FEMS Microbiology Letters 99 (1992) 193–198

Published by Elsevier

FEMSLE 05182

Multiple transformation of *Saccharomyces cerevisiae* by protoplast fusion

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Received 3 July 1992
Revision received 21 September 1992
Accepted 22 September 1992

Key words: Transformation; Protoplast fusion; *Saccharomyces*

1. SUMMARY

A technique for the multiple transformation of yeast by protoplast fusion is described. This involved the PEG-induced fusion of protoplasts from cells which had been treated with chromosome-fragmenting agents (in this case cupferron and hydroxylamine) with protoplasts of triply auxotrophic cells. The recovery of transformants was increased significantly if one of the amino acid requirements of the recipient strain was included in the selection medium. Transformants isolated on supplemented media remained auxotrophic for that requirement. Prototrophic, uninucleate transformants had a DNA content and cellular volume similar to that of the parental strains. Possible mechanisms of gene transfer are discussed. This technique offers the possibility of transferring desirable characteristics from one yeast strain to another without altering the ploidy level of the recipient strain.

2. INTRODUCTION

Protoplast fusion has been used as a tool for studying many aspects of the biochemistry and genetics of yeast and fungi [1–6]. Fusion is generally performed between protoplasts of complementary auxotrophic strains and selection is imposed for the isolation of prototrophic hybrids. As an alternative to this procedure we investigated the possibility of transferring genes by protoplast fusion from cells killed by the use of chromosome-fragmenting chemicals to protoplasts of viable auxotrophic cells. The chromosome-fragmenting chemicals employed here were cupferron (*N*-nitrosophenylhydroxylamine) and hydroxylamine, both of which have fungicidal and fungistatic effects on cells of *Saccharomyces cerevisiae* [7]. Hydroxylamine inhibits ribonucleotide reductase in bacterial and mammalian cells by destroying a tyrosyl-free radical at the active site [8] and this induces a deoxyribonucleotide pool

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imbalance which has been proposed to be a signal for the production of an endonuclease that produces double strand breaks in DNA [9]. Cupferron acts in a similar manner and also functions as a chelating agent capable of forming complexes with iron and copper. The chromosome-fragmenting effects of cupferron were discovered using root cells of *Vicia faba* [10], inducing damage to the chromatids [11].

3. MATERIALS AND METHODS

3.1. Yeast strains

The strains of *S. cerevisiae* employed in this work were JJ1A (haploid, Mat a, *arg1, thr1*) and JX6510C (haploid, Mat a, *his4-58, met8-1, ade2-1*). These auxotrophs are completely stable and revertants to prototrophy at any of the loci were not observed during the course of the work described here.

3.2. Media and culture conditions

Cells were routinely grown in 50 ml of YEPD (2% (w/v) glucose (BDH), 2% (w/v) bactopeptone (Difco) and 1% (w/v) yeast extract (Oxoid)) in 100-ml conical flasks at 30°C and 200 rpm in an orbital incubator. Minimal medium (MM) contained 2% (w/v) glucose, 0.17% (w/v) yeast nitrogen base (without amino acids and ammonium sulphate) (Difco) and 0.5% (w/v) ammonium sulphate. Where appropriate, amino acid supplements were added at a final concentration of 20 mg/l. All media were solidified by the use of 2% (w/v) technical agar (Oxoid). Where necessary, media were rendered osmotically stable by the addition of 0.8 M sorbitol (BDH). Culture media were sterilised by autoclaving at a temperature of 121°C and a pressure of 100 kPa for 17 min. Amino acids and chromosome-fragmenting chemicals were filter-sterilised prior to use.

3.3. Protoplast preparation and reversion

Protoplast preparation and reversion conditions were described previously [12]. Two genetically complementary strains were grown to mid-exponential phase in YEPD at 30°C. Strain JJ1A was treated with concentrations of cupferron or hydroxylamine sufficient to reduce the viability to zero [7]. Cells were harvested, washed twice in distilled water and resuspended at a concentration of 3×10^7/ml in protoplasting medium at 30°C for 30 min. The protoplasting medium contained 1.5 mg/ml Novozym 234 (Novo Industri, Denmark), 0.017 ml/ml Suc d'*Helix pomatia* (IBF Biotechnics, France) and 0.8 M sorbitol. Protoplasts of cells not treated with cupferron or hydroxylamine could be induced to revert to the cellular state by embedding in molten (48°C) osmotically stabilised media.

3.4. Protoplast fusion

The fusion of protoplasts of the complementary auxotrophic strains was achieved by resuspending 1×10^7 protoplasts of each strain in 1 ml 40% (w/v) polyethylene glycol (PEG) 3350 (Sigma) and 0.1 ml 1.0 M calcium chloride and allowing to stand for 6 min at room temperature. The suspension was subsequently diluted with 5 ml of 0.8 M sorbitol. After a further 6 min, protoplasts were harvested, washed and resuspended in 0.8 M sorbitol prior to being plated onto selection media at a density of 1×10^6 complementary protoplast pairs per plate. The transformation frequency was based upon the number of transformants obtained per fusion.

