The *pyrE* Gene as a Bidirectional Selection Marker in *Bifidobacterium Longum* 105-A

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We constructed a deletion mutant of the *pyrE* gene in *Bifidobacterium longum* 105-A. A *pyrE* knockout cassette was cloned into pKKT427, a *Bifidobacterium-Escherichia coli* shuttle vector, and then introduced into *B. longum* 105-A by electroporation. The transformants were propagated and spread onto MRS plates containing 5-fluoroorotic acid (5-FOA) and uracil. 5-FOA-resistant mutants were obtained at a frequency of 4.7×10^{-5} integrations per cell. To perform *pyrE* gene complementation, the *pyrE* gene was amplified by PCR and used to construct a complementation plasmid (pKKT427-*pyrE*$^+$). *B. longum* 105-A Δ*pyrE* harboring this plasmid could not grow on MRS plates containing 5-FOA, uracil and spectinomycin. We also developed a chemically defined medium (bifidobacterial minimal medium; BMM) containing inorganic salts, glucose, vitamins, isoleucine and tyrosine for positive selection of *pyrE* transformants. *B. longum* 105-A Δ*pyrE* could not grow on BMM agar, but the same strain harboring pKKT427-*pyrE*$^+$ could. Thus, *pyrE* can be used as a counterselection marker in *B. longum* 105-A and potentially other *Bifidobacterium* species as well. We demonstrated the effectiveness of this system by constructing a knockout mutant of the *xynF* gene in *B. longum* 105-A by using the *pyrE* gene as a counterselection marker. This *pyrE*-based selection system will contribute to genetic studies of bifidobacteria.

Key words: pyrimidine metabolism, gene knockout, homologous recombination, 5-FOA, *B. longum* 105-A, bifidobacterial minimal medium (BMM), gene inactivation

INTRODUCTION

Bifidobacteria are high GC-content, Gram-positive anaerobes that belong to the Actinobacteria phylum [1]. They inhabit the intestinal tracts of many animals, including humans. The genome sequences of bifidobacteria, such as *B. longum* and *B. adolescentis*, are available [2, 3]. However, only a few genetic investigations have been reported for bifidobacteria, because sufficient gene manipulation techniques had not yet been developed for this genus. Recently, some useful gene manipulation techniques have been developed [4–7].

Generally, the genetic approaches for anaerobes are difficult and very limited, because of their sensitivity to O_2 and their low transformation efficiency. The primary reason for low transformation efficiency is attributed to restriction-modification systems, which defend the cells from genetic invasion. To improve transformation efficiency, we developed the plasmid artificial modification (PAM) method [4, 5]. Briefly, a shuttle vector was prepared from *E. coli,* in which the genes encoding modification enzymes are cloned and expressed. The plasmid became resistant to digestion by the restriction enzyme during the transformation.

We have also constructed temperature-sensitive plasmid (pKO403) that replicates at 37°C but not at 42°C [6]. A gene knockout experiment was successfully performed using pKO403 in *B. longum* 105-A.

In order to perform gene inactivation by homologous recombination and selection of transformants, antibiotic selection markers have been generally used in some microorganisms. Some antibiotic selection markers have been reported, including genes for spectinomycin (Sp) [8], tetracycline [9], erythromycin and chloramphenicol (Cm) resistance [10], for the selection of transformants in *Bifidobacterium*. However, only Sp (75 μg/ml) gave reproducible, low-background results in our observation. Due to the limited number of available selection markers, multiple gene knockout experiments are hard to perform. To overcome the limited availability of selection markers,

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we focused on a counterselection marker, which allowed us to conduct a sequential multigene knockout technique by marker recycling.

In some microorganisms, several counterselection markers are available [11]. Genes related to the pyrimidine metabolic pathway have been found in most microorganisms (Fig. 1). In the case of *Thermococcus kodakaraensis*, the *pyrE* and *pyrF* genes encode orotate phosphoribosyl transferase (OPRTase) and orotidine 5′-monophosphate decarboxylase (OMPase), respectively [12]. The *pyrE* or *pyrF* genes and their orthologs (*URA5* and *URA3,* respectively) are also widely used as counterselection markers for gene manipulation in *T. kodakaraensis*, *Rhodobacter capsulatus* and yeasts [12–17].

Orotic acid, an intermediate in pyrimidine metabolism, is converted to orotidine 5′-monophosphate (OMP) by OPRTase, which is encoded by *pyrE*. 5-Fluoroorotic acid (5-FOA), an analog of orotic acid, is metabolized by the same enzyme. OPRTase (PyrE) can convert 5-FOA into 5-fluoroorotidine monophosphate (5-FOMP) instead of OMP. Then, 5-FOMP is converted to 5-fluorouridine monophosphate (5-FUMP), instead of UMP, by OMPase (PyrF). Accumulation of 5-FUMP is toxic and leads to cell death. In contrast, *pyrE*$^{-}$ or *pyrF*$^{-}$ cells can grow in the presence of 5-FOA, since pyrimidine metabolism is blocked at the corresponding steps so that 5-FUMP cannot be synthesized and accumulated in the cells (Fig. 1).

In addition, *pyrE* or *pyrF* mutants show uracil auxotrophy in a chemically defined medium without pyrimidine compounds, such as uracil, thymine, cytosine, or their nucleotide derivatives. In these mutant strains, the *pyrE* or *pyrF* genes are useful as positive selection markers. Mutant strains harboring the plasmids carrying these marker genes are able to grow in the same medium.

