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 ∆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 ∆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 O₂ 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,
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-fluoroorotic acid, 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 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.

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, dihydroorotate (EC:3.5.2.3); pyrD, dihydroorotodehhydrigenase; pyrG, CTP synthetase; pyrH, uridylate kinase (EC:2.7.4.22); pyrI, aspartate carbamoyltransferase regulatory subunit (EC:2.1.3.2); pyrK, dihydroorotate dehydrigenase 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.
MATERIALS AND METHODS

Bacterial strains and media

The bacterial strains and plasmids used in this study are listed in Table 1. *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-*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 BamHI/NotI-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).

Table 1. Bacterial strains and plasmids used

| Strain or plasmid | Description* | Source |
|-------------------|--------------|--------|
| **Bacterial strains** |             |        |
| *Escherichia coli* TOP10 | F-, mcrA(mrr, hsdRMS-merC), ϕ80(lacZ)ΔM15 ΔlacX74, recA1, araD139, (ara-leu)7697, galU, galK, rpsL(Str'), endA1, uppG | Invitrogen |
| *Bifidobacterium longum* 105-A | Wildtype | 24 |
| *B. longum* 105-A-*pyrE* | 5-FOA Ura−; ΔpyrE | This study |
| *B. longum* 105-A-*pyrE*/pKKT427-*pyrE*+ | 5-FOA Ura−; ΔpyrE | This study |
| *B. longum* 105-A-*pyrE*/pKEC58-*ΔxynF* | 5-FOA Ura− Sp+ Cm−; ΔpyrE ΔxynF | This study |
| *B. longum* 105-A-*pyrE*-*ΔxynF* | 5-FOA Ura− Sp+ Cm−; ΔpyrE ΔxynF | This study |
| **Plasmids** |             |        |
| pKKT427 | Sp−; 3.9 kb shuttle vector between *Bifidobacterium* and *E. coli* | 4, 5, 22 |
| pKKT427-*pyrE* | Sp−; pKKT427 carrying the up- and downstream flanking regions of *pyrE* | This study |
| pKKT427-*pyrE*+ | Sp−; pKKT427 carrying the putative *pyrE* gene, promoter, and terminator | This study |
| pBAD28 | Cm−; 5.8 kb plasmid for *E. coli* | 23 |
| pKEC58 | Cm−; same as pKKT427-*pyrE*+ but Sp− was replaced by the Cm− | This study |
| pKEC58-*ΔxynF* | Sp− and Cm−; pKEC58 carrying the *xynF* deletion DNA fragments. Sp− was sandwiched by up- and downstream regions of the *xynF* gene | This study |

* 5-FOA and Ura−, resistant to 5-fluoroorotic acid and uracil auxotrophy, respectively.
| Exp. | Aim | Primer set* | Thermal cycling program |
|------|-----|-------------|------------------------|
| 1    | Const. pKKT427-ΔpyrE amplify upstream 1 kb of pyrE | Up-pyrE-Fw: GCCCTGTGACCCGATCGACGCAAATCTGGGCGCGCGCGAAATGG Up-pyrE-Rv: GACGAGATCGTGGAGTAAACAGATCAGCTGTCTTCAATCTAAAAACAAAG | 94°C 120 sec [94°C 15 sec–53°C 30 sec–68°C 60 sec] × 30 cycles |
| 2    | Const. pKKT427-ΔpyrE amplify downstream 1 kb of pyrE | Down-pyrE-Fw: CTTTGTGTTTATGATTGAAAGACGAGATGTTCTTACCTACATCGATGTC Down-pyrE-Rv: GCCCTGAGGCGGCGCGCTTCTGGAATGGGGAAGATCGACGAG | 94°C 120 sec [94°C 15 sec–53°C 30 sec–68°C 60 sec] × 30 cycles |
| 3    | Const. pKKT427-ΔpyrE overlap PCR #1 & #2 | Up-pyrE-Fw: same as Exp. 1, Fw Up-pyrE-Rv: 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 ΔpyrE in B. longum genome | pyrE 1200-Fw: AGCCGATCATCTCTGTCTGCGGACC pyrE 1200-Rv: AACAGCATCGCCGATCTCGACGAGC | 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: TACTGAAGCTCAAGCTTGGAAAGTATAAATAAAAAAGCGGCT pyrE pt-Rv: GCCTGACATGACGTAACTCGACGACCTGTGGAG | 94°C 120 sec [94°C 15 sec–56°C 30 sec–68°C 60 sec] × 30 cycles |
| 6    | Const. pKEC58 amplify Cm' of pBAD33 | Cm' Fw: AAAAATATATATATGAAACACCTAACACCTAATCGG Cm' Rv: AGGCTTGAGCTGACGTGATGTCGTCGGCCTGCTGCTGC | 94°C 120 sec [94°C 15 sec–55°C 30 sec–68°C 120 sec] × 30 cycles |
| 7    | Const. pKEC58-ΔxynF amplify upstream of xynF | Up-xynF-Fw: GCCCTGTGACCCGATCGACGCAAATCTGGGCGCGCGCGAAATGG Up-xynF-Rv: ATTTATATAATTGAAATCTGCAATTTTTAACCCTTCACTGATAAG | 94°C 120 sec [94°C 15 sec–55°C 30 sec–68°C 60 sec] × 30 cycles |
| 8    | Const. pKEC58-ΔxynF amplify downstream of xynF | Down-xynF-Fw: GAACGAGAATCCGCAATTCTTGCGGAGACCGCTGTTGTTGC Down-xynF-Rv: GCCCTGAGGCGGCGCGCCTGCTCAGAATTGACGCGC | 94°C 120 sec [94°C 15 sec–55°C 30 sec–68°C 60 sec] × 30 cycles |
| 9    | Const. pKEC58-ΔxynF amplify Sp' gene | Sp'-Fw: CATGAAAGGTTAAAAAGACGTAATCCATTAAATAAATAAACAAC Sp'-Rv: CAACAGGCTGCGCCGATATTGTTGCTGTATCTGATGATACAT | 94°C 120 sec [94°C 15 sec–55°C 30 sec–68°C 120 sec] × 30 cycles |
| 10   | Const. pKEC58-ΔxynF overlap PCR of #7, 8, 9 | Up-xynF-Fw: same as Exp. 7, Fw Up-xynF-Rv: 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 ΔxynF in B. longum genome | xynF' 1200-Fw: CAAAGCTTGGCAAGGAAATGATGA xynF' 1200-Rv: AAAGAATAAACGACGCGGCAAC | 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 HindIII-digested pKKT427 using the In-Fusion method as described above.

