

Development of a Double-Crossover Markerless Gene Deletion System in *Bifidobacterium longum*: Functional Analysis of the α -Galactosidase Gene for Raffinose Assimilation

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Functional analysis of *Bifidobacterium* genes is essential for understanding host-*Bifidobacterium* interactions with beneficial effects on human health; however, the lack of an effective targeted gene inactivation system in bifidobacteria has prevented the development of functional genomics in this bacterium. Here, we report the development of a markerless gene deletion system involving a double crossover in *Bifidobacterium longum*. Incompatible plasmid vectors were used to facilitate a second crossover step. The conditional replication vector pBS423- Δ repA, which lacks the plasmid replication gene *repA*, was integrated into the target gene by a first crossover event. Subsequently, the replicative plasmid pTBR101-CM, which harbors *repA*, was introduced into this integrant to facilitate the second crossover step and subsequent elimination of the excised conditional replication vector from the cells by plasmid incompatibility. The proposed system was confirmed to work as expected in *B. longum* 105-A using the chromosomal full-length β -galactosidase gene as a target. Markerless gene deletion was tested using the *aga* gene, which encodes α -galactosidase, whose substrates include raffinose. Almost all the pTBR101-CM-transformed strains became double-crossover recombinants after subculture, and 4 out of the 270 double-crossover recombinants had lost the ability to assimilate raffinose. Genotype analysis of these strains revealed markerless gene deletion of *aga*. Carbohydrate assimilation analysis and α -galactosidase activity measurement were conducted using both the representative mutant and a plasmid-based *aga*-complemented strain. These functional analyses revealed that *aga* is the only gene encoding a functional α -galactosidase enzyme in *B. longum* 105-A.

Bifidobacteria are generally known as beneficial microbes that produce health-promoting effects by interacting with their hosts (20, 23). Some bifidobacteria are commercially used as probiotics in yogurt products and as pharmaceutical agents. To better understand bifidobacteria, the genome sequences of several probiotic bifidobacterial species, including *Bifidobacterium longum* NCC2705 (46), *B. longum* DJO10A (26), *B. longum* subsp. *infantis* ATCC 15697 (47), *Bifidobacterium breve* UCC2003 (34), and *Bifidobacterium animalis* subsp. *lactis* DSM 10140 and BI-04 (4), have been analyzed. Although knowledge of the genome sequences enables the prediction of bifidobacterial gene function, the clarification of the detailed mechanisms of interactions is not possible with genomic information alone. The most effective approach for directly analyzing gene function is targeted gene mutagenesis. However, this approach is still not easily applicable to studies of bifidobacteria (14), despite recent progress (15, 32–34, 41).

Currently, *Escherichia coli*-*Bifidobacterium* shuttle vectors based on cryptic plasmids have been developed, but they have relatively low transformation frequencies (14). As for gene mutagenesis systems, the construction of vector-insertion mutants by single crossover and gene disruption mutants by double crossover through allelic exchange between drug resistance genes and target genes has been reported (15, 32–34, 41). Markerless gene deletion, which leaves no exogenous DNA in mutated alleles, has not yet been achieved in bifidobacteria. Markerless gene deletion has several advantages: (i) it is stable, (ii) it avoids polar effects on the expression of adjacent genes, and (iii) it enables multiple gene deletions in a genome.

Markerless gene deletion using the double-crossover method has been achieved in various bacteria (19, 28, 31, 35, 38). Gener-

ally, a low frequency of the second crossover event is an obstacle to successful gene deletion, and strategies for efficiently selecting second-crossover recombinants are required. Conditional replication vectors such as thermosensitive replication vectors (7, 17, 35, 45) and counterselectable marker genes, e.g., *sacB* (31, 35, 38) and *pheS*, encoding the phenylalanyl-tRNA synthase α -subunit (5, 50), have been used to overcome this problem in other bacteria. A similar system should be applicable in bifidobacteria, but reliable conditional replication vectors and suitable counterselectable marker genes are currently unavailable.

To achieve markerless gene deletion in the bifidobacterial genome, a strategy based on conditional plasmid replication was conceived (Fig. 1). The plasmid for the first crossover functions as a conditional replication vector as a result of the deletion of the gene encoding the replication protein RepA. This plasmid can only replicate in bifidobacterial cells when RepA is provided in *trans*. When this plasmid is integrated into the target gene, supplying RepA is expected to induce the conditional replication of the integrated plasmid. This will result in severe growth defects (17), presumably due to the interference with chromosome replication as replication starts from not only the chromosome's origin

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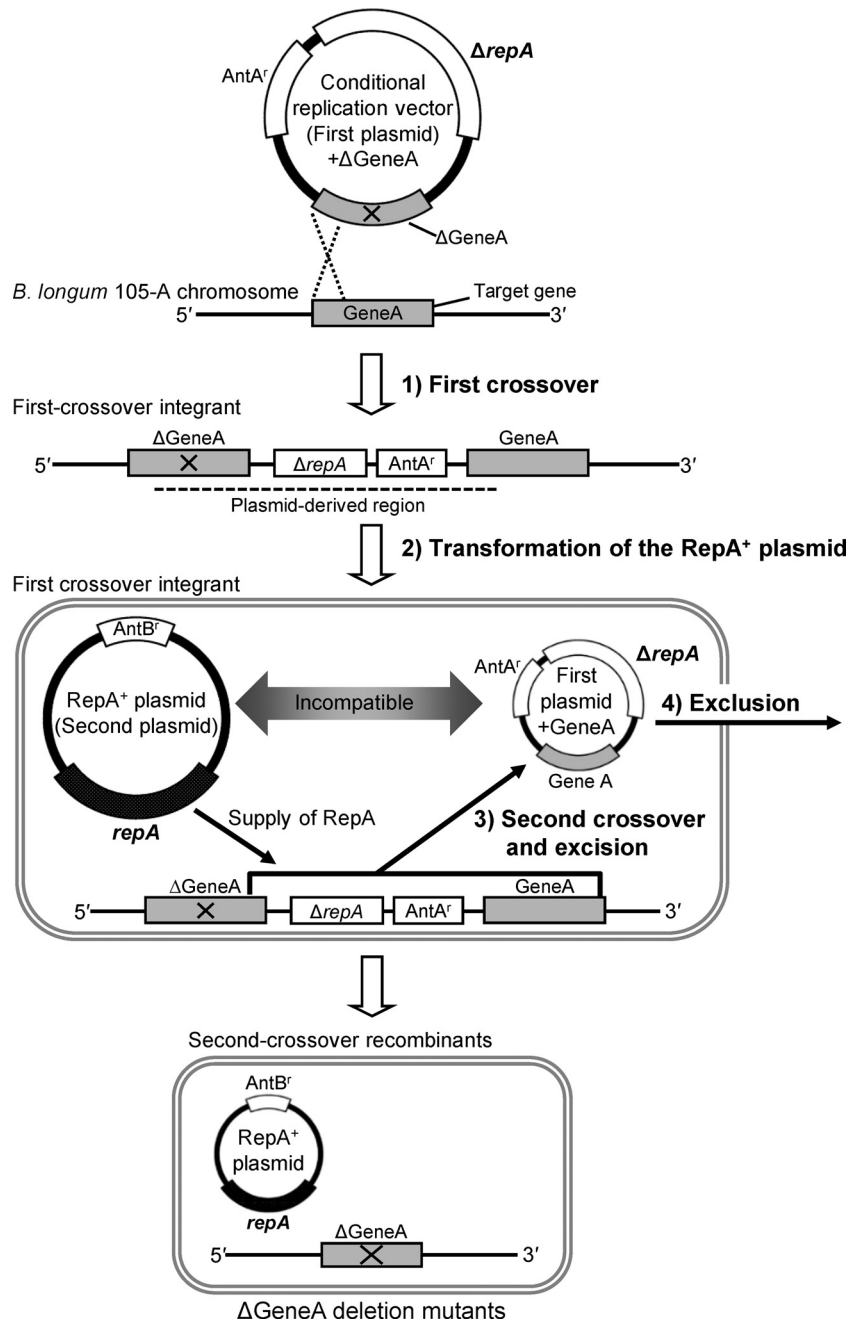