In molecular genetic experiments, if the *pyrE* (or *pyrF*) mutant strains are available, these genes can be applied as positive selection markers by auxotrophy in Ura^{-} minimal medium and negative selection (= counterselection) markers in 5-FOA-supplemented medium. Thus, these types of markers are called bidirectional selection markers and can be applied in multigene knockout experiments. After constructing a knockout mutant using a *pyrE* (or *pyrF*) marker, the marker can be cured by addition of 5-FOA. The same marker can be reused in knocking out the next target gene. Such marker recycling is already widely used in some microorganisms for sequential gene knockout [15, 16].

In our recent study, the *pyrE* gene was knocked out using a temperature-sensitive plasmid for evaluating the frequency of gene knockout [6]. In this study, we aimed to generate *pyrE* knockout mutants and investigate their properties. Furthermore, we developed a new chemically defined medium (bifidobacterial minimal medium; BMM) for *pyrE*-based positive selection and also for investigation of metabolism of *Bifidobacterium*.

This selection system should be a powerful genetic tool in studies of *Bifidobacterium* requiring efficient gene knockout construction. In this paper, we demonstrated the utility of this system by constructing a knockout mutant of the *xynF* gene in *B. longum* 105-A by using the *pyrE* gene as a counterselection marker.

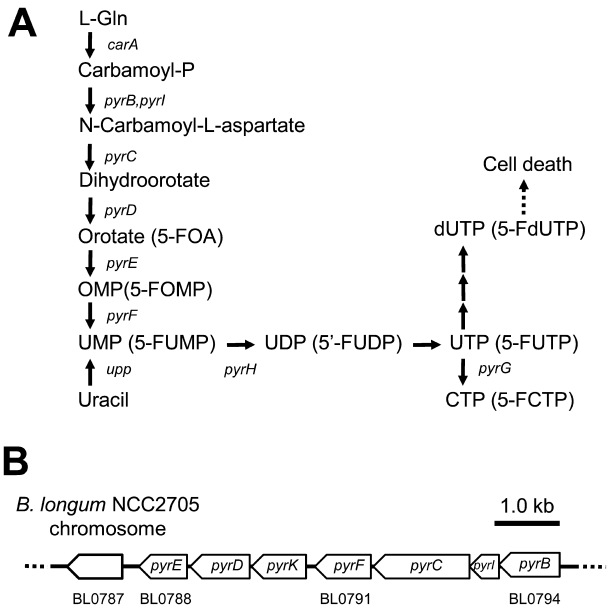

Fig. 1. Pyrimidine metabolism in *B. longum*.
(A) Orotic acid and 5-FOA are metabolized by the same enzymes. *pyrE* encodes orotate phosphoribosyl transferase (OPRTase, EC:2.4.2.10), and *pyrF* encodes orotidine 5′-monophosphate decarboxylase (OMPase, EC:4.1.1.23). 5-FOA, an analog of orotic acid, is sequentially converted to 5-fluoroorotidine monophosphate (5-FOMP) by *pyrE* and then 5-fluorouridine monophosphate (5-FUMP) by *pyrF*. 5-FUMP is converted to pyrimidine-containing nucleotides in further steps and is toxic to cells. The following abbreviations of genes were used: *carA*, carbamoyl phosphate synthase small subunit (EC:6.3.5.5); *pyrB*, aspartate carbamoyltransferase catalytic subunit (EC:2.1.3.2); *pyrC*, dihydroorotase (EC:3.5.2.3); *pyrD*, dihydroorotate dehydrogenase; *pyrG*, CTP synthetase; *pyrH*, uridylate kinase (EC:2.7.4.22); *pyrI*, aspartate carbamoyltransferase regulatory subunit (EC:2.1.3.2); *pyrK*, dihydroorotate dehydrogenase electron transfer subunit; *upp*, uracil phosphoribosyl transferase (EC:2.4.2.9). (B) Genomic organization of pyrimidine metabolic genes. *pyrB-F* and *pyrI* are clustered in the *B. longum* NCC2705 genome. *pyrG* (BL0874) and *pyrH* (BL1505) are located upstream and downstream of these genes, respectively.

Table 1. Bacterial strains and plasmids used

Strain or plasmid	Description[a]	Source
Bacterial strains		
Escherichia coli TOP10	F$^-$, *mcrA*(*mrr, hsdRMS-mcrBC*), φ80(*lacZ)*ΔM15 Δ*lacX*74, *recA*1, *araD139*, *(ara-leu)7697, galU, galK, rpsL(Str^r), endA1, nupG*	Invitrogen
Bifidobacterium longum 105-A	Wildtype	24
B. longum 105-A Δ*pyrE*	5-FOA^r Ura$^-$; Δ*pyrE*	This study
B. longum 105-A Δ*pyrE*/pKKT427-*pyrE*$^+$	5-FOA^s Ura$^+$; *pyrE*$^+$	This study
B. longum 105-A Δ*pyrE*/pKEC58-Δ*xynF*	5-FOA^s Ura$^+$ Sp^r Cm^r; Δ*pyrE*	This study
B. longum 105-A Δ*pyrE* Δ*xynF*	5-FOA^r Ura$^-$ Sp^r Cm^s; Δ*pyrE* Δ*xynF*	This study
Plasmids		
pKKT427	Sp^r; 3.9 kb shuttle vector between *Bifidobacterium* and *E. coli*	4, 5, 22
pKKT427-Δ*pyrE*	Sp^r; pKKT427 carrying the up- and downstream flanking regions of *pyrE*	This study
pKKT427-*pyrE*$^+$	Sp^r; pKKT427 carrying the putative *pyrE* gene, promoter, and terminator	This study
pBAD28	Cm^r; 5.8 kb plasmid for *E. coli*	23
pKEC58	Cm^r; same as pKKT427-*pyrE*$^+$ but Sp^r was replaced by the Cm^r	This study
pKEC58-Δ*xynF*	Sp^r and Cm^r; pKEC58 carrying the *xynF* deletion DNA fragments. Sp^r was sandwiched by up- and downstream regions of the *xynF* gene	This study

[a] 5-FOA^r and Ura$^-$, resistant to 5-fluoroorotic acid and uracil auxotrophy, respectively.

