Liposome fragment-mediated introduction of multiple plasmids into *Bacillus subtilis*

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**ABSTRACT**

Transformation of microorganisms by plasmid introduction is one of the central techniques in modern biotechnology. However, applicable transformation methods for simultaneous introduction of multiple plasmids are still limiting. Here, we reported a liposome-mediated method that efficiently introduces multiple plasmids into *B. subtilis*. In this method, liposomes containing three kinds of plasmids were mixed with *B. subtilis* protoplasts in the presence of 36% polyethylene glycol (PEG), and the resultant protoplasts were grown in cell wall-re-generation media. We found that the rates of introduction of multiple plasmids were significantly increased in the presence of liposomes. We also found that an intact liposome structure was not required for introduction, and the presence of phosphatidylglycerol (PG) was important for efficient introduction of multiple plasmids.

Therefore, the liposome- or liposome fragment-mediated transformation method reported here can advance studies utilizing multiple plasmids.

**1. Introduction**

Transformation of microorganisms through plasmid introduction is one of the central techniques of modern biotechnology. To date, various methods for plasmid introduction have been developed, such as methods utilizing the natural competency of bacteria [1], heat shock [2], electroportation [3], PEG-mediated protoplast [4], and conjugal transfer [5]. For gram-positive bacteria, such as *Bacillus subtilis*, the methods of utilizing natural competency [6,7] and electroportation [8] are widely employed.

However, each method has its shortcomings. For example, electroportation requires expensive equipment. In the transformation method utilizing natural competency of *B. subtilis*, the plasmid to be replicated in the cell must be a multimer. This is because the introduced DNA, which is a linearized single strand, must be circularized through homologous recombination using the plasmid’s repetitive sequences [9,10]. Furthermore, both methods are not suitable for simultaneous introduction of multiple plasmids.

The introduction of multiple plasmids is of great interest. Recently, increasing numbers of foreign genes have been introduced to microorganisms to produce useful molecules or to design molecular circuits in the cell [11,12]. In most cases, the foreign genes were encoded in a few plasmids or a long linear DNA to be introduced into the genome [13–18]. However, generally, larger plasmids or linear DNA are more difficult to construct, handle, and introduce into the cell. An alternative method is to separate foreign genes into several plasmids and introduce at once. This method will allow for easy modification of genes or combinatorial selection of different mutant genes [19]. Based on this idea, we developed a method to introduce multiple plasmids into *B. subtilis*, a model organism of gram-positive bacteria.

In this study, we used liposomes to enhance the introduction efficiency of multiple plasmids. Liposomes are artificial lipid vesicles, known to encapsulate plasmids and enhance the introduction efficiency in mammalian cells [20]. We encapsulated three kinds of plasmids in liposomes and mixed with *B. subtilis* protoplast in the presence of PEG. Here, we obtained the following three findings: 1) the simultaneous introduction ratios of three plasmids were significantly increased when encapsulated in liposomes; 2) intact vesicular structure of the liposome was not necessary for the enhanced introduction ratio; and 3) lipid composition was significantly important for the introduction ratio.
2. Materials and methods

2.1. Medium

Composition of the regeneration medium was prepared as previously described, with some modifications [21]. The preparation method is briefly described below. First, 500 mL solution A was prepared by dissolving disodium succinate hexahydrate (189 g) in distilled water and autoclave sterilization at 121 °C for 20 min. Second, 460 mL solution B was prepared by dissolving K$_2$HPO$_4$ (7 g), KH$_2$PO$_4$ (3 g), sodium caseinate (2.5 g), polyvinylpyrrolidone (60 g), casamino acid (10 g), tryptophan (0.2 g), glucose (10 g), tryptic soy broth (30 g) with heat and sterilization by filtration. Third, the solutions A and B were mixed. Subsequently, 1 M MgCl$_2$ (40 mL) was added at less than 30 °C to prepare a 2 × stock solution and stored at 4 °C. Before use, the stock solution was warmed at 65 °C and mixed with an equivalent volume of 1.6% agar solution, which was prepared by mixing agar and distilled water followed by autoclaving at 121 °C for 20 min. Aliquots (4 mL) were separated and kept at 55 °C for enclosing the protoplast. The remaining medium was cooled to around 55 °C before the addition of chloramphenicol (5 μg/mL) and plating.