FIG 1 Outline of markerless gene deletion system in *Bifidobacterium longum* 105-A. *GeneA* represents a model target gene for markerless gene deletion. $\Delta GeneA$ lacks the internal region of *GeneA* and is cloned in the conditional replication vector (first plasmid), which lacks the *repA* gene encoding the plasmid replication initiation protein RepA (indicated as $\Delta repA$). The region absent from $\Delta GeneA$ is identified by a cross. Theoretically, two types of *GeneA* alleles can be generated by the first crossover, and $\Delta GeneA$ deletion mutants and wild-type revertants can arise in the second-crossover recombinants. Here, only the simplified scheme for the generation of $\Delta GeneA$ deletion mutants was described to represent the concept. In the first crossover, the first plasmid harboring $\Delta GeneA$ was introduced into the host strain *B. longum* 105-A. Here, the 5' region of $\Delta GeneA$ is used as a homologous region for the first crossover. The first-crossover integrant harbors both the *GeneA* and $\Delta GeneA$ alleles in its chromosome. The region derived from the first plasmid harboring $\Delta GeneA$ in the chromosome of the first-crossover integrants is identified by a dashed line. For transformation of the $RepA^+$ plasmid, the introduction of the *repA*-harboring second plasmid ($RepA^+$ plasmid) into the first-crossover integrant results in the supply of the plasmid replication protein RepA to the cells. During the second crossover and excision, the integrated first plasmid is expected to be excised with *GeneA* from the chromosome as a result of the second crossover event facilitated by RepA. In exclusion, the excised first plasmid with *GeneA* has been excluded due to the incompatibility between two plasmids. Objective $\Delta GeneA$ deletion mutants can be found among the second-crossover recombinants. $AntA^r$, resistance gene for antibiotic A; $AntB^r$, resistance gene for antibiotic B.

of replication but also the origin in the integrated plasmid. Under these conditions, only the cells in which a second crossover has occurred, excising the integrated plasmid from the chromosome and leaving only the chromosomal replication origin, can survive.

To supply RepA, a second plasmid harboring the *repA* gene is introduced into first-crossover integrants. Second-crossover recombinants that lost the first plasmid can be efficiently isolated through selection with appropriate antibiotics, as the second plas-

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description ^a	Reference or source
<i>Escherichia coli</i>		
DH5 α	F ⁻ ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>recA1 endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>phoA supE44</i> λ ⁻ <i>thi-1 gyrA96 relA1</i>	Invitrogen
HMS174	F ⁻ λ ⁻ <i>recA1 IN(rrnD-rrnE)1 rph-1 rpoB331</i> (Rif ^r) <i>hsdR19</i>	8
JM109	<i>recA1 endA1 gyrA96 thi hsdR17</i> (r _K ⁻ m _K ⁺) <i>e14(mcrA) supE44 relA1</i> Δ (<i>lac-proAB</i>) F' [<i>traD36 proAB</i> ⁺ <i>lacI</i> ^q <i>lacZ</i> Δ M15]	TaKaRa Bio Inc.
<i>Bifidobacterium longum</i>		
105-A	Human fecal isolate, transformation host strain	29
105-A β gal::pBS423- Δ repA β gal	First-crossover integrant of pBS423- Δ repA β gal into chromosomal β -Gal gene locus	This study
105-A <i>aga</i> ::pBS423- Δ repA Δ aga	First-crossover integrant of pBS423- Δ repA Δ aga into chromosomal <i>aga</i> locus	This study
105-A Δ aga	<i>aga</i> deletion mutant of 105-A	This study
105-A Δ aga/pAga135	Plasmid-based <i>aga</i> complementation strain of 105-A Δ aga by introduction of pAga135	This study
105-A Δ aga/pKKT427	Empty vector (pKKT427)-introduced derivative of 105-A Δ aga	This study
BK25	Human fecal isolate harboring cryptic plasmid pTB4	13
Plasmids		
pAga135	6.4 kbp, pKKT427 derivative containing <i>aga</i> and 135-bp upstream region including putative <i>aga</i> promoter, Sp ^r	This study
pBS423	4.9 kbp, <i>E. coli</i> - <i>Bifidobacterium</i> shuttle vector, pMB1 <i>ori</i> pTB4 <i>ori</i> <i>repA</i> ⁺ Sp ^r	This study
pBS423- Δ repA	4.4 kbp, pTB4 <i>ori</i> Δ repA Sp ^r	This study
pBS423- Δ repA Δ aga	6.2 kbp, derivative of pBS423- Δ repA containing integrated fragment of 5' and 3' regions of <i>aga</i> , Sp ^r	This study
pBS423- Δ repA β gal	9.0 kbp, derivative of pBS423- Δ repA containing the β -Gal gene, Sp ^r	This study
pBR322	4.4 kbp, <i>E. coli</i> cloning vector, <i>rop</i> Tc ^r Ap ^r	TaKaRa Bio Inc.
pBRASTA101	5.0 kbp, <i>E. coli</i> - <i>Bifidobacterium</i> shuttle vector, pMB1 <i>ori</i> pTB6 <i>ori</i> Sp ^r	48
pC194	2.9 kbp, small R plasmid found in <i>Staphylococcus aureus</i> , Cm ^r	18
pGEM-T Easy	3.0 kbp, TA cloning vector, Ap ^r	Promega Corp.
pGEM-T- <i>aga</i>	7.9 kbp, 4.9 kbp of <i>B. longum</i> 105-A genomic DNA fragment containing <i>aga</i> cloned in pGEM-T Easy	This study
pKKT427	3.9 kbp, <i>E. coli</i> - <i>Bifidobacterium</i> shuttle vector, ColE1 <i>ori</i> pTB6 <i>ori</i> Sp ^r	51
pTANS19	1.9 kbp, <i>E. coli</i> cloning vector, pMB1 <i>ori</i> Sp ^r	This study
pTB4	2.9 kbp, cryptic plasmid in <i>B. longum</i> BK25	This study
pTBR101	7.3 kbp, <i>E. coli</i> - <i>Bifidobacterium</i> shuttle vector, pMB1 <i>ori</i> pTB4 <i>ori</i> <i>repA</i> ⁺ Ap ^r effective only in <i>E. coli</i>	This study
pTBR101-CM	8.4 kbp, Cm ^r gene cloned in pTBR101, pMB1 <i>ori</i> pTB4 <i>ori</i> <i>repA</i> ⁺ Ap ^r effective only in <i>E. coli</i>	This study

^a Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Sp^r, spectinomycin resistance; Tc^r, tetracycline resistance.