MATERIALS AND METHODS

Bacterial strains and media

The bacterial strains and plasmids used in this study are listed in Table 1. *B. longum* 105-A was used as the host strain. *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA, USA) was used as the cloning host for plasmid construction. *B. longum* was grown anaerobically in MRS medium (BD, Franklin Lakes, NJ, USA) at 37°C. *E. coli* was grown on LB medium (10 g tryptone, 5 g yeast extract and 5 g NaCl per l) at 37°C. For plate culture, 1.5% agar was added to the medium before autoclaving. Sp (75 μg/ml), Cm (30 μg/ml), 5-FOA (500 μg/ml) and uracil (200 μg/ml) were added as needed. A minimal medium was prepared based on the minimal medium for *Bacteroides* [18, 19]. Yeast extract (BD, Franklin Lakes, NJ, USA), vitamins and amino acids were added as required.

Molecular techniques

Genomic DNA of *B. longum* 105-A and its derivatives were extracted and purified as previously described [20]. Plasmid extractions from *E. coli* strains were performed using a QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany). DNA digestion with restriction enzymes was performed according to the manufacturer's protocol (Takara Bio, Japan). DNA sequencing of plasmid and genomic DNA was performed on an ABI 3100 DNA sequencer using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

PCR conditions

The PCR conditions and primer sequences used in this study for generating each plasmid or confirming the deletion of target genes are described in Table 2. KOD-plus DNA polymerase (Toyobo, Osaka, Japan) was used in all experiments as described by the manufacturer's protocol. The PCR products were analyzed by 1% (w/v) agarose gel electrophoresis. For cloning, the target fragments were extracted from the gel using a NucleoSpin kit (Nippon Genetics, Tokyo, Japan).

Plasmid construction

The pKKT427-Δ*pyrE* plasmid was constructed as follows. The PCR primers were designed according to the putative *pyrE* gene (BL0788) and the flanking genomic regions of *B. longum* NCC2705 (GenBank Accession no. AE014295). To obtain the *pyrE* deletion DNA fragments, upstream (1.0 kb) and downstream regions (1.0 kb) of the putative *pyrE* gene of *B. longum* 105-A was amplified by PCR as summarized in Table 2 (Exp. 1 and 2). The produced DNA fragments were connected by an overlap PCR (Table 2, Exp. 3) [21]. The connected PCR product was cloned into a *Bam*HI/*Not*I-digested pKKT427, a *Bifidobacterium-E. coli* shuttle vector [4, 5, 22], using an In-Fusion Dry-Down PCR Cloning Kit (Clontech, Mountain View, CA, USA).

The plasmid pKKT427-*pyrE*$^+$ was constructed as follows. The 835 bp DNA fragment, including the putative promoter (79 bp), open reading frame (ORF) (696 bp) and putative terminator (60 bp) of the *pyrE* gene in *B. longum* 105-A, was PCR amplified (Table 2, Exp.

Table 2. Summary of PCR experiments

Exp.	Aim		Primer set*	Thermal cycling program
1	Const. pKKT427-Δ*pyrE* amplify upstream 1 kb of *pyrE*	Up-*pyrE*-Fw: Up-*pyrE*-Rv:	GCCTGTCGACGGATCGACCATAATCGGCCCGGCCCAATGG GACAGACTTGGAGGTAAGGAACATCTCGTCTTCAATCTAAAAACCAAAAG	94°C 120 sec [94°C 15 sec–53°C 30 sec–68°C 60 sec] ×30 cycles
2	Const. pKKT427-Δ*pyrE* amplify downstream 1 kb of *pyrE*	Down-*pyrE*-Fw: Down-*pyrE*-Rv:	CTTTTGGTTTTTAGATTGAAGACGAGATGTTCCTTACCTCCAAGTCTGTC GCCCTGAGGGCGGCCCTCGGATGGGGGAAGTGACGATGAG	94°C 120 sec [94°C 15 sec–53°C 30 sec–68°C 60 sec] ×30 cycles
3	Const. pKKT427-Δ*pyrE* overlap PCR #1 & #2	Up-*pyrE*-Fw: Down-*pyrE*-Rv:	same as Exp. 1, Fw same as Exp. 2, Rv	94°C 120 sec [94°C 15 sec–53°C 30 sec–68°C 120 sec] ×30 cycles
4	Confirm Δ*pyrE* in *B. longum* genome	*pyrE* 1200-Fw: *pyrE* 1200-Rv:	AGCCGATCATTCTGTCCTGCGGACC AACAGCATGCCGATGATCGACGAGC	94°C 120 sec [94°C 15 sec–57°C 30 sec–68°C 180 sec] ×30 cycles
5	Const. pKKT427-*pyrE* amplify *pyrE*$^{+}$ gene	*pyrE* pt-Fw: *pyrE* pt-Rv:	TACTGAGCTCAAGCTTGGAACGTAATAAAAAAAGCGGGCT GCCTGCATGCAAGCTAACTCGACGACCTGTTGGAC	94°C 120 sec [94°C 15 sec–56°C 30 sec–68°C 60 sec] ×30 cycles
6	Const. pKEC58 amplify Cm^{r} of pBAD33	Cm^{r} Fw: Cm^{r} Rv:	AAAGTATATATGAGTGAACAACACTCAACCCTATCTCGG AAGCTTGAGCTCAGTTGATGTCCGGCGGTGCTTTTGCCGT	94°C 120 sec [94°C 15 sec–55°C 30 sec–68°C 120 sec] ×30 cycles
7	Const. pKEC58-Δ*xynF* amplify upstream of *xynF*	Up-*xynF*-Fw: Up-*xynF*-Rv:	GCCTGTCGACGGATCTTCCGGCATTCAGAACGCATATGAC ATTTAATGGGAATTCAAGTCTTTTAACCTTTCATGATAAG	94°C 120 sec [94°C 15 sec–55°C 30 sec–68°C 60 sec] ×30 cycles
8	Const. pKEC58-Δ*xynF* amplify downstream of *xynF*	Down-*xynF*-Fw: Down-*xynF*-Rv:	GAACGAAAATCGACCAATATCGCCGCAGCCTGTTGGTTGC GCCCTGAGGGCGGCCCCAAAAATCAATTTTCAGAATGCGC	94°C 120 sec [94°C 15 sec–55°C 30 sec–68°C 60 sec] ×30 cycles
9	Const. pKEC58-Δ*xynF* amplify Sp^{r} gene	Sp^{r}-Fw: Sp^{r}-Rv:	CATGAAAGGTTAAAAGACTTGAATTCCCATTAAATAATAAAACAA CAACAGGCTGCGGCGATATTGGTCGATTTTCGTTCGTGAATACAT	94°C 120 sec [94°C 15 sec–55°C 30 sec–68°C 120 sec] ×30 cycles
10	Const. pKEC58-Δ*xynF* overlap PCR of #7, 8, 9	Up-*xynF*-Fw: Down-*xynF*-Rv:	same as Exp. 7, Fw same as Exp. 8, Rv	94°C 120 sec [94°C 15 sec–55°C 30 sec–68°C 120 sec] ×30 cycles
11	Confirm Δ*xynF* in *B. longum* genome	*xynF* 1200-Fw: *xynF* 1200-Rv:	CAAACGGTGGCAAAGGAATTGATGA AAAGATAAACGACGCCCAAC	94°C 120 sec [98°C 10 sec–68°C 300 sec] ×25 cycles

