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A Monokaryotization Method and its Use for Genetic Studies in Wood-rotting Basidiomycetes

H. Leal-Lara and G. Eger-Hummel

Institut für Pharmazeutische Technologie der Universität Marburg (Federal Republic of Germany)

Summary. A monokaryotization method allows the recovery of the two monokaryotic components (=neohaplonts) from a dikaryon within a sample of few mycelial fragments. This relatively gentle, simple and rapid procedure makes use of glycine containing solutions low in phosphate and magnesium ions. It proved to be successful in all 14 strains (among them two multiple mutants) of the five species tested. No variant or mutant was found among the neohaplonts, not even in a sample of 1018 isolates from a single dikaryon. The method is useful for genetical studies and breeding work with sterile strains.

Key words: Basidiomycetes – Monokaryotization – Neohaplonts – Genetic studies – Breeding

Introduction

The breeding potential of the edible mushroom *Pleurotus ostreatus* has already been described (Eger et al. 1976) with emphasis on the strain "42×11", which has sporeless fruiting bodies. Using conventional methods, it was impossible to elucidate the inheritance of sporelessness. We therefore tried a new approach via monokaryotization.

P. ostreatus is a species in which the parental nuclei do not fuse after the mating of two compatible monokaryotic isolates but stay in pairs, one per hyphal compartment, and divide conjugatedly. Zygotes are formed in the fruiting bodies shortly before spore formation. The dikaryotic hyphae have clamp connections at each cross wall so that the monokaryotic, respectively dikaryotic state, can easily be recognized at magnifications of about 100 times under the microscope.

Dissociation of dikaryons into monokaryons has been repeatedly obtained by mechanical and/or chemical treatment. Harder (1927a) introduced micro-surgery. Only a low percentage of cells survived and only one nuclear type was recovered in two species (Harder 1927a, b; Fries and Aschan 1952). Among the isolated monokaryons, termed "neohaplonts" by Fries and Aschan (1952), variants were found (Aschan 1952; Fries and Aschan 1952; Papazian 1955). Ginterová (1973) observed that in shaked, submerged cultures of *Pleurotus ostreatus* one nucleus was eliminated. Wessels et al. (1976) found that protoplasts from a dikaryon of *Schizophyllum commune* reverted into monokaryotic mycelia at a rate of 20 to 40%. Miles and Raper (1956) used sodium taurocholate and cholic acid on one to several strains of each of 13 wood-rotting Basidiomycetes with varying success. Takemaru (1964a) confirmed the monokaryotization effect of sodium cholate and oxgall on four species, each requiring different concentrations. However, in the experiments of Kerruish and Da Costa (1963), sodium taurocholate was only active in one of 24 wood-pathogens, none of seven other toxicants showed a general monokaryotizing effect. A high level of nuclear selection resulted in some cases, as was also reported by Takemaru (1964b). Amburgey (1967) confirmed these results with similar chemicals on other species. Under treatment with polymixin, Parag (1961) noticed selective killing of the less resistant nucleus from various dikaryons of *Schizophyllum commune*. None of the chemicals so far tried had a general monokaryotizing effect, none of the methods used appeared to be satisfactory.

A common feature associated with a successful disruption of the dikaryotic organization by chemicals is growth retardation. Kerruish and Da Costa (1963) suggested that retardation may provide some selective mechanism to favour growth of the neohaplont(s) over that of the parent dikaryon. We accomplished monokaryotization under the influence of growth retarding conditions without use of toxins.

Materials and Methods

Strains

Pleurotus ostreatus (Jacq. ex Fr.) Kummer: 'FX' is one of the numerous subcultures from a strain obtained in 1964 from Block et al. (1958). 'G' and 'M' are cultures from plectenchyma of specimens from Westfalen, Germany, and Michigan, USA, respectively. 'So 3004' is a commercial strain of "Somycel"

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(F-78 600 Maisons-Laffitte). Dikaryons obtained from matings of single-spore isolates are '42×11' (Eger et al. 1976), 'FX 868×381' (von Netzer 1979), 'FX 88×136', 'G 1×4' and 'G 11×14', 'M V-10' and 'M V-81' are slow growing mutants with several fruiting abnormalities. *Pleurotus cornucopiae* Fr. ex Paul. and *P. eryngii* (Dc. ex Fr.) Quel. were kindly provided by F. Zadražil (Braunschweig). *Kuehneromyces mutabilis* (Schff. ex Fr.) Sing. and *Flammulina velutipes* (Curt. ex Fr.) Sing. are from Eger's culture collection.

Methods

One mycelial culture of about 4 cm diameter, grown on 3 mm thick plates of deproteinated malt extract agar (= DPMA, Eger et al. 1976) was blended with 50 ml sterile distilled water of 4 °C in a "Waring" blender in a "semimicro-container" with cooling jacket for 2½ min at high speed (= 20 500 U/min in unloaded condition). Hyphal fragments of one to a few living cells resulted. Portions of this "homogenate" were injected into the bottom of 50 ml Erlenmeyer flasks containing 25 ml testing solution. They were incubated at 22±2 °C without agitation and avoiding unnecessary movements. The testing solutions were prepared with glucose monohydrate (Merck 8342) and chemicals of "pro analysis" grade if not otherwise specified. They were either filter-sterilized (Seitz-Ek) or autoclaved at 121 °C for 15 min. Each solution was tested at least twice in triplicates.