2.2. Culture and protoplast preparation

The protoplast preparation method was modified from a previous study [22]. The overnight culture of B. subtilis strain (ISW1214), purchased from Takara (Japan), was prepared in 5 mL of LB medium at 37 °C. The culture (50 μL) was inoculated into fresh LB medium (5 mL) and incubated at 37 °C with shaking at 200 rpm until reaching the mid-log phase (OD$_{600}$ = 0.5). Cells in the mid-log-phase culture (500 μL) were centrifuged at 9 k × g for 3 min and suspended in 250 μL SMM buffer (0.5 M sucrose, 0.02 M maleate buffer (pH 6.4), 0.02 M magnesium chloride) [23]. The suspension was mixed with 250 μL SMM buffer containing 4 μg/μL lysozyme (Sigma Aldrich) and incubated at 40 °C for approximately 15 min until achieving even light scattering. After centrifugation at 2 k × g for 10 min at 4 °C, the cells were suspended in 500 μL NB/MSM (13 g/L nutrient broth (Difco), 20 mM magnesium chloride, 0.5 M sucrose, 20 mM maleic acid) [24] containing 1.5 mg/mL fosfomycin (Wako Chemical, Japan) and incubated at 30 °C for 1 h. The resultant cells were used as protoplasts.

2.3. Plasmids

Three plasmids, pHY300PLK-gfp (tetracycline (Tc) resistant, 5.6 kbp), pSEQ243 (neomycin (Nm) resistant, 5.6 kbp), pHTo1-bgal (chloramphenicol (Cm) resistant, 11 kbp), were used in this study. pSEQ243 [25] was purchased from Bacillus Gene Stock Center. pHTo1-bgal was purchased from MoBiTec (Germany). pHY300PLK-gfp was constructed as follows. First, the vector fragment was prepared by PCR using pHY300PLK (Takara, Japan) as a template with primers, primers v1 and v2. Second, two DNA fragments were prepared by PCR with primers (primers i1 and i2) using pETf5Stag, which encodes GFPuv5 [26], as a template and by PCR with primers (primers i3 and i4) without templates. The two fragments were connected by PCR with primers i3 and i2, and the resultant fragments were ligated with the vector fragment using the In-Fusion cloning kit (Takara, Japan). The primer sequences are shown in Table S1.

2.4. Liposome preparation

Liposomes used in this study were prepared by the freeze-dried empty liposome (FDEL) method [27] as briefly described below. Lipids, 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (PE), 1-palmitoyl-2-oleoyl-phosphatidylglycerol (PG), and 1-palmitoyl-2-oleoyl-glycerol-3-phosphatidylycerine (PC), were purchased from Avanti Polar Lipids. Each lipid (PE and PG for experiments shown in Figs. 2 and 3) was dissolved in chloroform at 13.3 mM each, and fluorescence-labeled lipid, PE-ATTO 633 (ATTO-TEC, Germany), was added at 6.67 μg/mL. After drying the solvent with a rotary evaporator, distilled water was added to achieve a lipid concentration of 12 mM. Subsequently, the mixture was vigorously shaken to create liposomes. The mixture was sonicated and freeze-dried under vacuum after dividing into aliquots (40 μL). The freeze-dried liposomes were rehydrated with the plasmid solution containing three plasmids (1.5–2.5 nM each). The resultant liposomes were centrifuged at 8 k × g for 3 min after the addition of 200 μL 0.9% NaCl solution, and the liposome precipitates were suspended in 100 μL LB medium for use as the liposome solution. Following, the concentration of the plasmids was measured, and the plasmid concentration was typically 13 pM of the liposome sample prepared with the 2.0 nM plasmid solution.

2.5. PEG treatment

Briefly, 40% PEG was prepared by dissolving PEG6000 (Nacalai, Japan) in distilled water at 65 °C. The protoplasts were centrifuged at 2 k × g for 10 min at 4 °C, and the resultant protoplast precipitates were suspended in the liposome solution (2 μL). After addition of 40% PEG (18 μL) and gentle mixing, the mixture was placed on ice for 5 min and then diluted with NB/MSM (200 μL).

2.6. Colony formation of protoplasts and antibiotics resistant assay

The colony formation method is the same as previously described [21]. Briefly, after incubation of the PEG-treated protoplasts at 30 °C for 2 h, the protoplasts were first mixed with 4 mL of the dissolved regeneration medium at 55 °C to enclose the protoplast into the gel. Following, it was spread on the solid regeneration plate medium containing 5 μg/mL Cm. The plates were wrapped and incubated at 30 °C for more than 2 days before counting the colony number. For analysis of Nm and Tc resistant ratios, 20 colonies on the regeneration plates were randomly selected and streaked onto LB plate medium containing 10 μg/mL Tc, 5 μg/mL Nm, or both. When the colony number was less than 20, all colonies were selected. After a three-day incubation at 30 °C, growth on each medium was evaluated.