3.5. DNA estimation

DNA was extracted from mid-exponential phase cells with perchloric acid and quantified using the diphenylamine assay [13]. Calf thymus DNA (Sigma) was used to prepare standard curves.

3.6. Mitotic segregation of fusants

The hybrid strains were induced to undergo mitotic segregation by growing for 4 days in GLM (2% (w/v) glucose, 0.5% (w/v) yeast extract and 0.2% (w/v) magnesium sulphate) supplemented with 500 mg/ml *p*-fluorophenylalanine. Treated cells were subsequently washed and plated onto YEPD. Segregants could be detected by their inability to grow on MM or, if selected on MM supplemented with an amino acid, the original selection medium.

3.7. Determination of yeast cell volume

The cell volumes of stationary phase parental and fusant strains were determined using the equation for the volume of a prolate ellipsoid:

$$V = 1.33\pi ab^2$$

where a is equal to half the length of the long axis and b is equal to half the length of the short axis. In all cases the dimensions of 50 cells were measured.

4. RESULTS

4.1. Toxicity of cupferron and hydroxylamine

Previous work from this laboratory demonstrated the fungistatic and fungicidal effects of cupferron and hydroxylamine on cells of *S. cerevisiae* [7]. In order to determine the concentration and exposure times necessary to kill all cells in a culture, kill curves were prepared using exponential phase cells of strain JJ1A. Cells were harvested, washed and resuspended at a density of 5×10^7/ml in YEPD supplemented with different concentrations of the two DNA-fragmenting chemicals for various periods of time. Over the course of the incubation samples were withdrawn, diluted and plated onto YEPD to enable the percentage viability to be ascertained. Results (not shown) from these trials established that treatment of cells with a concentration of 7.4×10^{-2} M cupferron or 1.0 M hydroxylamine for 3.5 h was sufficient to reduce culture viability to zero.

4.2. Construction of transformed strains

Cells of strain JJ1A were killed by treatment with cupferron of hydroxylamine as described above. Treated cells of JJ1A and non-treated cells of JX651OC were harvested, washed and converted to protoplasts (Section 3.3). Protoplast fusion was achieved using the PEG-$CaCl_2$ fusogen (Section 3.4) and hybrids were selected by embedding protoplasts in MM or MM plus one of the auxotrophic requirements of JX651OC, the recipient strain. The results (Table 1) indicate that in the fusions where JJ1A was treated with hydroxylamine or cupferron a higher number of transformants was obtained when the selection media contained one of the amino acid requirements of strain JX651OC. This may be attributable to the fact that for complementation to occur on MM plus a single amino acid only two genetic sequences must be transferred from JJ1A to JX651OC, whereas on unsupplemented MM all three genes must be present in the transformant. When, for example, MM + ade was used as the selection medium, the transformant yield was almost 600 times greater than that achieved on MM alone when hydroxylamine-treated JJ1A was used in the fusion as the donor strain. In the case of cupferron-treated JJ1A, the transformant yield on MM + ade was approximately 90 times greater than that achieved on MM alone. Similar differences were observed with the other media supplemented with amino acids. As is apparent from Table 1, treatment of JJ1A with chromosome-fragmenting chemicals leads to a lower yield of transformants than when untreated cells are used in a fusion and selection of transformants is performed on MM. No transformants were observed

Table 1

Yield of transformants from fusions of *S. cerevisiae* JJ1A and JX651OC

Fusant strains	Treatment	Selection medium	Transformation frequency ($\times 10^{-4}$%)
JJ1A + JX651OC	Control	MM	4.60
JJ1A * + JX651OC	Hydroxylamine	MM	0.10
		MM + ade	61.0
		MM + his	1.66
		MM + met	1.38
JJ1A * + JX651OC	Cupferron	MM	0.35
		MM + ade	30.0
		MM + his	2.60
		MM + met	2.40

Protoplasts were induced to fuse using PEG and calcium chloride (Section 3.4). Transformants were selected on MM or MM supplemented with one of the amino acids of the recipient strain (JX651OC). Strain JJ1A was treated with hydroxylamine or cupferron prior to protoplasting (Section 4.1) The transformation frequency is based upon the number of transformants obtained per fusion. An asterisk (*) indicates strain treated with chromosome-fragmenting chemical.

Table 2

Cellular dimensions and DNA content of parental strains and transformants

Strain	Cell dimensions			DNA content ($\mu g/10^9$ cells)
	Length (μm)	Width (μm)	Volume (μm^3)	
JJ1A	6.92	6.08	133.6	7.5 ± 0.7
JX651OC	6.50	6.00	122.2	7.0 ± 0.5
Diploid	8.84	7.52	261.1	13.0 ± 1.0
TR01	6.64	6.12	129.8	7.8 ± 0.9
TR02	6.74	6.00	126.7	8.0 ± 0.5
TR03	6.55	6.00	123.1	8.0 ± 0.6
TR04	6.50	6.10	126.3	8.0 ± 0.6
TR05	6.62	5.90	120.4	8.0 ± 0.5

The dimensions of 50 cells of each strain were determined (Section 3.7). DNA was extracted and quantified as described (Section 3.5). The diploid strain was formed by the fusion of protoplasts of JJ1A and JX651OC and selected on MM. All the transformants were formed by the fusion of cupferron-treated JJ1A protoplasts and JX651OC and selected on MM.

on any of the selection media if PEG and $CaCl_2$ were omitted from the fusion protocol.