In the testing solutions living fragments grew into visible mycelial pellets. The periphery of the pellets was inspected for clamps under the microscope every three days until they exceeded 3 mm. The monokaryotization effect per pellet was scored roughly as being 100% (no clamps observed), 0% (no monokaryotic hypha found), 50% (approximately as many monokaryotic as dikaryotic hyphae), more monokaryotic than dikaryotic hyphae and vice versa. The contents of the flasks which were recorded as having 100% monokaryotic pellets were blended for 2½ min at high speed as described above. To obtain isolated colonies the fragments were plated on DPMA in several dilution steps. The colonies were checked for clamps, growth morphology, mating type and marker genes. Growing and fruiting conditions were as described by Eger et al. (1976).

Experiments and Results

The minimal medium for cultivation of wood-rotting basidiomycetes (Raper and Hoffmann 1974) was modified by omitting ingredients or replacing them by other chemicals. The media were inoculated with 20 µl "homogenate" of *P. ostreatus* dikaryon "42×11". Growth was poorer than in the unaltered minimal medium and the mycelial pellets were dikaryotic with the exception of those growing in a solution containing only 20 g/l glucose and 1 to 5.6 g/l glycine in distilled water. There, small monokaryotic sectors were observed occasionally. When the glucose-glycine solutions were autoclaved instead of filter sterilized, growth was further retarded and 100% monokaryotization obtained. Glycine from different sources (Merck, Serva) and batches caused 100% monokaryotization and only slight variation in growth (Table 1). Glycine-glucose solutions supplemented with amino acids (singly or in combinations) or with "Peptone P" or "Meat-Peptone", gave likewise 100% monokaryotization. Peptones with relatively low glycine and high phosphate and mineral content were ineffective (Table 1). Addition of 10 mg/l K_2HPO_4 and 5 mg/l KH_2PO_4 to glycine-glucose solutions reduced the monokaryotization to approximately 50%. Addition of 5 mg/l $MgSO_4 \cdot 7\,H_2O$ alone or in combination with 10 mg/l K_2HPO_4 and 5 mg/l KH_2PO_4 allowed only small monokaryotic sectors to occur occasionally. Higher levels of magnesium ions and phosphates neutralized the monokaryotization effect completely. Monokaryotization by glycine and "Peptone P" was also drastically reduced when the volume of inoculum was increased. With 500 µl only dikaryotic pellets were obtained. However, in the case of "Meat Peptone" (30 g/l), increase from 20 to 150 µl gradually improved mycelial growth so that monokaryotic pellets were already visible after 12 days. If instead of 25 ml only 10 ml or less of monokaryotizing solutions were used, the monokaryotization was incomplete. Upon longer periods of cultivation the growing pellets made contact and dikaryotic sectors appeared regardless of the liquid volume. Mycelium which finally grew on the surface of the solutions was dikaryotic.

From six experiments with *P. ostreatus* '42×11' the monokaryotic pellets were further investigated. Hyphal tips were isolated under the dissection microscope or the total pellets blended (see Methods) and plated on DPMA. The hyphal fragments developed exclusively into clampless colonies. When checked for mating factors and growth characteristics, two types of mycelia were found in each trial (Table 2). They corresponded

Table 1. Monokaryotization effect of autoclaved solutions (15 min, 121 °C) on 20 µl inoculum of dikaryon "42×11" of *Pleurotus ostreatus* and number of days required for growth into visible pellets

Ingredients per liter dist. water in addition to 20 g glucose	Effect	Days
Glycine (Merck, Serva, three batches each): 3 to 8 g	100%	18 – 20
Glycine, 5 g; Peptone P (Oxoid L-49), 5 g	100%	6 – 7
Peptone P: 2.5 to 20 g	100%	6 – 7
Peptone P: 25.0 to 30 g	100%	12
Meat Peptone (Merck 7224):		
5 g	100%	80
10 g	100%	40
15 to 30 g	100%	30
Other Peptones: 2.5 to 30 g		
Oxoid L-34, L-44, CM 129	0%	4
Difco: 588686, 524533	0%	4
Merck: 7214, 7284	0%	4
BBL: 11919	0%	4

Table 2. Neohaplonts recovered from dikaryon "42×11" of *Pleurotus ostreatus* after monokaryotization in autoclaved solutions with 1% "Peptone P" or 0.5% glycine and 2% glucose (w/v) in dist. water

Monokaryotized with	Recovered neohaplonts				
	Exp.	Total	Number of type		χ^2 [a]
	No.	number	"42"	"11"	
Peptone P	1	44	19	25	0.818
	2	27	16	11	0.925
	3	45	32	13	8.022
	4	35	20	15	0.714
	5	44	22	22	0.000
Glycine	6	31	15	16	0.032

χ^2 For significance at 5% = 3.84, at 1% = 6.63
[a] For one degree of freedom

to the parental monokaryons '42' or '11'. Only in one experiment the ratio of the two neohaplont types differed significantly from a 1 : 1 ratio.

