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Improvement of electroporation-mediated transformation efficiency for a *Bifidobacterium* strain to a reproducibly high level

Min Ju Park[a], Myeong Soo Park[b,c,*], Geun Eog Ji[a,b,**]

[a] Department of Food and Nutrition, Research Institute of Human Ecology, Seoul National University, Seoul 151-742, Republic of Korea
[b] Research Center, BIFIDO Co., Ltd, Hongcheon 205-804, Republic of Korea
[c] Department of Hotel Culinary Arts, Yeonsung University, Anyang 430-749, Republic of Korea

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ABSTRACT

Bifidobacteria are representative probiotics which are defined as live microorganisms that confer a health benefit on the host. Because of their safety and healthfulness when applied to humans, bifidobacteria are suitable as genetically engineered bacteria for applications to benefit human physiology and pathology. However, molecular biological studies of bifidobacteria have been limited due to insufficient genetic tools including effective transformation methods. The aim of this study is to improve the electroporation-mediated transformation efficiency of bifidobacteria to a reproducibly high level. The crucial factors that determine electroporation efficiency are the restriction-modification system, together with the cell wall and cell membrane structure of the bacteria. We optimized the bifidobacterial electroporation conditions by focusing on these factors as well as the amount of plasmid DNA used, the electrical parameters and the bacterial growth phase. As a result, the electroporation efficiency of *B. bifidum* BGN4 drastically and consistently increased from 10^3 to 10^5 CFU / μg DNA. The most significant factor for increasing the electroporation efficiency was the cell wall weakening mediated by NaCl, which improved the electroporation frequency by 20 times. Because the optimized electrotransformation conditions reported here should be widely applicable to other *Bifidobacterium* species, these could promote the extensive genetic manipulation of the various *Bifidobacterium* species in future studies.

1. Introduction

The human intestine is densely populated by as many as 10^{13} to 10^{14} microorganisms (Suau et al., 1999). Most of these are obligate anaerobes including the clostridia, eubacteria, and bacteroides groups and the genus *Bifidobacterium*. *Bifidobacterium* is one of the dominant organisms in the gut microflora of healthy children and adults comprising > 1% of the total bacterial count (Blaut et al., 2002; Matsuki et al., 2004a; Matsuki et al., 2004b).

Bifidobacteria are Gram-positive, anaerobic, catalase-negative and fermentative bacteria which have beneficial effects on human health and diseases including physiological functions, nutrition, anti-cancer, immunological responses and resistance to infection (Aoki et al., 2016; Argnani et al., 1996; Jia et al., 2008; Kim and Ji, 2006; Ku et al., 2016; Lee et al., 2002; Mitsuoka, 1990; Sivan et al., 2015; Ventura et al., 2009; You et al., 2004). Because of these advantages, bifidobacteria are considered to be representative probiotics which are defined as live microorganisms that confer a health benefit on the host (Sanders, 2008). Therefore, bifidobacteria are suitable as a genetically engineered bacteria to augment human for use in human physiology and pathology. However, molecular biological studies on bifidobacteria have been limited by the insufficient genetic tools including effective transformation methods. Even though electroporation, a highly efficient transformation method, is used for bifidobacteria, the electroporation efficiency of bifidobacteria is usually < 10^4 CFU / μg DNA in previous studies (Argnani et al., 1996; Guglielmetti et al., 2007; Park et al., 1999a; Rossi et al., 1996; Sangrador-Vegas et al., 2007; Shkoporov et al., 2008).

In this study, we aimed to improve the electroporation-mediated transformation efficiency of bifidobacteria to a reproducibly high level. The crucial factors that determine electroporation efficiency are the restriction-modification system (R-M system), cell wall and cell

* Corresponding author at: Department of Food and Nutrition, College of Human Ecology, Seoul National Univ., Daehak-dong, Gwanak-gu, Seoul, Republic of Korea.
** Corresponding author at: School of Food Science, Yeonsung University, 37beongil 34, Yangwharo, Manan-gu, Anyang-si, Gyeonggi-do, 430-749, Republic of Korea.
E-mail addresses: mspark@yeonsung.ac.kr (M.S. Park), geji@snu.ac.kr (G.E. Ji).

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Table 1
Bacterial strains and plasmids used in this study.

Bacterial strain	Source or reference
Escherichia coli DH5α	Lab stock
Bifidobacterium bifidum BGN4	Isolated from breast-fed infant feces (Park et al., 1999)
Bifidobacterium bifidum KCTC 3440	Purchased from Korean Collection for Type Culture
Bifidobacterium bifidum KCTC 3418	Purchased from Korean Collection for Type Culture
Bifidobacterium breve KCTC 3419	Purchased from Korean Collection for Type Culture
Bifidobacterium pseudocatenulatum SJ32	Isolated from healthy human feces (Park, Ji, Ko, Jung, Ustunol and Pestka, 1999)
Bifidobacterium longum RD65	Isolated from healthy human feces
Bifidobacterium longum RD72	Isolated from healthy human feces
Bifidobacterium lactis RD68	Isolated from healthy human feces
Plasmid	Characteristics
pBES2	7.6 kbp, Ap^R, Cm^R

membrane structure of bacteria. We optimized the bifidobacterial electroporation conditions by focusing on these factors. In addition, we evaluated whether the optimized electroporation methods can be applied to other *Bifidobacterium* species. To the best of our knowledge, this is the first study to use cell wall-weakening agents and a cell membrane permeabilizing agent on bifidobacteria to enhance the electroporation efficiency.

2. Materials and methods

2.1.1. Bacterial strains and plasmid DNA

The various species of *Bifidobacterium* and plasmid used in this study are listed in Table 1. Bifidobacteria were grown at 37 °C in MRS medium (BD Difco™, Sparks, MD, USA) containing 0.05% L-cysteine·HCl.

The shuttle vector pBES2 was used in this study. pBES2 was constructed from a cryptic plasmid from *Bifidobacterium longum* MG1 ligated to pUC19 and possesses a chloramphenicol-resistance marker (Park et al., 2003).

2.1.2. DNA manipulation

Plasmid DNA was extracted from *Escherichia coli* DH5α transformants harboring pBES2 with a Plasmid Purification Mini Kit (Nucleogen, Gyeonggi-do, South Korea) and methylated *in vitro* by CpG (M.SssI) and GpC (M.CviPI) methyltransferases (NEB, Ipswich, MA, USA). The amount of DNA was measured by a Qubit 4 Fluorometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). To identify the plasmid from the transformants, plasmid DNA was extracted with a Plasmid Purification Mini Kit (Nucleogen) following an initial lysis step. Cells were resuspended in lysis buffer supplemented with lysozyme (20 mg / ml) and incubated at 37 °C for 1 h. The extracted DNA was digested with restriction enzyme *Xba*I (Fermentas, Waltham, MA, USA) and was identified by comparing the restriction patterns with the original *E. coli* derived plasmid DNA.

