JOURNAL OF BACTERIOLOGY, Feb. 1980, p. 565-569
0021-9193/80/02-0565/05$02.00/0
Vol. 141, No. 2

High-Frequency Heterokaryon Formation by *Mucor racemosus*

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Heterokaryons from *Mucor racemosus* were produced from two auxotrophic strains of the fungus. Germlings were converted to spheroplasts by using commercial chitinase and purified *Myxobacter* AL-1 chitosanase. Spheroplasts from the auxotrophic strains were mixed and fused in solutions of polyethylene glycol and $CaCl_2$. Under optimal conditions, prototrophic heterokaryons were formed at a frequency of about 5%.

Mucor racemosus, a dimorphic zygomycete, is capable of growth in two alternate vegetative modes. Under certain conditions, the synthesis of cell wall polymers such as chitin is localized at relatively few points in the cell leading to a filamentous hyphal morphology (3). Under other cultural conditions, wall synthesis occurs equally in all dimensions, giving rise to spherical yeast cells which divide by budding. In either case, the composition of the cell wall remains relatively constant (2). Therefore, it is apparent that regulation of the sites of wall synthesis play a crucial role in morphogenesis in *Mucor*.

Morphogenetic responses in *Mucor* (yeast-to-mycelia or mycelia-to-yeast transitions) can be evoked easily and by a multiplicity of conditions or agents (17). In addition, developmental mutants exhibiting abberrant morphologies can be isolated (16). Therefore, the system is quite amenable to experimental analysis as a model system of primitive eucaryotic morphogenesis. Until recently, however, no suitable genetic system was available for the analysis of mutants. We have described a method of spheroplast fusion which results in the formation of heterokaryons at frequencies of 10^{-4} to 10^{-5} (9). Although such a frequency is useful for complementation and dominance studies on selectable genetic markers, it is of less value for the analysis of unselectable developmental mutations.

The present study was undertaken to increase the frequency of heterokaryon formation to levels at which selection of complemented fusion products would not be necessary. If a high frequency were attainable, complementation of developmental mutations might be possible simply by morphological criteria. We report here that with polyethylene glycol (PEG) and calcium as fusion stimulants, the frequency of heterokaryon formation can attain about 5%.

Solutions of PEG and calcium have been found to greatly enhance the fusion of plant protoplasts (11). PEG has also been used to stimulate fusion of bacterial protoplasts *in Bacillus subtilis* (14) and *Streptomyces* (12). PEG-stimulated fusion of fungal spheroplasts has been shown in *Aspergillus nidulans* (5-7), *Saccharomyces cerevisae* (18), *Schizosaccharomyces pombe* (15), and *Penicillium* sp. (1). In addition, PEG and calcium have been used to stimulate the transformation of *S. cerevisae* by chimeric *Escherichia coli* plasmids containing yeast genes (10).

MATERIALS AND METHODS

Organisms. *M. racemosus* (*M. lusitanicus* ATCC 12166) was the parental strain for all auxotrophs. The leucine auxotroph Leu-1B and the adenine auxotroph (Ade-1) were provided by J. Peters. The auxotrophs were derived by mutagenesis with *N*-methyl-*N'*-nitrosoguanidine and were selected by a differential freeze killing technique (13). Sporangiospore inocula for all strains were prepared as previously described (4). *Myxobacter* AL-1 was donated by R. S. Wolfe.

Media and culture conditions. The defined amino acid-free yeast nitrogen base medium (YNB) contained per liter: glucose, 10 g; $(NH_4)_2SO_4$, 1.5 g; glutamic acid, 1.5 g; and amino acid-free yeast nitrogen base (Difco Laboratories), 0.5 g. The YNB medium was supplemented with 1 mM adenine sulfate for the growth of Ade-1, 1 mM leucine for the growth of Leu-1B, or 1 mM arginine for arginine auxotrophs. Liquid YNB was adjusted to pH 4.5 with 1 M H_2SO_4.

Fused spheroplasts were plated on hypertonic YNB agar medium, pH 3.0, containing 0.6 M sorbitol and supplemented when necessary with 1 mM adenine sulfate and 1 mM leucine or 1 mM arginine. Solid agar media at pH 3.0 were prepared by autoclaving double-strength medium components adjusted to pH 3.0 and 4.0% agar (Difco) separately, to prevent agar hydrolysis. The pH 3.0 medium induces colonial mycelial growth.

