university of groningen

University Medical Center Groningen

University of Groningen

Highly-efficient electrotransformation of the yeast Hansenula polymorpha

Faber, Klaas Nico; Haima, Peter; Harder, Wim; Veenhuis, Marten; AB, Geert

Published in:
Current Genetics

DOI:
10.1007/BF00351482

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1994

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Faber, K. N., Haima, P., Harder, W., Veenhuis, M., & AB, G. (1994). Highly-efficient electrotransformation of the yeast Hansenula polymorpha. *Current Genetics*, *25*(4). https://doi.org/10.1007/BF00351482

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Download date: 30-10-2020

Curr Genet (1994) 25: 305–310

Current Genetics

© Springer-Verlag 1994

Highly-efficient electrotransformation of the yeast *Hansenula polymorpha*

Klaas Nico Faber[1], Peter Haima[1]*, Wim Harder[1], Marten Veenhuis[1], Geert AB[2]

[1] Laboratory for Electron Microscopy, Biological Centre, University of Groningen, Kerklaan 30, NL-9751 NN Haren, The Netherlands
[2] Department of Biochemistry, University of Groningen, Nijenborgh NL-9747 AG Groningen, The Netherlands

Received: 13 August 1993

Abstract. A highly-efficient method for transformation of the methylotrophic yeast *Hansenula polymorpha* has been developed. Routinely, transformation frequencies of up to 1.7×10^6/µg plasmid DNA were obtained by applying an electric pulse of the exponential decay type of 7.5 kV/cm to a highly-concentrated cell mixture during 5 ms. Efficient transformation was dependent on: (1) pre-treatment of the cells with the reducing agent dithiotreitol, (2) the use of sucrose as an osmotic stabilizer in an ionic electroporation buffer, and (3) the use of cells grown to the mid-logarithmic phase. Important parameters for optimizing the transformation frequencies were field strength, pulse duration, and cell concentration during the electric pulse. In contrast to electrotransformation protocols described for *Saccharomyces cerevisiae* and *Candida maltosa*, transformation frequencies (transformants per µg DNA) for *H. polymorpha* remained high when large amounts (up to 10 µg) of plasmid DNA were added. This feature renders this procedure pre-eminently advantageous for gene cloning experiments when high numbers of transformants are needed.

Key words: Transformation – Electroporation – Methylotrophic yeasts – *Hansenula polymorpha* – *Pichia methanolica*

Introduction

Methanol utilization in the methylotrophic yeast *Hansenula polymorpha* is accompanied by strong proliferation of peroxisomes and high-level expression of the peroxisomal matrix enzymes, alcohol oxidase (AOX) and dihydroxyaceton synthase (DAS). As a result of these features the organism is of considerable academic and commercial interest. Firstly, peroxisome biogenesis is strictly regulated by the growth conditions applied and thus can be easily manipulated (for a recent review, see Veenhuis 1992). Secondly, the strong endogenous promoters can be used to control high-level expression of heterologous genes (reviewed by Buckholz and Gleeson 1991). Although basic genetic procedures have been established for *H. polymorpha* (Gleeson et al. 1986, 1988; Roggenkamp et al. 1986; Thikhomirova et al. 1986; Berardi and Thomas 1990; Sierkstra et al. 1991; Faber et al. 1992) a very efficient transformation procedure is still lacking. We have encountered this limitation during functional gene library complementation of mutants of *H. polymorpha* which are impaired in peroxisome biogenesis and function (Cregg et al. 1990; Waterham et al. 1992; Titorenko et al. 1992, 1993). Therefore, we have investigated whether *H. polymorpha* can be transformed by electroporation. Recently, this technique has been successfully applied to introduce foreign DNA into several yeast species, including *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Yarrowia lipolytica*, and several *Candida* species (Meilhoc et al. 1990; Becker et al. 1991; Kasüske et al. 1992; Rohrer and Picataggio 1992; Nuttley et al. 1993). The advantage of electrotransformation is that it combines simplicity with high transformation frequencies. In this paper we describe an electrotransformation procedure optimized for the yeast *H. polymorpha*. This protocol gave rise to 1.7×10^6 transformants per µg plasmid DNA, which is a 300-fold increase of transformation frequency compared to our previously-described procedure (Faber et al. 1992). Moreover, the present procedure appeared to be applicable for introducing plasmid DNA into another methylotrophic yeast, *Pichia methanolica*.