2.1.3. Preparation of electrocompetent cells

Overnight cultures in MRS broth containing 0.05% L-cysteine·HCl were diluted into 50 ml of fresh MRS broth supplemented with 0.05% L-cysteine·HCl, 0.2 M sucrose (final concentration) and increasing concentrations of NaCl (0, 0.05, 0.1 or 0.2 M, final concentration) and glycine (0, 0.75, 1, 1.25 or 1.5%) for some experiments. The inoculated 50 ml MRS broth with the additives were anaerobically incubated at 37 °C until the OD_{600} reached from 0.2 to 0.7. The bacteria were harvested by centrifugation and washed three times with 40 ml of electroporation buffer (0.5 M sucrose and 1 mM ammonium citrate, pH 6.0). The final cell pellet was resuspended in 0.5 ml of ice-cold electroporation buffer.

2.1.4. Electroporation

A 0.1-ml volume of cell suspension was mixed with 1, 50, 100, 500 or 1000 ng of plasmid DNA, respectively, and kept on ice for 30 min. and then transferred to a pre-cooled Gene Pulser cuvette (Bio-Rad, Hercules, CA, USA). If necessary, cell suspensions mixed with DNA were incubated in the presence of 0.5, 1, 1.5 or 2% ethanol (*v*/v) for 10 min shortly before the pulse delivery. The cuvette was pulsed at various field strengths and parallel resistances using the Gene Pulser Xcell Microbial Electroporation System (Bio-Rad). Following the electroporation, 0.9 ml of MRS broth supplemented with 0.05% L-cysteine·HCl and 0.2 M sucrose (final concentration) was added to the bacteria and incubated at 37 °C for 3 h under anaerobic condition. The bacteria were then plated onto MRS agar containing 3 μg / ml of chloramphenicol. The plates were incubated for 36 h under anaerobic condition.

3. Results

To enhance the bifidobacterial electrotransformation efficiency, we first chose *Bifidobacterium bifidum* BGN4 as the experimental strain. *B. bifidum* BGN4 is used as a probiotic strain in global food markets because of its reported benefits. BGN4 exhibits an outstanding colon cell adhesive ability among other bifidobacteria (Kim et al., 2003; Ku et al., 2009), significant immunoregulatory capacities (Hong et al., 2009; Kim et al., 2005; Kim et al., 2007; Lee et al., 2002; Lee et al., 2006) and anticancer effects (Ku et al., 2009; You et al., 2004). In addition to these features, BGN4 originates from human and is therefore suitable for use as genetically modified probiotics.

In this study, we proceeded with the experiments by applying the optimal conditions of the previous step to the next step. The following sequence of conditions were investigated: 1) *in vitro* methylation, 2) amount of plasmid DNA, 3) electrical parameters, 4) bacterial growth phase, 5) cell wall weakening agent, and 6) cell membrane permeabilizing agent. Each step was repeated three times independently. The transformation efficiency was expressed as the number of transformants per μg of DNA added. Several colonies per plate were randomly selected and the plasmid DNA in the transformants was identified by comparing the restriction patterns with the original *E. coli* derived plasmid DNA or by sequencing. Results were compared using a nonparametric one-way ANOVA test and post-hoc test (Duncan's multiple range test). $P < .05$ was considered as statistically significant. Because this study aimed to develop a high-efficiency electroporation protocol, we adopted the experimental method if it showed a consistently high electroporation efficiency in all three experiments, even if it was not statistically significant.

3.1.1. In vitro methylation

The restriction-modification (R-M) systems of BGN4 have not been studied in detail. We investigated which commercial methyltransferase is the most appropriate to protect against the possible R-M systems

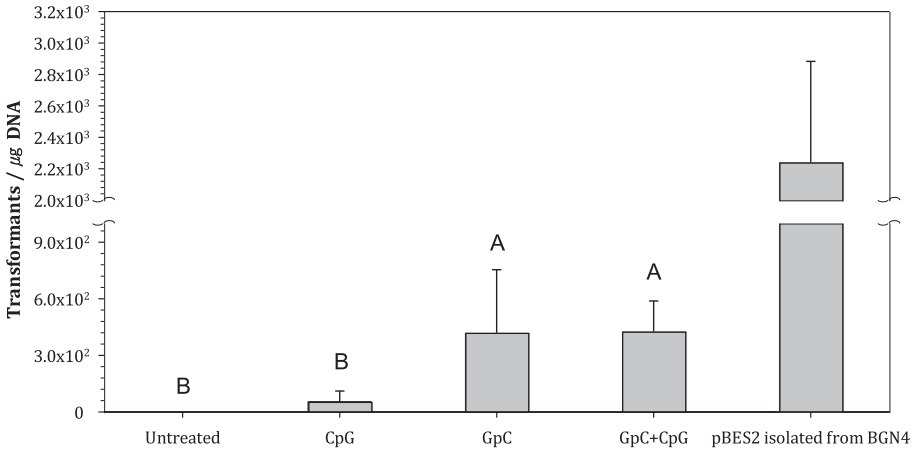

Fig. 1. Effect of *in vitro* methylation on the electrotransformation of *B. bifidum* BGN4. Cells were incubated in MRS broth supplemented with 0.05% L-cysteine·HCl and 0.2 M sucrose (final concentration) at 37 °C until an OD_{600} reached 0.4. CpG / GpC / double methylated / untreated plasmid DNA (pBES2) or the corresponding plasmid isolated from BGN4 of 50 ng was added to the competent cell suspension and electroporation was conducted under 12.5 kV / cm field strength, 200 Ω resistance and 25 μF capacitance. Experiments were repeated three times independently. All the groups except pBES2 isolatd from BGN4 were analyzed by one-way ANOVA. Result for GpC methylated DNA was significantly higher than the results of CpG methylated and untreated DNA.

present in BGN4 (Fig. 1.). GpC methylated plasmid DNA was 8 times more effective than the CpG methylated one, whereas the untreated one yielded no transformants. Double methylated DNA showed no additive effect. This means that BGN4 has an R-M system which can be effectively avoided by *in vitro* GpC methylation rather than by CpG methylation. Therefore, we decided to use GpC methylated DNA in the next steps of this study. The transformation efficiency of pBES2 isolated from BGN4 and GpC methylated pBES2 was compared to determine how much GpC methylation blocks the R-M systems in BGN4 (Fig. 1.). pBES2 isolated from BGN4 gave a 5.3 fold higher electroporation frequency as compared to the corresponding GpC methylated plasmid. As a result, GpC methylation does not completely cover the R-M systems of BGN4, which reflects the fact that *in vitro* methylation is convenient and effective but may have low versatility (Suzuki, 2012).

3.1.2. Amount of plasmid DNA

Various amounts of plasmid DNA were added to the electrocompetent cells to determine the optimal amount of DNA for the electroporation. As the amount of DNA increased, the number of transformants also tended to increase, but the transformation efficiency was not the same (Fig. 2B.). The largest number of transformants was obtained from the largest amount of DNA (1 μg) used in this study and the greatest electrotransformation efficiency was obtained when 50 ng of plasmid DNA was used. To minimize the amount of time and work and maximize the electrotransformation efficiency, we decided to use 50 ng of DNA. Although the standard deviation is high, this is the early stage of the optimization; thus, many factors that affect the transformation efficiency were not yet controlled for, and there are batch-to-batch variations in the electroporation process. In addition, because the transformation efficiency should be adjusted to 1 μg DNA, as the amount of inserted DNA becomes smaller, the standard deviation becomes greater. The raw data show that the tendencies between each independent experiment are not different (Fig. 2A.).