4.3. Analysis of transformed strains

Transformed strains that had been selected on MM plus an amino acid remained auxotrophic for that amino acid. Morphological and physiological characters of five prototrophic transformants formed by the fusion of JX651OC and cupferron treated JJ1A and isolated on MM selection medium were examined. Such transformants were selected for analysis since these would contain more genetic information from JJ1A than strains selected on MM plus an amino acid. Cell volumes were measured and compared with those of the parental strains and a known diploid strain formed by the fusion of strains JJ1A and JX651OC. The transformants were uninucleate and similar in volume to the parental strains (Table 2). Transformants with cell volumes greater than those shown here have never been isolated.

Extraction and quantification of DNA from the parentals, the transformants and the diploid strains indicated that the transformants had a DNA content similar to that of the parental strains. This indicates that either the transfer of only small chromosomal fragments, coding for the three nutritional requirements of the recipient strain, had occurred or if larger segments had been transferred this happened by homologous recombination. This observation is important since it indicates that the modified protoplast fusion/transformation procedure avoids diploidisation.

The stability of the transformant strains was confirmed by the inability to induce mitotic segregation of recombinant phenotypes by growing in GLM supplemented with *p*-fluorophenylalanine for 4 days. No segregants were detected after such treatment, which indicated that transferred gene fragments had been integrated into the recipient genome and expressed.

5. DISCUSSION

Intraspecific protoplast fusion between strains of yeasts initially results in the formation of a transient heterokaryon. Subsequently, there are a number of potential products. An unstable heterokaryotic state may persist in the presence of a selection pressure, the absence of which facilitates segregation to the parental genomes [14]. In certain instances the hybrids may contain the entire genome of one parent together with a few chromosomes of the other [15,16]. Under conditions where nuclear complementation is not essential for the survival of the hybrid, loss of one of the haploid nuclei may occur to give a cybrid, a haploid cell with a cytoplasm originating from both parents [1,17]. Alternatively, under stringent selection conditions karyogamy may occur to produce a diploid cell with a mixed cytoplasm.

We have devised a modification of the conventional protoplast fusion procedure in which cells of one of the haploid strains (the donor strain) have been killed by treatment with the chromosome-fragmenting agents cupferron or hydroxylamine. The chromosome-breaking properties of these two chemicals have been well established in other systems [8,10,11]. It has been demonstrated (unpublished results) using contour clamped homogeneous-electric field electrophoresis (CHEF) that treatment of *S. cerevisiae* with these agents destroys the normal chromosome banding pat-

tern, thus indicating that chromosome fragmentation is occurring here also. Fusion of protoplasts prepared from *S. cerevisiae* JJ1A, treated with cupferron or hydroxylamine, with protoplasts of *S. cerevisiae* JX651OC and selected on MM results in a yield of transformants considerably lower than that achieved when viable, untreated protoplasts of both parents are used. However, in fusions where chemically treated JJ1A is used, the yield of transformants achieved on MM plus an amino acid was considerably greater than that achieved on unsupplemented MM (Table 1). This is due to the fact that for complementation to occur on these media two genes must be transferred to the recipient strain whereas on MM the three appropriate sequences must be present in the genome for complementation. All of the transformants examined microscopically were similar in size to haploid rather than diploid cells. The DNA content of the five prototrophic transformants was very similar to that of the haploid parental strains. The stability of the transformant prototrophy suggested that the stable integration of the genetic sequences had occurred [18].

The haploid nature of the transformants indicates that transfer of the genes or small genetic sequences necessary to complement the auxotrophic requirements of the viable cell had occurred. This view is supported by the observation that significantly greater numbers of transformants could be obtained if the selection medium was supplemented with one of the auxotrophic requirements of the recipient strain. The data presented suggest the gene transfer event occurs with an incidence of about 1 in 100 protoplast fusion events. The stability of the prototrophic condition precludes the possibility that small chromosome fragments are maintained independently in the transformants. One possibility is that residual intact chromosomes may be exchanged following karyogamy and that the effect of chemical pretreatment may merely prevent the establishment of diploids. An alternative possibility is that the chromosome fragments may be incorporated into the recipient genome by a gene conversion process. Further work is necessary to distinguish between these two possibilities. Whichever mechanism operates, this technique has the potential for transferring desirable characteristics to industrial yeast strains without significantly altering the ploidy level.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to the Health Research Board and Eolas (Irish Science and Technology Agency) for financial support.

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