Induced Fusion of Fungal Protoplasts following Treatment with Polyethylene Glycol

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INTRODUCTION

Although protoplast fusion is of current interest because of its possibilities in pure and applied genetics, only a few reports exist on intraspecific fusion between fungal protoplasts and subsequent selection of heterokaryons. Naturally-occurring fusion between protoplasts of *Geotrichum candidum* (Ferenczy, Kevei & Zsolt, 1974) and *Aspergillus nidulans* (Ferenczy, Kevei & Szegedi, 1975) has been reported. Binding & Weber (1974) described fusion between protoplasts of *Phycomyces blakesleeanus* induced by seawater or calcium ions at high pH, but the frequency of heterokaryon formation was low in all cases. We here report heterokaryon formation, at high frequency, after induced intraspecific fusion between protoplasts of auxotrophs of *Penicillium chrysogenum*, *P. patulum*, *P. roquefortii*, *A. nidulans*, *A. niger* and *Cephalosporium acremonium*, and fusion between protoplasts of *P. chrysogenum* and *P. notatum* auxotrophs, using solutions containing polyethylene glycol (PEG) mol.wt. 6000.

METHODS

Organisms and growth conditions. Auxotrophic mutants of *Penicillium chrysogenum* Wis. 49-2105, *P. roquefortii* (University of Nottingham Collection) and mutant 8650 of *Cephalosporium acremonium* ATCC11550 were selected after *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine treatment (Adelberg, Mandel & Chen, 1965) and checked for their stability over several vegetative generations. An auxotrophic strain of *Penicillium notatum* IM138632 was obtained from the Commonwealth Institute, Kew, Surrey, and *Aspergillus nidulans* mutants G040 (*biA1*; *wA3*; *arg-9*) and G0112 (*yA2*; *pyroA4*; *methB3*) were obtained from the Department of Genetics, University of Glasgow. *Penicillium patulum* auxotrophs were kindly supplied by Mr A. R. Cottrell (ICI Ltd, Manchester) and mutants of *Aspergillus niger* by Dr I. H. Pallett (Beecham Pharmaceuticals, Surrey).

All strains were maintained on a complete medium (Anné, Eyssen & De Somer, 1974), except *A. nidulans* and *A. niger* which were grown on Vogel's medium N (Vogel, 1964) supplemented with glucose (10 g/l), Difco Bacto-peptone (5.0 g/l), Difco yeast extract (2.5 g/l) and the specific requirements for the strains (biotin, 0.02 mg/l; pyridoxine, 0.5 mg/l; arginine, 200 mg/l; methionine, 50 mg/l; inositol, 0.5 mg/l), and *C. acremonium* which was grown on a complete sporulation medium (Lemke & Nash, 1972).

Mutant symbols. Strains with different requirements and spore colour were used. The

symbols for the mutants other than *A. nidulans* were as follows: *whi* (white spore colour); *yl* (yellow spore colour); *met* (methionine); *cys* (cysteine); *met/cys* (methionine or cysteine); *lys* (lysine); *pro/arg* (proline or arginine); *leu* (leucine); *met/thr* (methionine or threonine); *val* (valine); *trp* (tryptophan); *ino* (inositol); *pyr* (pyridoxine).

