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Homokaryotization of *Agaricus bitorquis* (Quel.) Sacc. and *Agaricus bisporus* (Lange) Imb.

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Summary. After treatment of one strain of *A. bitorquis* and 12 strains of *A. bisporus* in modified monokaryotization solution, three types of mycelia were received: one is the original di- or heterokaryon, the other two were proven to be neohaplonts in *A. bitorquis* and two strains of *A. bisporus*. In neohaplonts of good fruiting strains, homokaryotic fruiting was observed.

Key words: Homokaryotization technique – *Agaricus* sp.

Introduction

H. Leal-Lara and G. Eger-Hummel (1982) described a monokaryotization method for dikaryotic wood-rotting basidiomycetes. In contrast to previous surgical (Harder 1927a; Fries and Aschan 1952) or chemical monokaryotization methods (Miles and Raper 1956; Kerruish and DaCosta 1963; Takemaru 1964), the new method with autoclaved glycine/glucose or peptone P/glucose solutions, respectively, leads to complete and symmetric monokaryotization, that means, both types of parental nuclei can be recovered in similar amounts.

So far, all the monokaryotized mycelia have been dikaryons with clamp connections. Miles and Raper (1956) supposed that monokaryotization by sodium taurocholate or cholic acid is caused by irregularities in the formation of clamps. Under the influence of these chemicals, hooks do not fuse with subterminal hyphal compartments, but grow out to monokaryotic branches. Irregularities in clamp formation were occasionally observed prior to monokaryotization in the solutions used by Leal-Lara and Eger-Hummel. Therefore, it can be presumed that prevention of the fusion of the hook with the subterminal hyphal compartments might be a basic principle in the monokaryotization methods applied so far.

If this is true, the method of Leal-Lara and Eger-Hummel would not be applicable to such dikaryons or heterokaryons without clamp connections as *A. bitorquis* and *A. bisporus*. It is the objective of this investigation to prove or reject that hypothesis.

Materials and methods

Strains

A. bitorquis 4/23 and 8/64, sterile single spore mycelia, were obtained from Dr. G. Fritsche, Proefstation voor de Champignoncultuur, Horst, The Netherlands. 4/23 grows with longer straight hyphal compartments. Its mycelium appears adpressed when compared to the dense and fluffy mycelium of 8/64. The colonies of both homokaryons are of the same extension and uniformity. They were paired to the dikaryon 4/23×8/64, the mycelium of which forms strands and rings, and grows faster than the homokaryotic ones.

A. bisporus, now also referred to *A. brunnescens* (Elliott 1983), was available from several sources. The four homokaryons 169, compatible with 337 and 253, compatible with 335 were also obtained from Dr. Fritsche. On malt agar, 169 has fluffy aerial mycelium and produces brown pigments in the medium. The growth of 337 is very slow and colonies appear with adpressed hyphae. Mycelia of strain 253 and 335 are shown in Fig. 1.

The homokaryons were paired to fertile heterokaryons 169×337 and 253×335, which grow much better than the corresponding homokaryons (about twice as rapid as the faster growing homokaryons 253 or 169, respectively). Microscopically, the heterokaryons reveal stronger hyphae; branches form smaller angles than in homokaryons. The early fruiting heterokaryon 310a, ATCC 36930, and the comparatively late fruiting heterokaryon 71a, ATCC 36929, are from the culture collection of Dr. Eger-Hummel, Marburg. AB-1 to AB-10 are commercial strains, which were either isolated as "tissue-cultures" from fruit bodies or kindly provided by spawnmakers.

