Glass Bead-based Genetic Transformation: An Efficient Method for Transformation of Thraustochytrid Microorganisms

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Abstract: Here, we describe a new method for genetic transformation of thraustochytrids, well-known producers of polyunsaturated fatty acids (PUFAs) like docosahexaenoic acid, by combining mild glass (zirconia) bead treatment and electroporation. Because the cell wall is a barrier against transfer of exogenous DNA into cells, gentle vortexing of cells with glass beads was performed prior to electroporation for partial cell wall disruption. G418-resistant transformants of thraustochytrid cells (Aurantiochytrium limacinum strain SR21 and thraustochytrid strain 12B) were successfully obtained with good reproducibility. The method reported here is simpler than methods using enzymes to generate spheroplasts and may provide advantages for PUFA production by using genetically modified thraustochytrids.

Key words: glass bead treatment, electroporation, genetic transformation, thraustochytrid

1 INTRODUCTION

Thraustochytrids are marine eukaryotic microorganisms that have attracted great attention owing to their capacity for accumulation of polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA), in large amounts and their ability to biosynthesize useful substances, such as squalene and astaxanthin. Genetic manipulation techniques, such as introduction and destruction of genes, are necessary for elucidation of biosynthetic pathways and molecular breeding for fermentative production of useful substances. To date, electroporation, the particle gun method and transformation using infection with Agrobacterium tumefaciens have been reported to be successful for transformation of thraustochytrids with foreign genes. However, among the strains currently being studied, many strains cannot be transformed or have poor transformation efficiency using existing methods.

Although Aurantiochytrium limacinum strain SR21 and thraustochytrid strain 12B have been reported to show high production of DHA, transformed strains have not yet been established. Therefore, in this study, improvement of the transformation method was examined using these two strains. From our analysis, we were able to improve the transformation efficiency of thraustochytrids using glass bead treatment prior to electroporation.

2 EXPERIMENTAL PROCEDURE

2.1 Cultivation of thraustochytrids

Thraustochytrid strain 12B (strain 12B) was isolated from the mangrove area of Okinawa, Japan, and was deposited at the NITE Biological Resource Center (NBRC), National Institute of Technology and Evaluation (NITE), Japan, as strain number NITE P-68. Aurantiochytrium limacinum strain SR21 (strain SR21: ATCC MYA-1381) was a gener-

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ous gift from Dr. Toshihiro Yokochi (AIST, Japan). The thraustochytrids used in this study were cultivated at 30°C in BY+790 medium containing peptone (1.0 g/L), yeast extract (1.0 g/L), and d-(-)-glucose (5.0 g/L) in artificial seawater (ASW) with a 50% salt concentration (approximately 1.5% NaCl; My Sea, Jamarine Laboratory, Osaka, Japan) with shaking or on BY + 790 medium with agar (15 g/L; referred to as BY + 790 plates).

2.2 Antibiotic resistance of strain 12B and strain SR21

Thraustochytrid cells were spread on BY + 790 plates containing 0.1, 0.2, 0.5, or 1.0 mg/mL G418 disulfate (Nacalai Tesque, Kyoto, Japan) and cultivated at 30°C to determine the concentration that could completely inhibit their growth.

2.3 Construction of an antibiotic resistance gene expression cassette

Genomic DNA of strain 12B was prepared using DNA-su-
isui-F (Rizo, Inc., Tsukuba, Ibaraki, Japan). With reference to the draft whole-genome sequence of strain 12B, which we will report in detail elsewhere, we amplified the 1500-bp region upstream from the putative translation start codon and the 1500-bp region downstream of the translation stop codon of the putative elongation factor α (EF-1α) gene of strain 12B by polymerase chain reaction (PCR) with the following primer sets: EF-1α upper Fw primer (5’-GAATTCCCAGGCCCCCTCATGTTGGGCTG-GACCTG-3’)/EF-1α upper Rv primer (5’-TTTGGTTGTTGCT- GCTAGTGCCTGGAAC-3’) and EF-1α down Fw primer (5’-AGATCCGCGGTGCTAGCAG-3’)/EF-1α down Rv primer (5’-ACTAGTGGATCCCCCCTTGGATCGTATTGTGTC-3’). KOD-Plus-Neo (TOYOBO, Osaka, Japan) was used, with genomic DNA of strain 12B as the template.

