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Enhanced intraspecific protoplast fusion in yeast

K. Kavanagh, M. Walsh and P.A. Whittaker

Department of Biology, Maynooth College, County Kildare, Republic of Ireland

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1. SUMMARY

Intraspecific hybrid production from the polyethylene glycol induced fusion of yeast protoplasts was greatly increased when calcium propionate was included as the source of the requisite Ca^{2+}. The use of calcium propionate, as opposed to the more commonly employed calcium chloride, resulted in substantially greater yields of hybrids from intraspecific fusions of protoplasts of *Saccharomyces cerevisiae* and *Candida albicans*. It is postulated that the ability of calcium propionate to enhance the fusion frequency is due to the anion binding to the etheric oxygen of PEG and potentiating the fusogenicity of the polymer.

2. INTRODUCTION

Fungal protoplast fusion may be induced by electrical [1] or chemical means. The most commonly employed chemical means of achieving fusion has been the use of polyethylene glycol (PEG), the fusogenic properties of which, in conjunction with calcium chloride, were first demonstrated using plant protoplasts [2]. This combination has been widely used to produce intraspecific [3], interspecific [4,5] and intergeneric [6] yeast hybrids. The chemically induced fusion of yeast protoplasts has also facilitated the creation of a parasexual cycle for the asexual pathogenic yeast *Candida albicans* [7,8].

The fusogenic potential of PEG is attributed to its ability to dehydrate protoplasts [9] and to induce the formation of aggregates in which intimate membrane contact is achieved between adjacent protoplasts [10]. The calcium cation is considered to potentiate fusion by creating areas of membrane instability at the points of closest contact, thus facilitating the coalesence of adjacent cytoplasms [11,12]. Calcium may also interact with the PEG polymer and enable the polymer to bind to negatively charged components of the protoplast membrane [13]. Subsequent resuspension of the protoplasts in an isotonic buffer ensures rehydration which has the effect of expanding the inter-protoplast cytoplasmic continuities [10]. Fol-

Correspondence to: K. Kavanagh, Department of Biology, Maynooth College, County Kildare, Republic of Ireland.

lowing protoplast fusion, karyogamy may proceed or the nuclei may co-exist in the cytoplasm as an heterokaryon.

Calcium chloride has been the most frequently employed source of the requisite exogenous Ca^{2+} for the PEG induced fusion process [14,15]. This paper details an investigation of the potential of a range of other calcium salts to supply the necessary Ca^{2+} for fusion and postulates a mechanism to explain the enhanced fusogenic properties observed with certain salts.

3. MATERIALS AND METHODS

3.1. Strains

The following strains were used in this work: *Saccharomyces cerevisiae* JJ1A (a, arg^-, thr^-) and JX651OC (a, ade^r, thr^-). *Candida albicans* ATCC 44987 (his^-, arg^-) and ATCC 44990 (ade^r, thr^-)

3.2. Media

Cultures were grown routinely in YEPD (2% (w/v) glucose, 2% (w/v) Bacto peptone (Difco) and 1% (w/v) yeast extract (Oxoid)) at 30°C and 200 rpm. Hybrids were detected by embedding fused protoplasts in molten (48°C) osmotically stabilized minimal medium (3% (w/v) Bacto agar, 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 and overlaying onto a thin layer of the same medium in a Petri dish).

3.3. Buffer

Osmotically stabilized MP buffer was used to maintain protoplast integrity. This contained 0.5 M KCl for protoplasts of *C. albicans* or 0.8 M sorbitol for those of *Saccharomyces cerevisiae* in addition to 0.1 M NaCl and 0.1 M acetic acid. The pH was adjusted to 5.5 with 0.1 M NaOH prior to autoclaving.

3.4. Protoplast preparation

Exponential phase cells were harvested, washed and resuspended at a density of 3×10^7/ml in 6 ml of the appropriate osmotically stabilized buffer containing 1.45 mg/ml Novozym 234 (Novo Industri, Denmark) and 0.017 ml/ml Suc d'Helix pomatia (IBF Biotechnics, France). The resulting mixture was incubated at 30°C for 45 min, which was sufficient to ensure that over 95% of the cells were rendered osmotically fragile. Protoplasts were then harvested and washed before being resuspended in 2 ml of buffer.