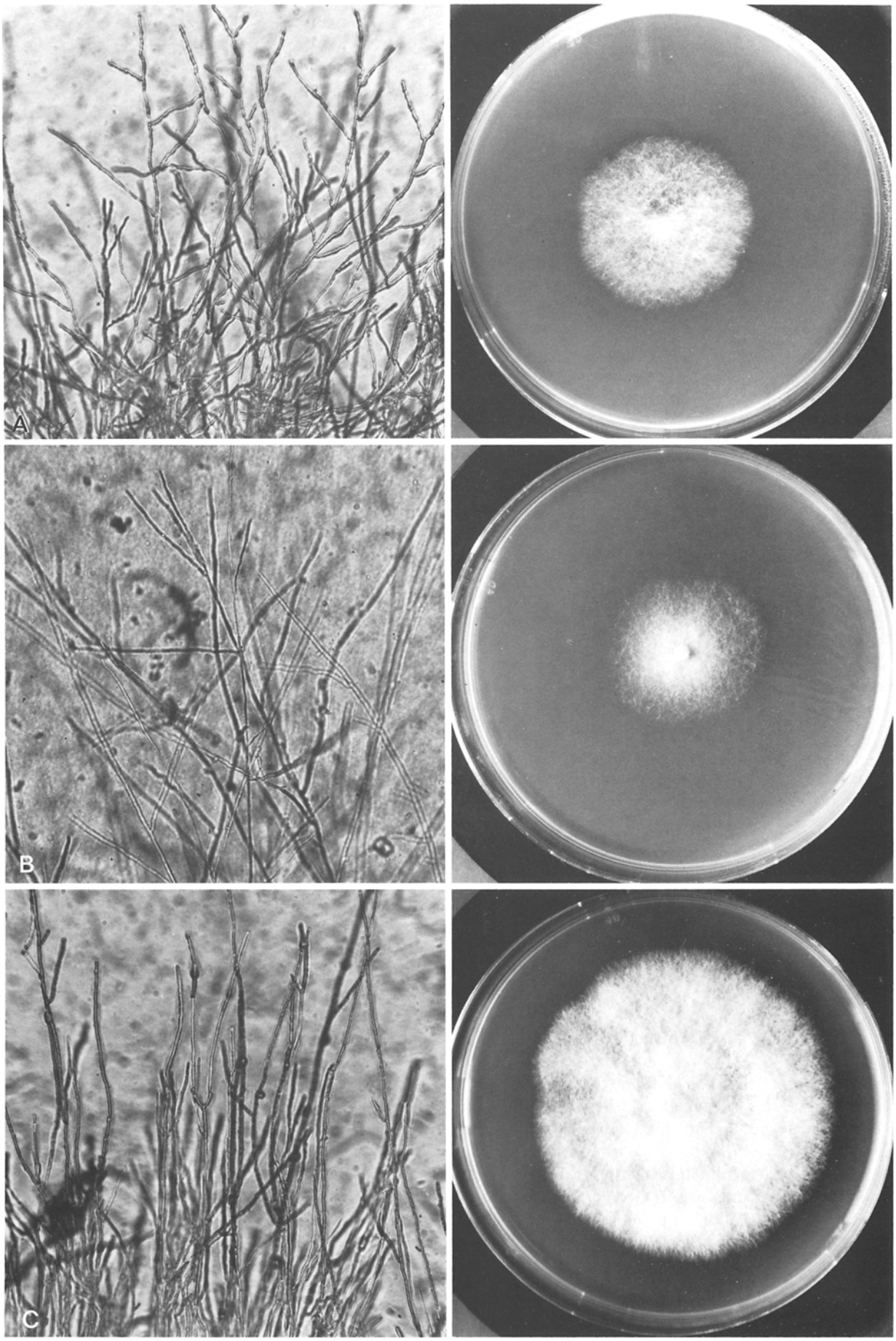

Fig. 1A–C. Microscopical (*left*) and macroscopical (*right*) characteristics of *A. bisporus*-homokaryons 253 (**A**) and 335 (**B**) as well as their mating – the heterokaryon 253 × 335 (**C**)