Materials and methods

Strains and plasmids. H. polymorpha strains A16 (Veale et al. 1992), *leu1.1* (Gleeson et al. 1986), *leu2-356* (Thikhomirova et al. 1986) and LR9 (Roggenkamp et al. 1986) were used for transformation with the autonomous replicating plasmids pHIP6 (see Fig. 1),

Present address: Nichols Institute Diagnostics, Nieuwe Weg 172, 6603 BT Wijchen, The Netherlands

Correspondence to: G. AB

pHIP3, pHRP2 (Faber et al. 1992) and pHARS1 (Roggenkamp et al. 1986). *P. methanolica* C512 (*leu⁻his⁻*) was transformed with pHIP6.

Growth conditions. H. polymorpha strains and *P. methanolica* were grown in YPD medium (1% Yeast extract, 1% Bacto-peptone, 1% glucose) at 37° C and 30° C, respectively. Selective medium (YND) contained 0.67% Bacto yeast nitrogen base without amino acids (DIFCO) and 1% glucose and was supplemented with 30 mg/l histidine when necessary.

Standard molecular methods. Plasmid DNA from *H. polymorpha* for re-transformation of *E. coli* DH1 (*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*) was isolated as described by Hoffman and Winston (1987). All recombinant DNA manipulations were performed using standard methods according to Sambrook et al. (1989). Restriction endonucleases, T4 DNA ligase, and Klenow enzyme were obtained from Boehringer Mannheim and used according to supplier's recommendations.

Electric pulse equipment. Electropulsing was carried out with the Electro Cell Manipulator 600 (ECM600) electroporation system from Biotechnologies and Experimental Research Inc. (BTX), using 2-mm cuvets. The electroporation method was optimized by varying the electric field strength in combination with different internal timing resistances (resulting in varying pulse lengths) at a fixed capacitance of 50 μF. Pulse field strength can be varied between 0 and 2.5 kV (0–12.5 kV/cm using 2-mm cuvets) and the internal timing resistance of the electroporator can be adjusted at ten values, R1–R10, ranging from 13 Ω to 1 048 Ω.

Standard electrotransformation procedure. Overnight cultures of *H. polymorpha* grown in non-selective YPD medium at 37° C, were diluted 100-fold in fresh, prewarmed, YPD medium (200 ml) and grown to an optical density at 663 nm (OD_{663}) of 1.2–1.5 (± 9×10^7 cells/ml). Cells were harvested by centrifugation at 3000 g for 10 min, resuspended in 0.2 vol (40 ml) of 50 mM potassium phosphate buffer pH 7.5 containing 25 mM of dithiotreitol (DTT) and incubated for 15 min at 37° C. Subsequently, the cells were washed twice with electroporation buffer STM (=270 mM sucrose, 10 mM Tris-HCl pH 7.5 and 1 mM $MgCl_2$); firstly with 1 vol (=200 ml), secondly with 0.5 vol (=100 ml) and kept at 0° C. Finally, the cells were resuspended in 0.005 vol (=1 ml) STM (0° C) to give approximately 2×10^{10} cells/ml. For long term storage of competent cells, batches of 60-μl cell suspensions were directly frozen in liquid N_2 and kept at –80° C. DNA was added to 60 μl of cell suspension and this mixture was tapped to the bottom of a prechilled 2-mm electroporation cuvet. Following the electric field pulse (7.5 kV/cm; 50 μF; 129 Ω resulting in pulse length of ±5 ms.), 1 ml of YPD medium (room temperature) was added to the cell/DNA mixture. The cell suspension was then incubated for 1 h at 37° C without agitation. Cells were harvested (5 min, 3000 g), washed once with 1 ml YND and subsequently resuspended (and diluted) in YND, spread on selective plates (YND) and incubated at 37° C. Transformants were quantified after 3–4 days.

