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Convenient Transformation of Anamorphic Basidiomycetous Yeasts Belonging to Genus *Pseudozyma* Induced by Electroporation

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A convenient procedure for carrying out transformation by electroporation was optimized for the genus *Pseudozyma*. Successful transformation was achieved using a plasmid, pUXV1, that confers resistance to hygromycin B; the maximum transformation efficiency was 48 transformants/μg of plasmid DNA. Transformants of *Pseudozyma antarctica* T-34 expressing a green fluorescent protein were obtained by the procedure.

[**Key words:** *Pseudozyma*, yeast, transformation, electroporation, green fluorescent protein (GFP), hygromycin B]

The microorganisms belonging to the genus *Pseudozyma* are anamorphic basidiomycetous yeasts, and provide us with unique production systems. *Pseudozyma antarctica*, *P. aphidis*, and *P. rugulosa* have been reported to efficiently produce mannosylerythritol lipids (MELs) at yields of over 100 g/*l* from vegetable oils (1). MELs are some of the most promising biosurfactants known, because they show not only excellent surface-active properties but also versatile biochemical actions including antitumor and cell differentiation activities with respect to different mammalian cells (2, 3). *Pseudozyma parantarctica* and *P. tsukubaensis* have been found to produce MELs from soybean oil (4). *Pseudozyma fusiformata* produces cellobiose lipids, namely, ustilagic acids, which exhibit high antimicrobial activity (5). *Pseudozyma flocculosa* produces a flocculosin, which is one type of cellobiose lipid and shows excellent antifungal activity (6).

Recently, the production of heterologous recombinant protein by *P. flocculosa* and *P. antarctica* has been reported (7). *P. antarctica* CBS 516.83 and *P. flocculosa* DAOM 196992, which are transformed to express active animal proteins such as green fluorescent protein and hen egg white lysozyme, secrete native proteins bearing a basic glycosylation structure typical of higher eukaryotic cells with relatively low levels of protease. Therefore, the *Pseudozyma* yeasts could naturally overcome some of the problems encountered with less evolved systems such as those of bacteria and ascomycetous fungi, and provide us with a wide range of industrial applications. However, there is little knowledge of the molecular events of such yeast cells. It is of great interest to develop genetic tools for the study and exploitation of the genus *Pseudozyma*.

Previously, a gene transfer system using plasmid DNA bearing a gene fragment that confers the resistance of *P. flocculosa* to hygromycin B under control of the *hsp70* promoter and a terminator from the basidiomycete *Ustilago maydis* was reported (8). However, the transformation procedure is only effective when using the yeast spheroplasts, which is considerably troublesome and time-consuming. One of the alternatives to using the spheroplast procedure is carrying out transformation by electroporation.

Electroporation is widely used for introducing foreign DNA into different organisms for strain manipulation and for gene-cloning experiments, because the technique is simple, rapid and highly efficient (9, 10). The technique has been applied not only to yeast cells (*e.g.*, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, and *Candida albicans*), but also to the filamentous fungi *Neurospora crassa* (10–16).

We thus focused our attention on the optimization of electroporation for the transformation of dimorphic yeasts of the genus *Pseudozyma*. Preliminary experiments indicated that the control of the cell form is crucial for an efficient transformation of the yeast *P. antarctica* T-34. That is, the yeast cells showed two forms, both yeast and fungus, and the latter form showed higher hygromycin B resistance (data not shown). To obtain the yeast form of the strain T-34 displaying sensitivity to hygromycin B, YM (0.5% peptone, 0.3% yeast extract, 0.3% malt extract and an adequate amount of glucose or glycerol) and YNB (0.67% yeast nitrogen base without amino acids and an adequate amount of glucose or glycerol) liquid media were separately applied to logarithmically grow cells for 1 d at 28°C (OD_{600}<1.0). The cells grown in YM medium containing 10% glycerol efficiently maintained the yeast form, whereas the cells grown under the other conditions essentially only showed the fungus form. A small number of fungus form cells in the YM medium were filtrated and removed using a satirized Miracloth filter (Calbiochem, San Diego, CA, USA), and the resulting yeast form cells were used as host cells in the following experiments.

Recently, the genomic similarity of *P. antarctica* T-34 to

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TABLE 1. Effects of pulse setting and amount of plasmid DNA on transformation

Pulse setting	DNA (μg)	Number of transformants
11.5 kV/cm, 25 μF	10	3.5±2.1
7.5 kV/cm, 25 μF	10	15.0±9.9
5.0 kV/cm, square wave, 1.0 ms pulse length, 2 pulses	10	55.0±21.2
	1.0	7.5±3.5
	0.1	0

TABLE 2. Effects of pretreatment with sorbitol, lithium acetate and dithiothreitol on transformation

	Transformants/μg of plasmid DNA			
	No treatment	10 mM DTT	100 mM lithium acetate	DTT and lithium acetate
No treatment	9.3±3.8	22.8±16.9	23.0±17.9	27.3±16.7
1 M sorbitol	34.0±14.3	28.3±16.1	32.5±16.3	28.0±15.1

the smut fungus *Ustilago maydis* was described on the basis of the result of expressed sequence tags analysis (17). In addition, the plasmid pSceI-Hyg, containing the hygromycin B selection cassette, which is composed of the *U. maydis* HSP70 promoter and terminator and the *Escherichia coli* hygromycin phosphotransferase gene (*hph*), is expressed in *P. antarctica* CBS 516.83 and *P. flocculosa* DAOM 196992 (7). In order to develop a transformation procedure for strain T-34 using electroporation, we thus used an autonomously replicating plasmid, pUXV1 ATCC 77463 (purchased from American Type Culture Collection, ATCC), for *U. maydis* containing the hygromycin B selection cassette (18).

