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Note

Improvement of the electro-transformed cell yield for *Bifidobacterium* sp. with large DNA

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Abstract In this study, the poor electro-transformant yield of large DNA in bifidobacteria was improved by increasing the DNA concentration, which was amplified by enhancing electroporation conditions: treating the cell wall weakening agent and cell membrane permeabilizing molecule as well as changing the electrical parameter. In the enhanced conditions, the electroporation frequency increased from 15 to 71 times compared to the initial conditions at the same DNA concentration. As the DNA concentration increased, the difference in the electroporation frequency between the two conditions became greater, and the curve of the enhanced conditions seemed to be saturated with a DNA concentration over 4 μg. The present study provided a clue to the recovery of the electroporation frequency with large DNA and formulated the relationship between the DNA concentration, the DNA size and the electroporation frequency in bifidobacteria. Therefore, this study will contribute to the expansion of molecular genetic studies of bifidobacteria.

Keywords: large dna, electroporation, bifidobacteria

Introduction

Electroporation, the most efficient method of bacterial transformation, has a strong correlation to a DNA size. It has been observed that the larger the DNA size, the lower the electroporation efficiency (Inoue et al., 1990; Ohse et al., 1995; Rhee et al., 2007; Sheng et al., 1995). Especially, in case of bacteria with recalcitrance for acquiring heterologous DNA, it is difficult to obtain the transformant even if plasmid DNA size exceeds only 10 kbp. Because the size of the subcloned plasmid DNA often exceeds 10 kbp, a decline in the transformation efficiency with an increase in DNA size can be a hurdle for molecular biological studies. There have been several attempts to overcome this by adjusting electrical parameters (Rhee et al., 2007; Sheng et al., 1995) or using mutant host (Hanahan et al., 1991), but related studies have not been reported much to our knowledge.

Bifidobacteria are probiotics defined as live nonpathogenic microorganisms that provide health benefits to the host (Lewis et al., 2016). Furthermore, their safety and advantageous functional characteristics have been well documented with the increasing number of clinical trials (Borriello et al., 2003; Eskesen et al., 2015; Saez-Lara et al., 2015; Sáez-Lara et al., 2016). However, the absence of proper genetic tools, including efficient transformation protocols to study the molecular mechanism or to exploit the full potential of bifidobacteria through genetic modifications, has been the limiting factor. The poor transformation efficiency of bifidobacteria is due to the very high sensitivity to oxygen stress during competent cell preparation, thick and complex cell wall (Fischer et al., 1987) as well as restriction-modification barriers (Serafini et al., 2012). When plasmid DNA above 10 kbp is introduced into the bifidobacteria, the transformation efficiency becomes much lower and makes it very difficult to obtain even a single transformant. In order to construct a plasmid vector that is less than 10 kbp, the number or size of the gene to be inserted into the plasmid is restricted. Reducing the size of the *Bifidobacterium* shuttle vector is advantageous, but it has a limitation. Because most parts of *Bifidobacterium* plasmids are cryptic with unknown functions, the plasmids may not be replicated if these cryptic parts are deleted. Therefore, the low-level electroporation efficiency for *Bifidobacterium* species with large DNA should be improved and the aim of present study is to overcome this problem. As has been observed by several studies (Dunny et al., 1991; Pyne et al., 2013) and our previous study (Park et al., 2019), there was a concentration-dependent increase in the relationship between the number of transformants and plasmid DNA concentration in some bacteria genus. In this respect, we tried to improve the electro-transformed cell recovery for *Bifidobacterium* species with large plasmid DNA by increasing the amount of DNA.

Materials and Methods

The experiments were designed to confirm the electroporation

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frequency of large size DNA above 10 kbp under two different electroporation conditions across multiple plasmid DNA concentrations. One of the two conditions is a initial low-level method of electroporation efficiency used in our laboratory while the other is a high-level method recently enhanced for *Bifidobacterium bifidum* BGN4, an experimental strain used in this study (Park et al., 2019). The differences between the initial conditions and the enhanced conditions are the use of a cell wall weakening agent and cell membrane permeabilizer as well as the electrical parameter change. Detailed experimental methods of this study are described below.