pKKT427-pyrE' was modified to change the selection marker from the Sp' 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 Scal-digested pKKT427-pyrE' using the In-Fusion PCR method. The obtained plasmid was designated pKEC58.

The plasmid pKEC58-ΔxynF 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' gene was PCR amplified from pKKT427 (Table 2, Exp. 9). The three produced DNA fragments (upstream, Sp' 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 BamHI/NotI-digested pKEC58 using the In-Fusion method.

Gene knockout of pyrE

B. longum 105-A cells were transformed with pKKT427-ΔpyrE 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' colonies were selected and used for further analysis. Genomic DNA was extracted from 5-FOA' 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 ΔpyrE was transformed with pKEC58-ΔxynF by electroporation. After transformation, cells were cultivated on MRS plates containing Sp at 37°C for 2 days. Sp' transformants were inoculated in MRS liquid medium containing Sp. Propagated cells were diluted and spread on MRS plates containing 5-FOA and uracil. After incubation at 37°C for 2 days, 5-FOA' colonies were selected and used as the target of xynF knockout. Genomic DNA was extracted from candidates and ΔpyrE 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' and Sp' cells to the number of total cells were calculated.

Frequency evaluation of homologous recombination

The frequency of homologous recombination events was examined. Sp' cells harboring pKKT427-ΔpyrE 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' 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' cells to the number of total cells.

Integrations per cell by DCO (ipc) = the number of 5-FOA' cells the number of total cells + plasmid loss rate

Plasmid loss rate = the number of Sp' cells the number of total cells

To confirm the phenotypic observation, 5-FOA' colonies (B. longum 105-A ΔpyrE1 and B. longum 105-A ΔpyrE2) 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 OD490 of growth was measured using a plate reader (Immunomini NJ-2300, Cosmo Bio, Tokyo, Japan).
**1H NMR analysis**

The growth-promoting fraction was used for 1H NMR. 1H 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-ΔpyrE (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 at 37°C. The cells in which DCO homologous recombination had occurred between the plasmid and chromosome showed a 5-FOA' and Sp' 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-EcoT14I, 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 ΔpyrE1; and lane 3, *B. longum* 105-A ΔpyrE2.

**Development of chemically defined medium**

We examined the growth characteristics of the ΔpyrE mutants (*B. longum* 105-A ΔpyrE1 and *B. longum* 105-A ΔpyrE2) on MRS plates with or without 5-FOA and uracil (Fig. 3-A). The resistance of ΔpyrE 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...
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 ∆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             |
| CH₃COONa         | 5.0 g             |
| KH₂PO₄           | 4.5 g             |
| Na₃CO₃*          | 400 mg            |
| (NH₄)₂SO₄        | 400 mg            |
| MgSO₄            | 200 mg            |
| CaCl₂·2H₂O       | 250 mg            |
| MnCl₂·4H₂O       | 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, Na₂CO₃, 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 ΔpyrE was successfully complemented by the plasmid pKKT427-pyrE⁺. The ΔpyrE 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 ΔpyrE1 and ΔpyrE2 harboring pKKT427-pyrE⁺) grew normally on BMM plates, but the pyrE⁻ strain (B. longum 105-A ΔpyrE1/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-AxynF (Fig. 5-A), was constructed and introduced into B. longum 105-A ΔpyrE. To obtain gene knockout mutants, the transformants were inoculated on MRS plates containing 5-FOA, uracil and Sp. Spr 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⁺ 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 sequence analysis. As a result, deletion of this gene was confirmed (data not shown).

The phenotypes of the ΔxynF 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 ΔpyrE and B. longum 105-A ΔpyrE ΔxynF 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.
In the case of \textit{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/\(\mu\)g 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 \textit{B. longum} and \textit{B. adolescentis} strains, using the PAM method [4, 5]. We have already applied this method in some other strains and species of bifidobacteria, including \textit{B. longum} and \textit{B. breve}, and successfully obtained \textit{ApyrE} 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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