Methods

From the growing margin of a mycelial colony cultured on agar, small cubes were cut and, on a sterile slide, broken up by several strokes with a sterile razor-blade. After the addition of a drop of distilled water, mycelial fragments of 3 to 10 hyphal compartments were decanted by lifting one side of the slide and about 5 to 15 of them were transferred by a micro-pipette onto the bottom of a 50 ml Erlenmeyer flask containing 25 ml of a solution of 2% malt extract ("trocken, hell" DIAMALT AG, München) which was then sterilized for 20 min at 121 °C/2 bar. Avoiding agitation, the flasks were incubated at 23–24 °C (*A. bisporus*) or 28–29 °C (*A. bitorquis*), the respective optimal growth temperatures. Transfer to malt extract solution was necessary in order to adapt the strains to submerged culture. After 3 to 4 weeks, colonies of 3 to 5 mm diam were taken out, fragmented, decanted and transferred as described above into the following test solutions:

a) 40 mmol glycine + 100 mmol glucose monohydrate (MERCK; Darmstadt) per liter distilled water
b) 10 g neutralized meat peptone ("P" from OXOID Ltd., London) + 100 mmol glucose monohydrate per liter distilled water,

autoclaved for 20 min at 121 °C/2 bar.

The growth of *Agaricus* strains is very poor in solutions a) and b); therefore, after being allowed to cool down to room temperature, they were supplemented with autoclaved malt extract solution, resulting in a final concentration of 0.01%. Supplemented solutions a) and b) are referred to as c) and d), respectively.

After 30–60 days of incubation in test solutions, one or two mycelia, 2–3 mm in diameter, were fragmented and in several dilution steps streaked on agar plates supplemented with 2% malt extract. These plates were used whenever a solid medium was required. After 4–7 days of culture on the solid medium, 50–220 colonies were examined microscopically for their branching pattern and other characteristics, and then successively subcultured one or two times on agar. These cultures were finally classified according to their morphological characteristics. Three to five representatives of each class of presumed neohaplonts were paired with one another, and mating success was confirmed by subcultivation of resulting sectors (Fig. 2), comparison of subcultures with the original di- or heterokaryons and fertility tests. Fertility (in this investigation defined as formation of fruitbody primordia at least) was studied by "Halbschalentest" (Eger 1962).

Experiments and results

In contrast to the strains of wood-rotting fungi used by Leal-Lara (1980), *A. bitorquis* was very sensitive to fragmentation in a high-speed homogenizer. Cutting by hand, used in this investigations, was satisfactory and is now routinely applied in this laboratory for different species. *A. bitorquis* and *A. bisporus* strains, after submerged cultivation in malt extract or sterile filtered test solutions, fragmentation and transfer of hyphal fragments to solid medium, only regained their typical

