiated from *Lc. lactis* at a specific temperature (45°C) and by resistance to erythromycin (EmR), and (2) IL1403 showed a higher 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 conugal 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, fl ori and ampicillin-resistance gene) was amplified with the primers LAB184 and LAB185C, which contained the Aval (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             | JCM15610T Japan Collection of Microorganisms |
| subsp. indicus             | SAK This study: Laboratory collection |
| subsp. sunkii              | JCM17838T Japan Collection of Microorganisms |
| subsp. lactis              | JCM1248T Japan Collection of Microorganisms |
| subsp. delbrueckii         | JCM1012T Japan Collection of Microorganisms |

Plasmid Reference

| 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] |
| pGMβ1_int1, 32,778 bp This study |
| pGMβ1_int2, 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.
The non-deletion 30.8-kb plasmid was selected and analyzed according to a previous procedure [36]. The resulting 1950 bp fragment (1932 bp + primer) was ligated from 1–18 nt of D-lactate dehydrogenase of L. bulgaricus and situated at either end of the 987 bp region of the D-ldh gene (Fig. 1B). The 359 bp fragment at the 5′ upstream region of the D-ldh gene of strain ST1131 (Fig. 1B). As the conversion occurred through double homologous recombination, pGMβ ldh1 was constructed so as to contain an insertion fragment for D-ldh (+SacI) of L. bulgaricus (Fig. 1B). 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 987 bp region of the D-ldh gene conversion with pGMβ ldh1 and pGMβ ldh2 plasmids for gene conversion with D-lactate dehydrogenas (D-ldh) of L. bulgaricus (+SacI).

### Table 2. Primer list

| Primer | Sequence (5′–3′) | Template (Plasmid or chromosome ) | Length (bp) |
|--------|-----------------|---------------------------------|-------------|
| LAB011 | GGGCATTTAAAGCAGAACAAACT | erythromycin resistance gene of pAMβ1 | LAB011/LAB012C |
| LAB012C | GGGGTTTCTCATTCTGTTGAT | erythromycin resistance gene of pAMβ1 | 509 bp |
| LAB216 | GGGTGAACACACTCTGACGAC | 5′ upper region of D-lactate dehydrogenase of L. bulgaricus | LAB216/LAB011 |
| LAB084 | GGATGACTGCAACTAAACTA | from 1–18 nt of D-lactate dehydrogenase of S. thermophilus | LAB084/LAB085C |
| LAB085C | CCTTAGTTTTTGATGAACTAGCGAAAC | whole nucleotides of pGEM-T-Easy vector (+AvaI) | 987 bp |
| LAB105 | GCCCTTGAAGTGTCTAGGAAC | fragment (1) Forward: 5–3076 nt of pGMβ1 | LAB105/LAB106C |
| LAB106C | CGGGTTTCTCAATTTCTCCAG | fragment (1) Reverse: 5–3076 nt of pGMβ1 | 3,072 bp |
| LAB107 | GCCATTACATGTTGGATTAGTC | fragment (2) Forward: 3073–6322 nt of pGMβ1 | LAB107/LAB108C |
| LAB108C | GCACATGACTCACTCTAGGATTGT | fragment (2) Forward: 3073–6322 nt of pGMβ1 | 3,250 bp |
| LAB109 | CCTTAGAAAGCCTTAAGTGGTGTG | fragment (3) Forward: 6299–9395 nt of pGMβ1 | LAB109/LAB110C |
| LAB110C | GGTTGCTGCCACCTCTGCTT | fragment (3) Reverse: 6299–9395 nt of pGMβ1 | 3,097 bp |
| LAB111 | CCTCAATTGATGAACTGCAAAAC | fragment (4) Forward: 9393–12377 nt of pGMβ1 | LAB111/LAB112C |
| LAB112C | CGAGCAGGTTCCAGGGGAAAATATTAC | fragment (4) Reverse: 9393–12377 nt of pGMβ1 | 2,985 bp |
| LAB113 | GCCCTTTTTCTAATTAGACGCTTTG | fragment (5) Forward: 12313–15426 nt of pGMβ1 | LAB113/LAB114C |
| LAB114C | GCCCTTTTTCTAATTAGACGCTTTG | fragment (5) Reverse: 12313–15426 nt of pGMβ1 | 3,114 bp |
| LAB115 | GGAGGATAAGTAGAATAGGATCAGA | fragment (6) Forward: 15389–18359 nt of pGMβ1 | LAB115/LAB116C |
| LAB116C | CGGACTAAGCGCTTAAATCTTCTT | fragment (6) Reverse: 15389–18359 nt of pGMβ1 | 2,970 bp |
| LAB117 | CGGCCCTTAGTCGCGAAAGAG | fragment (7) Forward: 18347–21446 nt of pGMβ1 | LAB117/LAB118C |
| LAB118C | GGTCTTACTCTTTCTCCTAATTTGAT | fragment (7) Reverse: 18347–21446 nt of pGMβ1 | 3,099 bp |
| LAB119 | CCCAAAGAACGACATTCAATTAG | fragment (8) Forward: 21428–24573 nt of pGMβ1 | LAB119/LAB120C |
| LAB120C | CCGCCCTAAAGAAGCATTAGAATAG | fragment (8) Reverse: 21428–24573 nt of pGMβ1 | 3,145 bp |
| LAB121 | GCTTCTACTCCTCTCTCCTAATTTGAT | fragment (9) Forward: 24540–40 nt of pGMβ1 | LAB121/LAB122C |
| LAB122C | GGACTGATTGCTTTACCTTACAT | fragment (9) Reverse: 24540–40 nt of pGMβ1 | 3,316 bp |
| LAB184 | CAACCTGGAAGCTATGGAATTTGCTGCGGCTTGG | whole nucleotides of pGEM-T-Easy vector (+AvaI) | LAB184/LAB185C |
| LAB185C | GCTTCTATTGCTTCTTCTTACTTACAT | whole nucleotides of pGEM-T-Easy vector (+AvaI) | 3,016 bp |
| LAB270 | CAGAGGCTCATTGCGTACCTTACAT | insertion fragment for L-ldh gene conversion with D-ldh of L. bulgaricus (+SacI) | LAB270/LAB271C |
| LAB271C | GGGTAGCTGTTCTCTTACATAAGCAGACAGAC | insertion fragment for L-ldh gene conversion with D-ldh of L. bulgaricus (+SacI) | 1,950 bp |
| LAB345 | CCTTATCATGATTAGCAAACGTATACGTTAAT | 3′ down region of D-lactate dehydrogenase of L. delbrueckii JCM1012 | LAB345/LAB346C |
| LAB346C | GGGCGAGCTGAATGGAATGGGATTCCTACCTGAG | 3′ down region of D-lactate dehydrogenase of L. delbrueckii JCM1012 | 516 bp |