Protoplast formation and fusion treatment. Protoplasts from *Penicillium*, *Cephalosporium* and *Aspergillus* were prepared as described previously (Anné *et al.*, 1974; Peberdy & Gibson, 1971), and separated from mycelial debris by filtration through a sintered glass filter (porosity 1). After washing in 0.7 M-NaCl, protoplasts were mixed (5×10^6 to 10^7 protoplasts of each auxotroph) and centrifuged (700 g, 10 min). The pelleted protoplasts were suspended in 1 ml of a pre-warmed (30 °C) solution of 30 % (w/v) PEG (Koch-Light) in 0.01 M- $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 0.05 M-glycine, adjusted to pH 7.5 with 0.01 M-NaOH. After incubation for 10 min at 30 °C the suspension was diluted with 6 ml of minimal medium adjusted to 0.6 M-NaCl (MM) (Anné *et al.*, 1974), and centrifuged (700 g, 5 min). Protoplasts were twice washed with 8 ml portions of 0.6 M-NaCl and finally resuspended in 5 ml of 0.7 M-NaCl. Serial dilutions were plated on solid MM to select nutritionally-complementing fused protoplasts. The total number of viable protoplasts before and after PEG treatment was determined by plating on solid MM supplemented with 0.2 % Difco yeast extract (PM). All plates were incubated at 30 °C.

RESULTS AND DISCUSSION

Immediately after the PEG solution was added, protoplasts adhered firmly and shrank, indicating the high osmotic pressure of the solution. During the subsequent dilution with MM, protoplasts regained their normal size although larger bodies were also observed which were assumed to originate from two or more fused protoplasts. Many protoplasts still adhered, but these protoplast aggregates separated completely after removal of PEG by the NaCl washes and still more fused protoplasts were then observed. PEG had no aggregating influence on fungal spores or mycelium.

Fusion between protoplasts of nutritionally-complementing strains was detected by formation of heterokaryons developing on solid MM. After 48 to 72 h, colonies appeared from PEG-treated mixed protoplasts on both MM and PM. Unfused protoplasts, spores or mycelium are unable to develop on these media, even after mixing the auxotrophic mutants at high density (10^7 spores or protoplasts on one plate), except that if protoplasts of *A. nidulans* or *P. patulum* auxotrophs were mixed and plated at high density ($> 10^6$) then cross-feeding occurred. However, at lower densities ($< 10^5$) no growth was observed on control plates. If protoplasts of the auxotrophic strains were separately treated with PEG solution, washed and then mixed before plating, again no heterokaryon formation was observed.

Fusion experiments between protoplasts of several auxotrophic mutants were done. The frequency of fusion, based on the ratio of the number of colonies developing on MM and on PM after PEG treatment, was 0.1 to 0.7 %, as determined from several experiments (Table 1). Comparison of numbers of protoplasts regenerating on PM, before and after PEG treatment, showed that only about 10 % of the protoplasts survived (Table 1). This loss of viability was probably due to lysis of protoplasts by PEG.

Colonies on MM sporulated after 4 to 6 days at 30 °C to give a mixture of spores of the two component strains used in the fusion treatment, and were identified as heterokaryons (Pontecorvo, 1956). In fusion experiments, using *P. chrysogenum* white and yellow nutritionally-complementing strains, some green sectors and outgrowths appeared in the mixed

Table 1. *Frequencies of heterokaryon formation before and after fusion treatment of mixtures of protoplasts of auxotrophic strains of different fungi*

Fusion treatment was carried out by suspending the protoplasts in solutions containing 30% (w/v) PEG, 0.01 M-CaCl₂.2H₂O and 0.05 M-glycine, pH 7.5 (30 °C, 10 min); after washing, dilutions were plated on to hypertonic MM and PM.

