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METHODS AND PROTOCOLS

A targeted gene knockout method using a newly constructed temperature-sensitive plasmid mediated homologous recombination in *Bifidobacterium longum*

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Abstract Bifidobacteria are the main component of the human microflora. We constructed a temperature-sensitive (Ts) plasmid by random mutagenesis of the *Bifidobacterium–Escherichia coli* shuttle vector pKKT427 using error-prone PCR. Mutant plasmids were introduced into *Bifidobacterium longum* 105-A and, after screening approximately 3,000 colonies, candidate clones that grew at 30 °C but not at 42 °C were selected. According to DNA sequence analysis of the Ts plasmid, five silent and one missense mutations were found in the *repB* region. The site-directed mutagenesis showed only the missense mutation to be relevant to the Ts phenotype. We designated this plasmid pKO403. The Ts phenotype was also observed in *B. longum* NCC2705 and *Bifidobacterium adolescentis* ATCC15703. Single-crossover homologous-recombination experiments were carried out to determine the relationship between the length of homologous sequences encoded on the plasmid and recombination frequency: fragments greater than 1 kb gave an efficiency of more than 10^3 integrations per cell. We performed gene knockout experiments using this Ts plasmid. We obtained gene knockout mutants of the *pyrE* region of *B. longum* 105-A, and determined that double-crossover homologous recombination occurred at an efficiency of 1.8 %. This knockout method also worked for the BL0033 gene in *B. longum* NCC2705.

Keywords Temperature-sensitive plasmid · Bifidobacteria · Mutagenesis · Gene knockout · Homologous recombination

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Introduction

Bifidobacteria are the main component of the human microflora and show various health-promoting effects (Mitsuoka 1990; Salminen et al. 1999). Whole-genome sequences of some bifidobacteria have been reported (Schell et al. 2002; Lee et al. 2008; Yasui et al. 2009a, b). Some genetic engineering tools, such as shuttle vectors (Missich et al. 1994), efficient transformation methods, and selection markers, have already been reported for bifidobacteria (Matsumura et al. 1997; Rossi et al. 1996; Florez et al. 2006; Yasui et al. 2009a, b). However, the elucidation of gene functions requires a reverse genetics approach that uses genetic engineering tools such as convenient shuttle vectors, efficient transformation, gene expression, and gene knockout techniques.

We have previously reported a compact (3.9 kb) *Bifidobacterium–Escherichia coli* shuttle vector pKKT427 (Yasui et al. 2009a, b). Low transformation efficiency is a bottleneck for gene manipulation. One reason for low transformation efficiency of plasmid pKKT427 is the restriction system of host cells that protects against the invasion of foreign DNA. In the case of bifidobacteria, transformation efficiency was increased from 10^0 to 10^5 transformants/μg DNA using the plasmid artificial modification (PAM) method (Yasui et al. 2009a, b; Suzuki and Yasui 2011).

However, one of the powerful tools for the generation of gene knockout mutants, the temperature-sensitive (Ts) plasmid, has not been reported although it has been commonly used in some microorganisms (Hamilton et al. 1989; Maguin et al. 1992; Biswas et al. 1993; Takamatsu et al. 2001; Fuchs et al. 2006; Chen et al. 2011). Ts plasmids are unable to replicate at high temperatures, causes bacterial cell death by losing selection marker gene such as antibiotics resistance. In the case of Ts plasmids containing homologous sequences and suitable selection markers, only the mutants that undergo homologous recombination are able to grow on a selection medium at a non-permissive high temperature.

Generally, the homologous recombination events occur with a very low frequency, such as 10^{-3}–10^{-5} integrations per cell (ipc), and a very high transformation efficiency (over 10^5 CFU/μg plasmid DNA) is required to obtain the recombinant (Suzuki and Yasui 2011). The higher transformation efficiency (10^5 CFU/μg) has been demonstrated in limited hosts, such as *Bifidobacterium longum* 105-A (10^5 CFU/μg) and *Bifidobacterium adolescentis* ATCC15703 (10^5 CFU/μg using PAM). However, it was not easy to obtain recombinants in these instances. In the case of *B. longum* NCC2705, which shows 10^4 CFU/μg efficiency, we took over 1 year to obtain one knockout mutant (Fukuda et al. 2011).

Ts plasmids, when available, do not require high transformation efficiencies. If at least one transformant is obtained, it can be propagated in a large bacterial population, such as 10^9 cells/mL. It is sufficient to enable a single-crossover (SCO) and also the more difficult double-crossover (DCO) homologous recombination. The gene knockout mutants are easily obtained in routine experiments using Ts plasmids.

In the present study, we report the construction of a Ts plasmid for *B. longum* by using error-prone PCR. Moreover, we demonstrate that the Ts plasmid works for gene knockout experiments in *B. longum* 105-A and NCC2705, which is one of the most important species for human health (Schell et al. 2002).

Materials and methods

Bacterial strains, plasmids, and growth conditions The bacterial strains and plasmids used in this study are listed in Table 1. *B. longum* were grown anaerobically on MRS medium (BD, Franklin Lakes, NJ, USA) at 37 °C (30 and 42 °C for handling of Ts mutants). *Escherichia coli* strains were grown on LB medium (per liter, 5 g yeast extract, 10 g tryptone, and 5 g NaCl) at 37 °C. For plate culture, 1.5 %

Table 1 Bacterial strains and plasmid

Strain or plasmid	Description	Source or reference
Strains		
Escherichia coli TOP10	F⁻,*mcrA*(*mrr, hsdRMS-mcrBC*), φ80(*lacZ*)ΔM15 Δ*lacX*74, *recA*1, *araD139*, *(ara-leu)7697*, *galU*, *galK*, *rpsL(StrR)*, *endA1*, *nupG*	Invitrogen
Escherichia coli XL10-Gold	Tet^r Δ*(mcrA)183* Δ*(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F' proAB lacIqZΔM15 Tn10 (Tetr) Amy Camr]*	Stratagene
Bifidobacteium longum 105-A	Wildtype	Matsumura et al.
Bifidobacterium longum NCC2705	Isolated from adult human feces (GenBank Accession no. AE014295)	Schell et al.
Bifidobacterium adolescentis ATCC15703	Type strain, isolated from adult human feces (GenBank Accession no. AP009256)	Yasui et al.
Plasmids		
pKKT427	Sp^r; 3.9 kb shuttle vector between *Bifidobacterium* and *E. coli*	Yasui et al.
pKO403	Sp^r; temperature-sensitive plasmid of pKKT427	This study
pBAD28	Cm^r; 5.8 kb plasmid	Guzman et al.
pKO403Cm	Cm^r; Sp^r gene of pKO403 was replaced by Cm^r gene	This study
pKO403Cm-ΔBL0033	7.7 kb plasmid for gene knockout of the BL0033 gene	This study
pKO403-sco 0.25	pKO403 containing 0.25 kb sequences of upstream of *pyrE* gene	This study
pKO403-sco 0.5	pKO403 containing 0.5 kb sequences of upstream of *pyrE* gene	This study
pKO403-sco 1.0	pKO403 containing 1.0 kb sequences of upstream of *pyrE* gene	This study
pKO403-sco 1.5	pKO403 containing 1.5 kb sequences of upstream of *pyrE* gene	This study
pKO403-sco 2.0	pKO403 containing 2.0 kb sequences of upstream of *pyrE* gene	This study
pKO403-sco 2.5	pKO403 containing 2.5 kb sequences of upstream of *pyrE* gene	This study
pKO403-sco 3.0	pKO403 containing 3.0 kb sequences of upstream of *pyrE* gene	This study