**Construction of pGMβ_{ldh1} and pGMβ_{ldh2} plasmids for gene conversion**

As the conversion occurred through double homologous recombination, pGMβ_{ldh1} 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_{ldh1} (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. delbrueckii.
indicus, and the other contained subsp. delbrueckii, subsp. lactis, and subsp. sunkii. Then plasmid pGMβldh2 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 JCM1012T chromosome as the template.

We used pGMβldh1 for L. bulgaricus and L. indicus, and pGMβldh2 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 (pGMβldh1, pGMβldh2, 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 JCM15610T.

![Fig. 1.](image)
L. indicus SAK and L. sunkii JCM17838 as the recipients. The next conjugation experiment was performed with L. indicus JCM15610 (pAMβ1) and L. sunkii JCM17838 (pAMβ1) as the donor and Lc. lactis IL1403 as the recipient. In this case, transconjugants of L. 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 (OD660= 0.4) could be used. Although the plasmid yield was extremely low, 10 ng plasmid was obtained from 400 mL of MRS culture medium (OD660=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 (pGMb ldh1, pGMb ldh2), 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 L. lactis was unable to grow at 45°C. Two variations of chromosomal integrations were expected for each integrated vector (pGMb ldh1 or pGMb ldh2), 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 LAB011 and LAB212C and labelled using DIG-High Prime DNA Labeling and Detection Starter Kit 1 (Sigma-Aldrich).

**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.

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**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 ldh1 (pGMb ldh2) occurred at the 3′ downstream region of D-ldh, and in the case of <2>, the first homologous recombination occurred at the 5′ upstream region of D-ldh.
**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 SUMICHRAL 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 electroperoration 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_{lab}1**

To validate the utility of pGMβ1 for conjugation, the conversion of the ldh gene in L. bulgaricus and the alteration of the stereochromy 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_{lab}1 (Fig. 1B) constructed in E. coli was electroperorated into Lc. lactis IL1403 and conjugation was performed from IL1403 (pGMβ_{lab}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_{lab}1 into the L. bulgaricus chromosome could be determined by PCR with the primers LAB011 (erm) 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 stereochromy 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_{lab}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.](image-url)

**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_{lab}2 to L. delbrueckii JCM1012, Lb. lactis JCM1248, and L. sunkii JCM17838, and during the transfer of pGMβ_{lab}1 to L. indicus JCM15610, Figure 4A shows there were two groups, one group showing high conjugation frequencies (L. indicus, pGMβ_{lab}1; L. sunkii, pGMβ_{lab}2) and one group showing low conjugation frequencies (L. bulgaricus, pGMβ_{lab}1; L. delbrueckii, pGMβ_{lab}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 and L. indicus SAK, or L. sunkii JCM17838 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, 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...
(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 pGMβ1 or pGMβ2 could be replicated in these strains (Fig. 4A). The *ldh*-converted mutants were detected in *L. delbrueckii*, which showed a lower 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* JCM15610T and *L. sunkii* JCM17838T 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<sup>8</sup>) of pAMβ1 confirmed that pAMβ1 could replicate in *L. sunkii* JCM17838<sup>T</sup>, 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<sup>−8</sup> for *L. indicus* and 10<sup>−6</sup> 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 overcome 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...
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 JCM15610T (pAMβ1) and L. sunkii JCM17838T (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 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 10-103-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 EmR strains of L. indicus JCM15610T and L. sunkii JCM17838T, 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 repilosome 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.

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