* Overlapping sequences to vector ends in the In-fusion cloning and overlap PCR are single and double underlined, respectively.

5) and then cloned into *Hind*III-digested pKKT427 using the In-Fusion method as described above.

pKKT427-*pyrE*$^{+}$ was modified to change the selection marker from the Sp^{r} gene to the Cm-resistance (Cm^{r}) gene. The Cm^{r} gene was PCR amplified from pBAD28 [23] (Table 2, Exp. 6). The Cm^{r} gene was cloned into *Sca*I-digested pKKT427-*pyrE*$^{+}$ using the In-Fusion PCR method. The obtained plasmid was designated pKEC58.

The plasmid pKEC58-Δ*xynF* was constructed as follows. PCR primers were designed according to the putative *xynF* gene (BL1544) and the flanking genomic regions of *B. longum* NCC2705. To obtain the *xynF* deletion DNA fragments, upstream (1.0 kb) and downstream regions (1.0 kb) of the putative *xynF* gene in *B. longum* 105-A, were amplified by PCR (Table 2, Exp.7 and 8, respectively). The Sp^{r} gene was PCR amplified from pKKT427 (Table 2, Exp. 9). The three produced DNA fragments (upstream, Sp^{r} gene and downstream) were connected by an overlap PCR [21] using the primers Up-*xynF*-Fw and Down-*xynF*-Rv (Table 2, Exp. 10). Approximately 3.1 kb DNA fragments were cloned into *Bam*HI/*Not*I-digested pKEC58 using the In-Fusion method.

Gene knockout of pyrE

B. longum 105-A cells were transformed with pKKT427-Δ*pyrE* by electroporation [24]. After transformation, cells were spread and cultured on MRS plates containing Sp. Transformants were selected and cultured in MRS liquid medium containing Sp. Cells were serially diluted and spread on MRS plates containing 5-FOA and uracil. After incubation at 37°C for 2 days, 5-FOA^{r} colonies were selected and used for further analysis. Genomic DNA was extracted from 5-FOA^{r} colonies and wild-type strains. The deletion of *pyrE* was confirmed by PCR using the *pyrE* 1200-Fw and *pyrE* 1200-Rv primers (Table 2, Exp. 4) from the genomic DNA template.

Gene knockout of xynF

B. longum 105-A Δ*pyrE* was transformed with pKEC58-Δ*xynF* by electroporation. After transformation, cells were cultivated on MRS plates containing Sp at 37°C for 2 days. Sp^{r} transformants were inoculated in MRS liquid medium containing Sp. Propagated cells were diluted and spread on MRS plates containing 5-FOA, uracil and Sp. After incubation at 37°C for 2 days, cells resistant to 5-FOA and Sp were selected as the target of *xynF* knockout. Genomic DNA was extracted from candidates and Δ*pyrE* strains. The deletion of *xynF* was confirmed by PCR (Table 2, Exp. 11) from the genomic DNA template.

Acquisition frequencies of *ΔxynF* mutants were estimated at the same time. The ratios of the number of 5-FOA^{r} and Sp^{r} cells to the number of total cells were calculated.

Frequency evaluation of homologous recombination

The frequency of homologous recombination events was examined. Sp^{r} cells harboring pKKT427-Δ*pyrE* were prepared and spread on MRS plates containing 5-FOA and uracil for selection of *pyrE* knockout mutants generated by DCO homologous recombination and nonselective MRS plates for viable cell count. These plates were cultured at 37°C for 2 days, and then the number of colonies was counted. The frequency of DCO homologous recombination (integrations per cell, ipc) was calculated as the ratio of the number of 5-FOA^{r} cells to the number of total cells. The number of total cells was corrected with the plasmid loss rate, which was estimated as the ratio of the number of Sp^{r} cells to the number of total cells.

$$\text{Integrations per cell by DCO (ipc)} = \frac{\text{the number of 5-FOA}^{r}\text{ cells}}{\text{the number of totall cells} \times \text{plasmid loss rate}}$$

$$\text{Plasmid loss rate} = \frac{\text{the number of Sp}^{r}\text{ cells}}{\text{the number of total cells}}$$

To confirm the phenotypic observation, 5-FOA^{r} colonies (*B. longum* 105-A Δ*pyrE*1 and *B. longum* 105-A Δ*pyrE*2) and wild-type strains were streaked on MRS plates containing uracil or 5-FOA and BMM plates with or without uracil. These plates were incubated at 37°C for 2 days.