agar was added to the medium before autoclaving. Spectinomycin hydrochloride (Sp; 75 μg/mL for *E. coli* and *B. longum*) and chloramphenicol (Cm; 10 μg/mL for *E. coli* and 1 μg/mL for *B. longum* NCC2705) were purchased from Wako Pure Chemical Industries (Osaka, Japan) and were added as needed. Plasmid pKKT427 carries a Sp resistance gene, a multiple-cloning site and two replication origins, including the *repB* gene from the *B. longum* plasmid pTB6 and the *ColE*1 *ori* from pUC19. For cloning, *E. coli* TOP10 (Invitrogen, Carlsbad, CA, USA) was used as the host cell. The electroporation of *B. longum* 105-A, *B. longum* NCC2705, and *B. adolescentis* ATCC15703 was performed with the following procedures. Bifidobacterial cells were washed two times with ice-cold sucrose buffer (0.05 M sucrose, 1 mM ammonium citrate, pH 6.0). One hundred nanograms of plasmid was added to 50 μl of washed cells and incubated on ice for 10 min. Cell mixtures were transferred to 0.2 cm of inter electrode distance cuvette (Bio-Rad, CA, USA). Electroporation was performed at 2.5 kV and 15 μF capacitor setting with the pulse controller at 335Ω parallel resistant with Easyject Optima Electroporation System (Equibio, Kent, UK). After electric pulsing, cell mixtures were transferred immediately to 1 mL of MRS liquid medium and incubated at 37 °C (30 °C for the handling Ts plasmid derivatives) for 3 h without shaking. Cells were spread on MRS plates containing antibiotic. Plasmid methylation was carried out using the PAM method (Yasui et al. 2009a, b).

Plasmid DNA isolation from B. longum and E. coli Plasmid isolation from 30 mL of bifidobacterial cells was performed using a QIAprep Spin Miniprep kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions, except for the use of 0.9 % (*w*/*v*) NaCl and 200 μg/mL lysozyme. Before adding P1 buffer, cells were washed with 0.9 % (*w*/*v*) NaCl. Lysozyme, 200 μL, was added and the cells were incubated at 37 °C for 30 min after resuspending with P1 buffer. Plasmid preparation from *E. coli* cells was carried out using a QIAprep Spin Miniprep kit following the manufacturer's instructions.

Plasmid mutagenesis and screening for Ts plasmids Mutagenesis of the target region was performed using an EZClone GeneMorph II Domain Mutagenesis kit (Stratagene, La Jolla, CA, USA). To construct the Ts plasmid, the replication unit of bifidobacteria (1,627 bp) containing the *repB* gene was PCR amplified from pKKT427 with Mutazyme II using the primers pTB6 Fw and pTB6 Rv (Table 2). The error-prone PCR conditions were as follows: 95 °C for 2 min; 30 cycles of 95 °C for 1 min, 55.4 °C for 1 min and 72 °C for 2 min; and 72 °C for 10 min. The PCR products generated were analyzed by 0.7 % (*w*/*v*) agarose gel electrophoresis, gel extracted, and purified using a Nucleo-Spin kit (Nippon Genetics, Tokyo, Japan). The purified PCR products were then used as the mutant megaprimer for the EZClone reaction, and thus mutations were introduced into the original plasmid. The EZClone reaction conditions were as follows: 95 °C for 1 min; and 25 cycles of 95 °C for 50 s, 60 °C for 50 s, and 68 °C for 8 min. To remove nonmutated template DNA, the reaction mixture was treated with *Dpn*I at 37 °C for 2 h. *E. coli* XL-10 Gold were then transformed with this mixture. Sp-resistant (Sp^r) transformants were

Table 2 Primers used in this study

Oligonucleotide	Sequence (5′ to 3′)
pTB6 Fw	GCCACCGTCGCCAAGGCTCTGGAAC
pTB6 Rv	GCATGGCCAGAACAACGCGCACAGG
G21T-Fw	TGAACACAACGTCAGGACCAGCGAT
G21T-Rv	AGAGCCTTGGCGACGGTGG
G883T-Fw	TCAAGTACGCCAAGGAGTTCTACCG
G883T-Rv	TCTTGAGGTCGGCGAACTCGG
C912G-Fw	GCCAAGCAGTACCGCAGCTCC
C912G-Rv	CCTGCGGTAGAACTCCTTGGCG
G1214A-Fw	ATACGGCGAGGTGTTCACGACCAC
G1214A-Rv	CCGGCAACGCTCGTCCAATGG
A1416T-Fw	TTCTGCCGCTGTTCGGCCTCA
A1416T-Rv	CGACCCATGGCAGGCGCAAA
G1423A-Fw	ACTGTTCGGCCTCACGCTGGT
G1423A-Rv	CGACCCATGGCAGGCGCAAAC
sco Fw	GCCTGTCGACGGATCCTTACCT CCAAGTCTGTCCTG
sco 0.25 Rv	GCCCTGAGGGCGGCCGCTCGGC CTGGCAGCCGTCTAT
sco 0.5 Rv	GCCCTGAGGGCGGCCGCGCCCT CGCCAATCTTATG
sco 1.0 Rv	GCCCTGAGGGCGGCCGCTGTGCA CCATCGACACCGTG
sco 1.5 Rv	GCCCTGAGGGCGGCCGCTTCAAC CTCAAGGAAGACGC
sco 2.0 Rv	GCCCTGAGGGCGGCCGCCAAGTAC TCCGAATCCATGACCGCC
sco 2.5 Rv	GCCCTGAGGGCGGCCGCCGACTTC GACCCGTGGCATGAGGAT
sco 3.0 Rv	GCCCTGAGGGCGGCCGCCAGTTCC ACTCCAAGGCTCGTAACA
Cm^r Fw	AAAGTATATATGAGTGAACAACAC TCAACCCTATCTCGG
Cm^r Rv	AAGCTTGAGCTCAGTTGATGTCCG GCGGTGCTTTTGCCGT
Sp^r Fw	GAATTCCCATTAAATAATAAAACA AAAAAA
Sp^r Rv	GGTCGATTTTCGTTCGTGAATACAT
Sp^r mid Fw	AATAGTGATCTTGACTTTTTAGTCG
BL0033-200 Fw	TGCTGGTACATCGCATCCGTCTGAC
BL0033-200 Rv	CGGCTCGTCCAGAATCAGCAGCTCG

pooled in LB medium containing Sp. The Ts plasmid library was generated by isolating plasmids from the pooled cells.