2.7. Measurement of plasmid concentration

To measure the plasmid concentration in the liposome solution, an aliquot of the liposome solution was subjected to DNA purification using the Purelink quick plasmid miniprep kit (ThermoFisher), and one of the plasmid concentrations was measured by quantitative PCR using primers, 5′-CGAGGGGGAATGGGATCC-3′ and 5′-CAACAGCACCACGT GACACC-3′. To compensate for loss during the purification procedure, the result was normalized to the average yield of the purification process (61%), which was estimated using three independent purification processes of a known plasmid concentration.

3. Results

3.1. Liposome-mediated plasmid introduction

The plasmid introduction method performed in this study is schematically shown in Fig. 1. B. subtilis strain, ISW1214, was cultured to the mid-log phase, treated with lysozyme, and further incubated in the presence of fosfomycin, cell wall inhibitor, at 30 °C for 1 h to prepare protoplasts. Liposomes were prepared by the FDEL method [27] using phospholipids (PE:PG = 1:1 in a molar concentration, unless otherwise noted). In the FDEL method, empty liposomes are prepared by freeze-drying and then rehydrating with solutions containing three kinds of plasmids, each of which contains a different antibiotic-resistant gene (Cm, Tc, or Nm). During the rehydration process, a part of each plasmid was incorporated into the liposomes. After washing out the external
plasmids, the liposomes were suspended in LB medium. The liposomes were mixed with the protoplast and incubated with 36% PEG on ice for 5 min. After dilution with NB/MSM medium and incubation at 30 °C for 2 h, the cells were enclosed in a soft agar medium and spread on the regeneration plate medium containing 5 μg/mL Cm. After more than 2 days of incubation at 30 °C, colony number was counted. Colonies appearing at this stage were expected to incorporate one of the three plasmids. To examine multiple plasmid incorporation, the Cm-resistant colonies were further streaked on LB plates containing Nm, Tc, or both to examine antibiotic resistance. As a control experiment, we also performed the same procedure with the same concentration of plasmids without liposomes.

We performed this experiment with plasmid concentrations (9.7, 13, and 16 pM each) with and without liposomes. The colony numbers on the initial Cm-containing plate were similar between those with and without liposomes (Fig. 2A), indicating that the introduction efficiency of a single plasmid was not affected by liposome presence. In contrast, the Tc or Nm resistant ratios of the colonies were significantly higher with liposomes than those without liposomes (Fig. 2B). We also detected some colonies that were resistant to both Tc and Nm in the presence of liposomes, implying that all three plasmids were simultaneously introduced. These results indicate that the presence of liposomes improves the efficiency of multi-plasmid introduction into B. subtilis cells. The differences in the Tc and Nm resistant ratios can be attributed to the different copy numbers of the plasmids (multicopy pHY300PLK-gfp [28] and low copy pSEQ243 [25]).

3.2. Liposome disruption experiment

We next examined whether intact liposome structure was necessary for plasmid introduction. After liposome preparation, we separated the liposomes into two fractions. After centrifugation of both fractions at 8 k × g for 3 min, one of the liposome precipitates was mixed with 1 μL chloroform to disrupt the liposomes and then diluted with 50 μL LB medium. Microscopic analysis revealed that no liposome-like structure was observed; however, the lipids formed droplets in this sample (Disruption+, Fig. 3A). The other liposome precipitate was suspended in 50 μL LB medium. Following, 1 μL chloroform was added. In this control sample, liposome-like structures, such as ring-like structures, were maintained (Disruption-, Fig. 3A). These results support that the prior addition of chloroform disrupts liposome structure. We then performed PEG treatment according to the method shown in Fig. 1 with these liposome samples. Both the initial Cm-resistant colonies (Fig. 3B) and restreaked Tc-
resistant colonies (Fig. 3C) were similar, irrespective of liposome disruption. These results indicate that intact liposome structure is not necessary for multiple plasmid introduction.

3.3. Effect of lipid composition

We next investigated the effect of lipid composition on the efficiency of multiple plasmid incorporation. In the previous experiment, we used lipid mixtures of PE and PG. In this experiment, we additionally used PC and prepared various lipid mixtures: each single lipid (PE, PC, PG), combinations of two lipids (PE/PG, PE/PC, PG/PC), and mixture of all lipids (PE/PG/PC) at an equivalent molar ratio. We performed the same procedures shown in Fig. 1 for all lipid compositions. The colony numbers on the initial Cm-containing plate varied depending on the lipid compositions (Fig. 4A). The largest number of colonies was observed for PG, while the smallest number of colonies was observed for PE/PC. The Tc- and Nm-resistant ratios were higher for PG, PE/PG, PG/PC, and PE/PG/PC, but no colonies were detected for PE (Fig. 4B). Since PG alone or PG containing lipids can form liposomes in water [29–31], these results suggest that PG-containing liposomes are more effective for multiple plasmid introduction.