Results

Prerequisities for electrotransformation of H. polymorpha

We have based our protocol on two electrotransformation procedures previously described for *S. cerevisiae* (Meilhoc et al. 1990; Becker et al. 1991). Application of these procedures to *H. polymorpha* revealed that pretreatment of the cells with DTT, in order to reduce cell wall proteins, is essential for transformation. Omission of this step resulted in no transformants at all. At a fixed incubation time of 15 min at 37° C, transformation frequencies increased linearly with the DTT concentration up to 25 mM, whereas higher concentrations of up to and including 40 mM gave no further improvement. The composition of the washing- and electroporation-buffer appeared to be crucial. Acceptable transformation frequencies were not observed when cells were washed and electroporated in sorbitol (1 M or 3 M) or glycerol (10% or 20%). However, high transformation frequencies were achieved using ionic electroporation buffers containing sucrose as the osmotic stabilizer (Meilhoc et al. 1990). Within the range of 0–540 mM tested, 270 mM of sucrose proved to yield optimal frequencies. Although pretreatment and washing of the cells was mainly according to Meilhoc et al. (1990), the characteristics of the pulse applied differed. In our experiments a pulse of the exponential decay type was utilized (Becker et al. 1991), whereas Meilhoc et al. (1990) used square-wave-type pulses.

Effect of field strength and pulse length on transformation efficiency

After having established the basic protocol, the effect of increasing field strengths at several time constants (pulse lengths) on transformation efficiency was examined using 1 μg of plasmid DNA (pHIP6, Fig. 1). The pulse lengths were varied by changing the timing resistance (R3 = 48 Ω; R5 = 129 Ω and R7 = 248 Ω) at a constant capacitance (50 μF). Under these conditions the resistance R3, R5 and R7 resulted in pulse durations of 2.2, 4.9 and 9.0 ms, respectively. At each voltage, one or more pulse durations were evaluated. As can be seen in Fig. 2, the total number of

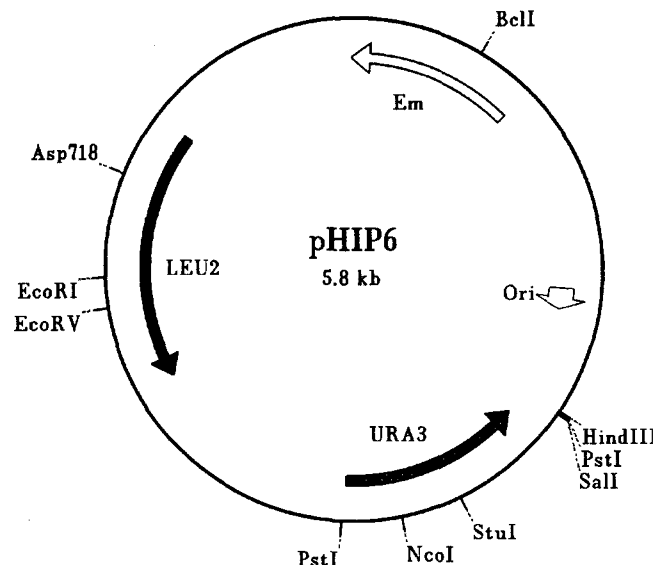

Fig. 1. Physical map of the *E. coli–H. polymorpha* shuttle vector pHIP6. The plasmid was constructed by inserting a 1.1-kb *Bam*HI fragment (sticky ends were filled-in) from YDp-U (Berben et al. 1991), containing the *S. cervisiae URA3* gene, into *Nco*I-*Bam*HI-cleaved (sticky ends were filled-in) pHIP3 (4.6 kb) (Faber et al. 1992). pHIP6 contains both the *URA3* and the *LEU2* gene from *S. cerevisiae* which are used as selectable markers in *H. polymorpha*. Moreover, the *S. cerevisiae LEU2* gene contains a sequence which promotes autonomous replication of plasmids in *H. polymorpha* (Berardi and Thomas 1990; Faber et al. 1992)

