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Chapter 5. Implementation of transposon mutagenesis in *Bifidobacterium*

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Abstract

Random transposon mutagenesis allows relatively rapid, genome-wide surveys to detect genes involved in functional traits, by performing screens of mutant libraries. This approach has been widely applied to identify genes responsible for activities of interest in multiple eukaryote and prokaryote organisms, although most studies on microorganisms have focused on pathogenic and clinically relevant bacteria. In this chapter we describe the implementation of an *in vitro* Tn5-based transposome strategy to generate a large collection of random mutants in the gut commensal *Bifidobacterium breve* UCC2003, and discuss considerations when applying this mutagenesis system to other *Bifidobacterium* species/strains of interest.

Keywords: Tn5, transposon, *Bifidobacterium*

1. Introduction

Bifidobacteria are Gram-positive commensal microorganisms whose presence in the human gastrointestinal tract has been associated with beneficial effects on host health (1). Consequently, significant scientific and commercial efforts have been made to discern the mechanisms responsible of their purported beneficial attributes and cross-talk with the human host and with other members of the intestinal microbiota (2). In this context, gene inactivation may be considered to represent the gold standard methodology to unequivocally prove the function of specific genes although most *Bifidobacterium* strains remain recalcitrant to genetic manipulation with only a few species and strains having been successfully mutated to date (3–6). An increasing number of genome-based studies has allowed insights into the reasons why genetic manipulation of this group of commensal microorganisms is so difficult, and has provided new opportunities to design tailor-made tools to achieve gene inactivation in various *Bifidobacterium* species/strains (4, 5, 7–10).

Transposon-based random mutagenesis approaches generally involve the *in vivo* delivery of a conditional vector carrying a transposon piece of DNA containing a selective marker (usually an antibiotic resistance cassette) and the transposase encoding gene. Tight control of transposase expression allows for the transposon to jump into (random positions in) the targeted genome, thereby inactivating any gene in which it had been inserted. Subsequent plasmid curing, which removes the transposase-encoding gene from the cells, fixes the transposon in the original insertion position of the host genome (11). This approach allows the generation of an unlimited

number of transposon insertion mutants from a single transformant carrying the delivery vector. However, substantial limitations exist to apply such an approach in bifidobacteria as conditional replicative vectors are currently not available for most species/strains. Besides, most heterologous expression systems in bifidobacteria have made use of strong constitutive promoters, which are not appropriate to control the expression of a transposase as high expression of a transposase in the presence of the corresponding transposon, may destabilize the transposon position and ultimately affect cell viability. An alternative to this *in vivo* transposon delivery approach was proposed early in 2002 when certain mobile genetic elements, i.e. Tn5 and Mu transposons, were shown to form functional transposon-transposase complexes by means of *in vitro* assembly. These complexes were reported to be stable enough to be directly electroporated into the targeted host, where they successfully transposed into the host DNA (12, 13). The practical implementation of this system to new strains requires achieving: (i) high transformation efficiencies in the selected host, and (ii) the use of an appropriate antibiotic selection marker within the transposon as it needs to be efficiently expressed to confer antibiotic resistance so as to allow selection for (single-copy) transposon insertion events. The development of these *in vitro* transposon delivery approaches represented a huge advancement as they offered new opportunities to tackle transposon-based random mutagenesis in microorganisms, such as bifidobacteria.

2. Materials

2.1. Materials and equipment for molecular biology techniques used for transposon construction, purification and assembly; and for identifying transposon insertion points in the mutants obtained

1. Standard molecular biology reagents (see **Note 1**): PCR master mix; appropriate restriction enzymes for transposon end pruning (PshAI or PvuII if using pMOD2-TetW), and, if relevant, for cloning a new antibiotic resistance cassette in between the Tn5 mosaic ends to create a new transposon (see **Note 2**); plasmid mini-preparation kits to conduct plasmid extraction from bacterial cultures ; PCR purification kits; agarose, 1X TAE buffer (40 mM Tris-HCl pH 7.6, 20 mM acetic acid, 1 mM EDTA), ethidium bromide.