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5α were cultured aerobically in LB medium (BD Difco, Sparks, MD, USA) at 37°C and *B. bifidum* BGN4 were cultivated anaerobically in MRS medium (BD Difco) containing 0.05% L-cysteine HCl at 37°C.

pB-P572G1 (11.2 kbp), pB-G7 (10.6 kbp), and pB-SKL (10.2 kbp), the plasmids in which β-galactosidase gene, α-galactosidase gene and sod-catalase genes, respectively, are subcloned into a *Bifidobacterium* shuttle vector pBES2, were used in this study.

DNA manipulation

Plasmid DNA was extracted from pB-P572G1, pB-G7 or pB-SKL-introduced *E. coli* DH5α with a Plasmid Purification Mini Kit (Nucleogen, Gyeonggi-do, South Korea) and methylated *in vitro* by GpC (M.CviPI) methyltransferases (NEB, Ipswich, MA, USA). DNA concentration was measured by a Qubit 4 Fluorometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Preparation of *B. bifidum* BGN4 cells for electroporation

Electroporation methods were divided into the initial method and the enhanced method, which was based on that of Park et al. (2019). Overnight cell cultures in MRS medium supplemented with 0.05% L-cysteine HCl were diluted into 50 mL of fresh MRS medium containing 0.05% L-cysteine HCl, 0.2 M sucrose (final concentration) and 0.2 M NaCl (final concentration, for the enhanced method only). The inoculated 50 mL MRS medium with the additives was anaerobically incubated at 37°C until the OD_{600} reached to 0.4. 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). Finally, they were resuspended in 0.5 mL of ice-cold electroporation buffer.

Electroporation

1, 50, 100, 500, 1,000, 2,000, 3,000, 4,000, or 5,000 ng of plasmid DNA was mixed with a 0.1 mL of cell suspension, kept on ice for 30 min, and then transferred into a pre-cooled Gene Pulser cuvette (Bio-Rad, Hercules, CA, USA). In the enhanced method, cell-DNA suspensions were incubated in the presence of 2% ethanol (v/v) acting as a cell membrane permeabilizing agent for 10 min before the pulse delivery. Electroporation was conducted under 12.5 (the initial method) or 15 (the enhanced method) kV/cm field strength at 200 Ω resistance and 25 μF capacitance using the Gene Pulser Xcell Microbial Electroporation System (Bio-Rad). Following the electroporation, a 0.9 mL volume of MRS medium containing 0.05% L-cysteine HCl and 0.2 M sucrose (final concentration) was added to the bacteria and they were incubated anaerobically at 37°C for 3 h. The bacteria were then plated onto MRS agar containing 3 μg/mL of chloramphenicol. The plates were cultured for 36 h under anaerobic condition.

Results and Discussion

As with previous reports (Inoue et al., 1990; Ohse et al., 1995; Rhee et al., 2007; Sheng et al., 1995), it was observed that the electroporation efficiency of the bifidobacteria is also strongly correlated with size dependent. 50 ng of pBES2 with 7.6 kbp, the original plasmid, yielded approximately 10^3 CFU/μg DNA in the initial method and approximately 10^5 CFU/μg DNA in the enhanced method (data not shown). However, pB-P572G1 with 11.2 kbp, which is the largest subcloned pBES2 used in this study, did not produce any transformants in both conditions at the same concentration (Fig. 1A). Even though moles of each plasmid is more important than mass for transformation efficiency (Hornstein et al., 2016), the difference in moles between 11.2 and 7.5 kbp DNA at the same concentration is 32% whereas the difference in the number of transformants was much greater. pB-G7 with 10.6 kbp and pB-SKL with 10.2 kbp also showed similar results (Fig. 1B, and Fig. 1C). These two large DNAs also failed to yield any transformants in the initial conditions and only yielded up to 20 colonies in the enhanced conditions when introduced into the host at a concentration of 50 ng. Thus, in all the subclones of pBES2 with over 10 kbp, there was a reduction in the electroporation frequency, which was greater than the difference in the moles,

Table 1. Bacterial strains and plasmids used in this study

Bacterial strain	Source and reference
Escherichia coli DH5α	Lab stock
Bifidobacterium bifidum BGN4	Isolated from breast-fed infant feces (Park et al., 1999)
Plasmid	**Characteristics and reference**
pBES2	7.6 kbp, Ap^R, Cm^R, *Bifidobacterium* - *E. coli* shuttle vector (Park et al., 2003)
pB-P572G1	11.2 kbp, Ap^R, Cm^R, pBES2 derivate, containing β-galactosidase expression cassette
pB-G7	10.6 kbp, Ap^R, Cm^R, pBES2 derivate, containing α-galactosidase expression cassette
pB-SKL	10.2 kbp, Ap^R, Cm^R, pBES2 derivate, containing sod-catalase expression cassette

