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Spheroplast Fusion and Heterokaryon Formation in *Mucor racemosus*

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Heterokaryons of *Mucor racemosus* were produced by fusion of spheroplasts from two auxotrophic strains of the fungus. Germinated sporangiospores were converted to spheroplasts by using commercial chitinase and *Myxobacter* AL-1 chitosanase. Spheroplasts from the auxotrophic strains were mixed in a buffered $Ca(NO_3)_2$ solution and fusion occurred. After cell wall regeneration, prototrophs were isolated. The frequency of heterokaryon formation was 1.45×10^{-4}. Prototrophic isolates segregated parental nuclei at a high frequency, indicating that heterokaryons had formed.

Mucor racemosus is a dimorphic fungus that grows in a mycelial form aerobically and as spherical, budding yeast cells when in a carbon dioxide atmosphere. In the course of our studies concerning morphogenesis in *Mucor,* we found it desirable to develop a system for genetic complementation of nutritional and developmental mutants. Complementation studies are critical for answering questions about the possible number of genes controlling a developmental process and for determining whether a mutation is dominant or recessive. We describe here a simple method for spheroplast production and heterokaryon formation in *M. racemosus.*

Genetic studies of the *Mucorales* have been hampered by the long dormancy of the sexual zygospores, the scarcity of genetic markers, and the difficulty in analyzing the progeny of each mating (5). In addition, exacting conditions are often required for mating. Consequently, complementation by heterokaryon formation has been the most useful tool for genetic manipulation. In *Phycomyces blakesleeanus,* heterokaryons can be formed by regeneration of a mixture of cytoplasm from the giant sporangiospores of different genetic constitution (5). In those species lacking giant cells, spheroplast fusion offers an alternate method for heterokaryon formation.

Spheroplast fusions in a variety of fungal genera have been described (6, 8). Recently, polyethylene glycol was introduced as a stimulant of spheroplast fusion (1, 2, 9, 10, 15). Members of the genus *Mucor* have been successfully converted to spheroplasts by utilizing the enzyme chitosanase (13, 14). Chitosan, a β-1,4-linked polymer of glucosamine, constitutes a major component of the vegetative cell wall of fungi belonging to the order *Mucorales* (3). Previous studies utilized chitosanase from a *Streptomyces* sp. to convert *Mucor* cells to spheroplasts (13, 14). In the studies reported here, we utilized a chitosanase from *Myxobacter* AL-1 (11), in combination with commercially available chitinase, to convert *Mucor* germlings to spheroplasts. We readily obtained the *Mycobacter* enzyme from relatively small cultures and could thus use it on a routine basis.

M. racemosus (*M. lusitanicus* ATCC 1216b) was used as the parental strain in this study. The leucine auxotroph Leu-1B and the adenine auxotroph Ade-1 used in the complementation experiments were provided by J. Peters (Department of Medical Microbiology, University of California, Irvine). Sporanigiospore inocula were prepared as previously described (7). *Myxobacter* AL-1 was provided by R. S. Wolfe (Department of Microbiology, University of Illinois).

Crude *Myxobacter* AL-1 chitosanase was prepared by a modification of the methods of Hedges and Wolfe (11). After citrate solubilization of the $ZnCl_2$ enzyme precipitate, the crude enzyme preparation was desalted on a column (2.5 by 50 cm) of Bio-Gel P-4 equilibrated with 0.02 M ammonium acetate buffer (pH 5.7) at 4°C. The fractions eluting in the void volume were pooled and lyophilized.

When sporangiospores were inoculated into liquid defined medium, they swelled and, within 10 h, produced germ tubes. Exposure of the germlings to an enzyme solution containing chitinase and chitosanase caused the release of spheroplasts that were apparent in the medium after about 20 min. Figure 1 shows the formation of two spheroplasts from a *Mucor* germling. Preferential degradation of the cell wall occurred at the apex of growing hyphae, although some