2. Replicative vector containing an antibiotic resistant Tn5 transposon, e.g. pMOD2-TetW from Ruiz et al. (14) or, alternatively, another customized transposon (see **Note 2**). This vector will be used as a source of the transposon piece of DNA and can be stably maintained into appropriate host cells as it does not contain the transposase-encoding gene (and therefore will not destabilize the transposon piece of DNA). If using pMOD2-TetW, most *E. coli* cloning hosts are capable to support its replication, although *endA*$^-$ strains are recommended in order to achieve high plasmid yields (see **Note 3**).

3. Oligonucleotides to amplify the Tn5 transposon from the replicative vector into which it is being maintained (e.g. pMOD2-TetW). If using pMOD2-TetW, pMOD<MCS>Fw (5'-*ATTCAGGCTGCGCAACTGT-3'*) and pMOD<MCS>Rev (5'-

GTCAGTGAGCGAGGAAGCGGAAG-3') can be used as previously described (Ruiz et al., 2013).

3. Purified EZ-Tn5 transposase (commercially available by Lucigen Corporation, Middleton, WI, US - www.lucigen.com -), it is a mutated version of the original Tn5 transposase that displays a transposition frequency which is 1,000 fold higher than the one exhibited by the original Tn5 transposase.

4. Molecular grade glycerol.

2.2. Microbiology reagents for culturing bacteria

1. Luria Bertani (LB) culture media: 10 g L^{-1} tryptone (Merck, Darmstadt, Alemania), 5 g L^{-1} yeast extract (Merck), 10 g L^{-1} sodium chloride (Merck), sterilized by autoclaving. This medium will be used for routinely growth of *E. coli* cells or, supplemented with 10 µg ml^{-1} tetracycline in order to select for *E. coli* strains harbouring pMOD2-TetW.

2. de-Man-Rogosa-Sharpe (MRS) adjusted to pH 6.8: Proteose Peptone (10 g L^{-1}) (Difco, Franklin Lakes, NJ, US), beef extract (10 g L^{-1}) (Difco), yeast extract (5 g L^{-1}) (Difco), polysorbate (tween 80) (1 ml L^{-1}) (Sigma Aldrich; St Louis, MO, US), tri-ammonium citrate (2 g L^{-1}) (Sigma Aldrich), magnesium sulfate heptahydrated (0.525 g L^{-1}) (Merck), manganese sulfate tetrahydrated (0.12 g L^{-1}) (Merck), di-potassium phosphate (3 g L^{-1}) (Merck), potassium phosphate (3 g L^{-1}) (Merck), pyruvic acid (0.2 g L^{-1}) (Sigma Aldrich), cysteine-HCl (0.3 g L^{-1}) (Sigma Aldrich), ferric sulphate heptahydrate (0.034 g L^{-1}) (Sigma Aldrich), sterilized by autoclaving. This medium will be used to grow the

appropriate *Bifidobacterium* strain for competent cell preparation prior to electroporation.

3. 10 % D-Lactose solution prepared in distilled water and sterilized by filtration (0.2 µm diameter pore) (see **Note 4**).

4. 10 % L-cysteine-HCl solution prepared in distilled water and sterilized by filtration (0.2 µm diameter pore).

5. Washing buffer for preparation of electrocompetent cells of *Bifidobacterium:* 1 mM citrate, 0.5 M sucrose buffer, pH 5.8, sterilized by autoclaving.

6. Reinforced Clostridial Medium (RCM) (Oxoid) for routine growth of *Bifidobacterium* cells, resuscitation and recovery of electrotransformed cells.

7. Reinforced Clostridial Agar (RCA) (Oxoid) plates supplemented with appropriate antibiotic concentrations for transposon insertion selection. In the particular case of using the Tn5-TetW transposon described for *B. breve* UCC2003 or *B. breve* NCFB2258 strains (14), use a final concentration of 10 µg ml^{-1} of tetracycline (see **Note 5**).

8. 10 mg ml^{-1} tetracycline stock solution dissolved in 50 % ethanol and sterilized by filtration (0.2 µm diameter pore). The filtered sterilized stock solution can be stored at -20ºC (see **Note 6**).

9. Plasmid capable of replicating into the selected *Bifidobacterium* host, preferably harbouring the same antibiotic resistance cassette than the transposon, to be used as a positive control to test transformation efficiency of the prepared competent cells. If

using pMOD2-TetW, the *E.coli-Bifidobacterium* shuttle vector pAM5 (15) can be used as a control plasmid.