HPLC conditions

Reverse-phase column chromatography was performed using an ODS column (Inertsil ODS-3V, 5 µm, 4.6 mm i.d. × 250 mm, GL Sciences, Tokyo, Japan). The HPLC system used was an LC-10ATVP with an SPD-M10A Diode Array Detector (Shimadzu, Kyoto, Japan). The mobile phase was composed of methanol (solvent A) and 0.05% trifluoroacetic acid (solvent B) with the following gradient elution: 20% A and 80% B initially, changing to 80% A and 20% B in 30 min. The column flow rate was 1 ml/min at 25°C with detection at 230 and 256 nm.

Growth assay using a 96-well microplate

The collected fractions were freeze-dried, redissolved in methanol at 5 mg/ml, individually added to a 96-well plate and air-dried, and then minimal medium was added. *B. longum* 105-A was cultured in these media at 37°C for 24 hr. The OD_{490} of growth was measured using a plate reader (Immunomini NJ-2300, Cosmo Bio, Tokyo, Japan).

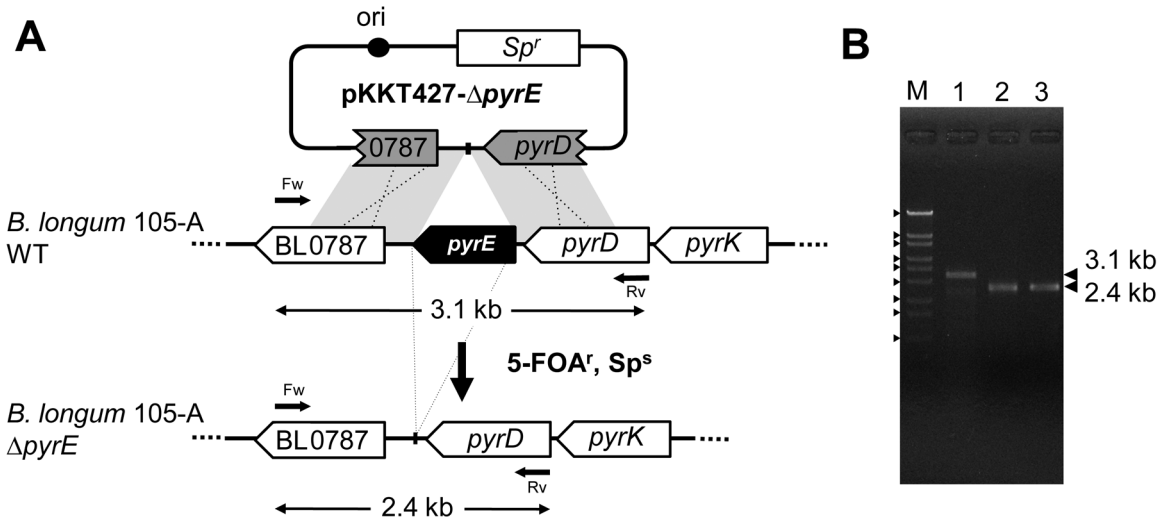

Fig. 2. Construction of *B. longum* 105-A *pyrE* knockout mutants.
(A) Schematic diagram of the construction of *pyrE* knockout mutants. The plasmid pKKT427-Δ*pyrE* was introduced into *B. longum* 105-A. Transformants were cultured on MRS plates containing 5-FOA and uracil at 37°C. The cells in which DCO homologous recombination had occurred between the plasmid and chromosome showed a 5-FOA^r and Sp^s phenotype. The arrows indicate primers, *pyrE* 1200-Fw, *pyrE* 1200-Rv and theoretical products of PCR confirmation for the *pyrE* deletion in Panel B. Ori indicates the origin of replication of the plasmid pTB6. (B) PCR analysis of *B. longum* 105-A wild-type and *B. longum* 105-A *pyrE* knockout mutants. The region flanking *pyrD*, BL0787, was amplified by PCR (Table 2, No.4). Lane M indicates the DNA marker, λ-*Eco*T14I, with the following size order from top to bottom: 19,329 bp, 7,743 bp, 6,223 bp, 4,254 bp, 3,472 bp, 2,690 bp, 1,882 bp, 1,489 bp and 925 bp. Lane 1, *B. longum* 105-A wild-type; lane 2, *B. longum* 105-A Δ*pyrE*1; and lane 3, *B. longum* 105-A Δ*pyrE*2.

^{1}H NMR analysis

The growth-promoting fraction was used for ^{1}H NMR. ^{1}H NMR spectra were recorded using an ECA500 (JEOL, Tokyo, Japan).