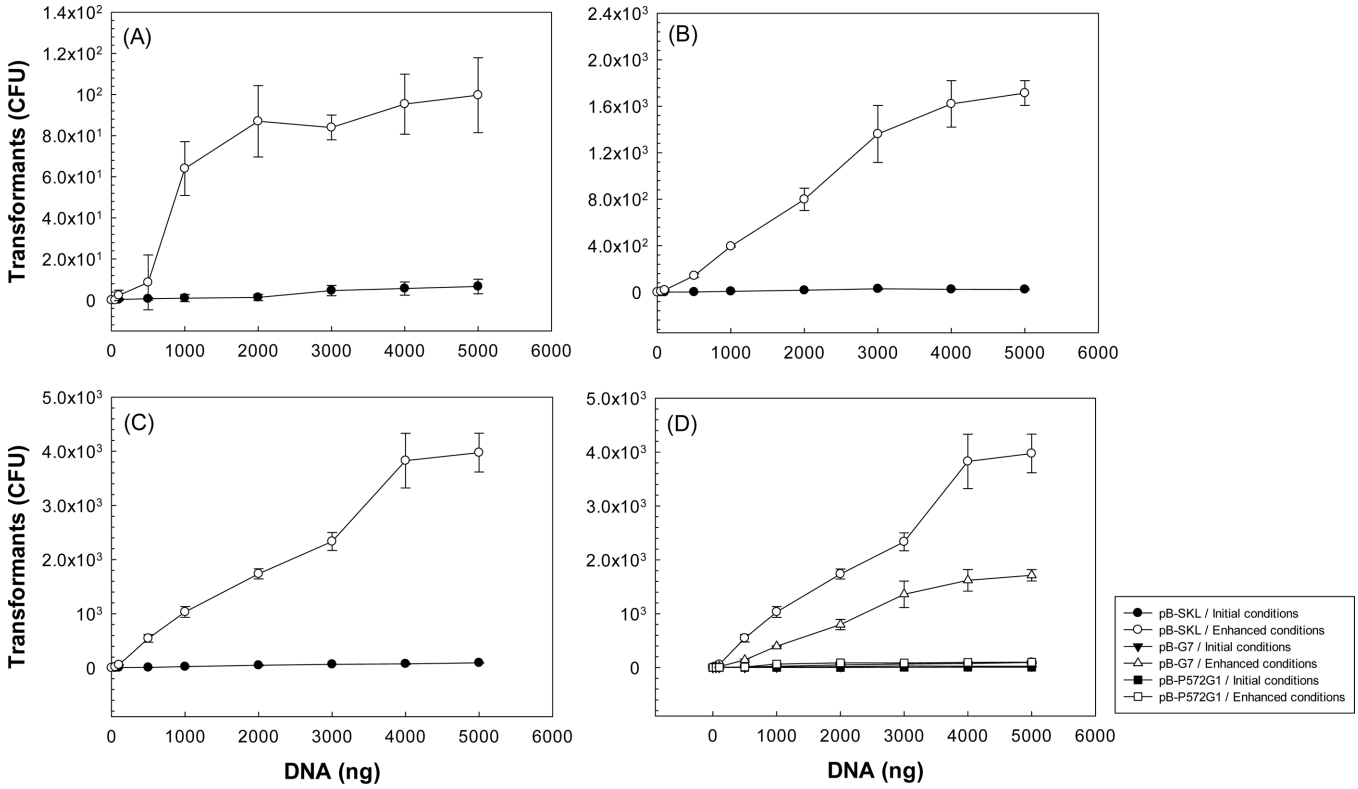

Fig. 1. Investigation of electro-transformation frequency of large size DNA under initial conditions (closed circle) and enhanced conditions (open circle) across multiple plasmid DNA concentrations. (A) pB-P572G1; (B) pB-G7; (C) pB-SKL. The results of (A), (B), and (C) were combined in (D).

when compared to the pBES2 with 7.6 kbp. The cause for the variability in the electroporation efficiency according to the DNA size has not been clarified yet. In addition to the moles, other important factors seem to affect this phenomenon.