mid is incompatible with the conditional replication vector. In this study, the proposed strategy was tested to introduce markerless gene deletion in the *B. longum* 105-A chromosome using the *aga* gene, which encodes α -galactosidase (α -Gal), as a target.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All chemicals were of analytical grade and were commercially available. Cultures of *B. longum* 105-A (29) were routinely grown in half-strength (1/2) MRS broth containing glucose as the fermentable carbohydrate source (Becton, Dickinson and Co., Franklin Lakes, NJ) (22) and supplemented with 0.02% (wt/vol) L-cysteine and 0.34% (wt/vol) sodium ascorbate (1/2 MRSCS). Carbohydrate assimilation by *B. longum* was examined in semisynthetic medium (SSM) containing raffinose, melibiose, or glucose as the sole carbohydrate source (20 g/liter) (39). The SSM, which did not contain L-tryptophan and L-asparagine, was autoclaved at 110°C for 10 min, and then its pH was adjusted to pH 6.8 with NaOH. Bacterial growth was monitored by measuring the optical density at 660 nm (OD₆₆₀) using a Mini Photo 518R (Taitec, Saitama, Japan) or UV-1800 (Shimadzu, Kyoto, Japan) spectrophotometer. When necessary, the following antibiotics were added to both the broth and the agar medium: ampicillin (100 μ g/

ml), chloramphenicol (Cm; 2.5 μ g/ml), rifampin (Rif; 0.1 μ g/ml), and spectinomycin (Sp; 75 μ g/ml). *Bifidobacterium* strains were cultured at 37°C under anaerobic conditions in an anaerobic chamber (N₂:CO₂:H₂ at 80:10:10; Coy Laboratory Products, Inc., Grass Lake, MI). Cultures of *E. coli* were grown aerobically in Luria-Bertani (LB) medium or SOC medium as described previously (44).

DNA manipulations. The oligonucleotide primers used in this study are listed in Table S1 in the supplemental material. Reagents used in molecular biological procedures were purchased from TaKaRa Bio, Inc. (Otsu, Japan), unless otherwise indicated. Chromosomal DNA was purified from cultured bifidobacterial cells using a previously described method (13) with one modification: prior to the extraction of chromosomal DNA using Isopiant II (Nippon Gene Co., Ltd., Tokyo, Japan), the cells were treated with 30 mg/ml lysozyme dissolved in a 25% (wt/vol) solution of sucrose at 37°C for 1 h. Molecular cloning in *E. coli* was conducted as described elsewhere (44). Plasmid DNA from *E. coli* was prepared using a QIAprep miniprep kit (Qiagen, Hilden, Germany). Plasmids were purified from *B. longum* cells as described previously (27). PCR was performed using Primestart HS DNA polymerase with GC buffer (TaKaRa Bio) according to the manufacturer's instructions. Amplified DNA fragments were purified using a MinElute PCR purification kit (Qiagen) or a MinElute gel extraction kit (Qiagen). Agarose gel electrophoresis was

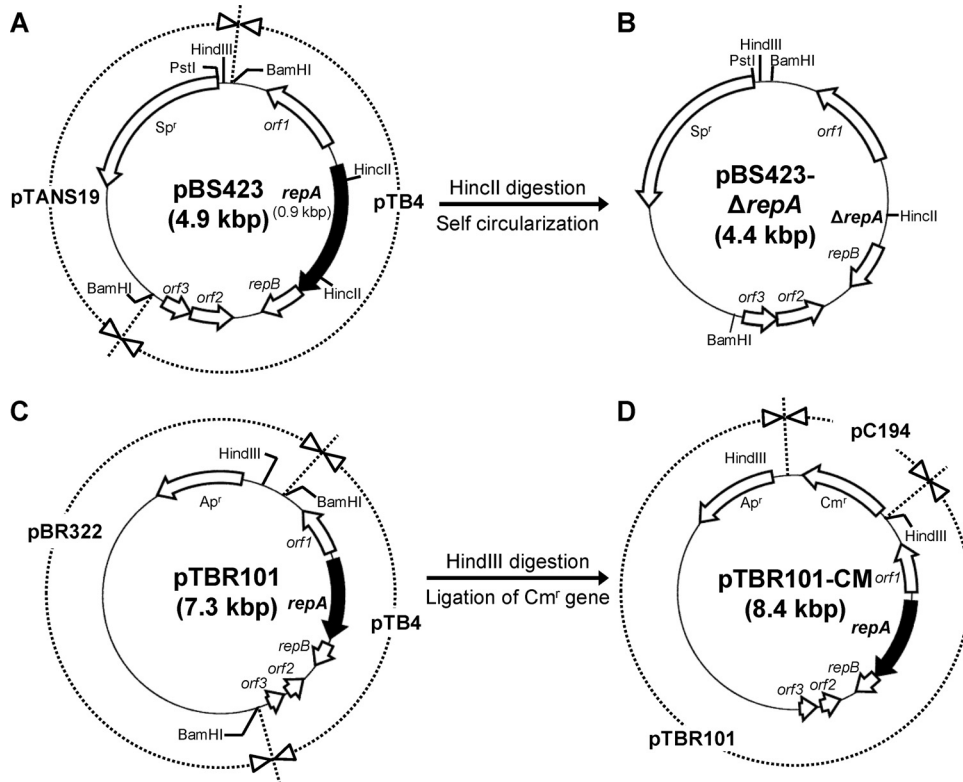


FIG 2 Structures of the plasmids used in markerless gene deletion. Genes are identified by open arrows, except for the *repA* gene, which is identified by black arrows. Representative restriction sites in the plasmids are shown. DNA regions in plasmids pBS423 (A), pTBR101 (C), and pTBR101-CM (D) are shown in the surrounding circle (dashed lines) of each plasmid and are divided according to the source of the DNA region. Names of derived genetic materials are shown on the surrounding circle. (A) The *E. coli*-*Bifidobacterium* shuttle vector pBS423 was constructed by cloning the cryptic plasmid pTB4 from *B. longum* BK25 into the BamHI site of pTANS19. (B) The conditional replication vector pBS423- Δ *repA* was constructed by the self-circularization of the 4.4-kbp HincII fragment of pBS423. Because pBS423- Δ *repA* lacks the *repA* gene, which encodes the replication initiation protein RepA, it cannot replicate in *B. longum* 105-A. (D) To construct pTBR101-CM, which is incompatible with pBS423- Δ *repA*, a chloramphenicol resistance gene (*Cm^r*) from pC194 (cryptic plasmid of *Staphylococcus aureus*) was cloned into the HindIII site of pTBR101 (C), which consists of pTB4 from *B. longum* BK25 and pBR322. Sp^r, spectinomycin resistance gene; Ap^r, ampicillin resistance gene.

conducted as described previously (13). Nucleotide sequences were determined by the dideoxy method at TaKaRa Bio and Operon Biotechnologies, Inc. (Tokyo, Japan). BLAST programs were used for sequence similarity analysis (1). Southern hybridization analysis was conducted as described previously (13). BamHI-digested chromosomal DNA (4 μ g) from each strain was analyzed. The 3' region of *aga* (1.1 kbp) was amplified from pBS423- Δ *repA* Δ *aga* with the primer pair *aga13f/aga8r* and used as a probe.

Electrotransformation of *B. longum* strains. Electrotransformation of *B. longum* was performed using a previously described method, with some modifications (3). In brief, *B. longum* 105-A and first-crossover integrants were cultured anaerobically until the OD₆₆₀ reached 0.5 to 0.6 in 1/2 MRSCS medium and 1/2 MRSCS-Sp medium, respectively, both of which were supplemented with 50 mM sucrose. Cells were then harvested by centrifugation at 32,300 \times g at 4°C for 15 min and washed twice with ice-cold buffered sucrose (1 mM ammonium citrate buffer containing 50 mM sucrose, pH 6.0) and resuspended in the buffered sucrose. Electrotransformation was conducted using Gene Pulser (Bio-Rad Laboratories, Hercules, CA) with settings of 12 kV/cm, 25 μ F, and 200 Ω . After an electric pulse, the cells were cultured anaerobically in 1/2 MRSCS supplemented with 50 mM sucrose for 3 h and spread onto 1/2 MRSCS agar medium (without sucrose supplementation) containing antibiotics corresponding to the plasmid used. The plates were incubated anaerobically at 37°C for 48 h. Sucrose in the 1/2 MRSCS medium and the buffered sucrose has been reported to improve the transformation efficiency in bifidobacteria (3).