This library was introduced into *B. longum* 105-A. After transformation, cells were spread on MRS plates containing Sp and incubated at 30 °C for 48 h. Then, Sp^r colonies were inoculated on MRS plates containing Sp. These plates were incubated at 30 and 42 °C. The colonies which failed to grow at 42 °C but grew at 30 °C were selected as candidate Ts mutants.

The growth of Ts mutants (*B. longum* 105-A/pKO403) and the control strain (*B. longum* 105-A/pKKT427) was monitored by Bio-plotter (Oriental Instruments, Tokyo, Japan). Both strains were cultured in MRS medium containing Sp at 30, 37, and 42 °C for 48 h.

DNA sequence analysis Oligonucleotide primers for sequencing were designed using IMC (In Silico Biology, Yokohama, Kanagawa, Japan). DNA sequencing of plasmid and genomic DNA was performed on an ABI 3100 DNA sequencer using Big Dye terminator ver. 3.1 (Applied Biosystems, Foster City, CA, USA).

Site-directed mutagenesis for single-base mutations Primers for site-directed mutagenesis are listed in Table 2. Inverse PCR was performed using pKKT427 as the template DNA with KOD-plus DNA polymerase (TOYOBO, Tokyo, Japan). The PCR was performed according to the manufacturer's instructions. *E. coli* TOP10 were transformed with each mutant plasmid, and the resulting strains were named G21T, G883T, C912G, G1214A, A1416T, and G1423A. To check the sequence, we performed DNA sequence analysis. These plasmids were used for the transformation of *B. longum* 105-A. After transformation, cells were inoculated into MRS liquid medium containing Sp. Propagated cells were spread on MRS plates containing Sp, and cultivated at 42 °C for 24 h and 30 °C for 48 h.

Host range of the Ts plasmid pKO403 was introduced into *B. longum* NCC2705 and *B. adolescentis* ATCC15703. Transformed cells were cultivated on MRS plates containing Sp at 30 °C for 48 h. Sp^r transformants were transferred to MRS liquid medium containing Sp and then propagated at 30 °C for 24 h. Cells were serially diluted and spread on MRS plates containing Sp. One plate was incubated at 30 °C and the other at 42 °C to evaluate the colony number.

Evaluation of plasmid stability B. longum 105-A harboring the Ts plasmid *(B. longum* 105-A/pKO403) was cultured in MRS liquid medium containing Sp at 30 °C for 24 h. Propagated cells were transferred into MRS liquid medium without Sp, and then incubated at 42 and 30 °C. Samples were taken every 3 h and diluted with MRS liquid medium without Sp. These samples were spread on MRS plates without Sp (for total cell yield) or with Sp (for the selection of cells harboring the plasmid). After incubation at 30 °C, colonies were counted and the ratio of Sp^r cells maintaining plasmid to Sp^s cells was calculated.

Construction of integration plasmids for SCO homologous recombination The integration plasmids were constructed as follows. PCR primers were designed according to the upstream flanking sequence of the putative *pyrE* gene (BL0788) in *B. longum* NCC2705. To obtain different lengths of homologous sequence fragments between 250 and 3,000 bp, PCR was performed with primers (Table 2) using KOD-Plus DNA polymerase. The PCR conditions were as follows: 94 °C for 2 min; and 30 cycles of 94 °C for 15 s, 60 °C for 30 s, and 68 °C for 1 min/kb. All PCR products were analyzed by agarose gel electrophoresis. Each PCR product was cloned into *BamH*I- and *Not*I-digested pKO403 using an In-Fusion Dry-Down PCR cloning kit (Clontech, Mountain View, CA, USA). The integration plasmids constructed were named pKO403-SCO 0.25, pKO403-SCO 0.5, pKO403-SCO 1.0, pKO403-SCO 1.5, pKO403-SCO 2.0, and pKO403-SCO 3.0 and were used in further analyses.

Measurement of SCO homologous recombination frequency Integration plasmids were introduced into *B. longum* 105-A by electroporation. Transformants were cultured on MRS plates containing Sp at 30 °C for 72 h. A single colony of each transformant harboring one of the above plasmids was inoculated in MRS liquid medium containing Sp. After cultivation at 30 °C, propagated cells were serially diluted, spread on MRS plates containing Sp, and incubated at 30 and 42 °C. The number of colonies on each plate was counted. The frequency of SCO homologous recombination was estimated as the ratio of the number of Sp^r cells that grew at 42 °C to the number of Sp^r cells that grew at 30 °C (total cells). These experiments were performed three times for each integration plasmid.

Frequency of gene knockout with one- or two-step DCO events A plasmid for evaluating the frequency of gene knockout was constructed. DNA fragments carrying 1.0 kb upstream and downstream flanking sequences of the putative *pyrE* gene in *B. longum* 105-A were PCR amplified and cloned into pKO403. This plasmid, pKO403-Δ*pyrE*, was introduced into *B. longum* 105-A. Transformants were cultivated on MRS plates containing Sp at 30 °C for 72 h. The resulting colonies were inoculated into MRS liquid medium containing Sp and incubated at 30 °C for 24 h. Propagated cells from MRS medium were spread on MRS plates containing 5-fluoroorotic acid (5-FOA; 500 μg/mL) and uracil (200 μg/mL) for the selection of mutants in which DCO homologous recombination had occurred. Cells from MRS medium containing Sp were

spread on MRS plates containing Sp and cultivated at 42 °C for the selection of mutants in which SCO homologous recombination had occurred. The number of colonies on each plate was counted. The total number of cells in each propagated colony was also counted by cultivation on MRS plates lacking Sp, 5-FOA, and uracil.