3.5. Protoplast fusion

Intraspecific protoplast fusion was achieved by resuspending 2×10^7 protoplasts of each complementary strain in a fusogen which consisted of 0.9 ml of PEG 3350 (Sigma, St. Louis, MO) and 0.1 ml of a calcium salt solution. PEG was used at a concentration of either 40% (w/v) or 60% (w/v) and was sterilized by filtration [16]. The fusogen was diluted after 6 minutes by the addition of 5 ml of the appropriate buffer. Protoplasts were harvested after a further 6 min, washed and resuspended in 2 ml of buffer prior to being embedded at a density of 1×10^6 complementary pairs per plate in selection medium (see SECTION 3.2). Mixtures of unfused parental protoplasts were employed as controls. All plates were incubated at 30°C for 7–10 days. The hybrid yield is based upon the number of hybrids obtained per fusion.

4. RESULTS

Previous work in this laboratory on optimizing the yield of hybrids from intraspecific fusions concentrated on means of reducing the toxicity of PEG to protoplast reversion and hybrid formation [16]. In an attempt to further enhance the hybrid production from such fusions, different calcium salts were employed as the source of the requisite Ca^{2+} in order to establish whether the source of the cation affected its potency as a facilitator of protoplast fusion. Solutions (1.0 M) of calcium chloride, calcium nitrate and calcium acetate were used in combination with PEG to induce the intraspecific fusion of protoplasts of the complementary auxotrophic strains of *S. cerevisiae* and *C. albicans*. The results of these fusions (Table 1) reveal that in the absence of PEG and Ca^{2+} no hybrids were obtained. The greatest

Table 1

Effect of calcium salts on the hybrid yield from PEG induced protoplast fusions

Fusogen	Hybrid yield ($\times 10^{-4}$%)			
	S. cerevisiae		*C. albicans*	
	A	B	A	B
Control	0.0	0.0	0.0	0.0
PEG + $CaCl_2$	4.54	7.86	13.7	6.25
PEG + $Ca(NO_3)_2$	12.4	12.6	17.2	47.1
PEG + $Ca(CH_3COO)_2$	58.14	16.2	200.3	128.2

Protoplasts of complementary strains of *S. cerevisiae* or *C. albicans* were mixed and induced to fuse by the addition of PEG and the appropriate calcium salt. Hybrids were detected by means of nutritional complementation. PEG was used at a concentration of either (A) 40% (w/v) or (B) 60% (w/v). The controls lacked PEG and Ca^{2+}. All values are the mean of five determinations.

Table 2

Influence of pH of calcium salt solution on hybrid production from PEG induced intraspecific fusion of protoplasts of *C. albicans* ATCC 44987 and ATCC 44990

Fusogen	Hybrid yield ($\times 10^{-4}$%)	
	A	B
Control	0.0	0.0
PEG + $CaCl_2$ (4.7) *	1.44	1.62
PEG + $CaCl_2$ (7.0) *	3.48	1.85
PEG + $Ca(CH_3COO)_2$ (4.7) *	1.92	1.76
PEG + $Ca(CH_3COO)_2$ (7.0) *	25.8	28.2

Calcium chloride and calcium acetate solutions were adjusted to pH 7.0 and 4.7, respectively. Fusion of protoplasts was induced either (A) 40% (w/v) or (B) 60% (w/v) PEG and the appropriate calcium salt solution. The controls lacked PEG and Ca^{2+}. The asterisk indicates the pH of the calcium salt solution. All values are the mean of five determinations.

yield of hybrids was evident when calcium acetate was used as the source of the Ca^{2+} although it has been demonstrated previously that the acetate anion displays no inherent fusogenicity [17]. This was apparent irrespective of the yeast strains or the concentration of PEG employed in the fusogen. The use of calcium nitrate, in conjunction with PEG, also produced an enhanced hybrid yield when compared with that obtained using calcium chloride.

