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Chapter 2

Bifidobacterium transformation

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Abstract

The protocol presented in this chapter describes a generic method for electro-transformation of *Bifidobacterium* spp., outlining a technique that is ideal for conferring selective properties onto strains as well as allowing the user to introduce or knock out/in selected genes for phenotypic characterisation purposes. We have generalized on the plasmid chosen for transformation and antibiotic selection marker, but the protocol is versatile in this respect and we are able to achieve transformation efficiencies up to 10^7 transformants/µg of DNA.

Key Words Probiotic, bifidobacterial, genetic accessibility, electroporation

1. Introduction

Electroporation as a technique is based on the imposition of a strong electrical field to increase cell membrane permeability *(1)*, thereby allowing the introduction of chemicals or nucleic acids (such as single and double stranded, circular or linear DNA) *(2-4)*. Introduction of DNA into target cells facilitates their "transformation" into derivatives carrying or expressing a novel function, or mutants in which a target gene was removed or (re)introduced *(5, 6)*. For microbiology the development and implementation of electroporation as a method of DNA introduction for genetic transformation purposes has been fundamental in the application of selective markers and the characterisation of hypothetical genes *(5, 7)*.

Being Gram-positive, obligate anaerobes, particular members of the genus *Bifidobacterium* are purported to exert beneficial effects to their host and as a result a large body of research has been published by scientists who are working to better characterise these rather fastidious microbes, which are sometimes difficult to cultivate *(8, 9)*. Members of the *Bifidobacterium* genus are ideal targets for genetic manipulation via electro-transformation, however, bifidobacteria are notoriously recalcitrant to genetic manipulation due to their extensive and diverse restriction/modification (RM) systems, thick cell wall, and sensitivity to oxygen *(10-12)*. Only recently these hurdles have been investigated and overcome *(5, 13, 14)*.

Here we describe a routine transformation methodology via electroporation for members of the genus *Bifidobacterium*. However, it should be noted that currently available literature suggests that the procedure for transformation may not be uniformly applicable for all

Bifidobacterium species and strains *(13, 15)*. The protocol described below should therefore be used as an initial guide to achieve transformation, and modifications may thus have to be tested in order to suit each individual *Bifidobacterium* spp. For example, modifications can be made to the carbohydrate in the growth medium, plasmid to be transformed, amount of plasmid DNA used, electroporation parameters, and recovery medium.

2. Materials

2.1. Reagents:

1. Luria Bertani (LB) culture medium/agar: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride (and 16 g/L agar when required), autoclave to sterilize solution.
2. Modified de-Man-Rogosa-Sharpe (mMRS) Media: 10 g/L Tryptone (Peptone from Casein), 2.5 g/L yeast extract, 3 g/L tryptose, 3 g/L potassium phosphate dibasic (K_2HPO_4), 3 g/L potassium phosphate monobasic (KH_2PO_4), 2 g/L tri-ammonium citrate, 0.2 g/L pyruvic acid (sodium pyruvate), 0.575 g/L magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), 0.12 g/L manganese (II) sulfate tetrahydrate ($MnSO_4.4H_2O$), 0.034 g/L iron (II) sulfate heptahydrate ($FeSO_4.7H_2O$), dissolve all components in distilled water using a magnetic stirrer and then add 1 mL/L Tween80, autoclave to sterilize solution (See Note 1).
3. 38 g/L Reinforced Clostridium Medium (RCM; available as a premix from Oxoid), autoclave to sterilize solution.
4. 52.6 g/L Reinforced Clostridium Agar (RCA; available as a premix from Oxoid), autoclave to sterilize solution.
5. 10 % glucose solution: prepared in distilled water and 0.2 μm filter sterilized. Store at 4°C and remake fresh weekly or as required.

6. 6 % L-cysteine-HCl solution: prepared in distilled water and 0.2 µm filter sterilized. Store at 4°C and remake fresh weekly or as required.
7. Glycerol stock tubes: 200 µL 100 % glycerol aliquoted into 2 mL screw cap tubes and sterilized by autoclaving.
8. 80 % glycerol stocking solution for competent cells: prepared in distilled water, autoclave to sterilize solution.
9. Thermo Scientific GeneJET Plasmid Miniprep Kit (or equivalent).
10. Antibiotic for selection of specific plasmid, filter sterilized (e.g.: 5 µg/mL Chloramphenicol, final concentration)
11. Sucrose-citrate wash buffer: 0.21 g Citric Acid dissolved in 800 mL distilled water, adjust pH to 5.8 (using NaOH), make up to volume to 1 L with distilled water. Divide solution into five 200 mL bottles and add 0.5 M Sucrose, equivalent to 34.2 g per 200 mL bottle, autoclave to sterilize solution.
12. 1x TAE buffer: 4.844 g/L Tris base, 1.21 mL/L acetic acid, and 0.372 g/L EDTA.
13. 1 % agarose dissolved in TAE by microwaving.

