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An efficient genetic transformation method for glycerol producer *Candida glycerinogenes*

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Summary

Transformation techniques generally require development before genetic and molecular studies of industrial yeast strains can commence. *Candida glycerinogenes* WL2002-5 has been used for industrial-scale glycerol production but has proven difficult to transform for molecular studies following previously published procedures. In the present study, phleomycin was used as drug-resistance marker based on perturbing plasma membranes and the lower minimum inhibitory concentration for *C. glycerinogenes*. We developed an efficient method for transformation of *C. glycerinogenes*, and parameters involved in transformation efficiency were optimized. Pretreatment of yeast cells with either dithiothreitol or lithium acetate enhanced the frequency of transformation markedly by electroporation. Significantly higher transformation efficiency was obtained when the electroporated cells were pretreated with phleomycin before plating. With this method, a maximal transformation frequency of 394 ± 50 transformants/μg plasmid DNA was obtained. The transformation method will foster genetic manipulation of this industrial strain.

Introduction

Candida glycerinogenes WL2002-5, a novel osmotolerant yeast strain isolated from a sugar factory effluent with high osmotic pressure, has been used for the commercial production of glycerol (Zhuge et al. 2001). Compared to other yeasts, *C. glycerinogenes* has several useful properties, such as tolerance to high glucose concentrations, rapid growth and ability to accumulate high extracellular glycerol concentrations. These properties might be due to this yeast possessing several specific genes involved in glycerol

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biosynthesis that differ from those found in other yeasts. Although some physiological and biochemical properties of *C. glycerinogenes* have been studied (Zhuge et al. 2001; Jin et al. 2003) and reviewed (Wang et al. 2001), our knowledge of the genetic and molecular properties lags far behind those of model yeasts such as *S. cerevisiae* because of the lack of effective genetic manipulation tools.

To transform yeast, several methods have been used to introduce exogenous DNA into cells, including the lithium acetate method (Ito et al. 1983; Gietz and Schiestl 2007), spheroplasting (Hinnen et al. 1978) and electroporation (Weaver et al. 1988; De Backer et al. 1999). As for *Candida albicans*, electroporation and the LiAc procedure were used for transformation and efficiency obtained was reliable and sufficient for genetic analyses of *C. albicans* (Kohler et al. 1997; Walther and Wendland 2003). However, in our hands, none of these general procedures yielded reliable transformation efficiency. In this paper, we developed a procedure to transform *C. glycerinogenes* that yielded an efficiency of ~400 transformants/μg for integrative linear DNA. With this electroporation method, genetic and molecular studies of *C. glycerinogenes* become possible.

Materials and methods

Strains, plasmids and cultivation conditions

C. glycerinogenes WL2002-5 (Zhuge et al. 2001) were routinely cultured in YPD (1% yeast extract, 2% peptone, 2% D-glucose). YPD containing 1 M sorbitol and supplemented with 150 μg phleomycin/ml (YPDS; CAYLA, Toulouse, France) was used as a selective medium. 5-FOA (Sigma-Aldrich, USA) supplemented in YPD was used at 2 mg/ml to verify *ura3* mutants. For solid media, 2% agar was added before autoclaving. *Escherichia coli* JM109 (Takara Bio, Shiga, Japan) used for the propagation of plasmids was cultured in low salt LB medium (0.5% yeast extract, 1% peptone, 0.5% NaCl). Plasmid pGAPZb (Invitrogen) imparting resistance to phleomycin was used to construct integrative vector.