In the work described above the pH of calcium acetate was 7.0, while that of calcium chloride was 4.7. In order to determine whether the increased hybrid yield evident with calcium acetate was a function of pH, fusions were performed using PEG and solutions of calcium chloride and calcium acetate adjusted to the same pH. The results (Table 2) of this series of experiments demonstrate that at pH 7.0 calcium acetate always facilitates the formation of greater numbers of *C. albicans* hybrids than calcium chloride, while at pH 4.7 both salts produce approximately equal numbers of hybrids. This phenomenon was also evident following fusions involving protoplasts of the complementary *S. cerevisiae* strains (data not shown). These results indicate that a low pH obviates the ability of calcium acetate to enhance the PEG-induced fusion process.

The fusogenic capabilities of calcium acetate and calcium chloride, in combination with PEG, have been compared previously (Table 1). In order to establish whether increasing the size of the anion would affect the hybrid yield, calcium propionate was employed as the source of the requisite Ca^{2+} in a number of fusions. The data obtained (Table 3) revealed that the use of calcium propionate, in conjunction with PEG, facilitated a greater hybrid yield than that which was evident when calcium chloride or calcium acetate were employed as the source of the Ca^{2+} cation. This was evident irrespective of the concentration of PEG employed and was also observed following fusions involving protoplasts of the *S. cerevisiae* strains (data not shown).

Table 3

Effect of calcium salts on intraspecific hybrid yield from fusions of protoplasts of *C. albicans* ATCC 44987 and ATCC 44990

Fusogen	Hybrid yield ($\times 10^{-4}$%)	
	A	B
Control	0.0	0.0
PEG + $CaCl_2$	3.32	15.3
PEG + $Ca(CH_3COO)_2$	50.6	71.4
PEG + $Ca(CH_3CH_2COO)_2$	85.0	131.3

Protoplasts were mixed and induced to fuse using PEG and one of the calcium salt solutions listed above. PEG was used at a concentration of either (A) 40% (w/v) or (B) 60% (w/v). The controls lacked PEG and Ca^{2+}. All values are the mean of five determinations.

5. DISCUSSION

The data presented in this paper demonstrate the superior fusogenic properties, compared with calcium chloride, of calcium acetate and calcium propionate in facilitating PEG-induced yeast hybrid formation. It has been established previously that the replacement of chloride by acetate produced no significant increase in the yield of hybrids during the electrofusion of protoplasts of *S. cerevisiae* [17]. This indicates that the acetate anion does not interact directly with yeast protoplasts and renders them more susceptible to fusion. The results presented here suggest that the enhanced hybrid yield observed with calcium propionate and calcium acetate may be due to the anions interacting with the PEG polymer and potentiating its fusogenicity. PEG is a 1,2-epoxide polymer formed by the catalytic polymerization of ethylene glycol [18]. The basic unit of the polymer consists of two methylene groups joined by an etheric oxygen. This oxygen is a powerful hydrogen bond acceptor and it is our proposition that the acetate and propionate anions bind to this due to electrostatic attraction and the increased stability which this conformation would engender. (The formation of a similar type of complex is the basis of Skoog's assay for the analytical determination of PEG [19]). Such an orientation would enable the carboxylic portion of the respective molecules to bind either directly or by means of the calcium cation to the protoplast membranes. This would have the effect of enhancing the fusogenic capacity of the PEG polymer by effectively rendering it more 'sticky'. The greater size of the propionate anion, compared with the acetate anion, is correlated with an elevated yield of hybrids. This is consistent with our explanation since the extra methylene group would position the carboxylic group further from the helical structure of the PEG polymer [18] and give it greater freedom to interact with the protoplast surface. Equivalent concentrations of Ca^{2+} from calcium acetate and calcium chloride produced different numbers of fusion products at pH 7.0. The reduced hybrid yield observed with calcium acetate at pH 4.7 (Table 2) may be due either to the hydrogen ions binding preferentially to the etheric oxygen and preventing the formation of the PEG-acetate anion complex or to protonation of the carboxylic acid groups. The enhanced hybrid yield observed when calcium nitrate was used as the source of the requisite Ca^{2+} may have been a result of the formation of a similar type of PEG-anion complex.

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