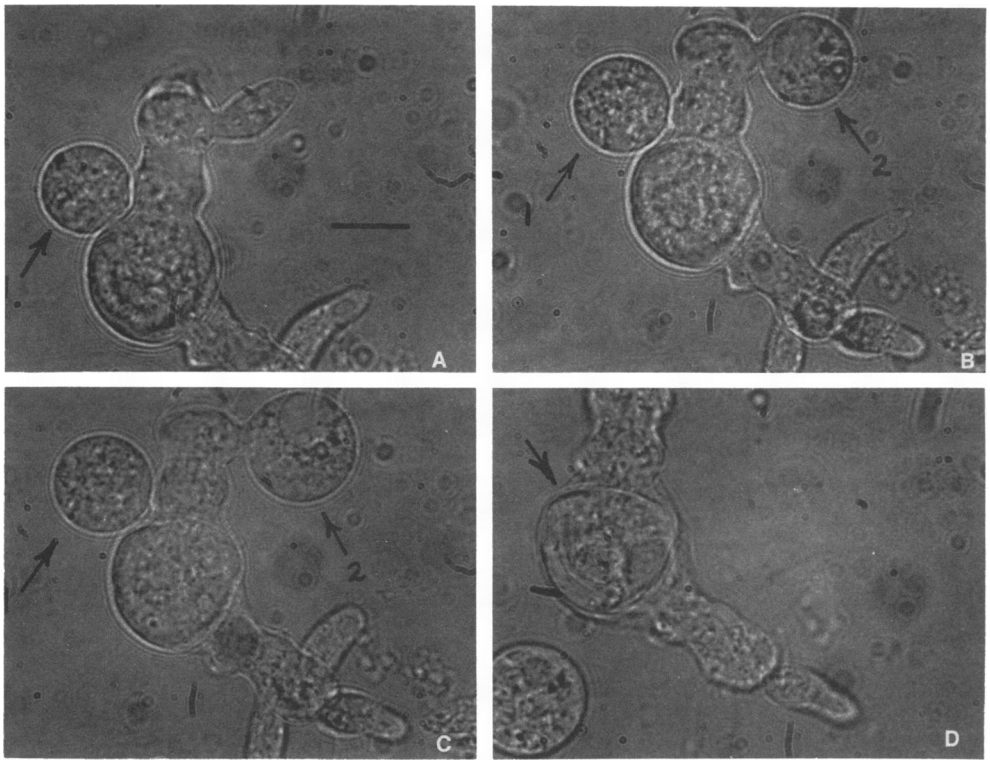

FIG. 1. *(A to C) Formation of two spheroplasts from a Mucor germling during incubation with chitinase and chitosanase. The positions of the spheroplasts are indicated by arrows. (D) shows the separation of a spheroplast from the mother cell. The arrow indicates its position before separation. Germlings were produced by incubating 10^6 spores per ml in a defined medium (7) supplemented with either 1 mM adenine or 1 mM leucine at 28°C on a shaker rotating at 200 rpm. Germlings were harvested and washed twice with 0.01 M sodium phosphate buffer containing 0.35 M sorbitol. Washed germlings were incubated at a density 10^6 cells per ml at 22°C in the same buffer containing 2 mg of crude chitosanase per ml and 1 mg of chitinase per ml (Nutritional Biochemicals Corp.). Bar represents 10 μm.*

spheroplast formation from the lateral walls was noted. Probably because of their unique composition, the sporangiospore walls were not attacked by the lytic enzymes (3). Presumably, the newly formed apical wall was more sensitive because it was the site of autolytic enzymes (4). During spheroplasting, the hyphal contents emptied into the newly formed spheroplast, which rounded, swelled, and eventually separated from the mother cell. Single germlings often gave rise to multiple spheroplasts. Since *Mucor* is coenocytic, these results suggest that the plasma membrane ruptured and refused during the process of multiple spheroplast extrusion. The osmotic sensitivity of *Mucor* spheroplasts is shown in Fig. 2. Dilution of the stabilizing medium with distilled water led to a progressive disintegration of cell integrity.

When spheroplasts from the two auxotrophic strains were mixed and incubated in $Ca(NO_3)_2$ solutions, prototrophs could be isolated after a period of cell wall regeneration. The average frequency of prototroph formation from two separate experiments was 1.45×10^{-4}. The reversion rates of the adenine and leucine auxotrophs as determined by plating spores from the strains on unsupplemented media were less than 4.2×10^{-8} and 3.5×10^{-8}, respectively.

The prototrophic isolates that arose after spheroplast fusion gave rise to sporangiospores of either parental phenotype and to prototrophs (Table 1, Fig. 3). Permanently complemented cells were not observed. After repeated transfer, prototrophic colonies continued to segregate spores with the parental phenotypes as well as prototrophic spores. These results indicate that heterokaryon formation rather than nuclear fusion occurred.