The best setting of the electric pulse was investigated using the cells grown in YM medium containing 10% glycerol and 10 μg of the plasmid pUXV1 ATCC 77463, using Bio-Rad Gene Pulser II with Pulse Controller Plus (Bio-Rad, Tokyo). The electroporated cells were immediately diluted in 0.5 ml of ice-cold 1 M sorbitol, and an aliquot (0.1 ml) was then spread onto a plate of YM medium containing 10% glycerol, 300 μg/ml hygromycin B and 3% agar. The colonies appearing within 5 d at 28°C were counted to estimate the transformation efficiency. Thirty to eighty of colonies resistant to hygromycin B (5.5 transformants/μg of plasmid DNA) were observed when the cells were pulsed twice with a square-wave electroporation pulse of 5.0 kV/cm and a pulse length of 1.0 ms, at a 5 s pulse interval (Table 1), while pulses of 11.5 kV/cm at a 25 μF setting and 7.5 kV/cm at a 25 μF setting provided 2–5 (0.4 transformants/μg of plasmid DNA) and 5–25 colonies (1.5 transformants/μg of plasmid DNA), respectively. The transformation frequency induced by the present electroporation method was significantly better than that induced by the spheroplast method (4 transformants/μg of plasmid DNA) reported previously (8).

The amount of plasmid DNA was also critical to the efficiency of transformation as described previously (19). The efficiency was then compared using different amounts of plasmid DNA (0.1, 1.0 and 10 μg) in 100 μl of reaction mixture (Table 1). The highest efficiency was obtained with 1.0 μg of plasmid DNA under the conditions employed, and the use of 10 μg of plasmid DNA resulted in a decrease in transformation frequency. Thus, 1.0 μg of plasmid DNA was set as a standard in our procedure.

In electroporation, pretreatment with lithium acetate and/or DTT enhances the transformation efficiency of *S. cerevisiae* (12). We thus evaluated the effect of pretreatment on the transformation efficiency of *P. antarctica* T-34. The cell pellets were collected by centrifugation at 4000×*g*, suspended in the pretreatment solution containing 10 mM Tris–HCl (pH 6.7), 100 mM lithium acetate and/or 10 mM dithiothreitol (DTT), and were then incubated at 30°C for 10 min. The pretreated cells were collected by centrifugation at 4000×*g*, and the pellet was suspended in ice-cold water. This step was repeated twice and the pellet was then suspended in a solution of 1 M ice-cold sorbitol. Finally, the cells were resuspended in 50 μl of 1 M sorbitol, and then used for the transformation. As shown in Table 2, pretreatment with 1 M sorbitol markedly improved the transformation frequency by more than 3.6-fold compared with the case of no treatment; the maximum transformation efficiency was 48 transformants/μg of pUXV1. On the other hand, pretreatment with 10 mM DTT and/or 100 mM lithium acetate decreased the positive effect of 1 M sorbitol. Consequently, the cells of *P. antarctica* T-34 were pretreated only with 1 M sorbitol before the high-intensity electric pulse was applied.

Interestingly, the cells were found to be more tolerant to hygromycin B after the cell suspension was stored at −80°C. This might have been due to an increase in the cell wall strength induced by low-temperature preservation. Consequently, the preservation of the cells at −80°C was unsuitable for the present transformation procedure.

Among the dimorphic yeast strains of the genus *Pseudozyma*, three strains, namely, *P. antarctica* JCM 10317, *P. aphidis* JCM 10318, and *P. rugulosa* JCM 10323, are also MEL producers, as described previously (4). They are genetically important for deciphering the key enzymes involved in the biosynthetic pathway of MELs. The newly developed transformation procedure was thus applied to these three strains. The three strains showed hygromycin B sensitivity, and no cell growth was observed on YM medium containing 300 μg/ml hygromycin B. According to the above protocol, the three strains were transformed with 1 μg of the plasmid pUXV1 ATCC 77463. As a result of the transformation, colonies resistant to hygromycin B were obtained. The transformation efficiencies of *P. antarctica* JCM 10317, *P. aphidis* JCM 10318 and *P. rugulosa* JCM 10323 were 6.6 transformants/μg of plasmid DNA, 1.6 transformants/μg of plasmid DNA and 10.8 transformants/μg of plasmid DNA, respectively.