Construction of incompatible plasmids for markerless gene deletion. Two incompatible plasmids, pBS423- Δ *repA* and pTBR101-CM, were constructed for markerless gene deletion. All of the plasmids named below were constructed by the introduction of ligated products into *E. coli* HMS174 (8) and subsequent selection on LB agar medium containing appropriate antibiotics. To construct pBS423- Δ *repA*, a conditional replication vector for the first crossover event, the Sp^r resistance (*Sp^r*) gene [Sp adenylyltransferase AAD(9)] from pBRASTA101 (48) was first ligated with a pMB1 *ori*-containing fragment from pUC18. The resulting plasmid was named pTANS19. The cryptic plasmid pTB4 from *B. longum* BK25 (13) was digested with BamHI and ligated into pTANS19. The resulting plasmid, *E. coli*-*Bifidobacterium* shuttle vector pBS423 (Fig. 2A), was digested with HincII to remove *repA*. HincII-digested pBS423 was separated into 4.4- and 0.5-kbp fragments (the latter contained a part of *repA*), and the 4.4-kbp fragment was self ligated. The resulting plasmid was named pBS423- Δ *repA* (*RepA⁻*, pTB4 *ori⁺*, Sp^r) (Fig. 2B) and was shown not to replicate in *B. longum* 105-A.

To construct pTBR101-CM, a *repA*-positive plasmid for facilitation of the second crossover step, bifidobacterial plasmid pTB4 was purified from *B. longum* BK25, digested with BamHI, and ligated into BamHI-digested pBR322. The resulting plasmid, *E. coli*-*Bifidobacterium* shuttle vector pTBR101 (Fig. 2C), was digested with HindIII. The HindIII linker fragment was ligated to the MspI-Sau3AI-digested DNA fragment from pC194 containing the *Cm^r* resistance (*Cm^r*) gene (18). The HindIII-digested fragment containing the *Cm^r* gene was ligated into

HindIII-digested pTBR101, and the resulting plasmid was named pTBR101-CM (RepA⁺, pTB4 ori⁺, Cm^r) (Fig. 2D).

Construction of vectors for the first crossover. Two first-crossover vectors were constructed for different objectives, pBS423- Δ repA β gal for validation of the integration and efficient excision/exclusion of the vector at the β -galactosidase (β -Gal) gene locus and pBS423- Δ repA Δ aga for the introduction of the markerless gene deletion in the *aga* locus.

For the construction of pBS423- Δ repA β gal, the full-length β -Gal gene of *B. longum* 105-A, which shows 99% nucleotide identity to BLLJ_1486 in *B. longum* subsp. *longum* JCM 1217^T (15), was used as the target gene. A 4.6-kbp fragment containing the full-length β -Gal gene was amplified by PCR, using *B. longum* 105-A chromosomal DNA as a template and the lacZ Fw/lacZ Rv primer pair. The amplified product was digested with PstI and ligated into PstI-digested pBS423- Δ repA. Ligated products were introduced into *E. coli* JM109 by electroporation. Transformants were selected on LB agar containing Sp and supplemented with X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside; 40 μ g/ml) and IPTG (isopropyl-1-thio- β -D-galactopyranoside; 0.1 mM). Colonies that showed blue color, indicating the successful cloning of the β -Gal gene, were isolated.

For the construction of pBS423- Δ repA Δ aga, the 5' and 3' regions of *aga*, but lacking the internal region of *aga* containing the predicted α -Gal catalytic site (Fig. 3A), were used for the construction. A 4.9-kbp fragment containing the full-length *aga* gene was amplified by PCR using *B. longum* 105-A chromosomal DNA as the template and the aga1f/aga2r oligonucleotide primer pair. The amplified fragment was purified and cloned into the TA cloning vector pGEM-T Easy (Promega Corp., Madison, WI). The resulting plasmid was named pGEM-T-*aga*. The 5' and 3' regions of the cloned fragment (0.9 and 1.1 kbp, respectively) were separately amplified by PCR using pGEM-T-*aga* as the template and aga1f/aga12r and aga13f/aga8r as the respective primer pairs. These fragments were fused together by performing a second amplification, covering the 25-bp overlapping region inserted at the 5' terminus of the primer aga13f, with the aga1f/aga8r primer pair. The amplified 2.0-kbp fragment was digested with PstI and ligated into PstI-digested pBS423- Δ repA. The ligated products were introduced into *E. coli* JM109, and transformants were selected on LB-Sp agar medium.

Construction of the first-crossover integrants. The first-crossover integrants were constructed by the introduction of the first-crossover vectors, either pBS423- Δ repA β gal or pBS423- Δ repA Δ aga, to *B. longum* 105-A by electroporation. Transformants were selected on 1/2 MRSCS-Sp agar medium. Integration of the first-crossover vector into an objective gene locus was verified by genomic PCR using following primers: pBS423- Δ repA β gal integrants SpR1/SpR3 and bgal1/UC18pBS423 (see Fig. S1A in the supplemental material) for the detection of the Sp^r gene and pBS423- Δ repA β gal integration sequence, respectively, and pBS423- Δ repA Δ aga integrants aga1f/aga8r, aga1f/TB4-1, and aga8r/SpR4 (Fig. 3A) for the detection of the pBS423- Δ repA Δ aga integration sequence. The validated strains were used as the first-crossover integrant of each vector.

Triggering of the second crossover by the introduction of pTBR101-CM. pTBR101-CM was introduced into the first-crossover integrant, either 105-A β gal::pBS423- Δ repA β gal or 105-A *aga*::pBS423- Δ repA Δ aga, by electroporation to facilitate the second crossover step through excision of integrated pBS423- Δ repA-derived first-crossover vectors. Transformants were selected on 1/2 MRSCS-Cm agar medium as the Cm-resistant colonies, and they were anaerobically subcultured overnight in 1/2 MRSCS-Cm medium. Subcultures were conducted twice. Excised plasmid was expected to be eliminated from the cells due to plasmid incompatibility during the subcultures. Second-crossover recombinants were selected by using an Sp-sensitive (Sp^s) phenotype as an indicator of the elimination of the excised plasmid. This was tested by a replica plating method using 1/2 MRSCS-Cm and 1/2 MRSCS-CmSp agar media. The excision of pBS423- Δ repA-derived first-crossover vectors was verified by genomic PCR using the following primer pairs: for pBS423- Δ repA β gal, SpR1/SpR3 and bgal1/UC18pBS423; for pBS423- Δ repA Δ aga, aga1f/aga8r, aga1f/TB4-1, and aga8r/SpR4. In principle, the second crossover event in 105-A β gal::pBS423- Δ repA β gal yields original wild-type 105-A

strain with respect to the β -Gal gene allele, and that in 105-A *aga*::pBS423- Δ repA Δ aga yields either wild-type revertant or a mutant with respect to the *aga* allele. To check if the recombination rates actually change when pTBR101-CM is introduced into the cell, control experiments were conducted in both of the first-crossover integrants without adding pTBR101-CM in the reaction mixture for the electroporation step (mock transformation experiment) for comparison. In this case, Cm was not added to the culture media.