DNA manipulations

Standard DNA manipulations were done as described by Sambrook and Russell (2001). For integrative transformation, disruption cassette with *CgURA3* locus was constructed as follows: 0.66 kb *CgURA3* gene (GenBank Accession No. AY623794) was amplified with primers CgUF: 5′-AGATCTATCAGAATCACACACTTCCC and CgUR: 5′-AGATCTTCCGTTCCAGTCTTTA CAAC (*Bgl*II site underlined) by polymerase chain reaction (PCR) using *C. glycerinogenes* genomic DNA as template and cut with *Bgl*II. The purified *CgURA3* was ligated into plasmid pGAPZb previously digested with *Bgl*II, in which phleomycin-resistant marker gene existed. The resulting plasmid was named pGACURA3 for transformation of *C. glycerinogenes*. A unique *Nde*I site in the *CgURA3* gene of the pGACU plasmid facilitated linearization, leaving 460–200 bp of flanking homology region, which is used for targeted integration (shown in Figure 1). Verification of phleomycin-resistant clones was carried out by PCR analysis according to the reported method (Walther and Wendland 2003). Primers Tf: 5′-GCAGCCCAAGAAACAACCAGTG-3′ and Tr: 5′-TTCAATTGATTGAAATAGGGAC-3′ were synthesized based on sequence of *CgURA3* and promoter GAP, respectively. PCR was performed using *C. glycerinogenes* WL2002-5 genomic DNA from the wild type and transformant strains as template with the primers Tf and Tr for 30 cycles at 94 °C for 50 s, 55 °C for 60 s, and at 72 °C for 90 s. The 1.1 kb PCR fragment of phleomycin-resistant gene amplified with C1F: 5′-ATCCCCCACACACCATAGCTT-3′ and C1R: 5′-GTTGGTCTCCAGCTTGCAAAT-3′ primers was used as a probe. Southern blot analysis was performed using the DIG High Prime DNA labeling and Detection Kit I (Boehringer, Mannheim, Germany) according to the supplier's manual.

Electroporation transformation procedure

Cells were grown in YPD medium at 30 °C with vigorous shaking (200 rpm). The OD_{600} was measured at indicated time points, cells were pelleted by centrifugation, resuspended in 25 ml distilled water containing the indicated concentration dithiothreitol (DTT) and/or lithium acetate (LiAc) and incubated at ambient temperature for 1 h with occasional shaking. Subsequent steps similar to those for the transformation of *S. cerevisiae* by electroporation (Becker and Guarente, 1991) were followed with slight modification. The cell suspension was washed twice in 25 ml ice-cold, sterilized water and once with 10 ml ice-cold 1 M sorbitol. The supernatant was carefully removed followed by centrifugation and the pellet was resuspended in 1 ml of 1 M sorbitol at a final cell concentration of approximately 2×10^9 cells/ml. This cell suspension was kept on ice until use. The cell suspension (100 μl) was mixed gently with appropriate purified plasmid DNA and incubated on ice for 5 min. The mixture was transferred to a 0.2 cm gap width electroporation