Spores arising from the prototrophic isolates were predominantly (92%) auxotrophic, indicating that the majority of the spores were probably uninucleate. The proportion of Ade^- segregants to Leu^- segregants differed from isolate to isolate (Table 1); the Leu^-/Ade^- ratio was always

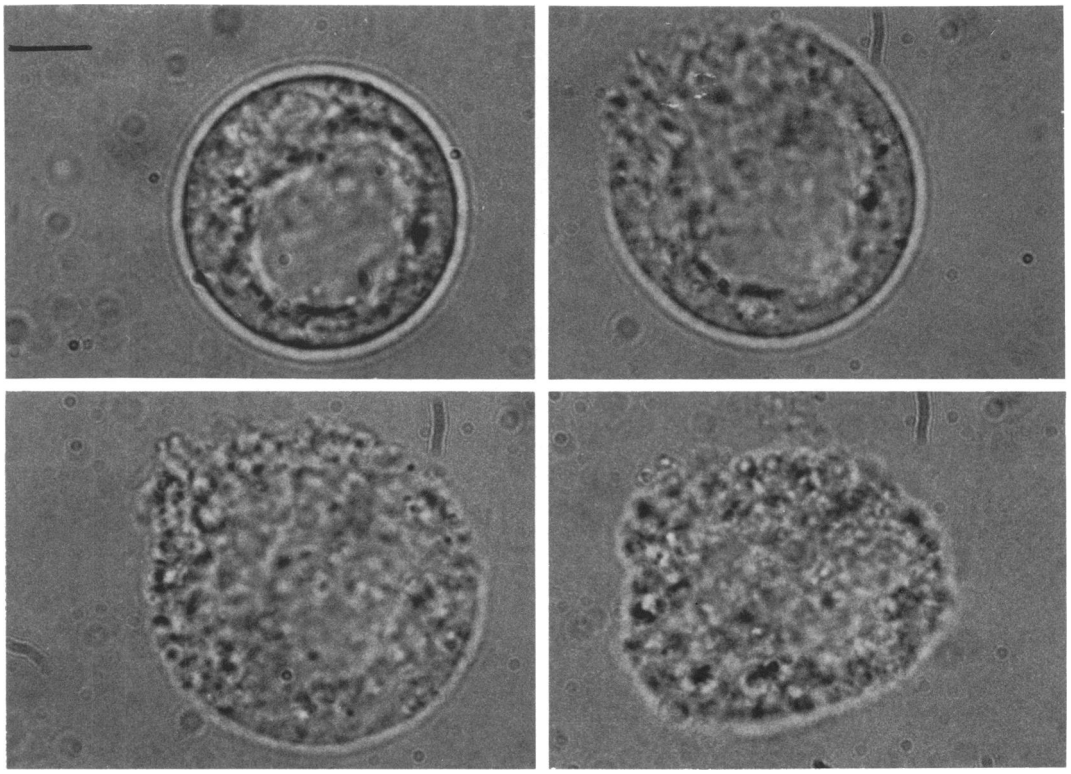

FIG. 2. *Osmotic lysis of a Mucor spheroplast. A progressive disintegration of cell integrity is evident as the osmotic pressure of the medium is decreased by flooding the slide with distilled water. Photographs were taken at 5- to 10-s intervals. Bar represents 10 μm.*

TABLE 1. *Results of replica plating the progeny of prototrophic isolates formed after spheroplast fusion*[a]

Phenotype	No. of heterokaryons formed											
	A-1[b]	A-2	A-3	B-1	B-2	B-3	B-4	B-5	B-6	B-7	B-8	B-12
$Ade^- Leu^+$	15	14	3	9	11	1	12	4	10	6	6	14
$Ade^+ Leu^-$	46	36	13	37	34	41	39	26	26	16	40	41
$Ade^+ Leu^+$	5	2	4	8	0	2	3	5	6	5	2	0