2.3. Other equipment required

1. Thermocycler.

2. Microcentrifuge.

3. Electroporator and electroporation cuvettes.

4. Device for spectrophotometric DNA quantification, e.g. Nanodrop or Qubit systems

5. Electrophoresis unit and transilluminator for DNA visualization.

6. Rotatory shaker incubator

7. Anaerobic work station (10 % H_2, 10 % CO_2, 80 % N_2).

8. Refrigerated centrifuge with rotor for bottles of at least 50 ml volume.

3. Methods

A schematic overview of the main steps which are detailed in the following sections is represented in Figure 1.

3.1. Transposon construction and preparation

1. A customized transposon can be constructed by cloning the desired antibiotic resistance cassette, or any alternative selective marker to identify transposon insertion events, in between the Tn5 mosaic ends recognized by the Tn5 transposase (Johnson

and Reznikoff, 1983) in an *E. coli* replicative vector (e.g. pMOD2 vector from Lucigen) (Figure 1). The *tetW* resistance cassette used in pMOD2-TetW was originally isolated from a *Bifidobacterium* species (17) and has been shown to be efficiently expressed to provide sufficient tetracycline resistance in order to achieve selection of clones harbouring a single chromosomal copy of the corresponding gene in multiple *Bifidobacterium* strains (4, 9). For the above reasons, this is a good marker to be included in a transposon to be used in tetracycline-sensitive *Bifidobacterium* strains (14).

2. Plasmid mini-preparations from the transposon containing construct (e.g. pMOD2-TetW or alternative customized constructs as indicated in Note 2) will be used to generate large quantities of a TetW-Tn5 transposon ready for assemblage with the purified EZ-Tn5 transposase. Plasmid miniprep extractions need to be performed on *E. coli* cells harbouring the transposon-containing plasmid (e.g. pMOD2-TetW). An appropriate volume of growing cells must be used according to guidelines of the plasmid mini-preparation kit provider. Extracted plasmid DNA must be verified by restriction profiling followed by agarose gel electrophoresis. For instance, to verify the pMOD2-TetW construct, digest 10 µl of a plasmid mini-preparation in a final reaction volume of 20 µl by using SphI and XbaI according to indications from the restriction enzymes provider. Following incubation for at least 1 hour at 37ºC, mix 10 µl of restricted plasmid DNA with 2 µl of commercial loading buffer dye and load on a 1 % agarose gel. Apply a voltage of 1.5 V/cm until the dye reaches about two thirds of the gel length; and stain the gel for 30 minutes with ethidium bromide (0.5 µg ml^{-1}). Visualize DNA bands in a UV transilluminator. SphI and XbaI digested pMOD2-TetW

should be visible as bright bands of 2.5 Kb (pMOD2 backbone) and 2.8 Kb (tetracycline resistance Tn5 transposon).

3. PCR-amplify the transposon piece of DNA using as a template the plasmid mini-preparation of the construct harbouring it, e.g. pMOD2-TetW, isolated from *E. coli* cells in the previous step. Oligonucleotides annealing immediately upstream of the inverted repeats flanking the Tn5 transposon in the plasmid harbouring the transposon must be used. If using a transposon constructed within a pMOD2 vector, as the one described above for bifidobacteria, oligonucleotides pMOD<MCS>Fw and pMOD<MCS>Rev (Lucigen) can be used. A suggested PCR cycling scheme is as follows: (i) denature the template at 94°C for 2 minutes; (ii) perform 30 cycles denaturing at 94°C for 30 seconds; annealing at 60°C for 45 seconds and extending at 72°C for 1 minute for every kb of expected product. Since a good amount of transposon DNA needs to be generated, it is recommended to perform at least 5-10 PCR reactions in a final volume of 50 µl each to guarantee a sufficiently large quantity of transposon DNA (as a general guideline, it would be recommended to generate at least 50-60 µg of PCR product).

3. To verify that PCR reactions have amplified a fragment of the desired size, load 5 µl of the PCR reactions in a 1 % agarose gel as previously described in step 2. The TetW-Tn5 transposon from Ruiz et al. (14) should produce a band of about 2.8 kb. Each PCR product must be purified on a silica column (see **Note 7**).