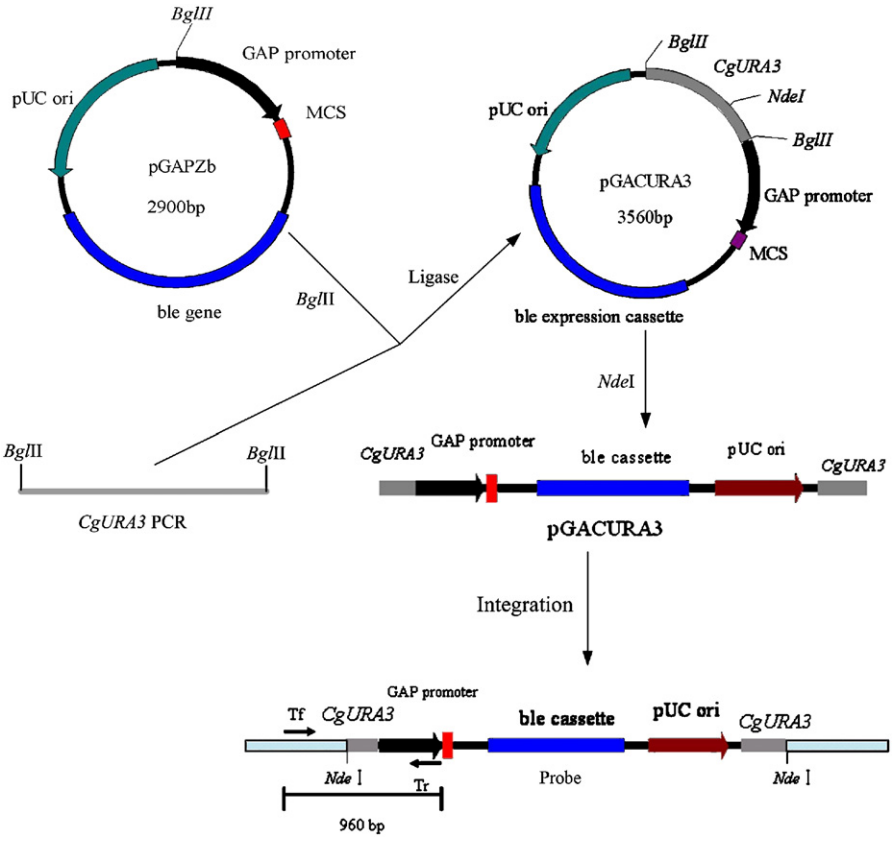

Figure 1. Construction procedure of pGACUA3 plasmid and schematic drawing of the integration with *C. glycerinogenes* chromosome at *CgURA3* site. The *ble* expression cassette endows resistance to phleomycin with TEF1 promoter from *S. cerevisiae* that drives expression of the *Sh ble* gene in *C. glycerinogenes* and EM7 promoter that drives expression of the *Sh ble* gene in *E.coli*. Positions of primers Tf and Tr used for analytical PCR verification are shown. Note that the position of the primer Tf is upstream of the *Nde*I restriction site in the *CgURA3* and Tr is located in the promoter GAP.

cuvette and pulsed at 1600 V, 25 μF and 200 Ω for 5 ms with a Eppendorf Multiporator (Eppendorf, Hamburg, Germany). The cells were immediately diluted in l ml ice-cold 1 M sorbitol and the contents of the cuvette were gently but thoroughly mixed by inversion. The cells were transferred into a sterile 15 ml tube, incubated without shaking at 30 °C for 1 h and then plated onto YPDS. Alternatively, the electroporated cells of *C. glycerinogenes* were added to 3 ml of YPD broth, incubated at 30 °C for 2 h, and then phleomycin (150 μg/ml) was added and incubated with shaking at 150 rpm for 3 h. Cells were harvested by centrifugation and spread onto YPDS plates and incubated at 30 °C.

Results and discussion

Antibiotic sensitivity of *C. glycerinogenes*

Yeasts isolated from nature are often more resistant to many drugs and chemical substances than laboratory strains (Chand-Goyal et al. 1999). We assessed the sensitivity of *C. glycerinogenes* to phleomycin (phleomycin), G418 (geneticin) and hygromycin B. Suspensions were prepared from log-phase growing cells and plated at a concentration of 10^6/ml on YPD plates containing different concentrations of phleomycin, G418 (Amresco, Solon, OH, USA), and hygromycin B (Roche, Basel, Switzerland). Phleomycin (100 μg/ml) completely inhibited growth, whereas some resistant colonies appeared on plates containing 500 μg/mL of G418 and hygromycin B. Furthermore, growth of *C. glycerinogenes* was also inhibited in liquid cultures containing 50 μg/ml phleomycin. Therefore, 150 μg/ml phleomycin was selected as a suitable concentration for screening of plasmids conferring resistance in transformants.

Improvement of transformation efficiency by treatment with dithiotreitol or lithium acetate

A voltage of 1600 V (200 Ω and 25 μF) yielded more transformants/μg DNA than any other pulse

when applied for 5 ms (data not shown). So, this pulse value was used for next investigation. In the presence of DTT or LiAc the transformation efficiency by electroporation of various yeast species including *S. cerevisiae* (Meilhoc et al. 1990; Thompson et al. 1998), *Kluyveromyces lactis* (Sanchez et al. 1993), *C. albicans* (De Backer et al. 1999), *Hansenula polymorpha* (Faber et al. 1994) and *Schizosaccharomyces pombe* (Suga and Hatakeyama 2001) has been improved. Therefore, the effect of DTT and LiAc on the transformation efficiency of *C. glycerinogenes* was investigated.

An optimal efficiency was obtained when cells were pretreated with 25 mM DTT and 100 mM LiAc (Table 1). These concentrations were higher than those reported for other yeasts (Thompson et al. 1998; De Backer et al. 1999; Pribylova and Sychrova 2003). However, a combination of DTT and LiAc at the optimal concentrations reduced transformation efficiency. On the other hand, Thompson et al. (1998) reported that pretreatment with DTT/LiAc for *S. cerevisiae* improved the transformation frequency more than treatment with either component alone. DTT and LiAc probably enhance pore formation and render the cell wall more permeable and the subsequent uptake of DNA by directly affecting cell wall structure.