3.1.3. Electrical parameters

The optimization of the electroporation process involves several important factors. The three main parameters are the wave form, which is the pulse shape, the field strength and the pulse length. The field strength is measured as the voltage delivered across an electrode gap and presented as the applied voltage divided by the gap size of the electroporation cuvette. The pulse length is the duration of time that the sample is exposed to the pulse. These parameters should be optimized for different species and even strains (Löfblom et al., 2007). The optimal electrical condition of *B. bifidum* BGN4 was found by adjusting these parameters. The Gene Pulser Xcell Microbial Electroporation System (Bio-Rad, USA) used in our laboratory supports three wave forms: a square wave pulse, exponential decay wave pulse, and time-constant pulse. Among these, the exponential decay wave pulse is routinely used for electroporation of bacteria. When it is used, the voltage rises rapidly to the peak voltage set then decreases over time. The pulse length in an exponential decay wave pulse is called the time constant which is not the same as the time-constant pulse and is defined as the time required for the voltage of the untruncated pulse to decline to 1 e^{-1} (~37%) of the peak amplitude (Löfblom et al., 2007; Shigekawa and Dower, 1988). The time constant is modified by adjusting the resistance and capacitance values. Hence, we found the optimum electrical conditions by adjusting the field strength and resistance while using the exponential decay wave pulse. The effects on the transformation efficiency for field strengths of 12.5 and 15 kV / cm and resistances between 50 and 600 Ω were evaluated. The best efficiency was obtained under a field strength of 15 kV / cm and a resistance of 200 Ω, which is approximately 2 times higher than that of the existing conditions used in the laboratory with a field strength of 12.5 kV / cm and a resistance of 200 Ω (Table 2).

3.1.4. Bacterial growth phase

We investigated the effects of the bacterial growth phase on the transformation efficiency. Overnight-grown BGN4 were inoculated into 50 ml of MRS broth supplemented with 0.05% L-cysteine·HCl and 0.2 M sucrose (final concentration) and cultured until the OD_{600} reached from 0.2 to 0.7. when the OD_{600} was 0.4 (early-exponential phase), the transformation frequency was notably increased, which is 2.9 times higher than the second highest efficiency of OD_{600} 0.3 and 15 times higher than the lowest efficiency of OD_{600} 0.7 (Fig. 3.).

3.1.5. Cell wall weakening agent

We added cell wall weakening agents to the medium to investigate their effects on the electroporation efficiency. Glycine has been frequently shown to increase the electroporation efficiency in some bacteria (Gerber and Solioz, 2007; Hashiba et al., 1990; Holo and Nes, 1989; Kim et al., 2005; Pyne et al., 2013; Thompson and Collins, 1996), and NaCl was used in one study (Palomino et al., 2010). Based on this, glycine and NaCl were each tested to determine whether these additives enhance the electroporation efficiency of *B. bifidum* BGN4. The highest glycine and NaCl concentrations were selected as 1.5% and 0.2 M, respectively, to minimize the inhibition of the bacterial growth and the experiments were performed at lower concentrations accordingly. When 0.2 M NaCl was used to treat the cells, an approximately 20-fold increase in the electroporation frequency was obtained compared to the control without any treatment (Fig. 4B.). However, glycine, already known to be effective for several bacteria, did not enhance transformation in our study with *B. bifidum* BGN4 (Fig. 4A.).

3.1.6. Cell membrane permeabilizing agent

Finally, we investigated whether ethanol which is known to be effective with some species of bacteria (Assad-García et al., 2008; Pyne

A

Amount of plasmid DNA	Expt. 1		Expt. 2		Expt. 3	
	Transformants	Transformation efficiency (CFU / μg DNA)	Transformants	Transformation efficiency (CFU / μg DNA)	Transformants	Transformation efficiency (CFU / μg DNA)
1 ng	1	1,000	0	0	0	0
50 ng	48	960	10	200	39	780
100 ng	55	550	12	120	53	530
500 ng	227	454	54	108	157	314
1,000 ng	500	500	175	175	160	160

B

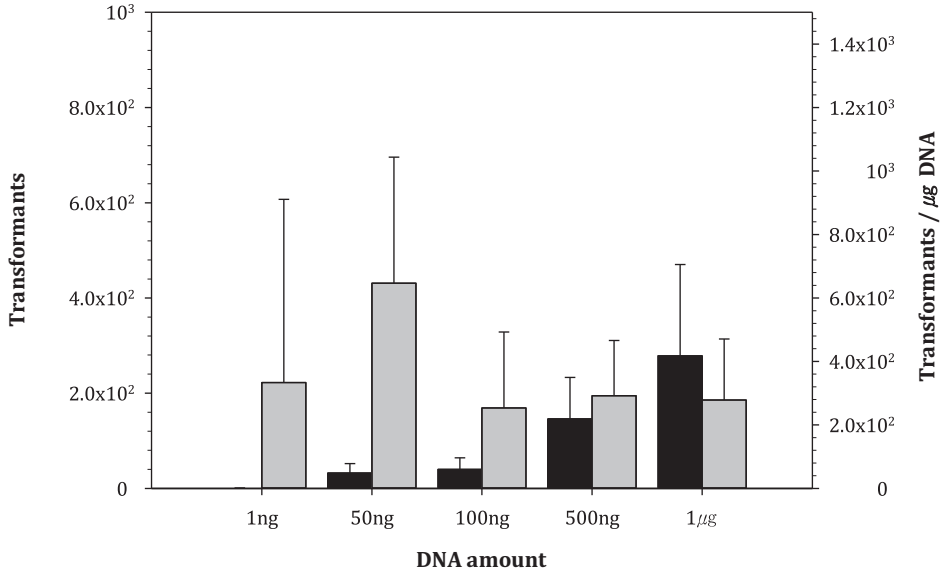

Fig. 2. Effect of amount of plasmid DNA on the electrotransformation of *B. bifidum* BGN4. Cells were incubated in MRS broth supplemented with 0.05% L-cysteine·HCl and 0.2 M sucrose (final concentration) at 37 °C until an OD_{600} reached 0.4. Separately, 1, 50, 100, 500, 1000 ng of GpC methylated plasmid DNA (pBES2) was added to the competent cell suspension and electroporation was conducted under 12.5 kV / cm field strength, 200 Ω resistance and 25 μF capacitance. Total number of transformants (dark bar) and electrotransformation efficiency (light bar) were quantified. Experiments were repeated three times independently.

et al., 2013; Sharma et al., 2007) improves the electrotransformation efficiency by altering the properties of the cell membrane. Because ethanol tolerance varies depending on the strain (Gold et al., 1992), we examined the tolerance of *B. bifidum* BGN4 by inoculating this strain with MRS medium containing various concentrations of ethanol. Because the growth of BGN4 was considerably retarded at above 2% (*v*/v) ethanol, the effect of ethanol on the electroporation efficiency was investigated at lower concentrations. Ten minutes prior to the electroporation, ethanol was added to a cell-DNA suspension. In the ethanol-treated groups, the electroporation efficiency generally tended to be higher than that in the untreated group. Among them, the 2% ethanol-treatment provided a 1.7-fold increase in the electroporation efficiency

Table 2
Effect of electrical parameters on the electrotransformation of *B. bifidum* BGN4. Cells were incubated in MRS broth supplemented with 0.05% L-cysteine·HCl and 0.2 M sucrose (final concentration) at 37 °C until an OD_{600} reached 0.4. GpC methylated plasmid DNA (pBES2) of 50 ng was added to the competent cell suspension and electroporation was conducted under indicated field strength and resistance at 25 μF capacitance. Experiments were repeated three times independently.