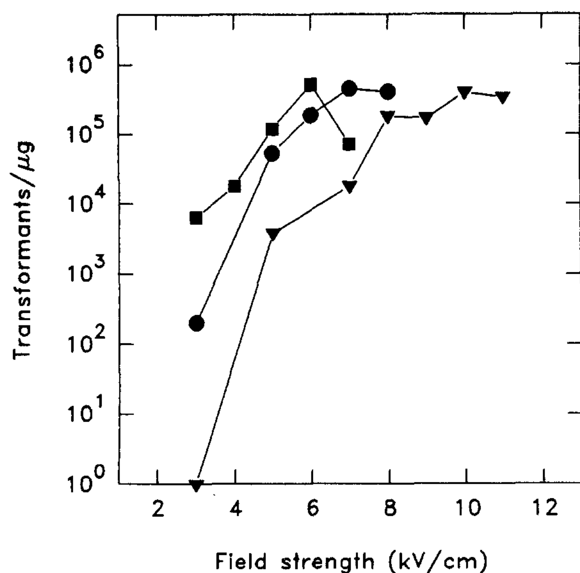

Fig. 2. Effect of field strength and pulse duration on transformation frequency of *H. polymorpha* A16. Cells were grown and treated prior to the electric pulse as described in Materials and methods. One microgram of pHIP6 was used for each transformation. Different pulse durations of approximately 2.2 (▼), 4.9 (●) and 9.0 ms (■) were obtained by adjusting the internal timing resistance to 48, 129 and 246 Ω, respectively

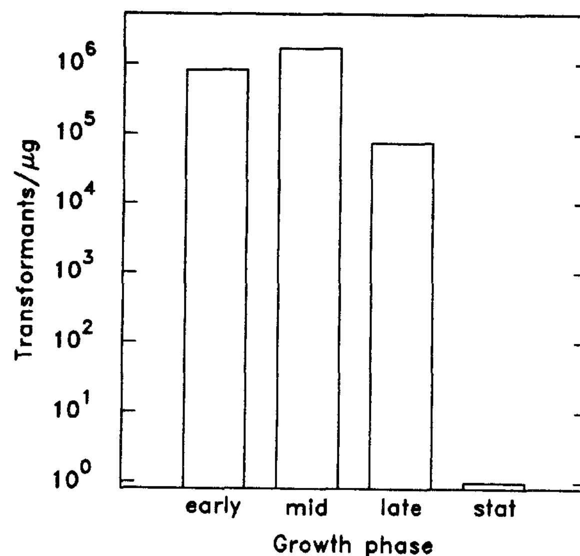

Fig. 3. Effect of growth phase on transformation frequency of *H. polymorpha* A16. Equal numbers of cells grown to the early- (OD_{663} = 0.6); mid- (OD_{663}= 1.2–2.5) or the late- (OD_{663} = 5.5) logarithmic phase, as well as cells from the stationary phase (OD_{663} = 9.0), were harvested and prepared as described in Materials and methods. One microgram of pHIP3 was added to 60 µl of cell suspension ($\pm 3 \times 10^{10}$ cell/ml). Pulse conditions, recovery, and plating of cells were as described in Materials and methods

transformants increased with increasing field strength, reaching a maximum of about 5×10^5 independent of the pulse length used. A drop in transformation frequency at higher field strength at a specific pulse length, as described for *S. cerevisiae* (Meilhoc et al. 1990), was not clearly seen, at least not within the range of adjusted field strengths applied, which was kept limited to avoid arching the electroporation cuvet.

Transformation efficiency is dependent on the growth phase of the cells

The effect of the growth phase of batch-cultured cells on the transformation frequency was studied. Cells were harvested from cultures grown to the early-, mid- and late-logarithmic growth phase or from the stationary phase of growth. Prior to electrotransformation, the cells were concentrated by centrifugation and resuspended in electroporation buffer to achieve equal cell densities (3×10^{10} cells/ml). The cells were then transformed with 1 µg of pHIP3. Maximum transformation frequencies were achieved with cells harvested at the mid-logarithmic growth phase (1.7×10^6), whereas no transformants were observed using cells from the stationary phase of growth (Fig. 3).