Effect of growth phase on transformation efficiency

To estimate the effect of the age of cells on the transformation efficiency, *C. glycerinogenes* cultured to mid-log, late-log and stationary phases were prepared for electroporation. When cells in mid-log phase were collected for preparation of competent cells, higher transformation efficiency (170 ± 15 transformants/μg DNA) was obtained than late-log (119 ± 21 transformants/μg DNA) and stationary-phase (98 ± 12 transformants/μg DNA) cells. In comparison with the effect of electric voltage and pretreatment procedures, growth phase appears to have a relatively minor effect on transformation efficiency.

Effect of cell and plasmid concentrations on transformation efficiency

The effect of cell concentration of *C. glycerinogenes* and linearized plasmid concentration on transformation efficiency revealed that efficiency increased significantly in proportion to cell concentration up to 2×10^9/ml (Table 2). We found that the optimum amount of DNA at the various cell concentrations was 200 ng of linearized plasmid DNA and the highest transformation efficiency of 180 ± 23 transformants/μg plasmid DNA was obtained. When the plasmid DNA was increased to 1 μg, the transformation efficiency decreased drastically although the total number of transformants was greatest at the highest DNA concentration.

Effect of treatment of the electroporated cells on transformation efficiency

We found that the minimum inhibition concentration differed significantly between liquid culture and solid plate medium. Furthermore, fewer colonies were observed when yeast cells were treated with phleomycin (150 μg/ml) before plating on YPD agar plates (data not shown). After transformation, the cells were cultured in 1 ml of ice-cold 1 M sorbitol for 1 h, and 3 ml of liquid YEPD medium was then added before incubation for 2 h. At last, phleomycin (150 μg/ml) was added to the transformants and incubated for 3 h. The results

Table 1. Effect of DTT or LiAc concentration on transformation efficiency

Pretreatment condition	Concentration (mM)	Transformants/μg DNA	Fold enhancement
None		69 ± 20	1.0
DTT	10	99 ± 18	1.4
	25	145 ± 42	2.1
	50	123 ± 21	1.8
LiAc	25	77 ± 21	1.1
	50	89 ± 15	1.3
	100	136 ± 33	1.9
	150	64 ± 27	0.9
DTT+ LiAc	25+100	33 ± 10	0.48

Cells of *C. glycerinogenes* were pretreated with DTT or LiAc at room temperature with shaking occasionally for 1 h. 0.1 μg linearized DNA was added to 100 μl cell and mixed. The results are the mean ± standard deviation of three independent experiments.

Table 2. Effect of cell concentration and amount of plasmid DNA on transformation efficiency

Cell concentration/ml	Transformants/μg DNA				
	DNA amount	50 ng	100 ng	200 ng	1 μg
8×10^7		27±8	28±11	54±20	21±10
2×10^8		42±12	64±20	76±18	38±9
8×10^8		69±15	121±24	138±11	56±15
2×10^9		72±9	154±30	180±23	97±24

Cells of *C. glycerinogenes* were collected and pretreated with 25 mM DTT at room temperature for 1 h. A series of cell concentrations was diluted with 1 M sorbitol before electroporation. The various amounts of DNA was added to 100 μl cell suspension. The results are the mean±standard deviation of three independent experiments.

Table 3. Enhancement of transformation efficiency of *C. glycerinogenes* by treatment of electroporated cells with 150 μg/ml phleomycin

Strains	Transformants/μg DNA			Fold increase
	Plasmid/restriction	NT	ET	
C. glycerinogenes	pGACU/ *Nde*I	160±24	394±50	2.5

Cells were collected, 200 ng of DNA was added to 100 μl cell suspension and electric pulse was held constant at 1600 V for 5 ms. phleomycin (150 μg/ml) was added to electroporated cells as described in the Materials and methods. The results are the mean±standard deviation of three independent experiments.

showed that when cells were treated with phleomycin after electroporation, the transformation efficiency increased markedly (Table 3). This procedure was previously found to be effective for the transformation of *Candida rugosa* (Tang et al. 2003) and indicates it may be effective to transform other yeast species by electroporation. However, it should be noted that this procedure added additional steps making it rather time-consuming.