Construction of the markerless *aga*-defective mutants. The second-crossover recombinants from 105-A *aga*::pBS423- Δ repA Δ aga were cultured in SSM supplemented with raffinose as the sole carbohydrate source using 96-well microtiter plates. Strains that showed no growth were selected as candidates for *aga* deletion. The genotypes of the candidates were checked by *aga*-specific PCR, which was conducted using chromosomal DNA as the template and the aga1f/aga8r primer pair. Subsequently, one candidate was cultured anaerobically overnight in 1/2 MRSCS-Rif broth at 37°C to cure pTBR101-CM, which was propagated in the cells. The culture was streaked onto 1/2 MRSCS agar medium. Plasmid-cured strains were selected as Cm-sensitive (Cm^s) colonies by replica plating using 1/2 MRSCS and 1/2 MRSCS-Cm agar media. After curing, detailed genotype analysis of the candidate *aga* deletion strain was conducted by Southern hybridization and the sequencing of the *aga* deletion region. The *aga* deletion mutant was named 105-A Δ aga.

Construction of the *aga*-complemented strain. The upstream region of *aga* containing the putative promoter was determined from the draft genome sequence of *B. longum* 105-A (H. Yoshikawa, S. Fukiya, and A. Yokota, unpublished results) and a previous report (21). A 2.5-kbp DNA fragment containing *aga* and an upstream region (135 bp) was amplified by PCR using pGEM-T-*aga* as the template and the aga20f/aga21r primer pair. The products were digested with SalI and ligated into SalI-digested pKKT427 (51) (Sp^r). The ligated products were introduced into *E. coli* DH5 α , and transformants were selected on LB-Sp agar medium. The resulting *aga* complementation plasmid was named pAga135. Purified pAga135 was subsequently introduced into 105-A Δ aga by electroporation, and an Sp-resistant strain was isolated on 1/2 MRSCS-Sp agar medium. The propagation of pAga135 was confirmed by agarose gel electrophoresis of the purified plasmid. The strain, named 105-A Δ aga/pAga135, was used as a plasmid-based *aga*-complemented strain. An empty vector-introduced strain, 105-A Δ aga/pKKT427, was similarly constructed through the introduction of the shuttle vector pKKT427 into 105-A Δ aga.

Measurement of α -Gal activity. Strains 105-A, 105-A Δ aga, 105-A Δ aga/pAga135, and 105-A Δ aga/pKKT427 were cultured overnight in 40 ml of 1/2 MRSCS (Sp was added when necessary). Cells were harvested by centrifugation at 19,000 \times g at 4°C for 15 min and washed with saline. The cells were resuspended in SSM containing 20 g/liter raffinose and incubated for 6 h under anaerobic conditions at 37°C to induce α -Gal expression. The cells were harvested by centrifugation, washed twice with ice-cold 50 mM potassium phosphate buffer (pH 6.0) containing 0.015% (wt/vol) dithiothreitol, and disrupted three times using a French pressure cell (Ohtake Works, Tokyo, Japan) at 125 MPa. Cell debris was removed by centrifugation. The resulting supernatant was used as a crude extract. Protein concentration was determined by the Bradford method using a Bio-Rad protein assay kit (Bio-Rad Laboratories). The measurement of α -Gal activity was performed according to a previously described method (36) in a 300- μ l reaction mixture containing 50 mM potassium phosphate buffer (pH 6.0), 5 mM *p*-nitrophenyl- α -D-galactopyranoside (25026-81; Nacalai Tesque, Inc., Kyoto, Japan) (as a substrate), and 60 μ l of crude extract, diluted appropriately in 50 mM potassium phosphate buffer (pH 6.0) containing 0.5 mg/ml bovine serum albumin. The enzyme reaction was started by adding the crude extract to the reaction mixture followed by incubation at 35°C for 5 or 10 min. The reaction was stopped by adding 600 μ l of 1 M Na₂CO₃. A blank reaction mixture was prepared by adding 600 μ l of 1 M Na₂CO₃ prior to the addition of the crude extract. The release of *p*-nitrophenol was monitored in a 1-cm-wide cuvette by measuring absorbance at 400 nm with a DU800 spectrophotometer (Beckman