Field strength (kV / cm)		Resistance (Ω)							
		50	100	150	200	300	400	500	600
12.5	Time constant (mS)	1.3	2.6	3.6	4.7	5.2	6.2	7.7	7.9
	Transformation efficiency (transformants / μg DNA)	387	533	160	2680	2330	113	2040	987
15	Time constant (mS)	1.3	2.5	3.7	4.7	6.5	7.9	8.5	9.8
	Transformation efficiency (transformants / μg DNA)	1040	2350	3540	4810	2290	607	53	40

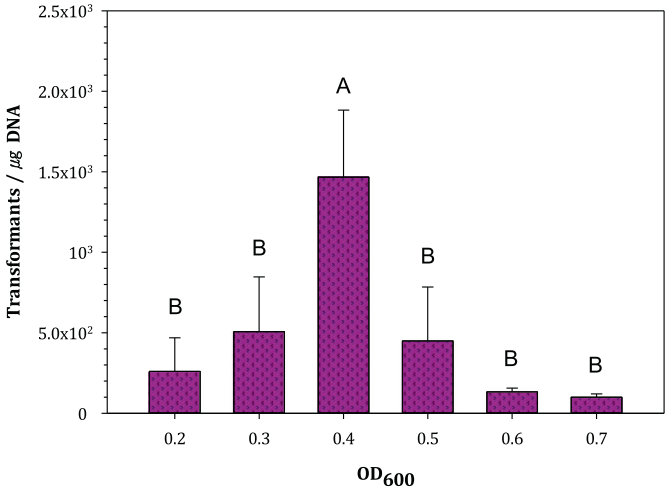

Fig. 3. Effect of bacterial growth phase on the electrotransformation of *B. bifidum* BGN4. Cells were incubated in MRS broth supplemented with 0.05% L-cysteine·HCl and 0.2 M sucrose (final concentration) at 37 °C until an OD_{600} reached from 0.2 to 0.7. GpC methylated plasmid DNA (pBES2) of 50 ng was added to the competent cell suspension and electroporation was conducted under 15 kV / cm field strength, 200 Ω resistance and 25 μF capacitance. Experiments were repeated three times independently. Result for OD_{600} 0.4 was statistically significantly higher (by one-way ANOVA) than the results of the others.

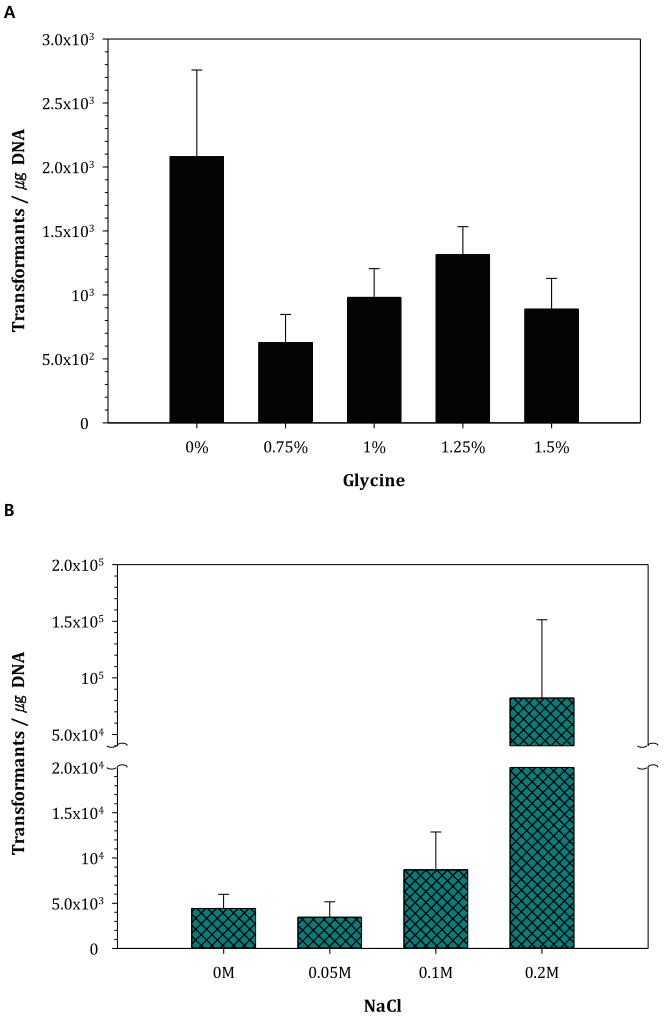

Fig. 4. Effect of cell wall weakening agents on the electrotransformation of *B. bifidum* BGN4. Cells were incubated in MRS broth supplemented with 0.05% L-cysteine·HCl, 0.2 M sucrose (final concentration) increasing concentrations of NaCl (0–0.2 M, final concentration) or glycine (0–1.5%) at 37 °C until an OD_{600} reached 0.4. GpC methylated plasmid DNA (pBES2) of 50 ng was added to the competent cell suspension and electroporation was conducted under 15 kV / cm field strength, 200 Ω resistance and 25 μF capacitance. A. Investigation of glycine concentrations. B. Investigation of NaCl concentrations. Experiments were repeated three times independently.

compared with the no ethanol treatment (Fig. 5).

During this study, we optimized the conditions for electroporation-mediated transformation of *Bifidobacterium*. The optimized conditions drastically improved the electrotransformation efficiency from 10^3 to 10^5 which is about a 72-fold increase from the existing conditions in our laboratory (Table 3). The differences between the initial method and the optimized method are the use of cell wall weakening agent and cell membrane permeabilizing molecule and the electrical parameter change, thereby these factors are key points to increase the electroporation efficiency of BGN4. Among them, using the optimization scheme employed herein, the cell wall weakening mediated by NaCl is the most significant factor, which improves the electroporation frequency by 20 times. In order to identify whether these methods can be applied to other *Bifidobacterium* species, two *B. bifidum* strains, *B. breve*, *B. pseudocatenulatum*, two *B. longum* strains and *B. lactis* were assayed. As a result, the electroporation efficiencies increased from 2.5 to 14 times as compared with the initial method and the transformants appeared in all tested species even the species which were not transformed under the initial method (Table 4).