Effect of cell concentration and DNA concentration on transformation efficiency

The effect of the cell concentration on the transformation frequency was determined. Cells were harvested, concentrated in electroporation buffer to cell densities ranging from 1.8×10^8 to 3×10^{10} cells/ml, and transformed with 1 µg pHIP6. The total number of transformants increased with increasing cell concentration (Fig. 4). Surprisingly, the yield of transformants per µg plasmid DNA increased more than proportional with the cell density, suggesting that the transformation is more efficient in highly-concentrated cell mixtures. Moreover, no saturation at very-high cell concentrations, as has been described for *S. cerevisiae* (Meilhoc et al. 1990), was observed.

In the previously reported DMSO-freezing-PEG procedure (Faber et al. 1992), the total number of transformants increased linearly up to 10 µg of added plasmid DNA (pHIP3). In addition, linearisation of the plasmids prior to transformation increased transformation frequency up to 15-fold. In order to determine whether this also holds for the present transformation procedure, plasmid pHIP3, either of the covalently closed circular form or of the linearized form after *Bam*HI digestion, was used for transforming *H. polymorpha* A16 (Fig. 5). As before, no saturation of the total number of transformants was reached within the range of plasmid DNA added (up to 10 µg); also linear plasmids were more efficient in transforming *H. polymorpha* than their circular counterparts, giving rise to 3.4×10^7 transformants/10 µg of (linear) pHIP3. Surprisingly, the stimulatory effect of linearization was most evident at high amounts of plasmid DNA and ranged from a 2-fold increase with 1 ng pHIP3 to a 25-fold increase at an input of 10 µg pHIP3.

Efficiency of transformation is strain dependent

Previously-reported transformation procedures for *H. polymorpha* (Gleeson et al. 1986; Roggenkamp et al. 1986;

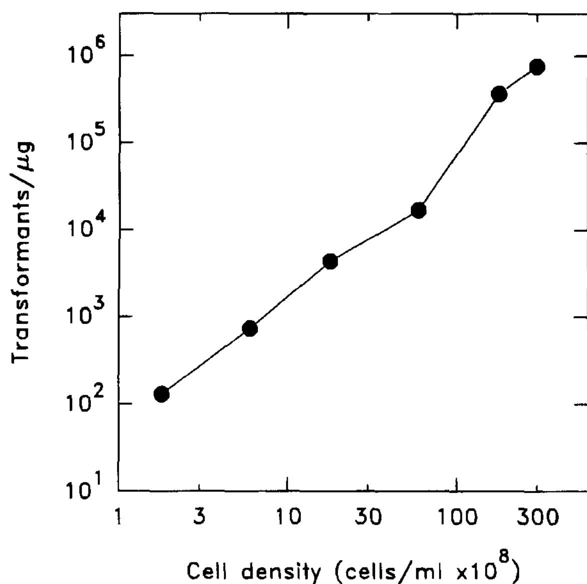

Fig. 4. Effect of cell concentration on transformation frequency. Results are displayed for strain A16 at a fixed sample volume of 60 µl. Pulse conditions and treatment of cells were as described in Materials and methods. Per transformation 1 µg of pHIP6 was used

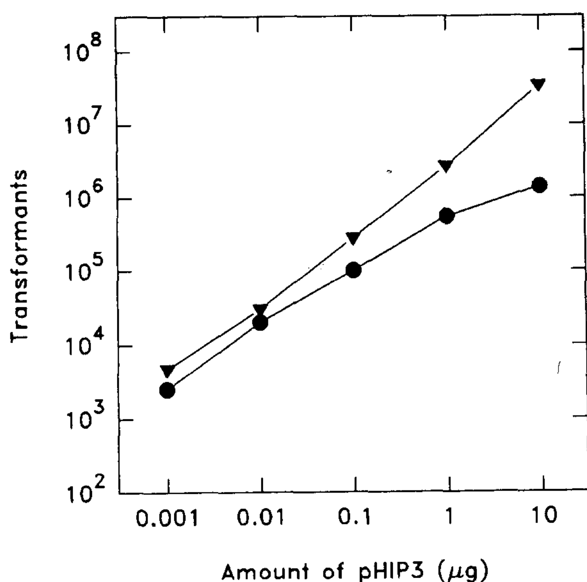

Fig. 5. Effect of amount of plasmid DNA, either of the covalently closed circular (●) or linear conformation (▼), on total number of transformants of *H. polymorpha* A16. Various amounts of pHIP3 were added to 60 µl of cell suspension, ranging from 1 ng to 10 µg. Pulse conditions and cell treatment were as described in Materials and methods