4. The efficiency of the transposition process is maximized in the presence of phosphorylated transposon ends. In order to get phosphorylated ends, the TetW-Tn5 transposon amplified from pMOD2-TetW as described in the previous step, can be

pruned by restriction with PshAI or PvuII, as these restriction sites are located immediately upstream of the transposon inverted repeats. The restriction reactions must be set up according to guidelines issued by the restriction enzyme provider (see **Note 8**). Following restriction, the transposon is cleaned up using a column PCR purification system and each DNA preparation is concentrated by eluting it in a small volume in the final step of the cleaning procedure (20 µl per purification column).

5. Pool all transposon preparations and measure DNA concentration for instance using spectrophotometric methods (e.g. nanodrop or Qubit based nucleic acid quantification). The concentration of transposon ends need to be adjusted to desired values taking into consideration the transposon length, by following instructions from the Tn5 transposase provider. The TetW-Tn5 transposon previously used for bifidobacteria was adjusted to a final concentration of 400 ng μl^{-1}.

6. The transposon-transposase complexes are assembled according to instructions from the transposase provider. As a standard guideline, mix 2 µl of transposon DNA preparation (dissolved in TE Buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA)); 4 µl of EZ-Tn5 transposase (1 U/µl) and 2 µl of 100 % glycerol, vortex and incubate at room temperature for 30 minutes. This preparation can be directly electroporated into freshly prepared *Bifidobacterium* competent cells, or stored at -20 ºC until use.

7. Assembled transposome complexes can then be electroporated into freshly prepared *Bifidobacterium* electrocompetent cells by following the instructions provided in the following section.

3.2. Preparation of electrocompetent cells of Bifidobacterium

In order to achieve maximum transformation efficiencies, electroporation needs to be conducted employing freshly prepared *Bifidobacterium* competent cells. Growth media, wash buffer and antibiotic containing plates are recommended to be prepared freshly the day before the experiment starts.

DAY 1

1. An isolated colony of the strain to be mutated is inoculated into 10 ml of RCM broth and grown overnight (~16 hours) at 37ºC in standing tubes into an anaerobic chamber (10 % CO_2, 10 % H_2, 80 % N_2 atmosphere).

2. 50 ml tubes containing de Man-Rogosa-Sharpe broth supplemented with a final concentration of 0.05 % L-cysteine (freshly added from a 10 % filter sterilized stock prepared in distilled water); and 1 % of an appropriate carbon source (for *B. breve* UCC2003 or *B. breve* NCFB2258, use D-lactose freshly added from a 10 % filter sterilized stock solution prepared in distilled water) can be pre-reduced and stored overnight in the same anaerobic chamber.

DAY 2

2. The pre-reduced and pre-warmed de Man-Rogosa-Sharpe broth supplemented with the carbon source and L-cysteine, is inoculated (2 % v/v) with the culture grown overnight. Incubate the standing tubes at 37ºC in the anaerobic chamber until the cultures reach an OD_{600nm} of about 0.5-0.7.

3. While the culture(s) for competent cell preparation are growing, the citrate-sucrose buffer must be placed in an ice box and an appropriate centrifuge, 1.5 ml eppendorf tubes, pipettes and electroporation cuvettes must be refrigerated by keeping them in an ice box.

4. Incubate cultures for competent cells preparation until they reach an optical density at 600 nm between 0.5-0.7 (5-7 hours). At this point, take the cultures out of the anaerobic incubator and spin down the cells at 4 000 g in a cold rotor (4ºC) for 10 minutes. From this point onwards the cells need to be kept ice-cold.

5. Wash the cells twice by decanting the supernatant and gently swirling the cell pellet into the same volume (50 ml) of ice-cold 1 mM citrate-0.5 M sucrose buffer.

6. Resuspend the washed cell pellet obtained from 50 ml of culture into 500 µl of ice-cold citrate-sucrose buffer.

7. In 1.5 ml eppendorf tubes dispense adequate amounts of Tn5 transposase-transposon complexes assembled as previously described in section 1. In parallel, in one of the tubes add 5 µl of a plasmid capable of replicating into the *Bifidobacterium* strain used, e.g. pAM5 (15), in order to be used as a positive control to verify the quality of the prepared competent cells and *Bifidobacterium* electrotransformation procedure.