Likewise, the transformation frequency among the three large plasmids (pB-SKL, pB-G7, and pB-P572G1) used in this study was determined according to the DNA size order and the difference was much greater than the difference in moles (Fig. 1D). Notably, the difference in DNA size between large plasmids is only by a few hundred bp, which is up to 8% difference when converted to moles, but the total number of transformants varied from 9.97×10^{1} to 3.98×10^{3} CFU for the enhanced method with 5 μg of DNA added. In detail, the electroporation frequency of pB-SKL (10.2 kbp), pB-G7 (10.6 kbp), and pB-P572G1 (11.2 kbp) was 3.98×10^{3}, 1.71×10^{3}, and 9.97×10^{1} CFU, respectively, for the enhanced method with 5 μg of DNA added. The difference in size between pB-SKL (10.2 kbp), pB-G7 (10.6 kbp) and pB-G7 (10.6 kbp), pB-P572G1 (11.2 kbp) was 3.9 and 5.7%, respectively, while the reduction in yield of the electro-transformed cells was 57 and 94.2%, respectively. Judging from this phenomenon, the electroporation efficiency reduction seems to be accelerated with a DNA size over 10 kbp in the genus *Bifidobacterium*.

The amount of DNA delivered to BGN4 increased from 1 ng to 5 μg to solve the electroporation frequency problem of large plasmid DNA. As expected, the transformed cell recovery of large DNA electroporation was improved by increasing the amount of DNA in both conditions (Fig. 1A, 1B, and 1C). However, there were significant differences between the two methods. In the initial low-level method, the threshold of the minimum amount of DNA that yields at least one transformant was high and the total number of transformants was extremely low, which are limitations. On the other hand, in the high-level method enhanced for BGN4, the transformant could be obtained at lower concentrations than with the initial method and the total number of transformants was relatively higher (Fig. 1A, 1B, and 1C). The difference in the electroporation frequency between the two methods ranges from as little as 15 times to as much as 71 times with a DNA concentration of 5 μg. As the amount of DNA increased, the gap between the two conditions became even greater, and the curve of the enhanced conditions appeared to be saturated with a DNA concentration over 4 μg. Briefly, it was observed that the low electroporation frequency of large plasmid DNA for bifidobacteria was improved by increasing the amount of DNA, but there was a limit to increase only the amount of DNA. Instead, increasing the amount of DNA after enhancement of the electroporation method by treating the cell wall weakening agent and cell membrane permeabilizing molecule as well as changing the electrical parameter for bifidobacterial strains yielded much more transformants at lower concentrations.

This study showed that it is possible to solve the problem of poor electro-transformed cell yield as the DNA size increases in the genus *Bifidobacterium*. We often experienced difficulties obtaining even a single transformant when subcloned plasmids larger than 10 kbp were electroporated into bifidobacteria. This

problem was overcome by simply adjusting the amount of plasmid DNA and this phenomenon was amplified by enhancing electroporation conditions. Treating DNA with commercial methyltransferase to get over the restriction-modification system of bifidobacteria limits the final DNA concentration and the amount of cell-DNA suspension that can be put into electroporation cuvette. Thus, the amount of added DNA for electroporation is limited. Increasing the DNA concentration after enhancing the electroporation method for each bifidobacterial strain will be the best way to obtain the maximum number of transformants from large size DNA within the limit. The electroporation method for *B. bifidum* BGN4 was enhanced through the use of a cell wall weakening agent and cell membrane permeabilizer as well as the electrical parameter change in our recent study (Park et al., 2019). Because the enhanced conditions were widely applied to other *Bifidobacterium* species, the strategy used in this study may also be applicable when introducing large DNA into other *Bifidobacterium* species. There is no previous study related to the improvement of electro-transformed cell yield of large DNA for bifidobacteria to our knowledge, which is why this study was conducted. It is meaningful that our study sheds light on the problem of the poor yield of electro-transformed cells when using large DNA and formulated the relationship between the DNA concentration, the DNA size and the electroporation frequency in bifidobacteria.

Conclusion

In present study, the poor yield of electro-transformed cells with large DNA in bifidobacteria was improved by increasing the DNA concentration, which was further improved from 15 to 71 times by enhancing the electroporation conditions: treating the cell wall weakening agent and cell membrane permeabilizing molecule as well as changing the electrical parameter. In addition, the relationship between the DNA concentration, the DNA size and the electroporation frequency in bifidobacteria was formulated for the first time.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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