4. Discussion

Electroporation-mediated transformation is generally more efficient than chemotransformation and is widely used with Gram-positive and Gram-negative bacteria (Itoh et al., 1994; Iwasaki et al., 1994; Wirth et al., 1989). The average electroporation efficiency is 10^4 - 10^5 CFU / μg DNA and even up to 10^{10} CFU / μg DNA for *E. coli* (Aune and Aachmann, 2010; Dower et al., 1988; Hanahan et al., 1991). However, bifidobacteria are vulnerable to oxygen and have a multilayered and complex cell wall (Fischer et al., 1987) as well as restriction-modification systems making it difficult to acquire a high electroporation efficiency (Serafini et al., 2012). Indeed, the electroporation efficiency of bifidobacteria was usually < 10^4 CFU / μg DNA in previous studies (Argnani et al., 1996; Guglielmetti et al., 2007; Park et al., 1999b; Rossi et al., 1996; Sangrador-Vegas et al., 2007; Shkoporov et al., 2008). Such a lack of efficient transformation methods has limited molecular-level studies and food-grade vector development of bifidobacteria. Therefore, we focused on finding highly efficient electroporation protocols for bifidobacteria in this study. First, we chose a suitable strain of bifidobacteria which accepts plasmid DNA. *B. bifidum* is predominant in the gut population of healthy breast-fed infants (Milani et al., 2013; Turroni et al., 2012). Because it colonizes initially in the infantile intestine, *B. bifidum* exists widely among the intestinal bifidobacterial population in healthy adults (Ku et al., 2016; Turroni et al., 2014). Among these bacterial strains, *B. bifidum* BGN4 *originates from the* feces of a breast-fed *infant* and is widely used as a probiotic strain in global food markets because of its reported benefits (Kim et al., 2003; Ku et al., 2009; Hong et al., 2009; Kim et al., 2007; Kim et al., 2007; Lee et al., 2002; Lee et al., 2006; You et al., 2004). In this respect, we decided to use *B. bifidum* BGN4 as the experimental strain.

The important factors determining the electroporation efficiency are the R-M system, cell wall and cell membrane of a bacteria, so we focused on them. At first, we tried to overcome the R-M systems of BGN4. R-M systems of several *Bifidobacterium* sp. have been determined by classical restriction analysis (Hartke et al., 1996; Khosaka et al., 1983; Khosaka et al., 1982; Kim et al., 2010; O'Connell Motherway et al., 2009) and by methylome analysis generated by single molecule real-

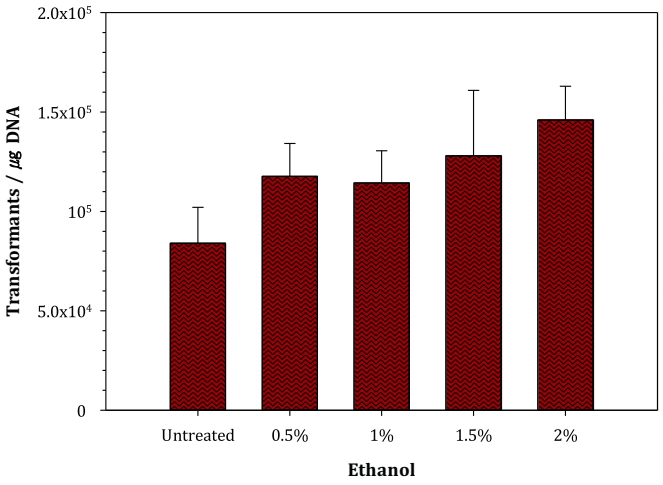

Fig. 5. Effect of cell membrane permeabilizing agent on the electrotransformation of *B. bifidum* BGN4. Cells were incubated in MRS broth supplemented with 0.05% L-cysteine·HCl, 0.2 M sucrose (final concentration) and 0.2 M NaCl (final concentration) at 37 °C until an OD_{600} reached 0.4. GpC methylated plasmid DNA (pBES2) of 50 ng was added to the competent cell suspension and the DNA-cell suspensions were supplemented with 0.5, 1, 1.5 or 2% ethanol (v/v) ten minutes prior to pulse delivery. Electroporation was conducted under 15 kV / cm field strength, 200 Ω resistance and 25 μF capacitance. Experiments were repeated three times independently.

Table 3
Comparison between optimized high-level electroporation method and initial low-level method.

Condition	Initial method	Optimized method
Bacterial growth phase	OD_{600} 0.4	OD_{600} 0.4
Cell wall weakening agent added to medium	–	0.2 M NaCl
Amount of plasmid DNA	50 ng	50 ng
Types of DNA methylation	GpC methylation	GpC methylation
Cell membrane permeabilizing agent added to cell-DNA suspension	–	2% Ethanol
Electrical parameters	12.5 kV / cm, 25 μF, 200 Ω	15 kV / cm, 25 μF, 200 Ω
Transformation efficiency (CFU / μg DNA)	2.04×10^3	1.46×10^5

Table 4
Comparison of electroporation efficiency of other *Bifidobacterium* species between initial method and optimized method.

Bacterial strain	Initial method	Optimized method
	Transformation efficiency (CFU / μg DNA)	Transformation efficiency (CFU / μg DNA)
B. bifidum KCTC 3440	$1.3 \pm 1.2 \times 10^1$	$3.3 \pm 2.3 \times 10^1$
B. bifidum KCTC 3418	0	$1.4 \pm 0.7 \times 10^2$
B. breve KCTC 3419	0	$7.3 \pm 2.3 \times 10^1$
B. pseudocatenulatum SJ32	$0.7 \pm 1.2 \times 10^1$	$1.0 \pm 0.4 \times 10^2$
B. longum RD65	0	$1.3 \pm 1.2 \times 10^1$
B. longum RD72	$2.0 \pm 1.7 \times 10^1$	$8.7 \pm 5.0 \times 10^1$
B. lactis RD68	$8.9 \pm 8.5 \times 10^3$	$4.3 \pm 0.3 \times 10^4$

time (SMRT) sequencing (Bottacini et al., 2017; Mary et al., 2014; O'Callaghan et al., 2015). However, the R-M systems of BGN4, even *B. bifidum*, has not been studied in detail to our knowledge. There are various strategies to block the R-M system of a transformation host: *in vitro* methylation using methyltransferases, *in vivo* methylation by expressing methyltransferase genes in *E. coli*, and removal of specific plasmid sites recognized by R-M systems (Suzuki, 2012). We first tried the most convenient method, *in vitro* methylation using methyltransferases and found that the R-M systems of BGN4 were effectively blocked by GpC methylation. GpC methylated plasmid DNA was 8 times more effective than the CpG methylated one, whereas the untreated one yielded no transformants. Double methylated DNA showed no additive effect (Fig. 1.). This means that the R-M systems of BGN4 are largely affected by *in vitro* GpC methylation rather than by CpG methylation. The transformation frequency of pBES2 isolated from BGN4 and GpC methylated pBES2 was compared to determine how much GpC methylation blocks the R-M systems in BGN4. There was a 5.3 fold higher electroporation efficiency for pBES2 isolated from BGN4 compared to the corresponding GpC methylated plasmid (Fig. 1.). As a result, because GpC methylation does not completely cover the R-M systems of BGN4, applying additional *in vitro* adenine methylation or *in vivo* methylation by expressing methyltransferase genes in *E. coli* is expected to yield a higher efficiency. The putative R-M systems in BGN4 were additionally analyzed through the REBASE website (http://rebase.neb.com/rebase) using the genome sequence of BGN4. Notably, the majority of the predicted recognition sites include the sequence where CpG methylation could work rather than GpC methylation (Supplementary Table 1). This is in contrast to the result shown in Fig. 1. Among the putative R-M systems of BGN4, there are several systems in which the recognition sequence is not predicted. We cautiously suppose that these less predicted R-M systems have a recognition site where GpC methylation can act and are relatively active in BGN4. To clarify this, it will be necessary to perform a methylome analysis of BGN4 through single molecule real-time (SMRT) sequencing.