Fusion mixtures	10 ⁻³ × Heterokaryon formation/ml on MM		10 ⁻⁶ × Protoplasts /ml regenerating on PM		Fusion frequency (%)
	Before PEG	After PEG	Before PEG	After PEG	
<i>P. chrysogenum</i>					
lys pro arg whi + leu met cys ylo	0	18.2	28.2	3.5	0.52
lys pro arg whi + lys met whi	0	0.0	33.6	3.62	0.00
lys pro arg whi + leu met thr ylo	0	22.0	24.8	3.10	0.70
leu met thr ylo + lys met whi	0	10.7	18.6	2.20	0.48
<i>P. patulum</i>					
met + pyr	0*	3.8	NC	2.3	0.17
met + ino	0*	5.7	NC	2.7	0.27
pyr + in	0*	13.0	NC	2.0	0.65
<i>P. roquefortii</i>					
val + lys	0	6.0	NC	3.1	0.19
<i>A. nidulans</i>					
GO40 + GO112	0*	2.15	10.8	0.79	0.27
<i>A. niger</i>					
met + ino	0	0.8	2.8	0.36	0.22
<i>C. acremonium</i>					
cys + met	0	24.5	71.0	7.0	0.35
cys + pyr	0	58.5	15.0	15.0	0.39
cys + trp	0	42.5	15.0	10.0	0.42
met + trp	0	9.0	6.4	5.0	0.18
<i>P. chrysogenum</i> + <i>P. notatum</i> (cys ylo)					
lys pro arg whi + cys ylo	0	9.22	52.5	5.05	0.18
leu met thr ylo + cys ylo	0	4.50	60.0	3.60	0.12
lys met whi + cys ylo	0	5.10	47.5	4.80	0.11
leu met cys ylo + cys ylo	0	0.00	40.5	4.70	0.00

NC, Not counted. * Only at low plating densities ($\leq 10^4$ protoplasts on a plate).

white-yellow colonies after 7 to 10 days' incubation. These sectors were confirmed as diploid by prototrophy and the colour of their spores (Pontecorvo, 1956) and by spore size and their doubled DNA content (Pontecorvo & Sermonti, 1954; MacDonald, Hutchinson & Gillett, 1965). Haploid and diploid spores, respectively, had mean diameters of 2.95 and 3.60 μ m, and contained 2.0 and 4.3 μ g DNA/ 10^8 spores (following the DNA extraction method of Heagy & Roper, 1952). Natural segregants appeared as rare white-yellow spots or sectors of auxotrophic spores in colonies of single spore isolates of the diploids on complete medium. Segregation patterns are now under investigation.

To show the versatility of PEG as a fusogenic agent in fungi other than *P. chrysogenum*, intraspecific heterokaryons were induced by PEG treatment of protoplasts of different auxotrophic species such as *P. patulum*, *P. roquefortii*, *A. nidulans*, *A. niger* and *C. acremonium* (Table 1). The fusion frequencies were always about the same.

These results demonstrated that heterokaryon formation on MM was obtained by fusion

of protoplasts, and was not due to cross-feeding and subsequent anastomosis between regenerating protoplasts. Fusion frequency was the same for species such as *A. nidulans* which easily form heterokaryons, as for species such as *C. acremonium* in which the heterokaryotic condition is not easily achieved by conventional methods (Nüesch, Treichler & Liersch, 1973). Also, no differences in fusion frequency were shown on MM plates with high ($\geq 10^4$) or with low ($< 10^3$) protoplast densities. If cross-feeding had occurred, plates with a larger number of protoplasts should have shown a higher fusion frequency, for they provide more opportunities for cross-feeding or for contact between germ tubes of regenerating protoplasts than do plates with a limited number of well-distributed protoplasts.

Heterokaryons between *P. chrysogenum* and *P. notatum* after PEG treatment may have shown that interspecific fusion can take place between closely related species, except that there is some doubt as to whether these are distinct species (Onions, personal communication).

Using *P. chrysogenum*, we also found heterokaryon formation after treatment of mixed auxotrophic protoplasts with polyethylene glycol of molecular weight 1540 or 4000 (Kao & Michayluk, 1974) and with calcium ions at high pH (Keller & Melchers 1973) – methods used for fusion between higher plant protoplasts – but frequencies were much lower. After treatment of protoplast mixtures with solutions of polyethylene glycol of molecular weights 6000, 4000 or 1540, fusion frequencies were 0.62, 0.48 and 0.072 %, respectively. Fusion induction by solutions containing 0.05 M- $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.05 M-glycine and 0.55 M-NaCl at pH 9.0, without polyethylene glycol, gave only 0.001 %.

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