Construction of the gene knockout plasmid To examine the effectiveness of pKO403, the gene BL0033 was knocked out by DCO homologous recombination. pKO403 was modified to change the selection marker from the Sp^r gene to the Cm-resistant (Cm^r) gene. The Cm^r gene was PCR amplified from pBAD28 (Guzman et al. 1995) using the Cm^r gene Fw and Rv primers (Table 2). The PCR product was cloned into *Sac*I digested pKO403 using the In-Fusion Dry-Down PCR cloning kit. *E. coli* FusionBlue were transformed with the In-Fusion reaction mixture. The plasmid was isolated and named pKO403Cm.

To construct the plasmid for BL0033 gene knockout, the 1.0 kb upstream and downstream flanking sequences of the putative BL0033 gene were PCR amplified from the chromosome of *B. longum* NCC2705. These DNA fragments and the Sp^r gene (1.2 kb) were connected by overlap PCR (Horton et al. 1989; Suzuki and Yasui 2011). Connected fragments (3.2 kb) were subcloned into pBluescript to generate pBlue-ΔBL0033. This plasmid was digested with *BamH*I and *Not*I. The digested fragment (3.2 kb) was ligated with *BamH*I- and *Not*I-digested pKO403Cm. After transformation of *E. coli* TOP10, Sp^r and Cm^r transformants were obtained. The plasmid extracted from these cells was designated pKO403Cm-ΔBL0033 (Fig. 6, scheme).

Gene knockout using the Ts plasmid pKO403Cm-ΔBL0033 was introduced into *B. longum* NCC2705. Sp^r transformants were selected after incubation at 30 °C for 72 h. These were inoculated in MRS liquid medium containing Sp and incubated at 30 °C for 24 h. Propagated cells were spread on MRS plates containing Sp. These plates were incubated at 42 °C for 30 h and 30 °C for 48 h. The colonies that grew after incubation at 42 °C were duplicated on Sp- and Cm-containing MRS plates and incubated at 37 °C for 24 h. Sp^r and Cm-sensitive (Cm^s) colonies were selected as gene knockout candidates and analyzed by colony PCR.

Colony PCR Colonies were used as template DNA, and PCR was performed with KOD-Plus DNA polymerase. The primers used are shown in Table 2. The PCR conditions were as follows: 94 °C for 2 min; and 30 cycles of 94 °C for 15 s, 50 °C or 57 °C for 30 s, and 68 °C for 2.5 min. PCR products were analyzed by agarose gel electrophoresis.

Results

Construction and screening of the Ts plasmid

To generate Ts derivative of plasmid pKKT427, a random mutagenesis technique was needed. The *Bifidobacterium–E. coli* shuttle vector pKKT427 (Yasui et al. 2009a, b) is carrying replication unit of a cryptic plasmid (pTB6) of *B. longum* which includes the *repB* gene, encoding an initiation protein of replication, origin for *E. coli* of pUC19, and the Sp-resistant gene. We first performed chemical mutagenesis for whole plasmid using hydroxylamine, which is commonly used for random mutagenesis (Humphreys et al. 1976; Nakamura et al. 2006). However, we were unable to obtain Ts replication mutants even though some silent mutations and Ts Sp^r mutations were observed by sequencing analyses (data not shown). Consequently, we performed error-prone PCR which targeted bifidobacterial replication unit from pTB6 (Matsumura et al. 1997) on pKKT427 as described in the "Materials and methods" section.

B. longum 105-A was transformed with the mutant plasmid library. By screening at different temperatures on plates containing Sp, colonies that grew at 30 °C but not at 42 °C were obtained as candidate Ts mutants. We screened approximately 3,000 colonies and obtained only one candidate Ts mutant. The candidate plasmid was isolated and analyzed by agarose gel electrophoresis (data not shown). We confirmed that the candidate plasmid showed a Ts phenotype by reintroduction into *B. longum* 105-A (Fig. 1a). Cultivation at 42 °C completely inhibited the growth of the strain harboring the candidate Ts plasmid (*B. longum* 105-A/pKO403) in the presence of Sp (Fig. 1a, lower left). The control strain harboring pKKT427 (*B. longum* 105-A/pKKT427) grew normally at 42 °C (Fig. 1a, upper left). Both strains grew normally when cultivated at 30 °C. The candidate Ts plasmid, which was designated pKO403, was used for further study.

We examined the growth characteristics of the Ts mutant in MRS liquid medium by measuring the optical density. The Ts mutant (*B. longum* 105-A/pKO403) grew almost as well as the control strain (*B. longum* 105-A/pKKT427) at 30 and 37 °C (Fig. 1b). In contrast, only the control strain was able to grow at 42 °C. These data were consistent with results obtained on agar plates (Fig. 1). Thus, pKO403 has the potential to be used as a powerful genetic tool for gene knockout experiments because the conditional suppression by temperature was feasible.

Sequence analysis of pKO403

pKO403 was sequenced to elucidate the number of mutations and their positions in the replication unit of bifidobacteria, which includes the *repB* gene and *Ori* V, comprising a