RESULTS AND DISCUSSION

Generation of a B. longum 105-A pyrE knockout mutant

In the *B. longum* NCC2705 genome, BL0788 and BL0791 CDS are annotated as *pyrE* and *pyrF*, respectively. To obtain a *pyrE* knockout mutant, we constructed a plasmid, pKKT427-Δ*pyrE* (Fig. 2-A). It was introduced into *B. longum* 105-A. For gene knockout experiments, the transformants were cultured in MRS liquid medium containing Sp, and propagated cells (100 µL culture ≈ 1×10^8 cells) were spread onto MRS plates containing 5-FOA and uracil. After incubation, 5-FOA^r colonies were obtained at about 200 colonies per plate. We selected two candidates (*B. longum* 105-A Δ*pyrE*1 and *B. longum* 105-A Δ*pyrE*2) for further analysis. Genomic DNA was extracted from these candidates to confirm the *pyrE* deletion by PCR (Fig. 2-B). The resulting PCR for both mutants gave fragments of 2.4 kb, whereas the DNA amplified from the wild-type *B. longum* 105-A resulted in a 3.1 kb fragment. The DNA sequences of the 2.4 kb fragments were analyzed to confirm correct deletion. A 696 bp region of the *pyrE* ORF was successfully knocked out in the candidates.

It was also possible that some spontaneous *pyrE* mutants (such as point mutations) were also selected during the 5-FOA selection. To estimate the frequency of spontaneous mutation, we tested 100 of the 5-FOA^r colonies by colony PCR. However, all colonies showed correct deletion. These results indicated that most 5-FOA^r colonies (>99%) were double crossover (DCO) recombination mutants. The calculated frequency of DCO homologous recombination was 4.7×10^{-5} ipc.

Development of chemically defined medium

We examined the growth characteristics of the Δ*pyrE* mutants (*B. longum* 105-A Δ*pyrE*1 and *B. longum* 105-A Δ*pyrE*2) on MRS plates with or without 5-FOA and uracil (Fig. 3-A). The resistance of Δ*pyrE* mutants to 5-FOA was clearly confirmed. However, uracil auxotrophy was not observed in MRS medium because MRS medium contains an adequate concentration of uracil or other pyrimidine compounds. Thus, we defined a new medium, BMM, based on a minimal medium for *Bacteroides* [18, 19]. Yeast extract, which is a component of the minimal medium, was analyzed to construct a chemically defined

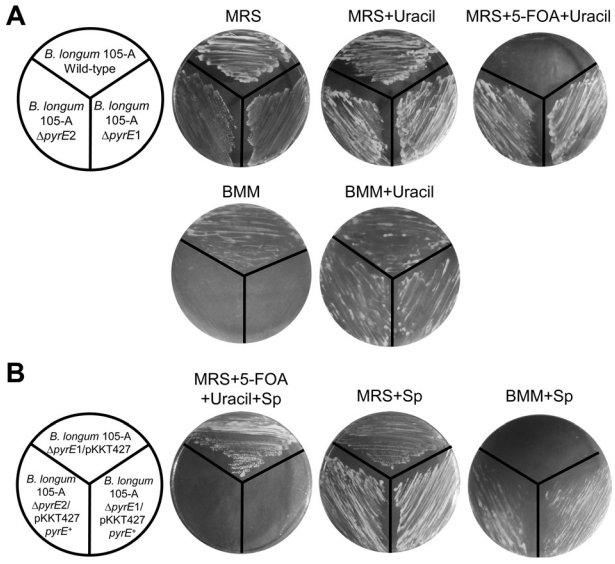

Fig. 3. Growth of transformants on the selection media.

(A) Growth of *pyrE* knockout mutants, *B. longum* 105-A Δ*pyrE*1 and *B. longum* 105-A Δ*pyrE*2, as compared with *B. longum* 105-A wild type. Each strain was spread on an MRS plate in the presence or absence of uracil (200 μg/ml) or with uracil and 5-FOA (500 μg/ml). (B) The *pyrE* deletion was complemented by pKKT427-*pyrE*$^{+}$. *B. longum* 105-A Δ*pyrE*1 and *B. longum* 105-A Δ*pyrE*2 were transformed with pKKT427-*pyrE*$^{+}$. Sp^{r} colonies were selected and streaked on an MRS plate containing Sp (75 μg/ml) or uracil and 5-FOA and on a BMM plate containing Sp. Sp is the selection marker for pKKT427-*pyrE*$^{+}$.

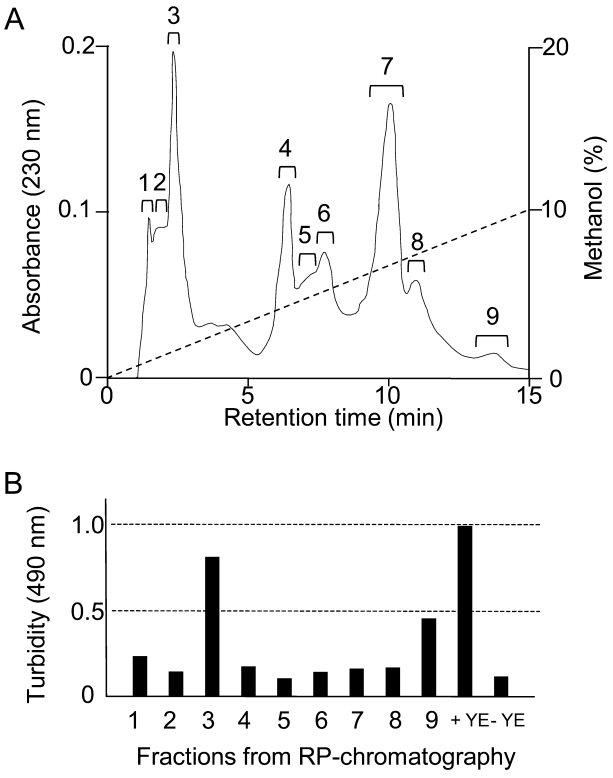

Fig. 4. Purification of growth-promoting compounds from yeast extract.