We next adjusted the initial electrical parameters which were the field strength and the resistance. The electroporation efficiency nearly doubled when the field strength was increased from 12.5 to 15 kV / cm at 200 Ω (Table 2), which is consistent with the studies that higher electric field strengths resulted in greater transformation efficiencies for *Campylobacter jejuni* (Miller et al., 1988) and for bifidobacteria (Argnani et al., 1996; Serafini et al., 2012) as long as the lethality does not exceed the threshold level. High-voltage pulses during electroporation are likely to destabilize the cell walls of Gram-positive bacteria (Pyne et al., 2013; Trevors et al., 1991). Indeed, the electroporation efficiency tended to increase overall at 15 kV / cm than at 12.5 kV / cm at resistances ranging from 50 to 400 Ω. However, once the resistance was 500 Ω or above which means the time constant is increased by that much, the efficiency was the opposite because of the excessive lethality (Table 2).

The electroporation efficiency is largely dependent on the structure and density of the cell wall (Aune and Aachmann, 2010); thus, increasing the fragility of the cell wall by using cell wall weakening agents usually improves the transformation efficiency (Gerber and Solioz, 2007; Hashiba et al., 1990; Holo and Nes, 1989; Kim et al., 2005; Palomino et al., 2010; Pyne et al., 2013; Thompson and Collins, 1996). In this study, we used glycine and NaCl as the cell wall weakening agents. The mechanisms by which these additives weaken the cell wall are somewhat different. Glycine replaces D- and L-alanine residues in peptidoglycan layers, weakening the cell wall due to reduction of cross-linking (Hammes et al., 1973). NaCl reduces peptidoglycan interpeptide bridges probably by interfering with the glycine addition process during peptidoglycan synthesis and results in loose cross-linking of the cell wall (Palomino et al., 2009; Vijaranakul et al., 1995). A limitation of using these agents is that they can be toxic to the cells depending on the species and strain (Aune and Aachmann, 2010). Therefore it is critical to find the optimal concentration of these additives to increase the electroporation efficiency without inhibiting the cell growth. Glycine and NaCl were added to the extent that they did not completely inhibit the cell growth, and NaCl, rather than glycine, worked well in increasing transformation frequency in BGN4 (Fig. 4). Although glycine is a widely used substance for increasing the electroporation efficiency of Gram-positive bacteria, it did not increase the

frequency in BGN4 at all. On the other hand, the use of NaCl as a cell wall weakening agent in electroporation has been reported rarely but it was effective for bifidobacteria. Because the structure of the cell wall is different for each species and strain, empirical screening for the type and concentration of a cell wall weakening agent suitable for the bacteria used in the experiments is necessary.

The cell membrane also acts as a physical obstacle during electroporation (Pyne et al., 2013). It has been reported that the addition of ethanol to cells increases the efficiency of electroporation (Assad-García et al., 2008; Pyne et al., 2013; Sharma et al., 2007). It is well known how ethanol acts on the cell membrane. Ethanol binds to the lipid-water interface of the phospholipid bilayers and weakens the hydrophobic barrier of the membrane, thereby increasing the fluidity and pore size of it (Barry and Gawrisch, 1994; Baskaran et al., 1995; Ingram, 1986; Weber and de Bont, 1996). Because ethanol is also toxic to cells at certain concentrations and ethanol tolerance varies depending on the strain, we first examined the **minimum** inhibitory concentration (MIC) of ethanol against BGN4. Because BGN4 growth was inhibited at an ethanol concentration above 2% (*v*/v), the effect of ethanol on the electroporation efficiency was investigated at lower concentrations. Though it is not as dramatic as the cell wall weakening agent, the permeabilization of the cell membrane with ethanol slightly increased the electroporation efficiency (Fig. 5).

As a result, the electroporation-mediated transformation efficiency of *B. bifidum* BGN4 increased from 10^3 to 10^5 CFU / μg DNA (Table 3). The optimized electrotransformation conditions were extensively applied to other *Bifidobacterium* species; two *B. bifidum* strains, *B. breve*, *B. pseudocatenulatum*, two *B. longum* strains and *B. lactis*. The electroporation efficiencies increased from 2.5 to 14 times as compared with the initial method and the transformants appeared in all tested species even the species which were not transformed under the initial method (Table 4). The differences between the initial method and the optimized method are the cell wall weakening agent and cell membrane permeabilizing agent treatment and the electrical parameter change, thereby these factors are key points to increase the electroporation efficiency of all tested *Bifidobacterium* species. To the best of our knowledge, this is the first study to apply cell wall-weakening agents and a cell membrane permeabilizing agent to bifidobacteria to enhance the electroporation efficiency. It is expected that further optimizing the concentrations of the additives in these newly optimized electroporation conditions considering the diversity of the cell walls and membrane structures of the *Bifidobacterium* strains would contribute to improvement of other bifidobacterial electroporation efficiencies.

5. Conclusions

In this paper, we described an efficient electroporation method for *Bifidobacterium* strain. The transformation efficiency of *B. bifidum* strain drastically and consistently increased from 10^3 to 10^5 CFU / μg DNA. Because the method was extensively applied to other *Bifidobacterium* species, this should allow for the advanced genetic manipulation of the various *Bifidobacterium* species in future studies.

Declarations of interest

none.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2018.11.019.