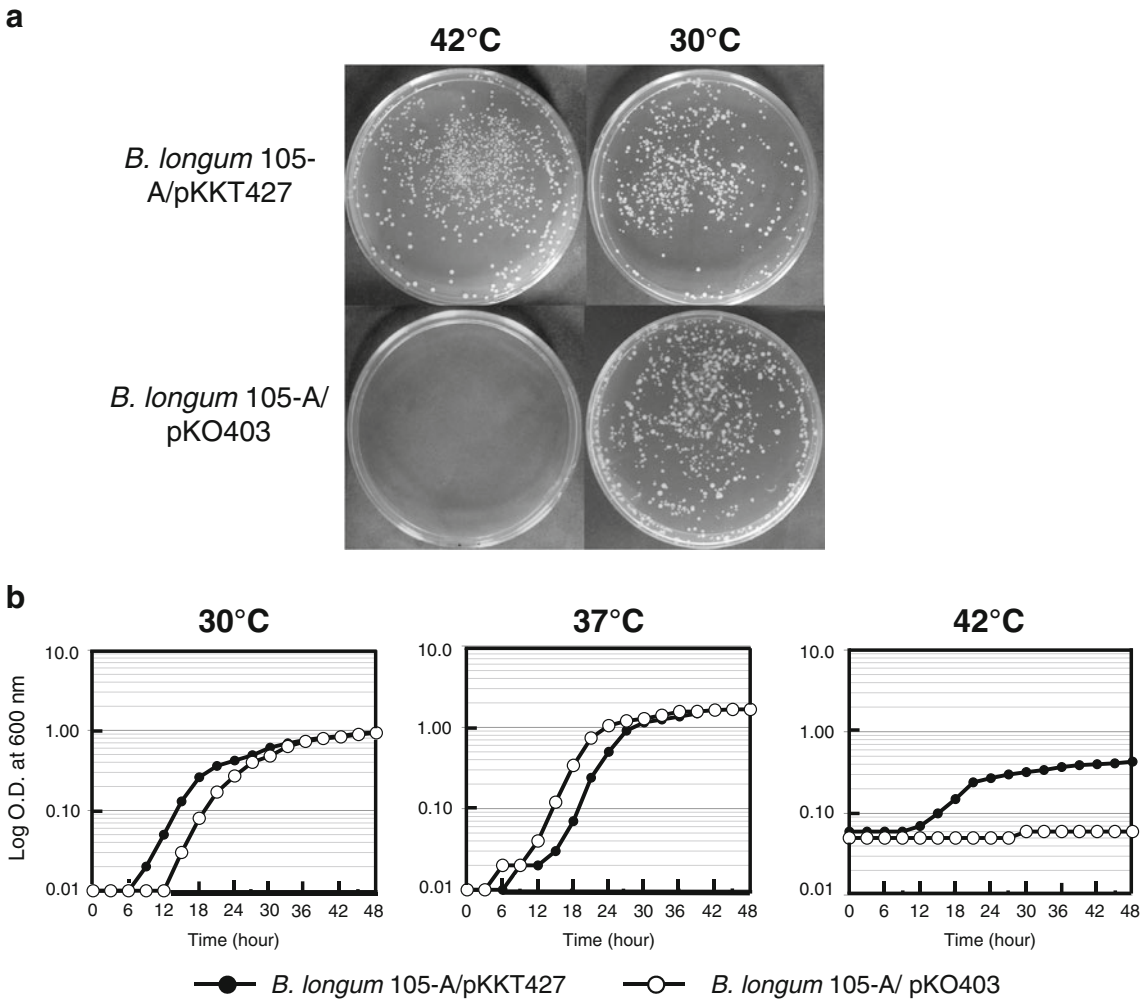

Fig. 1 Growth test of the Ts mutant. **a** Retransformation with pKO403. *B. longum* 105-A was transformed with pKO403. Transformants were cultured on MRS plates containing Sp at 42 and 30 °C. **b** Growth test in MRS liquid medium. The Ts mutant (*B. longum* 105-A/pKO403) was inoculated, and its growth at 30, 37, and 42 °C was measured. The generation times in the log phase at 30, 37, and 42 °C were 2.3, 3.0, and 5.0 h, respectively

single-strand origin (sso) and a double-strand origin (dso) as described (Matsumura et al. 1997). A total of six single-point mutations were found in this region (Fig. 2). Three mutations (G-883 to T, C-912 to G, and G-1214 to A) were located within the *repB* gene, which is required for plasmid replication. However, only the G-883 to T transition caused an amino acid substitution (Ser-86 to Ile) in the RepB protein. Other mutations (G-21 to T, A-1416 to T, and G-1423 to A) were observed in noncoding regions or were silent mutations. No mutations were observed outside of the target region for error-prone PCR. To investigate that the G-883 to T transition was responsible for the Ts replication of the plasmid, mutated plasmids each carrying a single-base mutation at G-21 to T, G-883 to T, C-912 to G, G-1214 to A, A-1416 to T, or G-1423 to A were constructed from wild-type pKKT427 and introduced into *B. longum* 105-A. The Ts phenotype of each transformant was evaluated by

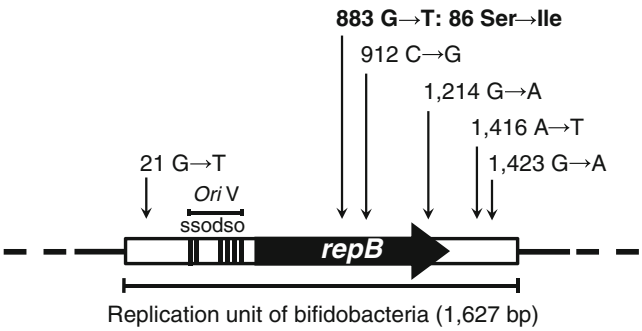

Fig. 2 Point mutations in pKO403. The entire sequence of pKO403 was analyzed. The replication unit of bifidobacteria is shown. *Arrows* indicate the positions of single-base mutations. The *repB* gene, which is required for plasmid replication initiation, is shown as a *solid arrow*. The positions of the single-strand origin (*sso*, *two stripes*) and the double-strand origin (*dso*, *four stripes*) are indicated by *black bars*

cultivation at 42 and 30 °C. Only the G-883 to T mutant (*B. longum* 105-A/pKKT427-mutant 883) presented a Ts phenotype at 42 °C (data not shown).

Evaluation of plasmid stability

Plasmid stability of pKO403 in *B. longum* 105-A was tested by measuring the plasmid loss rate as described in the "Materials and methods" section. As shown in Fig. 3, cultivation at 42 °C resulted in almost complete plasmid loss within 18 h. In contrast, the plasmid was stable in the host cells during cultivation at 30 °C. Thus, at 42 °C, plasmids were lost from host cells in the absence of Sp, and plasmid replication was inhibited at 42 °C.

Host range of the Ts plasmid

To examine whether pKO403 has a Ts phenotype in bifidobacterial strains other than *B. longum* 105-A, we tested the host range of pKO403 using *B. longum* NCC2705 and *B. adolescentis* ATCC15703. First, we confirmed that wild-type *B. longum* NCC2705 and *B. adolescentis* ATCC15703 grew normally at 42 °C. Transformation of *B. longum* NCC2705 with pKO403 was successful. However, no *B. adolescentis* ATCC15703 transformants were obtained. The transformation efficiency of this strain with pKKT427 is only 1–3×10 °CFU/μg DNA. However, the transformation efficiency can be elevated to 4×10^5 CFU/μg DNA using the PAM method (Yasui et al. 2009a, b). Transformation of *B. adolescentis* ATCC15703 with pKO403 using the PAM method produced Sp^r transformants at an efficiency of 6.7×10^3 CFU/μg DNA. Propagated cells of each transformant (*B. longum* NCC2705/pKO403 and *B. adolescentis* ATCC15703/pKO403) were spread on MRS plates containing Sp. Both transformants showed a Ts phenotype at 42 °C (data not shown).