We amplified the partial 18S rRNA gene of strain 12B by PCR with the following primer set: 18S rRNA Fw primer (5’-AAAGGTACCCTATGCTGCTGTCAGGAGCGGAGCCCA-3’)/18S rRNA Rv primer (5’-TAACCCTTCCCCCGAGATGTTCAACCTAC-3’). KOD-Plus-Neo was used, with genomic DNA of strain 12B as the template.

A neomycin-resistance gene as the antibiotic-resistance marker gene was amplified by PCR with the primer set Neo’ Fw primer (5’-CTAGCACCACAAACAAAAATGATTGAA-CAGATGGATTGCGACG-3’)/Neo’ Rv primer (5’-AGCC- GCGGAGGTACCTCATGGAAGAATCTGTAGCAGAAGCGGC-3’) specific to the pRES plasmid (TaKaRa Bio, Shiga, Japan) using KOD-Plus-Neo.

Using an In-Fusion HD Cloning Kit (TaKaRa Bio), directional cloning of these DNA fragments into the Smal site of pBluescript II SK (+) plasmid (Agilent Technologies, Santa Clara, CA, USA) was performed to generate the pBluescript_12B_Neo’_cassette (Fig. 1). Escherichia coli strain DH5α was used for the maintenance and multiplication of the plasmids. Transformation of thraustochytrids was performed using a linearized pBluescript_12B_Neo’_cassette vector, which was amplified by PCR with the primer set 18S rRNA inverse Fw primer (5’-TTAGCATATGCTTCTG-GAGCTGG-3’)/18S rRNA inverse Rv primer (5’-GGTAGGTTGACAGATTTGAGAG-3’).

2.4 Transformation by electroporation

Electroporation of thraustochytrids was carried out according to the method of Sakaguchi et al. using a Gene Pulser II apparatus (Bio-Rad, Hercules, CA, USA).

2.5 Agrobacterium-mediated transformation

An amplified neomycin resistance gene expression cassette was ligated into the Smal site of a binary vector pCAMBIA0390, which was obtained from CAMBIA, Canberra, Australia, to make pCAMBIA0390/12B_Neo’_cassette. E. coli strain DH5α was used for maintenance and multiplication of the plasmids. Agrobacterium tumefaciens strain LBA4404 (TaKaRa Bio) was transformed with this plasmid, and transformed Agrobacterium tumefaciens strain LBA4404 harboring pCAMBIA0390/12B_Neo’_cassette was used for the transformation of thraustochytrids according to methods described by Cheng et al.

2.6 Transformation using glass (zirconia) beads

Transformation using glass (zirconia) bead treatment was carried out according to the method of Kindle. Mid-log phase cells of thraustochytrids were collected by centrifugation, and 300 mg sterilized glass beads (0.5 mm in diameter; YTZ Ball; Nikkato, Osaka, Japan) was added to 0.4 mL of the cell suspension containing 5% PEG #6000. DNA was then added, and the samples were agitated at top speed on a Vortex Genie II mixer (M&S Instruments, Osaka, Japan) in 1.5-mL microcentrifuge tubes for 10–30 s. The collected
cells were spread on selective BY + 790 plates.

Alternatively, the bead-treated cells were washed with 50% ASW once and then with 50 mM sucrose solution three times. The washed cells were suspended in 50 mM sucrose solution and were adjusted to give an optical density (OD) at 600 nm of 8.0. Electroporation was then performed as described above.

2.7 PCR for confirmation of transformation

Colony PCR was carried out to check the insertion of the antibiotic-resistance gene in the putatively transformed thraustochytrid cells. The neomycin-resistance gene was amplified with the specific primer set Neo’ 30bp Fw primer (5’-ATGATTGAACAAGATGGATTTGACGCAGGT-3’)/Neo’ 30bp Rv primer (5’-TCAGAAGACTCGTCAAGAAGGC-GATAGAA-3’).