(A) Yeast extract powder was sequentially extracted with ethyl acetate, acetone and methanol. The methanol extract was separated by reverse-phase (RP) column chromatography using an ODS column as described in Materials and Methods. (B) Growth assays were performed in the basal medium supplemented with reverse-phase column chromatography fractions (panel A). The turbidity was measured using a 96-well plate reader at 490 nm. YE, yeast extract.

medium. Different volumes of yeast extract (5,000 mg, 500 mg and 50 mg/L) were added to the basal media. These media were used for cultivation of *B. longum* 105-A. As a result, normally growing colonies were detected in media containing more than 50 mg/L (data not shown).

To clarify additional factors to assist *B. longum* 105-A, the growth-promoting components were purified from yeast extract. Twenty-five grams of yeast extract powder was sequentially extracted with 500 mL each of ethyl acetate, acetone and methanol. The methanol fraction showed the strongest growth stimulation activity. Thus, we separated the fraction by reverse-phase column chromatography. As a result, nine assayed peaks were observed (Fig. 4-A). We examined the growth promoting activity for *B. longum* 105-A by adding each peak fraction to the basal media. The level of the third peak was the same as that for addition of yeast extract (Fig. 4-B). By the analysis using ^{1}H NMR, it was found that the fraction consisted of isoleucine and tyrosine. The growth of *B. longum* 105-A was confirmed by adding these compounds to the basal medium. Addition of isoleucine and tyrosine resulted in the cells showing successful growth on the agar plate. In contrast, the cells showed very weak growth using liquid medium. These data revealed that some unknown factor(s) was required for the growth of *B. longum* 105-A (Fig. 4 and Table 3). The unknown factor should be some compound present in trace amounts in agar and also pure agarose. We are investigating this as a possible new growth factor for bifidobacteria.

Uracil auxotrophy of the Δ*pyrE* mutants was examined on BMM plates with or without uracil. Consequently, these mutants were clearly confirmed to be uracil auxotrophic (Ura^{-}) mutants that could grow only in the presence of uracil (Fig. 3-A).

We also found that the acetone fraction of the yeast extract assisted the growth of *B. longum* NCC2705. For other strains, it would need to be optimized with supplemental amino acids or vitamins for each strain [25, 26].

Table 3. Composition of bifidobacterial minimal medium (BMM)

Components	Conc. (per liter)
Salts	
NaCl	9.0 g
CH_3COONa	5.0 g
KH_2PO_4	4.5 g
Na_2CO_3*	400 mg
$(NH_4)_2SO_4$	400 mg
$MgSO_4$	200 mg
$CaCl_2 \cdot 2H_2O$	250 mg
$MnCl_2 \cdot 4H_2O$	100 mg
Carbon source	
D-Glucose*	20 g
*Vitamins**	
L-ascorbate (Na)	340 mg
Inositol	1400 µg
4-aminobenzoic acd	800 µg
Nicotinic acid	600 µg
Biotin	300 µg
Choline	300 µg
Pantothenic acid	300 µg
Riboflavin	120 µg
Pyridoxine	40 µg
Folic acid	1.5 µg
Amino acids	
Cysteine·HCl*	500 mg
Isoleucine	64.5 mg
Tyrosine	87.0 mg

*Glucose, Na_2CO_3, vitamins and cysteine·HCl were sterilized with 0.33 µm filters (Sterivex, Millipore, Billerica, MA, USA) and added after the medium was autoclaved and cooled.

Complementation of pKKT427-pyrE$^+$

We performed complementation experiments using the *pyrE* gene as a selection marker. The knockout mutants (above) harboring pKKT427-*pyrE*$^+$ were streaked on MRS plates with or without 5-FOA, uracil and Sp. The uracil auxotrophy of *B. longum* 105-A Δ*pyrE* was successfully complemented by the plasmid pKKT427-*pyrE*$^+$. The Δ*pyrE* strains harboring pKKT427-*pyrE*$^+$ could not grow on MRS plates containing 5-FOA, uracil and Sp (Fig. 3-B, left), but the strain harboring pKKT427 grew normally. The complementation of the *pyrE* gene was observed on MRS plates. The *pyrE*$^+$ strains (*B. longum* 105-A Δ*pyrE*1 and Δ*pyrE*2 harboring pKKT427-*pyrE*$^+$) grew normally on BMM plates, but the *pyrE*$^-$ strain (*B. longum* 105-A Δ*pyrE1*/pKKT427) did not (Fig. 3-B, right). Thus, recombinant *pyrE* expressed a functional OPRTase, and BMM agar plates can be used for positive selection of *pyrE* transformants.

Gene knockout of the xynF gene

To evaluate the usefulness of a *pyrE*-based selection system, we demonstrated a gene knockout of *xynF*, which putatively encodes an extracellular exo-xylanase of *B. longum* 105-A, is conserved between *B. longum* strains and is annotated as BL1544 in *B. longum* NCC2705. We chose this as a target gene for the knockout study. β-1,4-Xylosidase is thought to be an essential enzyme in xylo-oligosaccharide metabolism, and *B. longum* consists of two possible β-1,4-xylosidase genes, *xynF* and BL0523 (classified in Family 31, possible α-glucosidase or β-xylosidase). The *xynF* gene encodes the ordinary β−xylosidase catalytic domain but also includes a putative signal peptide for secretion that is predicted to be an extracellular enzyme. The physiological roles of these genes are still unclear, and the knockout gene technique is essential to clarify such complex paralogue systems.

The gene knockout plasmid, pKEC58-Δ*xynF* (Fig. 5-A), was constructed and introduced into *B. longum* 105-A Δ*pyrE*. To obtain gene knockout mutants, the transformants were inoculated on MRS plates containing 5-FOA, uracil and Sp. Sp^r and 5-FOA^r colonies were obtained at a frequency of 8.5×10^{-4} ipc. To investigate the deletion of the *xynF* gene, 20 clones were analyzed by PCR. We confirmed the deletion of 5.3 kb of the *xynF* ORF by Sp^r gene integration (Fig. 5-B). These results indicate that gene knockout mutants might be easily constructed using this *pyrE*-based selection system. We also checked the deletion of the *xynF* gene by using sequence analysis. As a result, deletion of this gene was confirmed (data not shown).