Berardi and Thomas 1990; Dohmen et al. 1991; Faber et al. 1992) were usually developed for one specific strain of the organism. These procedures, however, often appear to be strongly strain-dependent and yield much lower transformation efficiencies when other strains are used. Therefore, we have compared the transformation efficiencies of our present method, for several common strains of *H. polymorpha*. Transformation frequency was determined with two different plasmids for each strain (Table 1). It appeared

Table 1. Total number of transformants obtained per strain per µg plasmid DNA. Pulse conditions and treatment of the cells are as described in Materials and methods

Strain	Plasmid		
	pHIP6	pHRP2	pHARS1
A16	7.5×10^5	2.3×10^5	–
leu1.1	6.0×10^4	3.0×10^4	–
leu2-356	2.9×10^5	7.6×10^4	–
LR9	1.7×10^4	–	5.5×10^4

that the transformation frequency was indeed strain-dependent and varied from 1.7×10^4 per µg of DNA for strain LR9 to 7.5×10^5 per µg of DNA for strain A16 with plasmid pHIP6. Selection was either for ura-prototrophy (LR9) or leu-prototrophy (A16, *leu1.1, leu2-356*). A second plasmid, pHRP2, which contained only one selectable gene, was also used to transform the different strains. For strain A16, *leu1.1* and *leu2-356*, pHRP2 gave about 2-fold lower frequencies, compared to pHIP6. In contrast, pHARS1 transformed strain LR9 at least 3-fold more efficiently then pHIP6. From each of the eight transformation events summarized in Table 1, several transformants were selected, plasmid DNA isolated and re-transformed to *E. coli* DH1. With several restriction enzymes, all the plasmids isolated from *E. coli* gave the same restriction pattern, as the original plasmid introduced into the *H. polymorpha* strain (data not shown). This indicates that no major rearrangements had occurred either during the transformation procedure or during maintenance of the plasmids in the different *H. polymorpha* strains.

As described for other yeasts, competent cells of *H. polymorpha* can be stored at –80°C. Although transformation frequencies using re-thawed cells dropped 2–5-fold, this is only a minor disadvantage in cases of routine transformation.

Transformation of P. methanolica

We have also investigated whether the procedure developed for *H. polymorpha* was applicable for transforming another methylotrophic yeast, *P. methanolica*. The same plasmid, pHIP6, could be used for transformation because the 2.2-kb *Xho*I-*Sal*I DNA fragment containing the *LEU2* gene from *S. cerevisiae* complements the *leu1* mutation of *P. methanolica* and contains an element which supports autonomous replication in this yeast (Tarutina and Tolstorukov 1992), as described for *H. polymorpha*. Cells were grown and treated prior to the electric pulse as described for *H. polymorpha*, except that growth and recovery of the cells were performed at 30°C. As shown in Fig. 6, 3.3×10^3 transformants were obtained per µg of pHIP6 at a field strength of 3 kV/cm and a pulse duration of 5 ms. Increase of the field strength resulted in a decrease of the number of transformants. Apparently, *P. methanolica* cells are more sensitive to electric pulses than are those of *H. polymorpha*. Probably, transformation frequencies may be further optimized by applying a broader range of pulse dura-