References

Aoki, R., Tsuchida, S., Arai, Y., Ohno, K., Nishijima, T., Mawatari, T., Mikami, Y., Ushida, K., 2016. Effect of Bifidobacterium animalis subsp. lactis GCL2505 on the physiological function of intestine in a rat model. Food Sci. Nutr. 4, 782–790.
Argnani, A., Leer, R.J., van Luijk, N., Pouwels, P.H., 1996. A convenient and reproducible method to genetically transform bacteria of the genus Bifidobacterium. Microbiology 142, 109–114.
Assad-García, J., Bonnin-Jusserand, M., Garmyn, D., Guzzo, J., Alexandre, H., Grandvalet, C., 2008. An improved protocol for electroporation of Oenococcus oeni ATCC BAA-1163 using ethanol as immediate membrane fluidizing agent. Lett. Appl. Microbiol. 47, 333–338.
Aune, T.E.V., Aachmann, F.L., 2010. Methodologies to increase the transformation efficiencies and the range of bacteria that can be transformed. Appl. Microbiol. Biotechnol. 85, 1301–1313.
Barry, J.A., Gawrisch, K., 1994. Direct NMR evidence for ethanol binding to the lipid-water interface of phospholipid bilayers. Biochemistry 33, 8082–8088.
Baskaran, S., Ahn, H.J., Lynd, L.R., 1995. Investigation of the ethanol tolerance of Clostridium thermosaccharolyticum in continuous culture. Biotechnol. Prog. 11, 276–281.
Blaut, M., Collins, M., Welling, G., Dore, J., Van Loo, J., De Vos, W., 2002. Molecular biological methods for studying the gut microbiota: the EU human gut flora project. Br. J. Nutr. 87, S203–S211.
Bottacini, F., Morrissey, R., Roberts, R.J., James, K., van Breen, J., Egan, M., Lambert, J., van Limpt, K., Knol, J., Motherway, M.O.C., 2017. Comparative genome and methylome analysis reveals restriction/modification system diversity in the gut commensal Bifidobacterium breve. Nucleic Acids Res. 46, 1860–1877.
Dower, W.J., Miller, J.F., Ragsdale, C.W., 1988. High efficiency transformation of E. coli by high voltage electroporation. Nucleic Acids Res. 16, 6127–6145.
Fischer, W., Bauer, W., Feigel, M., 1987. Analysis of the lipoteichoic-acid-like macroamphiphile from Bifidobacterium bifidum subspecies pennsylvanicum by one-and two-dimensional 1H-and 13C-NMR spectroscopy. FEBS J. 165, 647–652.
Gerber, S.D., Solioz, M., 2007. Efficient transformation of Lactococcus lactis IL1403 and generation of knock-out mutants by homologous recombination. J. Basic Microbiol. 47, 281–286.
Gold, R.S., Meagher, M., Hutkins, R., Conway, T., 1992. Ethanol tolerance and carbohydrate metabolism in lactobacilli. J. Ind. Microbiol. 10, 45–54.
Guglielmetti, S., Karp, M., Mora, D., Tamagnini, I., Parini, C., 2007. Molecular characterization of Bifidobacterium longum biovar longum NAL8 plasmids and construction of a novel replicon screening system. Appl. Microbiol. Biotechnol. 74, 1053.
Hammes, W., Schleifer, K., Kandler, O., 1973. Mode of action of glycine on the biosynthesis of peptidoglycan. J. Bacteriol. 116, 1029–1053.
Hanahan, D., Jessee, J., Bloom, F.R., 1991. Plasmid transformation of Escherichia coli and other bacteria. Methods Enzymol. 204, 63–113.
Hartke, A., Benachour, A., Boutibonnes, P., Auffray, Y., 1996. Characterisation of a complex restriction/modification system detected in a Bifidobacterium longum strain. Appl. Microbiol. Biotechnol. 45, 132–136.
Hashiba, H., Takiguchi, R., Ishii, S., Aoyama, K., 1990. Transformation of Lactobacillus helveticus subsp. jugurti with plasmid pLHR by electroporation. Agric. Biol. Chem. 54, 1537–1541.
Holo, H., Nes, I.F., 1989. High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp. cremoris grown with glycine in osmotically stabilized media. Appl. Environ. Microbiol. 55, 3119–3123.
Hong, K.S., Kang, H.W., Im, J.P., Ji, G.E., Kim, S.G., Jung, H.C., Song, I.S., Kim, J.S., 2009. Effect of probiotics on symptoms in Korean adults with irritable bowel syndrome. Gut Liver. 3, 101.
Ingram, L., 1986. Microbial tolerance to alcohols: role of the cell membrane. Trends Biotechnol. 4, 40–44.
Itoh, N., Kouzai, T., Koide, Y., 1994. Efficient transformation of Pseudomonas strains with pNI vectors by electroporation. Biosci. Biotechnol. Biochem. 58, 1306–1308.
Iwasaki, K., Uchiyama, H., Yagi, O., Kurabayashi, T., Ishizuka, K., Takamura, Y., 1994. Transformation of Pseudomonas putida by electroporation. Biosci. Biotechnol. Biochem. 58, 851–854.
Jia, W., Li, H., Zhao, L., Nicholson, J.K., 2008. Gut microbiota: a potential new territory for drug targeting. Nat. Rev. Drug Discov. 7, 123.
Khosaka, T., Sakurai, T., Takahashi, H., Saito, H., 1982. A new site-specific endonuclease BbeI from Bifidobacterium breve: Restriction endonucleases; 3′-cohesive termini; DNA sequence analysis. Gene 17, 117–122.
Khosaka, T., Kiwaki, M., Rak, B., 1983. Two site-specific endonucleases BinSI and BinSII from Bifidobacterium infantis. FEBS Lett. 163, 170–174.
Kim, N., Ji, G.-E., 2006. Modulatory activity of Bifidobacterium sp. BGN4 cell fractions on immune cells. J. Microbiol. Biotechnol. 16, 584.
Kim, I.-H., Park, M.-S., Ji, G.-E., 2003. Characterization of adhesion of Bifidobacterium sp. BGN4 to human enterocyte-like Caco-2 cells. J. Microbiol. Biotechnol. 13, 276–281.
Kim, Y., Han, K., Oh, S., You, S., Kim, S., 2005. Optimization of technical conditions for