Chitosanase production. *Myxobacter* AL-1 chitosanase was produced and purified by the method of Hedges and Wolfe (9). The purified enzyme showed

two bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and had a specific activity of 25,000 U/ml in the carboxymethyl cellulose assay (9).

Preparation of spheroplasts. A 40-ml amount of appropriately supplemented liquid YNB medium, in a 500-ml Erlenmeyer flask, was inoculated to 2.5×10^5 spores per ml. The culture was incubated 11 to 12 h for Ade-1 and 8 to 9 h for other auxotrophs at 28°C and 200 rpm on a rotatory shaker. Germlings were harvested by centrifugation and washed once in 0.02 M sodium phosphate buffer, pH 6.8, containing 0.6 M sorbitol. The washed germlings were incubated at a density of 1.25×10^6 cells per ml at 30°C in 1 ml of the above buffer containing 200 U of purified chitosanase per ml and 3 mg of chitinase (Nutritional Biochemicals) per ml. Microscopic observation revealed the presence of spheroplasts within 5 min. Incubation was continued for 1 h to achieve nearly 100% spheroplasts.

Fusion and regeneration. Spheroplasts of the two auxotrophs were mixed and washed three times with cold 0.02 M sodium phosphate buffer (pH 6.8) containing 0.6 M KCl. Washed spheroplasts were fused in 1 ml of PEG (average molecular weight 4,000 or 6,000; Sigma Chemical Co.) containing $CaCl_2$ for 20 min at room temperature. The concentration of PEG ranged from 15 to 54% (wt/vol), and the concentration of $CaCl_2$ ranged from 0 to 100 mM. The fusion mixture was serially diluted and spread with a glass rod on 4°C hypertonic YNB agar medium, pH 3.0, and 4°C hypertonic YNB agar medium, pH 3.0, supplemented with adenine and leucine or with arginine. Heterokaryon frequency was calculated by dividing the number of colonies per milliliter appearing after 24 h at 22°C on hypertonic YNB agar medium by the number of colonies per milliliter on supplemented hypertonic YNB agar medium.

RESULTS

Spheroplast formation. Essentially 100% conversion of germlings to spheroplasts occurred within 30 to 60 min under the optimized conditions given in Materials and Methods (Fig. 1). These conditions were derived from preliminary experiments in which the type of osmotic stabilizer (sorbitol, NaCl, or KCl), the molarity of the stabilizer, and the concentrations of lytic enzymes were varied. Spheroplast formation was monitored microscopically by the appearance of spherical, osmotically sensitive cells. Spheroplast formation was shown to be necessary for heterokaryon formation because no complementation could be detected by mixing intact auxotrophic germlings.

Regeneration and plating conditions. Our earlier method (8) of heterokaryon formation involved a period of cell wall regeneration in an osmotically stabilized liquid medium followed by plating on nonstabilized solid media. To achieve a more rapid and convenient procedure, direct plating of fusion mixtures on osmotically stabilized plates was attempted (Table 1). In this experiment, a fusion mixture of the leucine and