4. Discussion

In this study, we demonstrated that the efficiency of simultaneous introduction of multiple plasmids into B. subtilis protoplast is significantly increased with liposomes. This method can be useful for experiments that require multiple foreign genes in various combinations. In the field of synthetic biology, various foreign genes are introduced to microorganisms for bioproduction of useful molecules. In many cases, researchers have to search for appropriate gene sets from various organisms or mutant libraries through repeated trial-and-error [11]. When the introduced genes are encoded in a single plasmid, researchers have to construct a number of plasmids encoding every gene combination. However, with efficient simultaneous introduction of multiple plasmids, an alternative strategy is possible; we can introduce multiple

Fig. 2. Effect of liposomes on plasmid introduction. (A) Colony numbers observed on the initial Cm-containing plate. (B) Tc, Nm, and Tc + Nm resistance ratios of colonies appearing on the Cm-containing plates. Twenty colonies on the Cm-containing plate were streaked on Tc, Nm, or Tc + Nm-containing plates. For samples with less than 20 colonies, all colonies were assayed. The error bars represent the standard errors (N = 3). *Significant at p < 0.05.

Fig. 3. Effect of liposome disruption. (A) Fluorescent microscopy of liposome samples treated with (Disruption +) or without (Disruption -) chloroform. (B) Colony numbers observed on the initial Cm-containing plate. (C) Tc resistance ratios of colonies appearing on the Cm-containing plates. Twenty colonies on the Cm-containing plate were streaked on Tc -containing plates. The error bars represent the standard errors (N = 3).
plasmids encoding various genes and select the best combination. The method reported in this study paves the way for a new multiple gene screening method, although we admit that further investigation is required, such as application to organisms other than B. subtilis.

Lipid-mediated transformation method has been widely used for the transformation of mammalian cells, known as lipofection [20], but such method has been rarely utilized for bacteria [32,33]. That is probably because lipofection depends on the unique characteristics of mammalian cells, such as endocytosis and the naked cell membrane without cell walls. Furthermore, in previous bacterial studies, the incorporation of multiple plasmids has not been examined [32,33]. In this study, we successfully performed an efficient simultaneous introduction of up to three plasmids using liposomes and protoplasts. This study broadens applicability of the liposome-mediated transformation method.

One of the remaining questions in this study is the mechanism of multiple plasmid introduction. In this study, we found that an intact liposome structure is not required for the introduction of multiple plasmids (Fig. 3), and PG-containing lipids exhibited the best transformation efficiency. The plasmids encapsulated in the liposomes might bind to the charged PG-containing lipid membrane and be incorporated into the protoplast even after disruption. The PG requirement shown in Fig. 4 supports this notion because the PG-containing negatively charged membrane forms a complex with the negatively charged DNA in the presence of a divalent cation, such as magnesium ion [34]. Further, PEG treatment induces fusion of the membrane fragments with the protoplast; thus, the bound plasmids can be simultaneously introduced. To verify the mechanism, direct observation of membrane fusion would be required in a future study.

Although we reported the enhancement of multiple plasmids introduction by using liposome, the introduction ratio is still low (about 5% in PE/PG/PG in Fig. 4B). To improve this ratio, there are several possible strategies. First, the lipid composition can be further optimized because the lipid composition significantly affects the introduction ratio as demonstrated in Fig. 4. Another possible strategy is the optimization of the liposome preparation method. In this study, we used FDEL method, which produces multilamellar liposomes of various sizes. The liposome lamellarity and sizes can affect the efficiency of the plasmid introduction because the plasmid encapsulation ratio depends on the liposome sizes and the lamellarity should affect the fusion efficiency of the liposome to the protoplast. There are various methods for liposome preparation with different lamellarity and sizes, such as hydration method [27], the water-in-oil (w/o) emulsion-transfer method [35–37], and sonication [38] or extrusion methods [39]. Optimization of the liposome preparation method in the future can improve the ratio of multiple plasmids introduction.

Acknowledgement

We thank Professor Mitsuhiro Itaya of Keio University for providing the plasmids. We thank Professor Takashi Akamatsu of Sojo University for useful technical advice. We also thank Professor Fumio Matsuda for his support and discussion. This research was funded by ImpACT Program of Council for Science, Technology and Innovation (Cabinet Office, Government of Japan).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2019.100646.

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