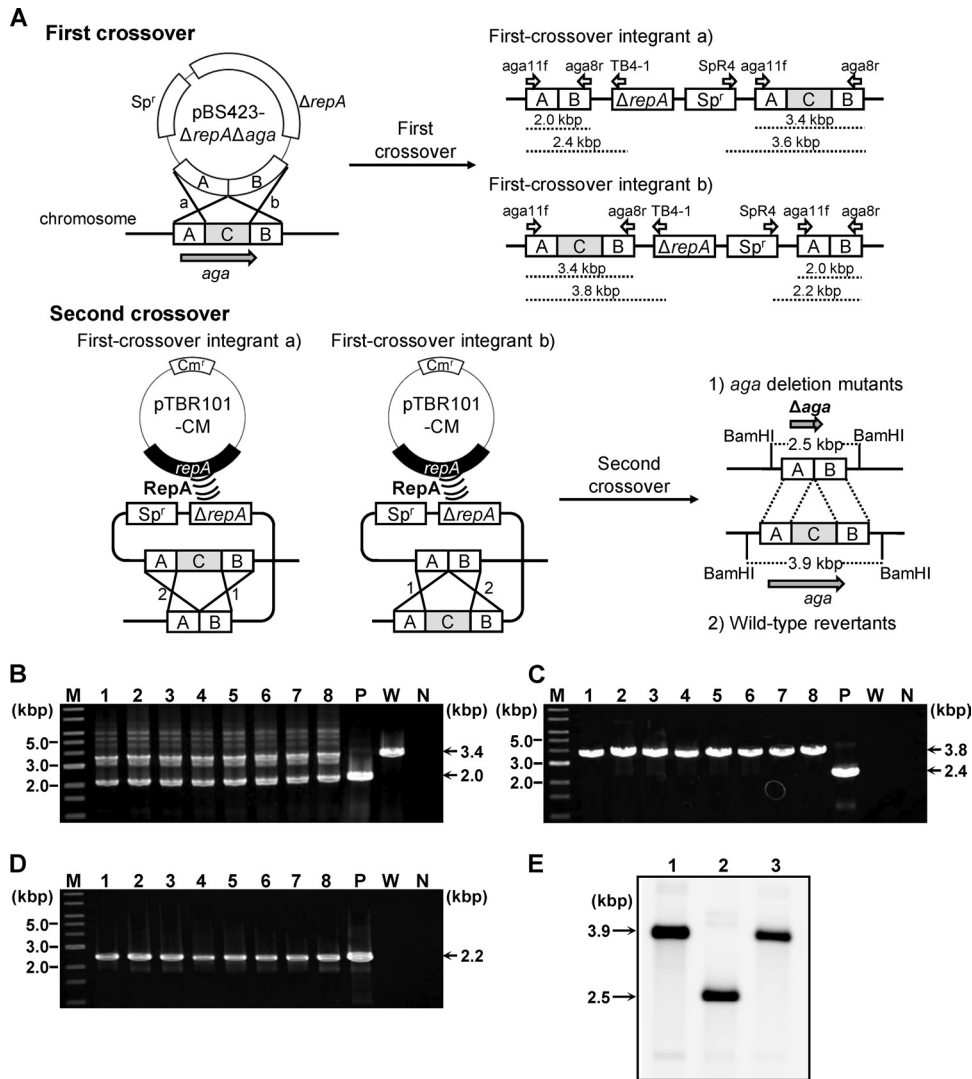


FIG 3 (A) Schematic of the markerless gene deletion procedures conducted in *B. longum* 105-A. The first crossover event is shown in the upper panel, and the second crossover event is in the lower panel. The first crossover between the target gene (*aga*) and the conditional replication vector pBS423- Δ *repA Δ *aga*, harboring the Δ *aga* allele, which lacks the internal region of *aga* (C in the chromosome structure), can take place with homologous region A or B, yielding two types of first-crossover integrants, designated a and b. Primers used to check the genotypes of first-crossover integrants are shown as open arrows, with names above the predicted structure of the target locus of each first-crossover integrant. The expected lengths of the amplicons generated using these primers are indicated by dashed lines below the predicted structures. The second crossover event, facilitated by the supply of RepA by the plasmid pTBR101-CM, can take place with homologous region A or B in the first-crossover integrants, designated a and b. The second crossover event yielded two types of strains, *aga* deletion mutants and wild-type revertants. Predicted restriction sites for BamHI and the predicted lengths of the restriction fragments are indicated in the structure of the *aga* locus for each strain. (B to D) Genotype analyses of the pBS423- Δ *repA Δ *aga* transformants to confirm integration of pBS423- Δ *repA Δ *aga* into the *aga* locus. Amplified fragments produced using the *aga*11f/*aga*8r primer pair (B), the *aga*11f/TB4-1 primer pair (C), and the SpR4/*aga*8r primer pair (D) were subjected to agarose gel electrophoresis. A 1-kb DNA ladder (New England BioLabs, Inc., Ipswich, MA) was used as a molecular mass marker (lane M). The sizes of representative marker fragments are shown to the left of each panel. Each lane shows amplified DNA generated from a DNA template: lanes 1 to 8, candidate first-crossover integrants; lane P, pBS423- Δ *repA Δ *aga* (positive control for amplification); lane W, *B. longum* 105-A (wild-type); and lane N, no template DNA (negative control for amplification). The sizes of the amplified fragments are shown to the right of each panel. (E) Southern hybridization analysis of markerless *aga* deletion. Samples of BamHI-digested chromosomal DNA (4 μ g) were electrophoresed in a 0.7% agarose gel. Each lane corresponds to a DNA sample from a *Bifidobacterium* strain: lane 1, *B. longum* 105-A; lane 2, 105-A Δ *aga*; lane 3, wild-type revertant (after the second crossover). Electrophoresed total DNA was transferred to a nylon membrane. The 3' region of *aga* (region B in panel A) was used as a probe. Hybridization signals were detected after exposure for 30 min. The sizes of the hybridized fragments are indicated to the left of the panel.****

Coulter Inc., Fullerton, CA). The absorbance value for the blank was subtracted from that for the samples. A molar extinction coefficient of $5,560 \text{ M}^{-1} \text{ cm}^{-1}$ for *p*-nitrophenol was used in the calculation. The specific activity was expressed as μmol of *p*-nitrophenol per min per mg protein.

Nucleotide sequence accession numbers. Nucleotide sequences of the plasmids constructed were deposited into the DDBJ/EMBL/GenBank

databases (accession no. [AB687999](#) for pBS423, including information for the deleted region in pBS423- Δ *repA*, and [AB688000](#) for pTBR101-CM).

RESULTS

Validation of the proposed system at β -Gal gene locus in *B. longum* 105-A. In the proposed system, the plasmid pBS423-

TABLE 2 Frequency of detection of Sp^s colonies during the culture of first-crossover integrants^a

Addition of pTBR101-CM to reaction mixture for electroporation		No. of tested colonies (A)	No. of Sp ^s second-crossover recombinants (B)	Detection frequency (%)
105-A β gal::				
pBS423- Δ repA β gal				
–		2,396	3	0.125
+		3,568	3,568	100
105-A <i>aga</i> ::				
pBS423- Δ repA Δ aga				
–		963	1	0.104
+		1,645	1,643	99.9

^a The reaction mixtures were supplemented with pTBR101-CM for electroporation (+) or left unsupplemented (–). Detection frequency was determined by the following equation: (number of Sp^s second-crossover recombinants/number of tested colonies) \times 100.

Δ repA (RepA[–] pTB4 *ori*⁺ Sp^r), which lacks the gene encoding the replication protein RepA, was used as the conditional replication vector. A second plasmid, pTBR101-CM (RepA⁺ pTB4 *ori*⁺ Cm^r), which was incompatible with pBS423- Δ repA, was used to supply RepA. Uncertainty as to the presence of sufficient recombination activity in *B. longum* 105-A led us to first examine whether integration and excision events readily occur using the full-length β -Gal gene as the target.

Sp^r transformants carrying pBS423- Δ repA β gal were obtained with an efficiency of 50 CFU/ μ g DNA. Genomic PCR analysis of the isolated transformants indicated that pBS423- Δ repA β gal was successfully integrated into the chromosomal β -Gal gene locus as a result of the first crossover (see Fig. S1B and C in the supplemental material). The introduction of pTBR101-CM into the first-crossover pBS423- Δ repA β gal integrants yielded transformants (7.5×10^2 CFU/ μ g DNA) on 1/2 MRSCS-Cm agar. After the subsequent culture of pTBR101-CM transformants in 1/2 MRSCS-Cm medium, 100% of the colonies tested were Sp^s (Table 2). In contrast, only 0.125% of the mock transformants without using pTBR101-CM showed a Sp^s phenotype after the culture in 1/2 MRSCS medium. Therefore, it was found that the introduction of pTBR101-CM dramatically increased the frequency of the appearance of the second-crossover recombinants.

The lack of amplification in genomic PCR analysis of selected Sp^s strains (see Fig. S1D and E in the supplemental material) indicated the successful excision of pBS423- Δ repA β gal from the chromosome by the second crossover. To confirm the elimination

of the excised pBS423- Δ repA by using incompatibility, plasmids in the second-crossover recombinants were extracted and introduced into *E. coli* DH5 α . Many transformants derived from pTBR101-CM appeared in the LB-Cm agar. In contrast, none of the transformants derived from pBS423- Δ repA were detected in LB-Sp agar. These results indicate that the excised pBS423- Δ repA was eliminated from cells because of plasmid incompatibility. Taken together, the proposed system was verified to function as expected.

Markerless deletion of *aga* in *B. longum* 105-A. This system was next applied for markerless gene deletion into the *aga* gene, which putatively encodes α -Gal (Fig. 3A). The α -Gal-encoding gene was selected as the target because α -Gal hydrolyzes raffinose, which is known to increase bifidobacterial populations in the intestinal tract of rats and humans (9, 10). As shown in Table 3, three genes annotated as α -Gal were found in the draft genome sequence of *B. longum* 105-A (Yoshikawa et al., unpublished). The contribution of each gene to the α -Gal activity in *B. longum* 105-A cells was not known. Among these genes, *aga* and *galA3* are conserved in the genomes of *B. longum* NCC2705 and *B. longum* DJO10A (Table 3). Furthermore, the molecular mass of the deduced *aga* polypeptide was similar to those of purified α -Gals from other bifidobacteria (approximately 80 kDa as a monomer) (16, 24, 49). Therefore, *aga* was inferred to contribute to α -Gal activity and raffinose assimilation in *B. longum* 105-A.

Fragments of both the 5' and 3' regions of *aga* (each approximately 1 kbp in length), which lacked the sequence for the putative active site of the protein, were used as the homologous regions to construct the conditional replication vector pBS423- Δ repA Δ aga. An outline of experimental procedures is illustrated in Fig. 3A. When pBS423- Δ repA Δ aga was introduced into *B. longum* 105-A by electroporation, Sp^r transformants were formed with an efficiency of 2.2×10^2 CFU/ μ g DNA. After single-colony isolation, strains harboring pBS423- Δ repA Δ aga integrated into the chromosomal *aga* locus were selected by *aga*-specific PCR (primer pair *aga11f/aga8r*). The amplicons of both wild-type (3.4 kbp) and mutant (2.0 kbp) *aga* alleles were identified in Sp^r transformants (Fig. 3B). To identify integration patterns, PCR was conducted using different primer pairs. Amplicons of 3.8 kbp (primer pair *aga11f/TB4-1*; Fig. 3C) and 2.2 kbp (primer pair *aga8r/SpR4*; Fig. 3D) were detected in the same transformants. These results demonstrated that the selected strains were first-crossover integrants of pBS423- Δ repA Δ aga (105-A *aga*::pBS423- Δ repA Δ aga) in which recombination had taken place in the 3' region of the *aga* locus (region B) (Fig. 3A).