3 RESULTS AND DISCUSSION

3.1 Selection of antibiotics and construction of an antibiotic-resistance gene expression cassette

Several antibiotics are known to inhibit the growth of thraustochytrids efficiently\(^5\). First, we investigated G418 resistance in strain 12B and strain SR21. Our results showed that 0.5 mg/mL G418 completely inhibited the growth of both strains in BY + 790 liquid medium and on BY + 790 plates (data not shown). Therefore, we constructed a putative neomycin-resistance gene expression cassette using upstream and downstream regions of the EF-1α gene of strain 12B (Fig. 1) with reference to the draft whole-genome sequence of the strain 12B, which we will report in detail elsewhere.

3.2 Transformation by electroporation

Using the constructed putative neomycin-resistance gene expression cassette, transformation of strain 12B by electroporation was carried out. Cell suspensions (60–70 μL) with 2 μg DNA were transferred to a 0.1-cm-gap cuvette and electroporated under pulse conditions with a capacitor capacity of 25 μF and a resistance value of 200 Ω at voltages of 500, 750, and 1500 V. Under these conditions, no transformants were observed on BY + 790 plates containing G418. Next, with pulse conditions of a capacitor capacity of 25 μF and a resistance value of 200 Ω at a voltage of 750 V, the cell suspension was electroporated twice. From this method, only one colony was observed on the BY + 790 plate containing G418. The resistance of this transformant to G418 was retained after several cultivations in BY + 790 liquid medium containing G418; therefore, we concluded that the transformation of strain 12B was successful. Furthermore, we confirmed that the neomycin-resistance gene was functioning, indicating that the upstream and downstream regions of the EF-1α gene of strain 12B functioned properly as the promoter and terminator, respectively.

However, it was not possible to obtain reproducibility after several trials of electroporation with the same conditions or by increasing the amount of DNA. Additionally, when using strain SR21 for electroporation under the same conditions, no transformant of strain SR21 was obtained. Consequently, we needed to further optimize the electroporation conditions for both strains.

3.3 Agrobacterium-mediated transformation

Next, gene introduction by conjugal transfer using Agrobacterium tumefaciens was attempted since the thraustochytrid Schizochytrium has previously been transformed using A. tumefaciens\(^6\). A. tumefaciens LBA4404 was transformed with pCAMBIA0390/12B_Neo cassette, and Agrobacterium-mediated transformation of strain 12B and strain SR21 was carried out according to the methods of Cheng et al.\(^5\) without snailase treatment. Under these conditions, no transformants were obtained from either strain.

In the study by Cheng et al.\(^5\), a medium called "Induction Medium" was used for Agrobacterium-mediated transformation of thraustochytrids. However, we used BY + 790 liquid medium instead of "Induction Medium" because the composition of this medium was not disclosed. Although this may explain why no transformants were obtained by Agrobacterium-mediated transformation, we hypothesized that the lack of snailase treatment may explain the lack of transformants. Therefore, further optimization was necessary to obtain thraustochytrid transformants using Agrobacterium-mediated transformation.

3.4 Transformation by glass (zirconia) bead treatment

Glass bead treatment of cells has been reported in genetic transformation of bacteria\(^9,10\), yeast\(^11\), and the green algae Chlamydomonas\(^8\). Therefore, we attempted to use this method to achieve transformation of thraustochytrids.

The cells were agitated for 10 or 30 s in the presence of 750 mg/mL of 0.5-mm zirconia beads and DNA carrying the neomycin-resistance gene expression cassette. After agitation, cells were immediately plated on BY + 790 plates with G418 to select colonies with the neomycin-resistance gene. However, no colonies appeared in samples that had been agitated, even in the presence of DNA.