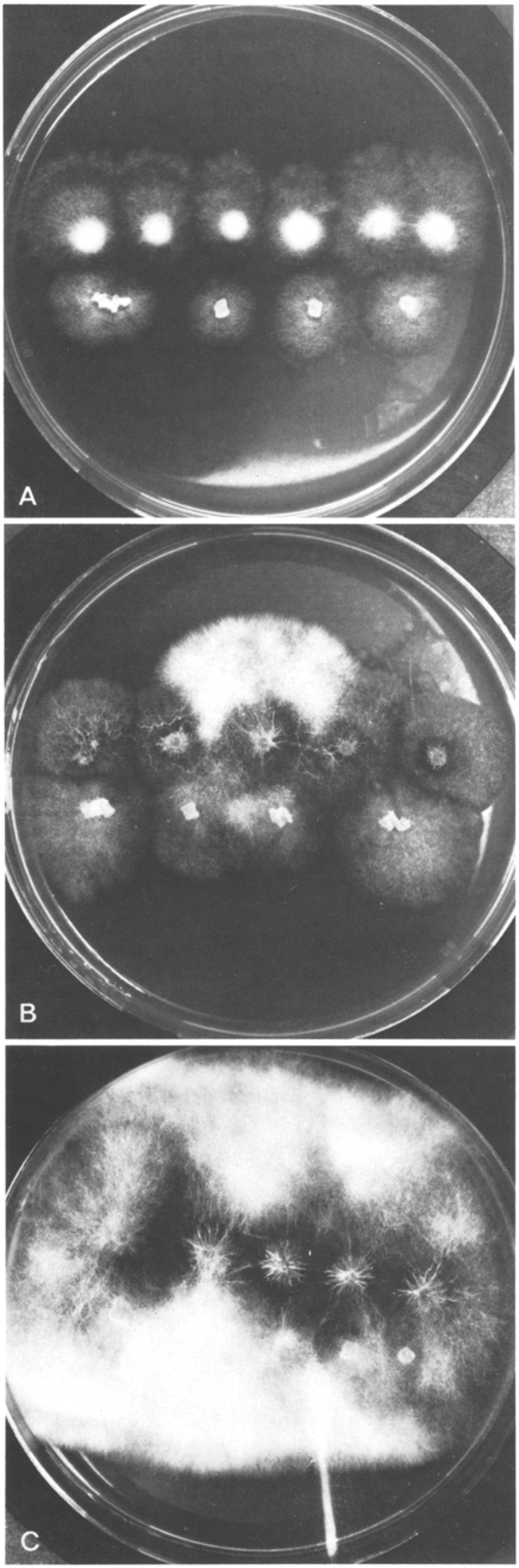

Fig. 2 A–C. Mating reaction of presumed neohaplonts of 310a. **A** No mating reaction so far; **B** Mating reaction on one side; **C** Mating reaction on both sides

micro- and macroscopic features during two to three successive subcultivations. *A. bitorquis* did not grow in test solutions a) or b). From solution c) and d) three types of mycelia were recovered in each experiment: one had the macro- and microscopic characters of the dikaryon, the other two corresponded to the known parental homokaryons (Table 1).

Results from the treatment of *A. bisporus* strains with known parental homokaryons are shown in Table 2; with unknown parental homokaryons in Table 3.

In *A. bisporus*, three types of mycelia were found in all four test solutions; the ratio of the types to each other, however, varied from experiment to experiment, as with *A. bitorquis*. When the parent homokaryons were known, it was relatively easy to recognize by comparison the heterokaryons and the single neohaplont types. There were difficulties, however, with strains of unknown parents because strain specific morphology is established sometimes only after several transfers onto solid medium and only the heterokaryon was available as reference strain. Since homokaryons had been considered to be sterile, confirmation of homokaryosis was attempted not only by mating experiments, but also by fertility tests.

In the case of strain 310a (Table 4), the presumed type I-neohaplont unexpectedly produced primordia and a small fruiting body. To prove that it was a real homokaryon, type I of 310a was placed into the test solution c) again. After 30–60 days, 220 individual mycelia were isolated, all of which were type I concerning growth rate and morphology. Ten of them – randomly chosen from the 220 – also produced primordia and fruiting bodies in the "Halbschalentest". They were paired with two neohaplonts of type II, Nh II_1 and Nh II_2, from other, independent homokaryotization experiments. The resulting 20 mycelia equalled the original strain 310a in morphology, growth rate and sporophore production. In addition, single spore progeny of one heterokaryotic and three presumed homokaryotic fruiting bodies were analysed. The first was heterogenous. The latter were homogenous in themselves, very similar to each other and significantly different from the one of the heterokaryotic fruiting body (for more detail see Dickhardt 1985). It can be concluded, therefore, that the presumed fertile neohaplont – 310a Nh I – was a parental homokaryon. It can be concluded further that in *A. bisporus*, sterility of a strain is not a reliable criterium for homokaryosis.

Finally, 10 commercial strains of *A. bisporus*, AB-1 to AB-10, were treated with either test solution c) or d). With two exceptions, one neohaplont of each heterokaryon formed at least primordia, in four cases also small fruit bodies.

Table 1. Recovery of neohaplonts and dikaryons in three experiments with *A. bitorquis*

Test solution	Incubation time (days)	No. of isolated mycelia	% neohaplonts		% dikaryons
			4/23	8/64	4/23×8/64
c	63	11	18	27	55
d	51	81	5	24	71
d	80	120	3	97	–

Table 2. Recovery of neohaplonts and heterokaryons in four experiments, each with two *A. bisporus* strains (parental homokaryons known)

Test solution	Incubation time (days)	No. of isolated mycelia	% neohaplonts		% heterokaryons
			Nh I	Nh II	
			253	335	253×335
a	40	165	19	10	81
b	62	120	31	35	34
c	31	100	10	32	58
d	49	100	41	4	55
			169	337	169×337
a	37	52	25	46	29
b	62	140	17	61	22
c	60	54	24	26	50
d	60	83	37	28	35