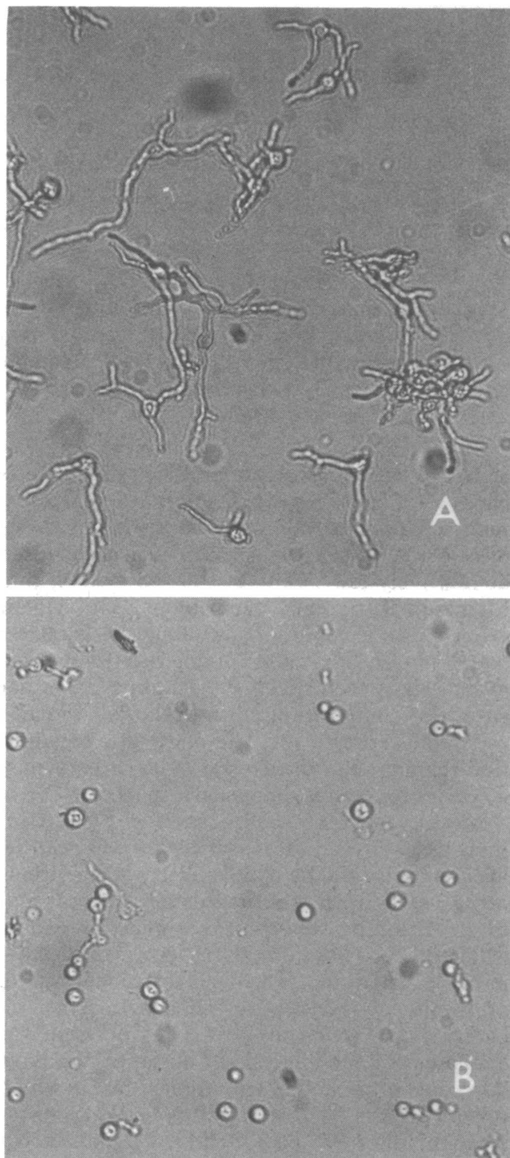

FIG. 1. *(A) Germlings of M. racemosus. (B) Mucor spheroplasts after treatment for 30 m with chitinase and chitosanase. The same field as in A was photographed.*

adenine auxtrophs in 25% PEG 4000–100 mM $CaCl_2$ was divided in half. One portion was centrifuged, and the PEG was replaced with YNB medium supplemented with adenine and leucine and containing 0.6 M sorbitol. After being washed twice with 0.02 M phosphate buffer, the cells were diluted and plated onto unstabilized YNB plates, pH 3.0. The other portion was diluted and plated directly onto stabilized YNB solid media as described in Materials and Meth-

ods. The two methods gave comparable results (Table 1). Plating was therefore adopted as standard procedure in subsequent experiments.

Fusion pH conditions. To determine the optimal pH for cell fusion, solutions of 45% PEG 4000 containing 60 mM $CaCl_2$ were prepared in a variety of buffers. These solutions were used for the fusion of Ade-1 and Leu-1 (Table 2). Compared with an unbuffered fusion mixture, all the buffered solutions resulted in decreased frequency of heterokaryon formation. Subsequent experiments did not utilize buffers during fusion.

Dependence on PEG concentration and molecular weight. Figure 2 shows the dependence of heterokaryon formation on the concentration of PEG 4000 in the presence of 100 mM $CaCl_2$. The curve represents the average from three independent experiments. A similar series of experiments using PEG 6000 in 100 mM $CaCl_2$ is shown in Fig. 3. The average frequency of heterokaryon formation in the two series of experiments was 3.3% ± 0.6% at 45% PEG 4000 and 4.8% ± 2.1% at 40% PEG 6000. Although PEG 6000 (Fig. 3) gave higher frequencies on average, we adopted 45% PEG 4000 as standard procedure in subsequent experiments, since it is easier to work with concentrated solutions of PEG 4000.

TABLE 1. *Comparison of the frequencies of heterokaryon formation by the liquid regeneration method and the hypertonic spread plate method*

Method	Frequency with:[a]		% Prototrophs
	YNB + Ade + Leu	YNB	
Liquid regeneration	2.0×10^5	2.4×10^2	0.12
Hypertonic spread plate			
0.6 M glucose	5.2×10^5	8.2×10^2	0.16
0.6 M KCl	3.8×10^5	5.4×10^2	0.14
0.6 M sorbitol	5.9×10^5	1.3×10^3	0.23

[a] Each value represents colony-forming units per milliliter.

TABLE 2. *Frequency of heterokaryon formation with buffered solutions of PEG containing $CaCl_2$*

Buffer[a]	Frequency with:[b]		% Prototrophs
	YNB + Ade + Leu	YNB	
Unbuffered, pH 5.2	3.9×10^5	1.1×10^4	2.8
Acetate, pH 4.0	1.9×10^5	5.0×10^2	0.3
Acetate, pH 5.0	2.4×10^5	2.4×10^3	1.0
MES, pH 5.0	3.0×10^5	1.0×10^4	1.8
MOPS, pH 6.0	2.7×10^5	1.9×10^3	0.7

[a] MES, Morpholineethanesulfonic acid; MOPS, morpholinepropanesulfonic acid.

[b] Each value represents colony-forming units per milliliter.