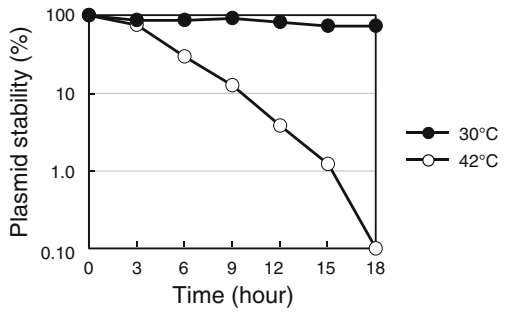

Fig. 3 Ts plasmid stability. *B. longum* 105-A/pKO403 cells were cultured at 42 and 30 °C without Sp. Samples were taken every 3 h and spread on MRS plates with or without Sp. Total cells and Sp^r cells were counted and the proportion of Sp^r cells was calculated. *Open circles* represent the ratio of Sp^r cells at 42 °C. *Closed circles* represent the ratio of Sp^r cells at 30 °C

SCO homologous recombination frequency

To begin to investigate the possibility of using the Ts plasmid as a genetic tool, its potential for integrating into the chromosome by single-crossover homologous recombination was assessed. We constructed pKO403-SCO integration plasmids (Fig. 4a and Table 1) carrying sequences homologous to the upstream flanking region of the putative *pyrE* gene in *B. longum* 105-A. Homologous sequences of various lengths were PCR amplified. Each integration plasmid was introduced into *B. longum* 105-A, and the frequency of SCO homologous recombination was evaluated (Fig. 4b). We found a proportional relationship between the log frequency of homologous recombination and the length of the homologous sequence within the range 250–2,000 bp. However, with longer homologous sequences, the frequency of SCO homologous recombination decreased slightly. Furthermore, the frequency using a 2,000-bp homologous sequence gave the highest rate at 4.6×10^{-3} ipc, and homologous recombination occurred efficiently using up to 1.0 kb of homologous sequence.

Frequency of gene knockout with one- or two-step DCO events

To obtain DCO knockout mutants, a DNA fragment carrying the Sp^r gene flanked by the genomic sequences upstream and downstream of the putative *pyrE* gene in *B. longum* 105-A was constructed by overlap PCR. This fragment was cloned into the *BamH*I and *Not*I sites of pKO403 to generate the plasmid pKO403-Δ*pyrE* (Fig. 5). The *pyrE* gene encodes orotate phosphoribosyl transferase, which is a member of the pyrimidine metabolic pathway. It is essential when cells are grown on minimal medium. However, it is toxic when cells are grown in the presence of 5-FOA (Hirashima et al. 2006). Thus, the *pyrE* gene is used as a bidirectional selection marker in some microorganisms, such as fission yeast. We have applied this system to bifidobacteria (Sakaguchi et al. 2012).

The two-step DCO procedure was performed as follows (Fig. 5a). At first step, SCO recombinants, which showed Sp^r, were selected by cultivation at 42 °C, and the frequency of SCO homologous recombination frequency was determined to be 1.3×10^{-3} ipc (Fig. 5a). Subsequently (second step), SCO mutants were cultivated in MRS liquid medium, and cells in which a secondary crossover homologous recombination event had occurred were selected as *pyrE* knockout mutants by cultivation on MRS plates containing 5-FOA and uracil. Some 5-FOA-resistant (5-FOA^r) colonies were obtained by this procedure, designated two-step DCO homologous recombination.

We also performed the simpler one-step DCO procedure (Fig. 5b). *B. longum*/pKO403-Δ*pyrE* 105-A was directly

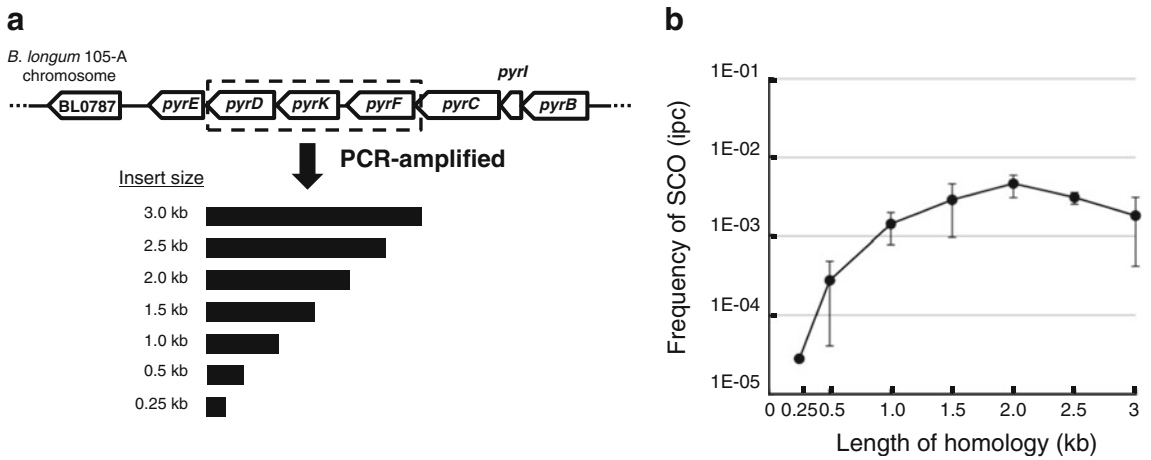

Fig. 4 The relationship between the length of homologous sequence and frequency of homologous recombination. **a** PCR amplification of homologous sequences. The upstream sequences of the *pyrE* gene were PCR amplified and cloned into pKO403. **b** Frequency of SCO homologous recombination

spread on MRS containing 5-FOA and uracil. The 5-FOA-resistant colonies obtained were tested for Sp resistance. DCO mutants would be expected to show 5-FOA resistance and Sp sensitivity in this procedure, which was designated one-step DCO homologous recombination.

Among 100 clones tested, all 5-FOA^r clones showed Sp sensitivity. Ten clones were analyzed by PCR and all showed the correct *pyrE* region knockout (data not shown). Thus, we considered that spontaneous mutations occurred in very fewer rate and were neglected in the following frequency determination.

Using approximately 1.0 kb length homologous region, the frequency achieved with the first homologous recombination was 1.3×10^{-3} ipc. Surprisingly, the frequency with one-step DCO homologous recombination was also almost at the same level, 1.4×10^{-3} ipc (Fig. 5).