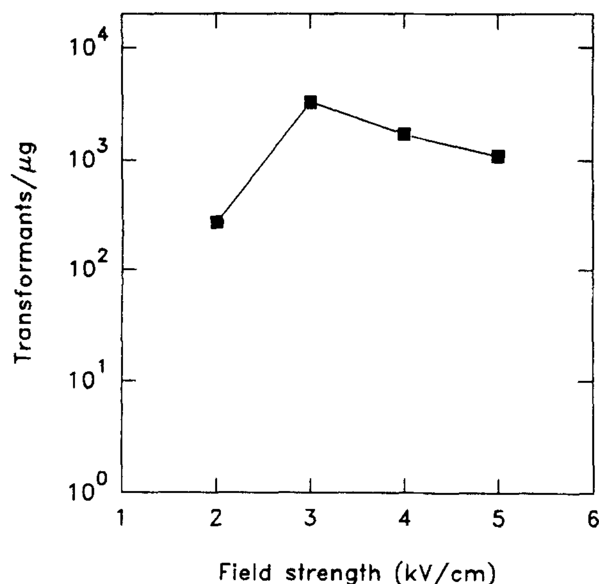

Fig. 6. Effect of field strength on transformation frequency of *P. methanolica* C512. Cells were grown in YPD at 30 °C and pretreated as described for *H. polymorpha* in Materials and methods. One microgram of pHIP6 was added to 60 µl of cell suspension and transferred to a prechilled 2-mm cuvet. The settings for the electric pulse were as described in Materials and methods, varying the field strength from 0.4 kV to 1.0 kV. After the pulse, 1 ml of YPD was added and cells were incubated for 1 h at 30 °C. Subsequently, the cells were washed with 2 ml of YND and plated on selective plates. Transformants were quantified after approximately 5 days of incubation at 30 °C

tions and varying the pretreatment of the cells prior to the pulse.

Discussion

In this study we describe a relatively-simple, highly-efficient electroporation procedure for transforming *H. polymorpha* with plasmid DNA. This procedure, applying a 5 ms pulse at a field strength of 7.5 kV/cm, yields transformation efficiencies of up to 1.7×10^6 transformants per µg of plasmid DNA. Essentially the same protocol was also successfully used to transform another methylotrophic yeast, *P. methanolica*, but yielded optimal transformation frequencies at a significantly lower field strength.

Our results, together with those of Meilhoc et al. (1990), suggest that the procedure is applicable for a broad range of yeast species and does not depend on the application of square-wave-type electric pulses. Prerequisities for efficient electrotransformation of *H. polymorpha* were pretreatment of the cells with the reducing agent DTT and use of an ionic electroporation buffer with sucrose as the osmotic stabilizer. Clear differences in transformation frequencies were observed upon transformation of various *H. polymorpha* strains with different plasmids. A maximum of 1.7×10^6 transformants was obtained when 1 µg of pHIP3 was used with *H. polymorpha* A16 at a cell density of approximately 3×10^{10} cells/ml. However, for each strain, transformation frequencies exceeding 5×10^4/µg were observed. This implies, that the present transformation procedure yields frequencies which are 100-fold higher compared to the values previously described (Gleeson et al. 1986; Roggenkamp et al. 1986; Thikhomirova et al. 1988; Berardi and Thomas 1990; Dohmen et al. 1991; Faber et al. 1992).

Furthermore, this study confirmed an earlier observation (Faber et al. 1992) that linear plasmids transformed *H. polymorpha* more efficiently than circular plasmids. This feature appears to be organism-specific, independent of the transformation procedure used. *H. polymorpha* appears to possess a very efficient system for the repair of double-stranded breaks in DNA molecules because, after transformation with linear plasmids, the transformed DNA was be present as circular autonomous replicating units in the cells (Faber et al. 1992).

In contrast to results obtained with *S. cerevisiae*, no distinct saturation of the total number of transformants was observed in relation to either the cell concentration or the amount of DNA added. For *S. cerevisiae*, it was suggested that only a small subfraction of the cells, probably dependent on the cell size and/or stage in the cell cycle, are electrocompetent. Such a sub-population of competent cells was also supposed to account for the optimum in transformation frequencies observed for *H. polymorpha* with a lithium acetate-based method (Berardi and Thomas 1990). For *S. cerevisiae*, maximal transformation frequencies were observed at an input of 10 ng of plasmid DNA (Meilhoc et al. 1990); a further increase in the amount of plasmid DNA added did not result in enhanced numbers of transformants. It can be calculated that in these experiments a maximum of 1 out of 2500 cells was in fact transformed, apparently encompassing the 'theoretical' pool of electrocompetent cells. In our present study however, a total number of 3.4×10^7 transformed *H. polymorpha* cells was obtained with 10 µg of linear pHIP3. Hence, 1 out of approximately 35 cells was transformed. This suggests that the pool of electrocompetent cells, if such a pool really exists, is significantly larger than supposed for *S. cerevisiae*. The observation that a high number of transformants can be obtained with one single transformation experiment makes our protocol and, with it the yeast *H. polymorpha*, pre-eminently suited for isolating genes by gene library cloning. At present, the procedure has already been successfully used in our laboratory for the isolation of genes which complement mutants impaired in peroxisome biogenesis/function (Cregg et al. 1990; Titorenko et al. 1992, 1993; Waterham et al. 1992) and which are now being analyzed. Moreover, the high transformation efficiency, described in this study, renders these Per^- mutants of *H. polymorpha* potentially attractive for screening gene libraries of higher eukaryotic species to identify functional *PER* homologues of these organisms.