A random sample of 15 neohaplonts of each type was mated in all possible combinations. The dikaryons obtained from them were indistinguishable from '42×11' in growth characteristics and fruit body morphology. In 23 additional experiments 426 different dikaryons were formed from neohaplonts. They could not be distinguished from the original '42×11' strain either. To accumulate possible small harmful effects, a dikaryon was synthesized from neohaplonts and then monokaryotized and resynthesized consecutively three times. In a total of 1018 neohaplonts not a single variant was observed.

Finally neohaplonts corresponding to the parental monokaryons '42' and '11' were mated each with single-spore isolates from several wild type strains. All compatible matings resulted in dikaryons which formed fruiting bodies with normal spore production.

Monokaryotization was also tried in 13 strains of five species as indicated in Table 3. Only monokaryotic pellets were obtained. After blending and plating on DPMA, in all cases two uniform classes were found which represented the component mating types. Table 3 demonstrates that there is a good chance to obtain both types of neohaplonts among a few isolates only.

Table 3. Neohaplonts recovered from various strains of basidiomycetes after monokaryotization in autoclaved solution with 1% "Peptone P" and 2% glucose (w/v) in dist. water

Dikaryons	Recovered neohaplonts			
	Total number	Number of type		χ^2 [b]
		1	2	
Pleurotus ostreatus				
SOMYCEL 3004	25	15	10	1.000
G	25	16	9[a]	1.960
G 1×4	18	14	4	–
G 11×14	19	12	7	1.310
M V-10	11	6	5	0.818
M V-81	9	7	2	–
FX	54	28	26[a]	0.070
FX 868×381	36	27	9	9.000
FX 88×136	34	22	12[a]	2.940
Pleurotus cornucopiae	34	20	14	1.058
Pleurotus eryngii	8	6	2	–
Kuehneromyces mutabilis	23	16	7	3.520
Flammulina velutipes	24	18	6[a]	6.000

[a] Neohaplont growing faster than its corresponding partner
[b] See Table 2

Discussion

A method for monokaryotization of certain basidiomycetes has been developed. Unlike earlier methods it does not require mechanical injury or toxic chemicals. Instead, it uses the growth retarding effects of phosphate and magnesium deficiencies in connection with relatively high concentration of glycine. "Peptone P", which is rich in glycine and poor in phosphates and minerals, alone or in combination with glycine, gave the fastest results (Table 1). Monokaryotization of 100% was obtained within one week in 12 strains (including the species *Pleurotus ostreatus, P. cornucopiae, P. eryngii, Kuehneromyces mutabilis, Flammulina velutipes*) and within three weeks in two severely impaired mutants of *P. ostreatus*. There is no indication for nuclear selection. In *P. ostreatus* 'G', 'FX', 'FX 88×136' and in *F. velutipes* the two types of neohaplonts differed in their linear growth rates (Table 3), yet the faster growing ones did not outnumber the slower ones. It deserves to be mentioned that the mutant strains 'M V-10' and 'M V-81' al-

so gave two types of neohaplonts, both of which were very poor growing and with abnormal morphology. Not a single variant or mutant was observed, even in the large samples of the experiments with *P. ostreatus* '42 × 11'.

The mechanism by which monokaryotization takes place is unknown. Submerged cultivation under a minimum liquid layer and small amount of inoculum seem to be essential. The amount of inoculum should always be checked. With one batch of "Peptone P" it was found that monokaryotization was incomplete when concentrations of 10 g/l and 20 µl inoculum were applied, but 100% when 5 µl inoculum was used. Possibly a substance which favours growth and the dikaryotic state is delivered by the inoculum. Growing mycelium might also excrete such a substance by which the successive reestablishment of the dikaryon with increasing amount of mycelium could be explained.

Our simple and rapid method facilitates investigations on cytoplasmic inheritance (Leal-Lara 1979). It makes possible genetic studies on sterile dikaryons if fertility is restored by mating neohaplonts with wild-type monokaryons as in the example of *P. ostreatus* '42 × 11'. It allows the breeding of sporeless strains for commercial mushroom cultivation (Eger and Leal-Lara 1978) and simplifies strain identification (Eger and Leal-Lara 1978; Eger 1979; Eger et al. 1979).

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Dr. H. Leal-Lara
Food Science Department
Faculty of Chemistry
University of Mexico, UNAM
Mexico 20 D.F. (Mexico)

Prof. Dr. G. Eger-Hummel
Institut für Pharmazeutische Technologie
Universität Marburg
Ketzerbach 63
D-355 Marburg (Federal Republic of Germany)