8. Add to each tube containing the transposase-transposon complexes or positive control plasmid DNA, 45 µl of freshly prepared electrocompetent cells. Mix by gently

pipetting up and down and transfer the whole volume to an ice cold electroporation cuvette (see **Note 9**).

9. Apply a pulse of 2.0 KV, 200 ohms, 25 µF in an electroporator (e.g. Gene Pulser II Porator Electroporation System from Bio Rad). Immediately following application of the electric pulse, aseptically add 950 µl of RCM and gently suspend the cells by pipetting up and down a few times.

10. Incubate the electroporation cuvettes with the cell suspensions for 30 minutes at 37ºC in the anaerobic incubator (see **Note 10**).

11. Plate 100 µl aliquots of electroporated cells onto freshly prepared plates of RCA supplemented with 10 µg ml^{-1} of tetracycline added from a filter-sterilized 1000X stock solution as previously described (section 2.2).

12. Incubate the plates anaerobically at 37ºC for 2-3 days.

2.3. Recovery of tetracycline-resistant colonies and identification of transposon insertion sites

If no antibiotic resistant colonies are grown following electroporation of transposome complexes, verify the presence of antibiotic resistant colonies in the reaction performed with the positive control plasmid DNA. If transformation efficiency of this control plasmid is under 10^4 cfu μg^{-1}, repeat the experiment or consider optimizing the competent cells preparation or transformation procedure (see **Note 11**).

If (sufficient) antibiotic resistant colonies are grown following electroporation of transposome complexes:

1. Using a sterile pipette tip, inoculate them into RCM broth supplemented with tetracycline (10 µg ml^{-1}) and cultivate them overnight anaerobically at 37ºC.

2. The following day, add 30 % sterile glycerol and stock the clones at -80ºC for further analysis.

3. The transposon presence in obtained clones can be verified through a) Southern blot hybridization; b) PCR using oligonucleotides targeting the transposon mosaic ends or targeting internal sequence fragments within the transposon.

4. Identification of transposon insertion sites in specific clones can be performed through inverse-PCR coupled to Sanger sequencing as previously described (14) (see **Note 12**). As a general guideline, the inverse-PCR procedure to identify the genome positions where a transposon is inserted in a specific clone includes the following steps: 1) extract *Bifidobacterium* DNA (see **Note 13**); 2) digest 1 µg of DNA in a final volume of 50 µl of reaction, using a restriction enzyme which does not cut within the transposon; 3) purify digested DNA; a suggested protocol is as follows: add 50 µl of distilled water and 100 µl of phenol/chloroform pH 8.0, mix and spin down (≥ 12 000 g, 10 minutes), transfer upper phase to a fresh tube, add 1/10 volumes of sodium acetate 3 M pH 5.8 and 1 volume of cold ethanol; spin down 30 minutes, ≥ 12 000 g, 4ºC; remove supernatant; wash with 70 % ethanol; air dry and resuspend in 10 µl of distilled water; 4) quantify DNA spectrophotometrically; 5) set up self-ligation

reactions, using 0.2 or 0.4 µg of restricted DNA in a final volume of 50 µl; 6) incubate ligations overnight at room temperature; 7) purify ligated DNA using the same procedure described in step 3; 8) set up PCR reactions using each ligation reaction as template DNA and oligonucleotides annealing with the transposon ends, outwards facing (if using the TetW-Tn5 previously used for bifidobacteria, the oligonucleotides i-PCR-Fw 5'- GCATACCGTACTGATCTG-3' and i-PCR-Rev 5'-CAATCATACCGGCTTCC-3' can be used) (see **Note 14**); 9) verify the PCR amplification by loading 5 µl in a 1 % agarose gel as previously described (section 3.1. step 2); 10) sequence the PCR products using nested oligonucleotides, located within the transposon ends, upstream of the position where the inverse-PCR oligonucleotides annealed e.g. if using the TetW-Tn5 transposon previously described for bifidobacteria, sequencing can be performed using oligonucleotides pMOD-fw-seq *5'-GCCAACGACTACGCACTAGCC-3'* and pMOD-rev-seq *5'-GAGCCAATATGCGAGAACACC-3'* (14).