TABLE 3 Annotated α -galactosidase genes in *B. longum* 105-A draft genome and their distribution in *B. longum* strains NCC2705 and DJO10A

Annotated α -Gal ^a gene in 105-A	<i>B. longum</i> strain						
	105-A			NCC2705		DJO10A	
	Length of gene (bp)	Calculated molecular mass ^b (kDa)	Nucleotide sequence identity ^c (%)	Annotated α -Gal ^a gene	Nucleotide sequence identity ^c (%)	Annotated α -Gal ^a gene	Nucleotide sequence identity ^c (%)
<i>aga</i> (<i>galA1</i>)	2,307	83.3	100	BL_1518 (<i>aga</i>)	98.1	BLD_1483 (<i>galA1</i>)	99.0
<i>galA2</i>	1,863	68.1	100	ND ^d	ND ^d	BLD_1496 (<i>galA2</i>)	99.8
<i>galA3</i>	1,410	52.4	100	BL_0177	98.7	BLD_1538 (<i>galA3</i>)	99.5

^a α -Gal, α -galactosidase.

^b The molecular mass of the deduced polypeptide was calculated.

^c Nucleotide sequence identity to the corresponding α -Gal gene in *B. longum* 105-A.

^d ND, not determined because the *galA2* homolog was absent from *B. longum* NCC2705.

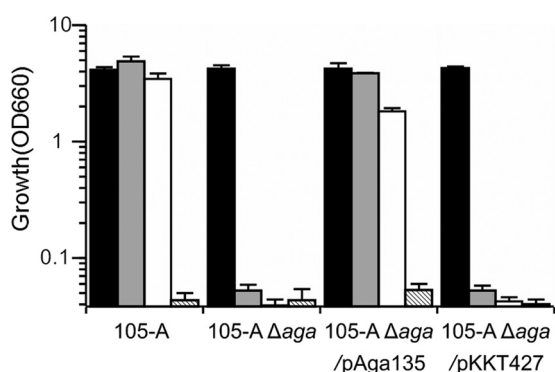


FIG 4 Growth profiles of *B. longum* strains 105-A, 105-A Δ aga, 105-A Δ aga/pAga135, and 105-A Δ aga/pKKT427. Bacteria were cultured anaerobically at 37°C for 24 h in semisynthetic medium containing glucose (filled bars), raffinose (gray bars), or melibiose (open bars) as the sole carbohydrate source. Striped bars indicate cultures without the addition of a carbohydrate source. Data are shown as means \pm standard errors of three independent experiments.

To construct the markerless *aga* deletion mutants, pTBR101-CM was introduced into 105-A *aga::pBS423- Δ repA Δ aga*, and Cm^r transformants were formed with an average efficiency of 3.9×10^2 CFU/ μ g DNA. Randomly selected Cm^r transformants (288 colonies) were subcultured in 1/2 MRSCS-Cm broth to select for second-crossover recombinants. The subcultures were checked for Sp^s and the inability to assimilate raffinose by the replica method. The results indicated a high frequency of the appearance of the second-crossover recombinants, as 270 of the 288 subcultured strains were Sp^s (94%). In this case, 4 of the 270 Sp^s strains (1.5%) lacked raffinose assimilation ability and were thus considered candidate *aga* deletion mutants. Genotype analysis of all of these candidates by *aga*-specific PCR supported their inability to grow on raffinose (data not shown). In addition, the same *aga*-specific PCR analysis of raffinose-assimilating Sp^s strains indicated that these strains were wild-type *aga* allele revertants (data not shown). In a separate experiment, the usefulness of pTBR101-CM was also confirmed at the *aga* locus. As shown in Table 2 when pTBR101-CM was not introduced into the integrant, the ratio of Sp^s colonies among the tested colonies was found to be dramatically decreased, indicating the facilitation of the second crossover step by the plasmid. One of the four candidate strains was selected and used to cure pTBR101-CM, which remained in the cells. Rif was employed as a curing agent because it was previously used to cure plasmids in *E. coli* (6, 30). After Rif treatment, 84% of the colonies grown on 1/2 MRSCS agar plates were Cm^s . The curing of the plasmid was also confirmed by the introduction of the extracted plasmid fraction from a Cm^s strain into *E. coli*, which yielded no Cm^r *E. coli* transformants. These results indicated that pTBR101-CM was successfully removed from the candidate strain by Rif treatment. The deletion of *aga* from the pTBR101-CM-cured candidate strain was further confirmed by Southern hybridization (Fig. 3E) and sequence analysis. These results demonstrated successful markerless *aga* gene deletion in *B. longum* 105-A. This mutant was named 105-A Δ aga.

Functional analyses of *aga* using *B. longum* 105-A Δ aga. To clarify the function of *aga*, the complementation of *aga* in 105-A Δ aga was conducted using the plasmid pAga135, which harbors the *aga* open reading frame and a 135-bp upstream region containing the putative *aga* promoter. A transformant, 105-A Δ aga/

pAga135, was used in the functional analysis, together with the wild-type 105-A strain, 105-A Δ aga, and the empty vector-introduced derivative 105-A Δ aga/pKKT427.

Carbohydrate assimilation analysis using SSM revealed that 105-A Δ aga was unable to grow after cultivation for 24 h on either melibiose or raffinose, two substrates for α -Gal (Fig. 4). In contrast, Δ aga/pAga135 showed growth levels comparable to those of 105-A on all tested carbohydrates. These results indicated that *aga* is the gene responsible for the assimilation of melibiose and raffinose.

α -Gal activity was next measured in cells incubated for 6 h in SSM containing raffinose as the sole carbohydrate. As expected, α -Gal activity was not detected in 105-A Δ aga (Table 4). α -Gal activity was successfully restored in Δ aga/pAga135 but not in 105-A Δ aga/pKKT427 (Table 4). Taken together, these results clearly identify *aga* as the only functional α -Gal gene necessary for the assimilation of α -galactosides such as melibiose and raffinose in *B. longum* 105-A. These results also suggest that the other annotated α -Gal genes in the *B. longum* 105-A genome, *galA2* and *galA3* (Table 3), make little contribution, if any, to α -Gal activity under the conditions used in this study.

DISCUSSION

In this study, a markerless gene deletion system was successfully developed using a double-crossover method in *B. longum* 105-A. Despite the emerging importance of bifidobacteria in human health, genetic manipulation systems for improving our understanding of these bacteria, especially efficient methods for targeted gene mutagenesis, have not yet been established. Several *B. breve* UCC2003 gene insertion mutants (32–34, 41), in which a nonreplicating vector containing internal DNA fragments of the target gene was integrated into the target gene by a single-crossover method, were recently reported. A mutant of *B. longum* NCC2705 has also been generated (15). In this mutant, gene disruption was achieved by a double-crossover method in which a region homologous to the target gene along with an inserted antibiotic resistance gene was cloned into a general *E. coli* vector, and allelic exchange was performed. These methods represent important progress in the functional analysis of bifidobacterial genes, although exogenous sequences (vector sequences and/or selection marker genes) were left in the mutated allele by both methods. In contrast, the proposed method is a markerless deletion method that does not leave any exogenous sequences in the mutated allele.

The following factors are considered to be the characteristics of the developed system.