Acknowledgements. We thank Dr. Ilya Tolstorukov (Moscow) for providing the *P. methanolica* strain C512. Klaas Nico Faber was supported by the Netherlands Technology Foundation (STW), which is subsidized by the Netherlands Organization for the Advancement of Scientific Research (NWO). Peter Haima was supported by Unilever Research Laboratories, Vlaardingen.

References

Becker DM, Guarente L (1991) Methods Enzymol 194: 182–187

Berben G, Dumont J, Gilliquet V, Bolle P-A, Hilger F (1991) Yeast 7: 475–477

Berardi E, Thomas DY (1990) Curr Genet 18: 169–170

Buckholz RG, Gleesen MAG (1991) Bio/Technology 9: 1067–1072

Cregg JM, Van der Klei IJ, Sulter GJ, Veenhuis M, Harder W (1990) Yeast 6: 87–97

Dohmen RJ, Strasser AWM, Höner CB, Hollenberg CP (1991) Yeast 7: 691–692

Faber KN, Swaving GJ, Faber F, AB G, Harder W, Veenhuis M, Haima P (1992) J Gen Microbiol 138: 2405–2416

Gleeson MAG, Ortori GS, Sudbery PE (1986) J Gen Microbiol 132: 3459–3465

Gleeson MAG, Sudbery PE (1988) Yeast 4: 293–303

Hoffman CS, Winston F (1987) Gene 57: 267–272

Kasüske A, Wedler H, Schulze S, Becher D (1992) Yeast 8: 691–697

Meilhoc E, Masson J-M, Teissié J (1990) Bio/Technology 8: 223–227

Nuttley WM, Brade AM, Gaillardin C, Eitzen GA, Glover JR, Aitchison JD, Rachubinski RA (1993) Yeast 9: 507–517

Roggenkamp R, Hansen H, Eckart M, Janowicz Z, Hollenberg CP (1986) Mol Gen Genet 202: 302–308

Rohrer TL, Picataggio SK (1992) Appl Microbiol Biotechnol 36: 650–654

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Sierkstra LN, Verbakel JMA, Verrips CT (1991) Curr Genet 19: 81–89

Tarutina M, Tolstorukov I (1992) Yeast 8 (special issue: 16th Int Conf Yeast Genet Mol Biol): 14–60 B

Thikhomirova LP, Iknomova RN, Kuznetsova EN (1986) Curr Genet 10: 741–747

Thikhomirova LP, Iknomova RN, Kuznetsova EN, Fodor II, Bystrykh LV, Aminova LR, Trotsenko YA (1988) J Basic Microbiol 28: 343-351

Titorenko VI, Waterham HR, Haima P, Harder W, Veenhuis M (1992) FEMS Microbiol Lett 95: 143–148

Titorenko VI, Waterham HR, Cregg JM, Harder W, Veenhuis M (1993) Proc Natl Acad Sci USA 90: 7470–7474

Veale RA, Guisseppin MLF, Van Eijk HMJ, Sudbery PE, Verrips CT (1992) Yeast 8: 361–372

Veenhuis M (1992) Cell Bioch Funct 10: 175–184

Waterham HR, Titorenko VI, Van der Klei IJ, Harder W, Veenhuis M (1992) Yeast 8: 961–972

Communicated by C. P. Hollenberg