[a] Fusion experiments were performed as follows. After 1 h of incubation with the lytic enzymes, 1-ml portions of the spheroplast suspension from the two auxotrophic mutants were mixed and centrifuged, and the sorbitol buffer was decanted and replaced with an equal volume of sterile 0.2 M $Ca(NO_3)_2$ in 0.05 M glycine-sodium hydroxide buffer (pH 9.0). The mixture was centrifuged to a pellet and incubated for 15 min at 22°C. The supernatant fluid was removed and replaced with an equal volume of sterile 0.2 M $Ca(NO_3)_2$ in 0.05 M glycine-sodium hydroxide buffer (pH 7.0). After further incubation at 22°C for 45 min, the spheroplast mixture was centrifuged, and the buffer was replaced with a regeneration medium consisting of the defined medium supplemented with 1 mM adenine, 1 mM leucine, and 0.35 M sorbitol. The regenerating spheroplasts were incubated for 8 h at room temperature with occasional agitation. The cells were then centrifuged and washed three times in 0.01 M sodium phosphate buffer containing 0.35 M sorbitol. The cells were plated on unsupplemented defined medium (pH 3.0) at a density of ca. 10^5 per plate and on defined medium supplemented with 1 mM adenine and 1 mM leucine (pH 3.0) at a ca. 300 per plate. The plates were incubated at room temperature and scored after 24 h. Presumptive heterokaryons growing on the unsupplemented medium were isolated and allowed to sporulate on defined medium plates (pH 4.5). The spores from these colonies were used for replica plating to confirm their heterokaryotic nature. The results presented are from 12 of 15 prototrophs isolated in a single fusion experiment.

[b] Isolate.

greater than 1, with the highest value at 41.0 and the lowest at 2.4. An average for the spores from all isolates yielded leucine auxotrophs, adenine auxotrophs, and complemented prototrophs in a ratio of 9.0:2.3:1.

The unequal segregation ratios of parental

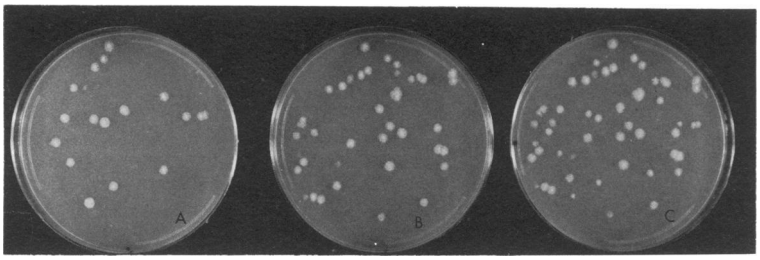

FIG. 3. *Results of replica plating of segregants of prototrophic isolate B-1 onto supplemented minimal media. The utility of acidified media (pH 3.0) to promote colonial growth of Mucor is also demonstrated. Masters for replica plating were produced by plating spores from prototrophic isolates onto a yeast extract-peptone-glucose medium (7) supplemented with 1 mM adenine. The plates were incubated at 22°C for 48 h under a 70% N_2–30% CO_2 atmosphere to induce yeast colonies. Each master plate was replicated onto three defined medium plates (pH 3.0) containing (1 mM each; in order of transfer): (A) adenine, (B) leucine, (C) adenine and leucine. The replica plates were incubated aerobically at 22°C and scored after 24 h. The efficiency of transfer in replica plating was about 90%. Consequently, colonies appearing on either of the singly supplemented media but not on the doubly supplemented medium were not scored.*

nuclei demonstrated in our work may have been due to any of several factors. The ratios may reflect slower replication of the Ade-1 nucleus compared with that of the Leu-1B nucleus. The nuclei of multinucleate cells of the fungi are not synchronized and may divide at different rates (12). The necessity to translocate adenine for DNA synthesis into these nuclei from other areas in the mycelium may have resulted in a slower replication rate. Alternatively, the heterokaryons may have contained more Leu-1B nuclei as a result of multiple spheroplast fusions. If this was the case, an unequal nuclear ratio would have resulted despite equal nuclear division rates. Of course, multiple fusions would not result in more Leu-1B nuclei unless the Leu-1B spheroplasts were more numerous or had a higher fusion tendency.

Our results demonstrate the relative ease with which complementation studies can be performed with *Mucor*. The cells are readily replica plated in the yeast phase when grown anaerobically. The use of acidified agar media (pH 3.0) promotes colonial morphology in the mycelial phase, allowing up to 150 colonies to be discerned. Colonial mycelia generated in this manner form sporangiospores and can be transferred easily. The use of chitinase and chitosanase to spheroplast *Mucor* and related cells suggests a number of other potential uses. Because both enzymes are readily obtainable, they would be useful in the preparation of membranes and other intracellular organelles requiring a gentle lysis procedure. It may also be feasible to utilize chitinase-chitosanase digestion as a counterselection against germinated spores to enrich for auxotrophic or nongerminating mutants. This would take advantage of the fact that spore walls have a unique composition and are insensitive to lytic enzymes (4).

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