Table 3. Recovery of presumed neohaplonts and heterokaryons in *A. bisporus* strains (parental homokaryons unknown)

Test solution	Incubation time (days)	No. of isolated mycelia	% presumed neohaplonts		% heterokaryons
			Nh I	Nh II	
					71a
c	44	123	4	12	84
c	54	115	40	3	57
					310a
c	44	120	48	2	50
c	54	135	51	6	43
c	65	90	36	7	57
					AB–1
c	40	120	92	5	3
c	54	118	55	34	11

Table 4. Fertility of presumed neohaplonts and the heterokaryons from them in comparison to original heterokaryons. P = primordia; F = fruit body; – = sterile

Strains	Original heterokaryons	Presumed neohaplonts		Heterokaryons from neohaplonts
		Nh I	Nh II	
253×335	F, F, F	–,–,–	–,–,–	P, F, F
71a	P, F, F	–,–,–	–,–,–	P, F, P
310a	F, F, F	P, P, F	–,–,–	F, F, F
AB–1	P, F, F	–,–,–	–,–,–	F, F, F

Discussion

The results show clearly that disintegration of strains into their mono- or homokaryons with autoclaved solutions containing glycin and glucose does not depend on either clamp connections or dikaryotic state. Heterokaryons of *A. bisporus* with several nuclei per hyphal compartment (Raper et al. 1972) can be homokaryotized as well. In these solutions, conjugated nuclei in a dikaryon as well as associated nuclei of different mating types in a heterokaryon leave their affiliation and separate into mono- and homokaryons, respectively.

The little understood mating reaction in basidiomycetes is reversed! We can conclude therefrom that these solutions interfere with an essential process of fungal morphogenesis.

In wood-rotting species, Leal-Lara and Eger-Hummel (1982) reported 100% monokaryotization in all cases. In *A. bitorquis* and *A. bisporus* the degree of homokaryotization was not as high. It should be kept in mind, however, that *Agaricus* species have growth requirements different from wood-rotting species, and that the test solutions used in these investigations might not have been quite adequate. The results suggest that in addition to the proper substitution of test solutions for the single strains – to allow growth, but not to disturb homokaryotization – the duration of treatment might be a crucial factor for obtaining a high quantity of neohaplonts and a 1 : 1 ratio of both types (compare Tables 1 and 3). Leal-Lara and Eger-Hummel (1982) suggest that inhibition of growth is essential for monokaryotization. In the experiments with *Agaricus* strains the degree of homokaryotization was not observed to be correlated to growth inhibition. In test solutions supplemented with malt extract, growth and homokaryotization were better than in malt free media.

In *A. bisporus*, the morphology of neohaplonts within the classes varies more than in *P. ostreatus* (Leal-Lara 1980). This may be related to the multinucleate state: in the homokaryon as well as in the heterokaryon mutations do not become fully expressed as long as they are complemented with wild type genes.

The results reported here encourage further homokaryotization experiments, especially in *A. bisporus*. In this species two-spored basidia are predominantly found, but also three- and four spored ones occur (Miller 1971). According to Elliott (1972) single spore mycelia from two-spored isolates are supposed to be self-fertile, that means, heterokaryons, from triads ⅔ and from tetrades all single spore isolates are supposed to be cross fertile. Since spore germination is poor (7–60%, depending on the strain!), it is very tedious to get homokaryons for breeding purpose from random spores (Fritsche 1978), as well as picking tetrad-spores with the micromanipulator. Therefore, further elaboration of the homokaryotization method may become very useful for the breeding of new commercial strains.

So far, fruit body formation in *A. bisporus* strains has been considered to be an indication of heterokaryons (Elliott 1972; Miller 1971; Raper 1972) and sterility of mycelia from random spores an indication of being homokaryotic. Results from the homokaryotization experiments with the early fruiting strain 310a show that fertile homokaryons also occur in *A. bisporus*. Fruiting of one neohaplont each in 8 of 10 commercial strains supports the idea of Esser et al. that early and high yields in a commercial strain and fruiting of its homokaryons are correlated (Meinhardt and Esser 1981).

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