Construction of BL0033 gene knockout mutants using the Ts plasmid

To demonstrate the usefulness of the Ts plasmid, we performed another gene knockout experiment using the Ts

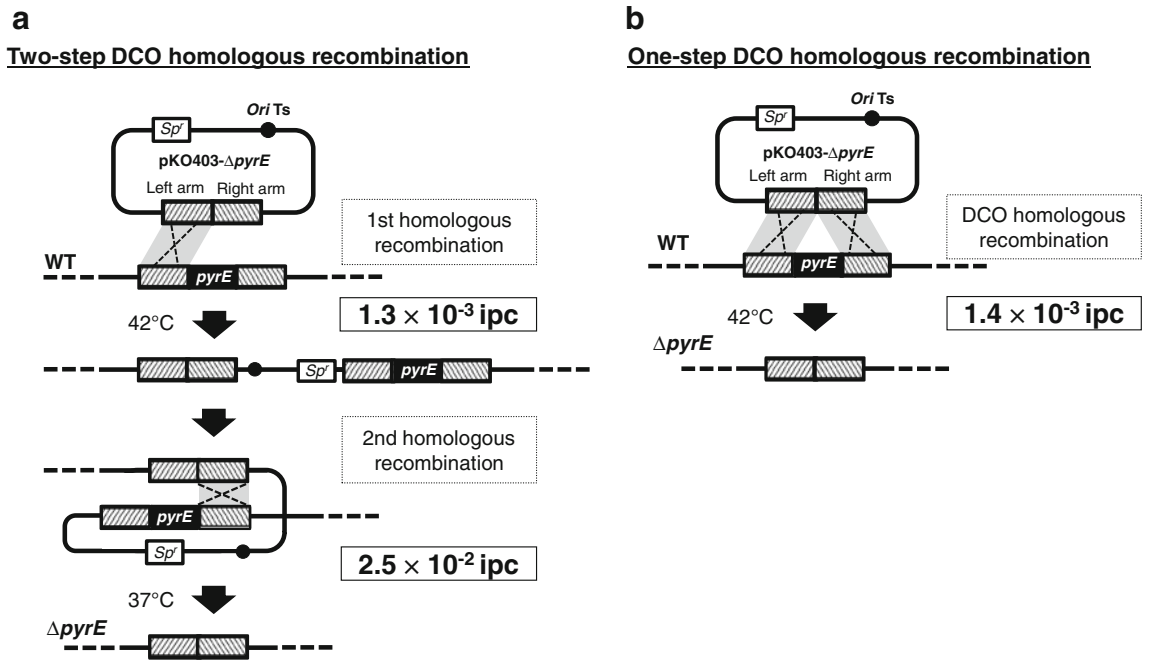

Fig. 5 Schematic presentation of gene knockout by SCO or DCO homologous recombination. Gene knockout mutants generated by two-step DCO (**a**) and one-step DCO (**b**) homologous recombination. In the two-step DCO, the first homologous recombination could also occur at the right arm (not indicated). WT and Δ*pyrE* indicate *B. longum* 105-A wild type and *B. longum* 105-A Δ*pyrE*, respectively

plasmid pKO403. We constructed a BL0033 gene knockout mutant in *B. longum* NCC2705. The BL0033 (NCBI Gene ID: 1021730) gene encodes a sugar ABC transporter solute-binding protein (Fukuda et al. 2011). The Sp resistance gene, sandwiched between 1.0 kb of the upstream and downstream regions of the BL0033 gene, was cloned into pKO403Cm to generate pKO403Cm-ΔBL0033 (Fig. 6).

pKO403Cm-ΔBL0033 was introduced into *B. longum* NCC2705, and Sp^r transformants were obtained. Cells propagated in MRS liquid medium were spread on MRS plates containing Sp. After incubation at 42 °C (non-permissive temperature) for 30 h, Sp^r colonies were obtained at a frequency of 1.1×10^{-3} ipc and were selected as candidates of SCO or DCO homologous recombination. Six hundred of these colonies were duplicated to Sp- and Cm-containing MRS plates. Colonies that showed an Sp^r and Cm^s phenotype were selected for analysis as gene knockout mutants having undergone one-step DCO homologous recombination (Fig. 6). These mutants were analyzed by colony PCR using primer set A, A-Fw, and A-Rv, and primer set B, B-Fw, and B-Rv (see Table 2). Gene knockout mutants would generate a DNA fragment of approximately 2 kb using both the A and B primer sets, but wild-type would not, because the A-Rv and B-Fw primers were located within the inserted Sp-resistant gene region (Fig. 6, scheme). The mutants amplified by only the A or only the B primer sets were putative SCO mutants. According to the PCR analysis, 11 of the 22 candidates with an Sp^r and Cm^s phenotype were DCO mutants (Fig. 6a, b). Six and five of the 22 candidates were putative SCO mutants and wild-type cells (no crossover), respectively. Thus, the frequency of DCO homologous recombination was approximately 1.8 % (11 DCO mutants among 600 tested clones) of Sp^r phenotype clones. We consider this frequency to be sufficient for practical purposes in the genetic manipulation of bifidobacteria.

Discussion

By the recent innovation in DNA sequencing technology, the whole genome nucleotide sequences have been revealed in many bacterial strains including bifidobacteria. Using informatic analyses, based on homology, the putative genes have been extracted and annotated using the accumulated knowledge, which had been experimentally studied mainly in some model organism such as *E. coli* and *Bacillus subtilis*. Very limited evidences are available in bifidobacteria. Almost half gene's functions are only predicted, and the others are still hypothetical. To accelerate functional analyses in bifidobacteria, the reliable reverse genetics tools,