Glass bead treatment itself may cause cell damage; indeed, cells agitated with glass beads for 30 s did not grow on BY + 790 plates containing no antibiotics, although those agitated for only 10 s were able to grow. Accordingly, we concluded that further optimization was necessary to obtain thraustochytrid transformants using this method.
3.5 Transformation by a combination of mild glass bead treatment and electroporation

After our attempts using various methods, we assumed that the cell wall was preventing the introduction of exogenous DNA into cells. In many cases, cells whose cell walls have been removed have been used as hosts for transformation. Although there are several methods for removing cell walls, such as enzyme treatment and the use of mutants lacking cell walls, we assumed that the mild glass bead treatment may disrupt the cell wall slightly. Therefore, electroporation was carried out after treating the cells with glass beads. Briefly, 750 mg/mL of zirconia beads was added to 0.4 mL of the cell suspension of strain 12B or strain SR21 in BY + 790 medium, and the cell suspension was agitated by vortexing with beads for 10 s. Supernatants were centrifuged, and collected cells were washed for electroporation. Electroporation was performed under pulse conditions with a capacitor capacity of 25 μF, a resistance value of 200 Ω, and a voltage of 500 V. After this combined glass bead treatment and electroporation, G418-resistant transformants of strain 12B and strain SR21 were observed on BY + 790 plates with antibiotics (Fig. 2). Because the G418 resistance of these transformants was retained after several cultivations in BY + 790 liquid medium containing G418, we confirmed that the transformation was successful. Furthermore, insertion of the neomycin-resistance gene expression cassette into genomic DNA of both strain 12B and strain SR21 was confirmed by PCR (data not shown).

Transformants were obtained with an efficiency of about 1.5 × 10⁷ – 1.5 × 10⁸ cfu/μg DNA for strain 12B and about 3.0 × 10⁴ – 1.5 × 10⁵ cfu/μg DNA for strain SR21 (Table 1). Reproducibility was confirmed by repeating cultivation of transformants in liquid medium with antibiotics, and the obtained transformants were very stable. Sakaguchi et al. reported that the efficiency of transformation depends on the method of gene transfer. For example, particle bombardment is suitable for transformation for *Thraustochytrium aureum* ATCC 34304, *Parietichytrium* sp. TA04Bh, and *Schizochytrium* sp. 204-06 m; however, few or no transformants were obtained using electroporation. Notably, for these strains, transformants could be obtained using the glass bead-based genetic transformation method described herein.

Based on these findings, we speculated that transfer of exogenous DNA into cells by electroporation rarely occurred using cells with a complete cell wall; however, transfer of DNA occurred relatively easily using cells with a partially damaged cell wall, e.g., that caused by mild agitation with glass beads.

At this time, it is still unclear whether the neomycin-resistance gene expression cassette was integrated into the chromosomal DNA by site-specific homologous recombination or random integration. Further studies are needed to clarify this mechanism.

### Table 1 Transformation frequencies of thraustochytrids.

| Method                        | Thraustochytrid strain 12B | *A. limacinum* strain SR21 |
|-------------------------------|-----------------------------|---------------------------|
| Electroporation⁴              | Extremely rare              | 0                         |
| Agrobacterium-mediated        | 0                           | 0                         |
| Agitation with glass beads    | 0                           | 0                         |
| + Electroporation⁵            | 1.5 × 10⁷ – 1.5 × 10⁸       | 3.0 × 10⁴ – 1.5 × 10⁵     |

⁴ Wild type cells were used. No process to remove the cell wall was performed.
⁵ Voltage: 750 V, capacitor capacity: 25 μF, resistance: 200 Ω, applied twice in a row.
⁶ 10 s-agitation and electroporation: Voltage: 500 V, capacitor capacity: 25 μF, resistance: 200 Ω.

4 CONCLUSION

In this paper, we showed that we could transform thraustochytrid strain 12B and *A. limacinum* strain SR21, which have not been able to be transformed by other known methods. For our method, we combined glass bead pretreatment and electroporation (Fig. 3). We assumed that the cell wall was one of the main barriers affecting gene transfer into these strains. To overcome this difficulty,
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we used a simple treatment, i.e., agitation with glass beads; this combined treatment was successful for transformation of thraustochytrids. This convenient method could facilitate the molecular breeding of thraustochytrids for the production of beneficial lipids and could be applicable for other microorganisms with robust cell walls.

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