the transformation of Lactobacillus acidophilus strains by electroporation. J. Appl. Microbiol. 99, 167–174.
Kim, N., Kunisawa, J., Kweon, M.N., Eog Ji, G., Kiyono, H., 2007. Oral feeding of Bifidobacterium bifidum (BGN4) prevents CD4(+) CD45RB(high) T cell-mediated inflammatory bowel disease by inhibition of disordered T cell activation. Clin. Immunol. 123, 30–39.
Kim, J.-Y., Wang, Y., Park, M.-S., Ji, G.-E., 2010. Improvement of transformation efficiency through in vitro methylation and SacII site mutation of plasmid vector in Bifidobacterium longum MG1. J. Microbiol. Biotechnol. 20, 1022–1026.
Ku, S., You, H.J., Ji, G.E., 2009. Enhancement of anti-tumorigenic polysaccharide production, adhesion, and branch formation of *Bifidobacterium bifidum* BGN4 by phytic acid. Food Sci. Biotechnol. 18, 749–754.
Ku, S., Park, M.S., Ji, G.E., You, H.J., 2016. Review on *Bifidobacterium bifidum* BGN4: Functionality and Nutraceutical applications as a Probiotic Microorganism. Int. J. Mol. Sci. 17, 1544.
Lee, M.J., Zang, Z., Choi, E.-Y., Shin, H.K., Ji, G.-E., 2002. Cytoskeleton reorganization and cytokine production of macrophages by bifidobacterial cells and cell-free extracts. J. Microbiol. Biotechnol. 12, 398–405.
Lee, S., Koo, N., Oh, S., 2006. Regulatory effect on specific IgE Response of Bifidobacterium Bifidum(bgn4 Strain) in Murine Model of Peanut Allergy. J. Allergy Clin. Immunol. 117, S204.
Löfblom, J., Kronqvist, N., Uhlén, M., Ståhl, S., Wernérus, H., 2007. Optimization of electroporation-mediated transformation: Staphylococcus carnosus as model organism. J. Appl. Microbiol. 102, 736–747.
Mary, O., Motherway, C., Watson, D., Bottacini, F., Clark, T.A., Roberts, R.J., Korlach, J., Garault, P., Chervaux, C., van Hylckama Vlieg, J.E., 2014. Identification of restriction-modification systems of *Bifidobacterium animalis* subsp. lactis CNCM I-2494 by SMRT sequencing and associated methylome analysis. PLoS One 9, e94875.
Matsuki, T., Watanabe, K., Fujimoto, J., Kado, Y., Takada, T., Matsumoto, K., Tanaka, R., 2004a. Quantitative PCR with 16S rRNA-gene-targeted species-specific primers for analysis of human intestinal bifidobacteria. Appl. Environ. Microbiol. 70, 167–173.
Matsuki, T., Watanabe, K., Fujimoto, J., Takada, T., Tanaka, R., 2004b. Use of 16S rRNA gene-targeted group-specific primers for real-time PCR analysis of predominant bacteria in human feces. Appl. Environ. Microbiol. 70, 7220–7228.
Milani, C., Hevia, A., Foroni, E., Duranti, S., Turroni, F., Lugli, G.A., Sanchez, B., Martin, R., Gueimonde, M., Van Sinderen, D., 2013. Assessing the fecal microbiota: an optimized ion torrent 16S rRNA gene-based analysis protocol. PLoS One 8, e68739.
Miller, J.F., Dower, W.J., Tompkins, L.S., 1988. High-voltage electroporation of bacteria: genetic transformation of Campylobacter jejuni with plasmid DNA. Proc. Natl. Acad. Sci. U. S. A. 85, 856–860.
Mitsuoka, T., 1990. Bifidobacteria and their role in human health. J. Ind. Microbiol. 6, 263–267.
O'Callaghan, A., Bottacini, F., Motherway, M.C., Van Sinderen, D., 2015. Pangenome analysis of Bifidobacterium longum and site-directed mutagenesis through by-pass of restriction-modification systems. BMC Genomics 16, 832.
O'Connell Motherway, M., O'Driscoll, J., Fitzgerald, G.F., Van Sinderen, D., 2009. Overcoming the restriction barrier to plasmid transformation and targeted mutagenesis in Bifidobacterium breve UCC2003. Microb. Biotechnol. 2, 321–332.
Palomino, M.M., Sanchez-Rivas, C., Ruzal, S.M., 2009. High salt stress in Bacillus subtilis: involvement of PBP4* as a peptidoglycan hydrolase. Res. Microbiol. 160, 117–124.
Palomino, M.M., Allievi, M.C., Prado-Acosta, M., Sanchez-Rivas, C., Ruzal, S.M., 2010. New method for electroporation of Lactobacillus species grown in high salt. J. Microbiol. Methods 83, 164–167.
Park, M.S., Shin, D.W., Lee, K.H., Ji, G.E., 1999a. Sequence analysis of plasmid pKJ50 from Bifidobacterium longum. Microbiology 145, 585–592.
Park, S.Y., Ji, G.E., Ko, Y.T., Jung, H.K., Ustunol, Z., Pestka, J.J., 1999b. Potentiation of hydrogen peroxide, nitric oxide, and cytokine production in RAW 264.7 macrophage cells exposed to human and commercial isolates of Bifidobacterium. Int. J. Food Microbiol. 46, 231–241.
Park, M.-S., Moon, H.-W., Ji, G.-E., 2003. Molecular characterization of plasmid from Bifidobacterium longum. J. Microbiol. Biotechnol. 13, 457–462.
Pyne, M.E., Moo-Young, M., Chung, D.A., Chou, C.P., 2013. Development of an electrotransformation protocol for genetic manipulation of Clostridium pasteurianum. Biotechnol. Biofuels. 6, 50.
Rossi, M., Brigidi, P., Rodriguez, A.G.V., Matteuzzi, D., 1996. Characterization of the plasmid pMB1 from Bifidobacterium longum and its use for shuttle vector construction. Res. Microbiol. 147, 133–143.
Sanders, M.E., 2008. Probiotics: definition, sources, selection, and uses. Clin. Infect. Dis. 46, S58–S61.
Sangrador-Vegas, A., Stanton, C., Van Sinderen, D., Fitzgerald, G., Ross, R., 2007. Characterization of plasmid pASV479 from Bifidobacterium pseudolongum subsp. globosum and its use for expression vector construction. Plasmid 58, 140–147.
Serafini, F., Turroni, F., Guglielmetti, S., Gioiosa, L., Foroni, E., Sanghez, V., Bartolomucci, A., Motherway, M.O.C., Palanza, P., van Sinderen, D., 2012. An efficient and reproducible method for transformation of genetically recalcitrant bifidobacteria. FEMS Microbiol. Lett. 333, 146–152.
Sharma, A.D., Singh, J., Gill, P.K., 2007. Ethanol mediated enhancement in bacterial transformation. Electron. J. Biotechnol. 10, 166–168.
Shigekawa, K., Dower, W.J., 1988. Electroporation of eukaryo and prokaryotes: a general approach to the introduction of macromolecules into cells. BioTechniques 6, 742–751.
Shkoporov, A.N., Efimov, B.A., Khokhlova, E.V., Steele, J.L., Kafarskaia, L.I., Smeianov, V.V., 2008. Characterization of plasmids from human infant Bifidobacterium strains: sequence analysis and construction of E. coli–Bifidobacterium shuttle vectors. Plasmid 60, 136–148.
Sivan, A., Corrales, L., Hubert, N., Williams, J.B., Aquino-Michaels, K., Earley, Z.M., Benyamin, F.W., Lei, Y.M., Jabri, B., Alegre, M.-L., 2015. Commensal Bifidobacterium promotes antitumor immunity and facilitates anti–PD-L1 efficacy. Science 350, 1084–1089.
Suau, A., Bonnet, R., Sutren, M., Godon, J.-J., Gibson, G.R., Collins, M.D., Doré, J., 1999. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl. Environ. Microbiol. 65, 4799–4807.
Suzuki, H., 2012. Host-mimicking strategies in DNA methylation for improved bacterial transformation. In: Methylation-from DNA, RNA and Histones to Diseases and Treatment. InTech.
Thompson, K., Collins, M.A., 1996. Improvement in electroporation efficiency for Lactobacillus plantarum by the inclusion of high concentrations of glycine in the growth medium. J. Microbiol. Methods 26, 73–79.
Trevors, J.T., Chassy, B.M., Dower, W.J., Blaschek, H.P., 1991. Electrotransformation of Bacteria by Plasmid DNA, Guide to Electroporation and Electrofusion. pp. 265–290.
Turroni, F., Peano, C., Pass, D.A., Foroni, E., Severgnini, M., Claesson, M.J., Kerr, C., Hourihane, J., Murray, D., Fuligni, F., 2012. Diversity of bifidobacteria within the infant gut microbiota. PLoS One 7, e36957.
Turroni, F., Duranti, S., Bottacini, F., Guglielmetti, S., Van Sinderen, D., Ventura, M., 2014. Bifidobacterium bifidum as an example of a specialized human gut commensal. Front. Microbiol. 5, 437.
Ventura, M., O'Flaherty, S., Claesson, M.J., Turroni, F., Klaenhammer, T.R., Van Sinderen, D., O'Toole, P.W., 2009. Genome-scale analyses of health-promoting bacteria: probiogenomics. Nat. Rev. Microbiol. 7, 61.
Vijaranakul, U., Nadakavukaren, M.J., De Jonge, B., Wilkinson, B.J., Jayaswal, R.K., 1995. Increased cell size and shortened peptidoglycan interpeptide bridge of NaCl-stressed Staphylococcus aureus and their reversal by glycine betaine. J. Bacteriol. 177, 5116–5121.
Weber, F.J., de Bont, J.A., 1996. Adaptation mechanisms of microorganisms to the toxic effects of organic solvents on membranes. Biochim. Biophys. Acta Biomembr. 1286, 225–245.
Wirth, R., Friesenegger, A., Fiedler, S., 1989. Transformation of various species of gram-negative bacteria belonging to 11 different genera by electroporation. Mol. Gen. Genet. 216, 175–177.
You, H.J., Oh, D.K., Ji, G.E., 2004. Anticancerogenic effect of a novel chiroinositol-containing polysaccharide from Bifidobacterium bifidum BGN4. FEMS Microbiol. Lett. 240, 131–136.