Integration of plasmid DNA into the genome of *C. glycerinogenes*

The integration of the phleomycin-resistance gene into the genome of transformants was verified by PCR and Southern blot (Figure 2). If the disruption cassette was integrated at the targeted locus, the 0.96 kb fragment corresponding from Tf to Tr should be amplified by PCR (Figure 1). As indicated in Figure 2A, diagnostic PCR with primers Tf and Tr using genomic DNA of phleomycin-resistance transformants as template showed that 0.96 kb product was amplified, whereas no product was found using untransformed *C. glycerinogenes* genomic DNA (Figure 2a). Southern blot revealed that eight of nine colonies had only one band and only one clone had two bands, which indicated that integration of only a single copy of the phleomycin-resistance gene occurred in most instances (Figure 2b). Untransformed *C. glycerinogenes* yielded no bands. Fewer transformants were observed for both transformation methods described above when the circular integrative plasmid was used for transformation (data not shown). Previous studied indicated it is diploid (our unpublished data), so 20 transformants were restreaked on 5-FOA medium and only 25% transformants tested grew normally, which indicated that fewer transformants were *ura3* disruption mutant; however, it should allow complementation be the reuse of the cloned *URA3*, we have no other suitable marker and plasmid for use. This is a critical point for our next investigation. Circular integrative vectors have previously been reported to result in lower transformation efficiency than that of linear integrative form for most yeasts (Gietz and Schiestl, 1994). In comparison with circular vectors which resulted in 28 transformants/μg DNA, a significant increase of transformation efficiency was observed in *C. parapsilosis*, which gave 229 transformants/μg DNA (Nosek et al. 2002). Moreover, *C. oleophila* and *Pfaffia rhodozyma* yielded no transformants with a circular integrative vector (Wery et al. 1997; Yehuda et al. 2001). A notable increase of transformation efficiencies with linearized integrative vectors was also observed in *S. pombe* (Keeney and Boeke 1994) and *Y. lipolytica* (Davidow et al. 1985).

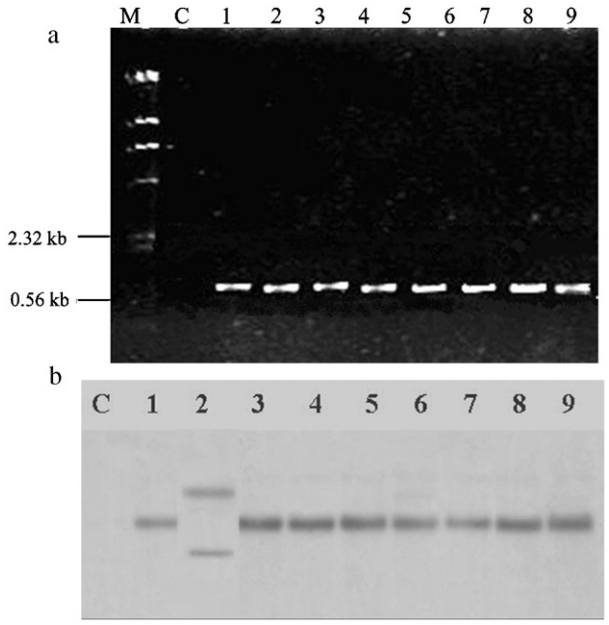

Figure 2. Diagnostic PCR and Southern blot on phleomycin-resistance transformants for confirmation of the integration of pGACU into *C. glycerinogenes* genome. (a) Random transformants was identified by PCR with primers of Tf and Tr using colonies genomic DNA and *C. glycerinogenes* genomic DNA as template. Lanes 1–9, phleomycin-resistance transformants; C, *C. glycerinogenes*; M, λ DNA/*Hin*d □ markers. (b) Genomic DNA from random transformants were digested with *Pst*I, without a digestion site in pGACU and the released fragments were separated by electrophoresis in 1% agarose and transferred to a Nylon membrane. A 1.1 kb PCR probe fragment was amplified with primers of C1F and C1R using pGAPZb as template was labelled with digoxigenin (DIG). C, wild type *C. glycerinogenes*; lanes 1–9, phleomycin-resistant transformants.

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