Notes

1. Molecular biology grade reagents need to be used for transposon construction and transposase-transposon assembly. Standard caution to prevent nuclease contamination of transposon DNA preparation needs to be taken (use molecular biology grade reagents, gloves and filter tips).

2. Virtually any piece of DNA can be included into a transposon, in between the mosaic ends recognized by the corresponding transposase. For further instructions and background information please check Lucigen guidelines.

3. If using pMOD2-TetW grow the *E. coli* host cells harbouring the vector in LB supplemented with 10 µg ml^{-1} of tetracycline. Grow *E. coli* cultures into sterile tubes, ensuring to leave a sufficient empty space in the tubes to allow for appropriate aeration during incubation. Grow the cultures by incubating them overnight at 37ºC in a rotary shaker (200 r.p.m.).

4. D-Lactose might need to be replaced by an alternative carbon source depending on the particular needs of the strain used (14). In order facilitate preparation of a 10 % stock solution of D-lactose, the suspension might need to be warmed up at 37ºC, e.g. incubate it in a water bath at 37ºC for 5 minutes and then vortex until the carbohydrate is fully dissolved in the suspension. Then filter sterilize (0.2 µm pore filters).

5. After autoclaving agar containing media, leave the bottles to cool down to approximately 50ºC before adding the antibiotic solution as it might be heat labile. Gently mix the medium bottles and pour the plates.

6. Discard the tetracycline solution stored at -20ºC if precipitation is observed.

7. Alternative methods to purify the PCR-amplified transposon so as to eliminate salts, and excess of nucleotides and primers, can be used (for example polyethylene glycol precipitation or standard ethanol precipitation).

8. When designing a new customized transposon construct, ensure the absence of PshAI or PvuII internal restriction sites in the constructed transposon. Alternatively, whenever these two sites have target sequences within the transposon sequence, it is

possible to PCR-amplify the transposon piece of DNA by employing a single phosphorylated oligonucleotide, annealing with the Tn5 mosaic end (*5'-CTGTCTCTTATACACATCT-3'*). If the transposon is amplified in this way from the pMOD-derived constructs, there is no need to trim the transposon ends with PshAI or PvuII restriction.

9. Avoid the presence of bubbles in the sample dispensed in the electroporation cuvette.

10. Time of incubation following electroporation might need to be adjusted depending on the strains used and transformation efficiencies. Longer times increase the number of antibiotic resistant colonies obtained, but this may be the result of growth rather than increased transposition efficiency.

11. When optimizing transposon mutagenesis strategies in new strains, it is critical to achieve high transformation efficiencies and to guarantee that the antibiotic selective marker included within the transposon is efficiently expressed in the selected host and allows accurate and reliable selection for transposition events. Type I and Type II endogenous restriction-modification systems encoded in the host genome have been demonstrated as key bottlenecks limiting transformation efficiencies in bifidobacteria (4, 9). Therefore, if the (recognition sites of the) restriction-modification systems of the host genome are known, it is advisable to avoid the presence of the corresponding restriction sites in the transposon sequence.

12. For alternative procedures to simultaneously identify transposon insertion points in large collections of transposon insertion mutants, see (18).

13. Multiple procedures have been available in order to extract DNA from *Bifidobacterium* cells. As a general guideline, cell pellets obtained from 6 ml of *Bifidobacterium* cells grown overnight need to be lysed enzymatically by suspending the cell pellets into TE buffer supplemented with lysozyme (30 mg ml^{-1}) and mutanolysin (5 U ml^{-1}) and incubated for at least 30 minutes at 37ºC. Then proceed with DNA purification procedures either using column-based kits or standard phenol/chloroform purification coupled to ethanol precipitation (19).

14. In the inverse-PCR programme, include a long elongation time in each cycle (at least 8 minutes).

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Figure Legends

Figure 1. Schematic overview of the main steps required to create transposon insertion mutants in bifidobacteria, by using EZ-tn5 transposome complexes and a customized tetracycline resistant Tn5- transposon encompassing a TetW casette.