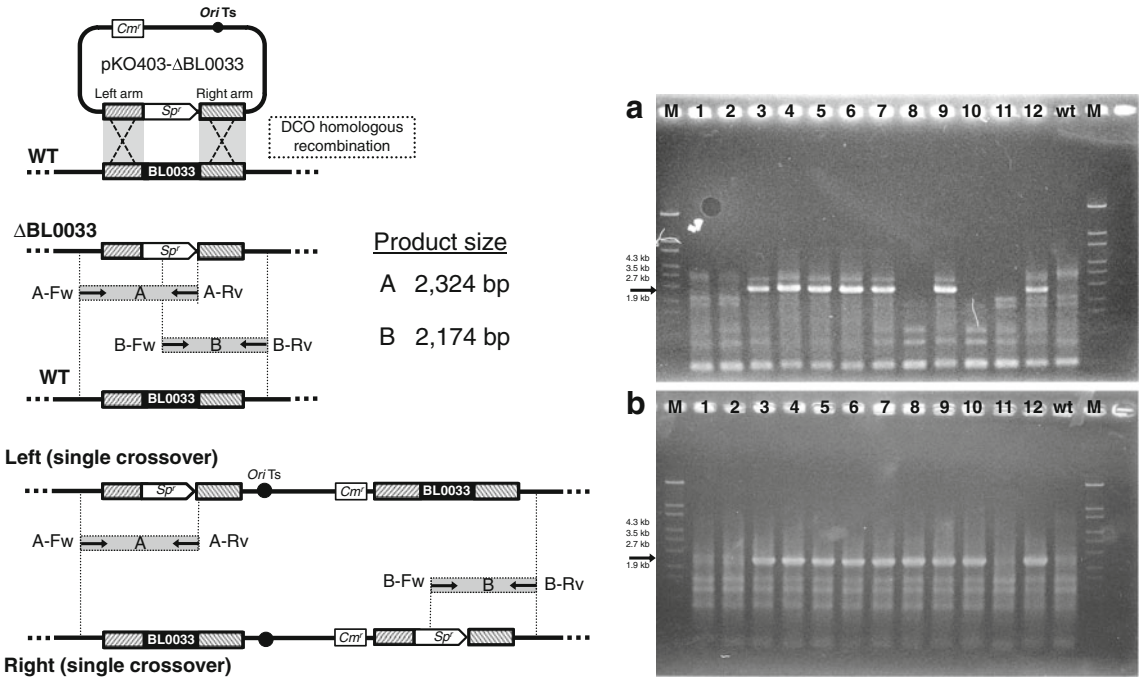

Fig. 6 Gene knockout of the BL0033 gene. Schematic diagram of SCO or DCO homologous recombination (*scheme panel*). SCO and DCO homologous recombination mutants were selected by cultivation at 42 °C. DCO mutants with an Sp^r and Cm^s phenotype were obtained by streaking on MRS plates. Knockout of the BL0033 gene was confirmed by colony PCR using primer sets A and B. *Arrows* indicate primers and their annealing positions. *Boxes bordered with a broken line* indicate the expected PCR products. Confirmation of BL0033 knockout (**a** and **b**). *Lane M* indicates the DNA marker, λ-*Eco*T14 I, with the following size order from *top to bottom*: 19, 7.8, 6.2, 4.3, 3.5, 2.7, 1.9, and 1.5 kb. *Lanes 1–12*, *B. longum* 105-A ΔBL0033 candidates. *Lane WT*, *B. longum* 105-A wild type. *Arrows* shows expected PCR products A and B

including gene knockout techniques, have been desired by the researchers.

If the homologous recombination frequency of SCO was 10^{-3} ipc, very higher transformation efficiency (>10^9 CFU/μg) was required to obtain gene knockout mutants because twice recombination ($10^{-3}\times10^{-3}$ ipc) are needed to generate around 10^2 mutant colonies in a practical experimental procedure. If such higher efficiency (>10^9 CFU/μg) was available, it should be possible to obtain DCO recombinants by using simple transformation without replicating plasmid such as in eukaryotes (Suzuki and Yasui 2011). In the case of bifidobacteria, and also most other bacteria, the transformation efficiencies are quite low, typically 10^0–10^3 CFU/μg DNA, because of the plasmid degradation by their restriction endo-nuclease system. We have been constructed through PAM method (Yasui et al. 2009a, b), which can dramatically improve the transformation efficiency; however, 10^4–10^5 efficiencies are maximal even if the method was employed.

The Ts replication plasmid has an obvious advantage to construct gene knockout mutant. If a Ts plasmid was available, it does not need such extremely higher transformation efficiency. It became possible to construct knockout mutants from single transformant. In *E. coli*, and *Lactcoccus*, etc., many reverse-genetic research have been undergone using the Ts plasmid. We have tried to get Ts plasmid to construct efficient gene knockout technique in bifidobacteria.

We obtained one Ts plasmid, pKO403, by using error-prone PCR. The sequence analysis of pKO403 revealed that six single-point mutations were found (Fig. 2). Among them, only one mutation (G-883 to T) caused amino acid substitution (Ser-86 to Ile). We hypothesized that this mutation was responsible for the Ts replication phenotype. It was confirmed by site-directed mutagenesis analyses.

We also analyzed the stability of pKO403. The population of Sp-resistant cells were decrease to 1/1,000 after 18 h cultivation at 42 °C. During the incubation, the cells should divide 10 times (=2^{10}, Fig. 3). This indicates the replication has been completely arrested by shifting temperature from 30 to 42 °C. This result indicating that this Ts plasmid can be applied for gene knockout experiments.

At host range of pKO403 section, three strains of bifidobacteria, *B. longum* 105-A, *B. longum* NCC2705, and *B. adolescentis* ATCC15703, harboring pKO403 were clearly exhibited Ts phenotype at 42 °C (data not shown). These data suggested that the Ts plasmid could be used as a tool for genetic engineering techniques in these species.

Then we performed gene integration in practically by SCO homologous recombination using this Ts plasmid. The results revealed a proportional relationship between the log frequency of homologous recombination and the length of the homologous sequence within the range 250–2,000 bp (Fig. 4). Similar logarithmic linear relationships were observed in *Lactococcus lactis* (Biswas et al. 1993). Around 10^{-3} ipc efficiency is needed to perform successful homologous recombination experiments (Suzuki and Yasui 2011). It was reported that 1.0 kb of homologous sequences were sufficient for SCO homologous recombination. Actually as demonstrated here, SCO recombinants were produced at the frequency of 1.4×10^{-3} ipc with 1.0 kb of homologous sequences (Fig. 4b). We propose that 1.0 kb length of homologous sequences are enough to accomplish gene knockout experiment efficiently.

To obtain DCO knockout mutants, methods for gene knockout experiments using Ts plasmids have usually been performed by two-step DCO homologous recombination (Fig. 5a). First, transformants harboring a Ts plasmid carrying homologous sequences for gene knockout are cultivated at non-permissive temperatures to obtain SCO mutants. Then, integrants are cultivated at the permissive temperature to stimulate secondary SCO homologous recombination resulting DCO mutant in the production of gene knockout mutants. This two-step DCO homologous recombination is commonly used in Gram-positive and Gram-negative bacteria (Hamilton et al. 1989; Biswas et al. 1993; Datsenko and Wanner 2000; Takamatsu et al. 2001; Yakhnin and Babitzke 2004; Fuchs et al. 2006; Nallapareddy et al. 2006; Sanchez et al. 2007; Zakataeva et al. 2010; Chen et al. 2011). In this method, it is necessary to perform SCO recombination twice. One-step DCO (Fig. 5b), in which the experimental procedure is greatly shortened, has also been attempted (Suzuki et al. 1987). We tried to evaluate the recombination efficiencies of these two methods. However, two-step DCO and one-step DCO homologous recombination occurred at almost the same frequency in our results, as described above. This finding suggests that the two recombination events were occurring concurrently or were closely related each other. Thus, we propose that the one-step DCO homologous recombination procedure is a more convenient method for obtaining a gene knockout mutant.

These results indicate that our Ts plasmid, pKO403, can be used as a versatile tool for gene knockout techniques in *B. longum*. Furthermore, gene knockout using a Ts plasmid also has potential in other bifidobacterial species. We hope that our method, described here, accelerates the functional analysis of bifidobacteria.

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