(i) Facilitation of the second crossover step. A low-frequency second crossover event is generally the bottleneck in gene mutagenesis based on the double-crossover system. In this study, a

TABLE 4 α -Galactosidase activities of *B. longum* 105-A and its derivatives

Strain	Sp act^a
105-A	4.44 \pm 0.36
105-A Δ aga	Not detected
105-A Δ aga/pAga135	1.30 \pm 0.09
105-A Δ aga/pKKT427	Not detected

^a The activities were measured after induction by raffinose as described in Materials and Methods. Results are mean values of α -galactosidase specific activity [$\mu\text{mol min}^{-1}$ (mg protein)⁻¹] with standard errors as determined in duplicate on three biological replicates.

conditional replication vector lacking *repA* [pBS423- Δ *repA* (Sp^r)] for integration into the chromosome (first crossover event) and a second plasmid to supply RepA (pTBR101-CM) were used to facilitate the second crossover step. As shown in Table 2, pTBR101-CM apparently increased the second-crossover frequency. Still, one can also consider the possibility that the introduced pTBR101-CM served simply as a strong selective pressure to the first-crossover integrants, allowing the preferential growth of preexisting spontaneous second-crossover recombinants after the electrotransformation step. However, this may not be the case, as there seemed to be no contamination of the spontaneous second-crossover recombinants in the competent cells (Sp was included in the medium for preparation of the competent cells). Though not shown in the results, colony PCR analysis of the pTBR101-CM transformants clearly indicated that the excision of the integrated plasmid from the chromosome had already occurred in the transformed colonies before subcultures. Taking these findings together, the supply of RepA was judged to increase the second-crossover frequency, which led to the facilitation of the second crossover step.

(ii) Exclusion of excised vector by plasmid incompatibility. Plasmid incompatibility has been used as a method to eliminate unnecessary plasmids from cells during bacterial gene mutagenesis by double-crossover recombination (11, 25, 37, 43). This system was applied to markerless gene deletion. The use of two incompatible vectors, pBS423- Δ *repA* and pTBR101-CM, enabled the excised vector pBS423- Δ *repA* (containing a wild-type or mutated allele) to be effectively excluded from cells. In principle, either plasmid could be excluded. However, in this case, retaining pTBR101-CM was the only option, because pBS423- Δ *repA* cannot replicate on its own. The Sp^s plus Cm^r combination ensured the acquisition of the desired recombinants.

(iii) Successful curing of pTBR101-CM by Rif. The remaining pTBR101-CM in cells was easily cured by Rif treatment, as has been reported for *E. coli* (6, 30). Successful curing of the replicative vector in the markerless gene deletion mutant means that multiple gene deletions are possible using the developed system. Multiple gene mutations have not been introduced into bifidobacteria and are theoretically limited by mutagenesis methods that leave antibiotic resistance markers, such as Sp (29), tetracycline (2), and erythromycin (42), in the mutated allele, owing to the limited availability of these markers in bifidobacteria.

Temperature-sensitive vectors harboring a temperature-sensitive *rep* gene have been commonly used for targeted gene mutagenesis in other bacteria (7, 17, 35, 45). However, no reliable temperature-sensitive vector is currently available for use in bifidobacteria. In addition, mutagenic effects of heat shock stress on the genome sequence are a concern, because heat shock-induced GroEL/GroES chaperone is known to protect error-prone DNA polymerases IV (Pol IV) and Pol V from degradation in *E. coli* (12). Homologs of the chaperone and the DNA polymerases are also found in the genome sequences of *B. longum* 105-A and other bifidobacteria.

Theoretically, after the introduction of gene mutations by the double-crossover method, the frequencies of the appearance of the wild-type and the mutant genotypes should be the same. However, it was found that the number of *aga* deletion mutants was far lower than that of revertants (4 versus 266). The reason for the low frequency of gene deletion mutants is unclear, but it is possible that there was a difference in recombination efficiency between

the homologous regions A and B used in *aga* deletion (Fig. 3A). Therefore, the recombination frequency was checked using plasmids consisting of homologous region A or B in pBS423- Δ *repA*. These plasmids were introduced into *B. longum* 105-A, and the appearance of first-crossover integrants was examined. However, no significant difference between homologous regions A and B was observed (data not shown). These results suggest that a difference in recombination frequency between these homologous regions was not a cause of the low occurrence of gene deletion mutants. An unexpected selective disadvantage associated with the *aga* deletion might be involved in this tendency. Further comparisons using other genes are needed to address this point.

aga was shown to be the sole gene responsible for α -Gal activity in *B. longum* 105-A. Successful complementation in both the growth on melibiose and the α -Gal enzyme activity by introduction of pAga135 into α -Gal-deficient *E. coli* JW4080 (*mela* mutant) also supported the observation (data not shown). Although gene deletion mutants for the other annotated α -Gal genes (*galA2* and *galA3*) were not constructed, it seemed that these genes are not expressed or do not encode proteins with α -Gal activity. The nucleotide similarity between *aga* and *galA2* or *galA3* was calculated to be 47 or 48%, respectively. The molecular sizes of the predicted polypeptides encoded by these genes were smaller than that of *aga* (Table 3) and those of purified α -Gals in other bifidobacteria (approximately 80 kDa) (16, 24, 49), suggesting that these genes are unlikely to encode proteins with α -Gal activity.

The applicability of the developed system to other bifidobacterial species is unclear. It may depend on the host range of the plasmids pBS423- Δ *repA* and pTBR101-CM, both of which were derived from a cryptic plasmid, pTB4, that is harbored by *B. longum* BK25 (13). Another factor will be the transformation efficiency of a given bifidobacterial strain. The average transformation efficiency of an *E. coli*-*Bifidobacterium* shuttle vector pBS423 into *B. longum* 105-A was 1.1×10^5 CFU/ μ g DNA, whereas in the case of *aga* deletion, the transformation efficiency of pBS423- Δ *repA* Δ *aga* for the construction of the first-crossover integrants decreased to 2.2×10^2 CFU/ μ g DNA. The average transformation efficiency of the replicative pTBR101-CM vector into the first-crossover integrants was 3.9×10^2 CFU/ μ g DNA. According to our calculation from previously reported data on host/vector systems, the median transformation efficiency in bifidobacteria was up to 10^3 CFU/ μ g DNA (14). It therefore appears that the application of the developed system to other bifidobacteria will be limited to the strains that have relatively high transformation efficiency, at least more than 10^4 CFU/ μ g DNA with the replicative vector pBS423. In fact, preliminary trials for the deletion of *aga* in *B. longum* subsp. *longum* JCM 1217^T, *B. longum* subsp. *infantis* JCM 1222^T, and *B. breve* JCM 1192^T were not successful, probably due to the low transformation efficiencies as well as appearance of false-positive transformants. The optimization of transformation conditions and antibiotic concentrations for transformant selection will allow markerless gene deletion in other strains. The methylation of the vectors using bifidobacterial modification enzymes is one way to increase the transformation efficiency by avoiding the cleavage of introduced DNA by restriction systems in bifidobacteria (33, 51).

The markerless gene deletion method reported here can be used for functional gene analysis as well as for other applications. For example, this method may have advantages in the construction of food-grade host strains, as the use of antibiotic resistance

genes has not been approved for the construction of food-grade hosts (40, 45). Moreover, the method may be used to insert exogenous genes into the bifidobacterial chromosome. It is anticipated that therapeutic proteins and hormones for medical treatments can be stably expressed in genetically engineered bifidobacteria (14).

In conclusion, a rational system for markerless gene deletion was successfully developed in *B. longum* 105-A. Although the applicability of this system to other bifidobacteria remains to be demonstrated and there is much room for improvement, we believe that this technique will accelerate functional gene analysis in bifidobacteria.

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