2.2. Equipment:

1. Nanodrop1000/Qubit (DNA quantification equivalent)
2. 1.5 mL tubes
3. 50 mL falcon tubes
4. 25 mL serological pipettes
5. Microcentrifuge
6. Gel-electrophoresis system
7. Transilluminator for gel imaging
8. Anaerobic work station (10 % hydrogen, 10 % carbon dioxide and 80 % nitrogen)

9. Refrigerated centrifuge with rotor for 50 mL falcon tubes
10. Electroporator and electroporation cuvettes (2 mm)
11. Spectrophotometer measuring optical density at a wavelength of 600 nm

3. Methods

1. Recover desired plasmid from relevant bacterial host (e.g.: *Escherichia coli*, see Note 2) achieved with Plasmid Miniprep Kit following the manufacturer's instructions.
2. Plasmid recovery can be confirmed by standard agarose gel electrophoresis.
3. Quantify extracted plasmid DNA using spectrophotometric methods (e.g.: Nanodrop or Qubit).
4. To prepare bifidobacterial competent cells, overnight cultures are first prepared in 10 mL RCM supplemented with 0.05 % L-cysteine stock solution (with additional carbohydrate if required- strain specific). Incubate cultures at 37°C anaerobically overnight (~16 hours) without shaking.
5. The following day inoculate 5 mL of the overnight culture into 40 mL of mMRS, with 1 % vol/vol addition of filter sterilised stock sugar (e.g.: glucose) and 0.05 % L-cysteine stock solution.
6. Incubate anaerobically at 37°C until optical density (OD_{600nm}) reaches 0.6-0.9 (~3 hours), monitor OD_{600nm} with a spectrophotometer by aseptically removing 1 mL of growing culture approximately every 1-2 hours.
7. Once an OD_{600nm} of 0.6-0.9 is reached, place cultures on ice for 20 minutes, inverting every 5 minutes (see Note 3).
8. Harvest cells by centrifugation: 4°C, 4052 x *g* for 10 min.
9. Discard supernatant into waste.

10. Wash cells with ice cold 25 mL sucrose-citrate wash buffer. To resuspend the cell pellet use the serological pipette to knock the pellet off the side of the tube after adding the ice cold buffer and gently mix.
11. Repeat steps 8-10.
12. During centrifugation, ensure that recovery medium RCM is pre-warmed to 37°C.
13. Prepare 1.5 mL tubes with aliquoted plasmid DNA (concentration can vary, we recommend starting with 200 ng) and label electroporation cuvettes, these should be chilled on ice before use.
14. After final wash and centrifugation of bifidobacterial cells, discard supernatant into waste.
15. Gently resuspend cells in 200 µL sucrose-citrate wash buffer. If freezing competent cells, add 200 µl of 80 % glycerol (see Note 4), dispense into pre-chilled labelled 1.5 mL tubes and store at -80°C.
16. Mix 50 µL of competent cells and plasmid DNA (e.g.: 200 ng), transfer total volume to an electroporation cuvette.
17. Prepare a negative control for electroporation by only adding 50 µL of competent cells to a cuvette.
18. Carry out electro-transformation as quickly as possible, using the following settings:
    - 25 µF
    - 200 Ohms
    - 2000 V
19. Following electroporation, resuspend cells in cuvette to a final volume of 1 mL with prewarmed RCM and incubate at 37°C anaerobically for 1 hour.

20. Plate 100 µL of transformed cells onto RCA (plating dilutions of the cell preparation are also recommended, e.g.: 10^{-1}, 10^{-2} and 10^{-3}) with appropriate selective antibiotic (e.g.: final concentration 5 µg/mL chloramphenicol).
21. Incubate plates anaerobically at 37°C for 2-3 days.
22. Colony counts can then be performed to determine the transformation efficiency. Transformation efficiency = number of colonies counted on plate / (µg plasmid DNA transformed / total dilution of DNA before plating)

4. Notes

1. Improved transformation efficiency has been observed when mMRS is 0.2 µm filter sterilized in comparison to autoclave sterilization. Filter sterilized medium should be stored at 4°C and remade fresh weekly or aliquoted and frozen at -30°C.

2. Suggested plasmids for bifidobacterial transformation are listed below in Table 1 (NB. See also chapter 15 of this book for information on plasmids that replicate in bifidobacteria). It should be noted that one of the biggest hurdles for successful transformation is resident restriction-modification (RM) systems *(10, 16).* Selection of a plasmid with fewer RM motifs (strain-specific) can drastically improve the recovery of transformants. Alternatively, a plasmid can be methylated chemically (eg: NEB, GpC Methyltransferase (M.CviPI)) or by first transforming a given plasmid into a methylase-positive strain such as *E. coli* EC101 (DAM+; methylates GATC sites) otherwise an *E. coli* strain in which a bifidobacterial methylase gene is expressed *(16-19)*. Transformation of EC101 and other *E. coli* strains are performed using methods published by Dower, Miller *(20)*.

Table 1. Example plasmids for *Bifidobacterium* transformation

Plasmid	**Relevant characteristics**	**Citation**
pNZ8048	Cm^R, pSH71 replicon, inducible nisA promoter	*(21, 22)*
pNZ44	Cm^R, pNZ8048 containing constitutive P44 promoter from *L. lactis*	*(23)*
pPKCM	Cm^R, Set of *E. coli-Bifidobacterium* shuttle vectors based on pBlueCm	*(24)*
pSKEm	Em^R, *E. coli-Bifidobacterium* shuttle vector derived from pErythromycin	*(24)*
pAM5	Tet^R, pBC1-pUC19 [tet(W)]	*(25)*

3. When preparing competent cells ensure that they are always kept chilled (on ice) to ensure cells remain receptive to plasmid DNA. This includes making the wash buffer at least the day before, storing at 4ºC overnight and then keeping on ice during the cell wash steps.

4. Improved transformation efficiency is always observed when freshly made competent cells are used. Use of frozen competent cells is still possible for electroporation but expect a decrease in transformation efficiency.

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