The phenotypes of the Δ*xynF* mutants were examined by means of BMM plates containing 5-FOA, uracil, Sp and xylo-oligosaccharides. Contrary to our expectation, both strains of *B. longum* 105-A Δ*pyrE* and *B. longum* 105-A Δ*pyrE* Δ*xynF* grew in this medium (data not shown). This result suggests that other β-1,4-xylosidase isozymes shows enough activity to utilize xylo-oligosaccharides in *B. longum* 105-A. Here, we demonstrated a *xynF* knockout experiment to prove the usefulness of *pyrE* as a counterselection marker. Further studies, including BL0523 knockout, are required to understand the functions of these genes and also xylan and xylo-oligosaccharide metabolism.

We have tried to construct genetic tools for bifidobacteria, such as high efficiency transformation methods [4, 5], and to improve gene expression by tuning promoter and ribosomal binding sequences [27, 28]. In this study, we reported a new selection marker in *Bifidobacterium*. Now we can use two gene knockout methods, Ts-plasmid [6] and *pyrE* bidirectional selection marker.

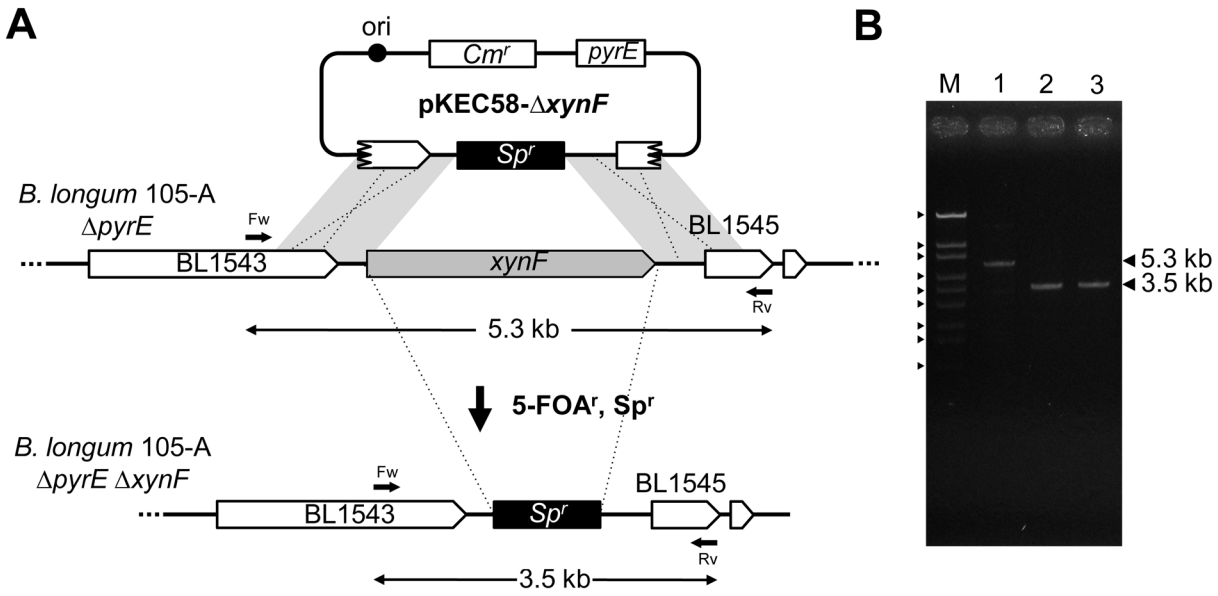

Fig. 5. Construction of *B. longum* 105-A *xynF* knockout mutants.
(A) Schematic diagram of the construction of *xynF* knockout mutants. The plasmid pKEC58-Δ*xynF* was introduced into *B. longum* 105-A Δ*pyrE*. Transformants were cultured on MRS plates containing 5-FOA, uracil and Sp at 37°C. The cells in which DCO homologous recombination had occurred between the plasmid and chromosome showed a 5-FOA^r and Sp^r phenotype. The arrows indicate primers, *xynF* 1200-Fw, *xynF* 1200-Rv and theoretical products of PCR. Ori indicates the origin of replication of the plasmid pTB6. (B) PCR analysis of *B. longum* 105-A Δ*pyrE* and *B. longum* 105-A *xynF* knockout mutants (Table 2, No. 10). Lane M: DNA size marker as described in Fig. 2. Lane 1, *B. longum* 105-A Δ*pyrE*; lane 2, *B. longum* 105-A Δ*xynF*1; and lane 3, *B. longum* 105-A Δ*xynF*2.

In the case of *xynF* gene knockout as described above, only 2 weeks are needed for construction, from primer design to PCR confirmation. In each experimental step, only 4–5 plates were needed, and around 100 colonies were obtained on the selection plate, in transformation and counterselection. Because of the high efficiency, around only 10 colonies were enough to screen for true knockout mutants by colony direct PCR confirmation.

In addition, a transformation efficiency of around 10^3 cfu/μg DNA is sufficient for the initial transformation experiment in this system because a few, such as 5–10, clones are enough to perform the subsequent procedure. We have achieved this efficiency with some strains, including *B. longum* and *B. adolescentis* strains, using the PAM method [4, 5]. We have already applied this method in some other strains and species of bifidobacteria, including *B. longum* and *B. breve*, and successfully obtained *ΔpyrE* mutants (data not shown). Using these quick and convenient tools, it becomes very easy to design reverse genetic experiments and perform strain engineering [29]. We hope these improvements accelerate genetic studies of bifidobacteria.

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