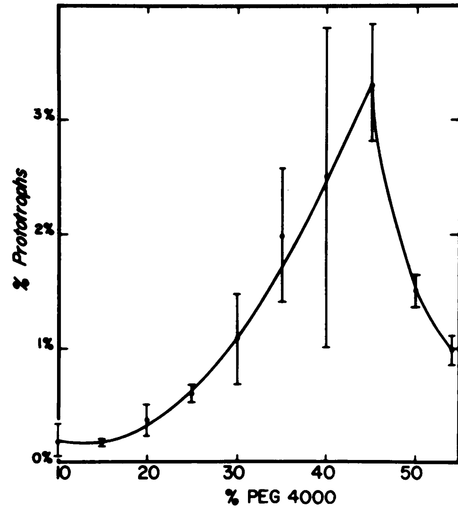

FIG. 2. *Dependence of heterokaryon formation from Ade-1 and Leu-1B on the concentration of PEG 4000 in the fusion mixture. The bars represent the standard deviation from three independent experiments.*

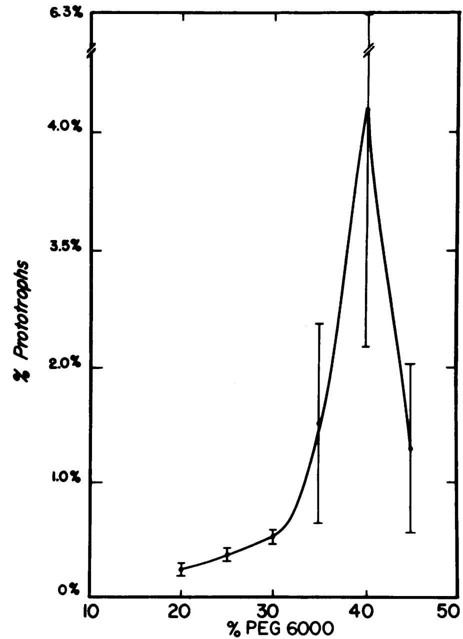

FIG. 3. *Dependence of heterokaryon formation from Ade-1 and Leu-1B on the concentration of PEG 6000 in the fusion mixture. The bars represent the standard deviation from three independent experiments.*

Dependence on calcium concentration. The optimal $CaCl_2$ concentration for cell fusion was determined by using 45% PEG 4000 solutions containing various concentrations of $CaCl_2$, ranging from 0 to 100 mM. Figure 4 shows the averaged results from three independent experiments. The average frequency of heterokaryon formation without $CaCl_2$ was 0.4%. The frequency was greatest at 60 mM $CaCl_2$, being 5.3%.

Complementation of other auxotrophic strains. Table 3 shows the results of fusion experiments performed with a variety of auxotrophic strains. No complementation was observed from fusion of cells of a single strain. The results also indicate that the complementation is not unique to the leucine and adenine auxotrophs, and that complementation within a pathway (ARG-1 × ARG-3) can be demonstrated.

DISCUSSION

Genetic studies of the *Mucorales* have been hampered by the long dormancy of the sexual zygospores, the scarcity of genetic markers, and difficulties in analysis of the progeny of a mating. In addition, exacting conditions are required for mating. Protoplast fusion and heterokaryon formation offers a means for complementation and dominance testing with these fungi. Since the vegetative cell walls of the members of the *Mucorales* are composed primarily of chitin and chitosan (2), the methods presented in this paper may be of general utility for this group of organisms.

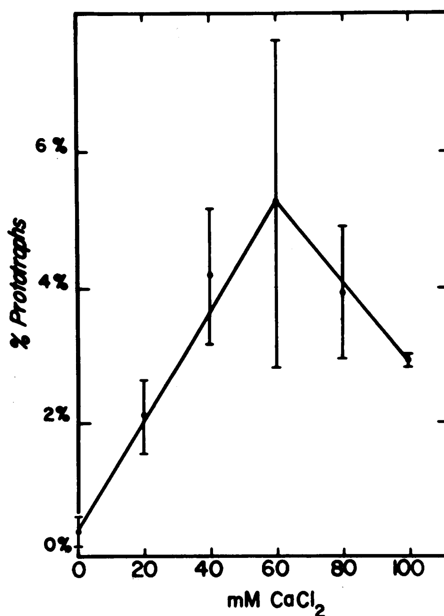

FIG. 4. *Dependence of heterokaryon formation from Ade-1 and Leu-1B on $CaCl_2$ concentration in 45% PEG 4000. The bars represent the standard deviation from three independent experiments.*

TABLE 3. *Complementation of other auxotrophs*

Auxotrophic strain	% Prototrophs
Ade-1 × Ade-1	0
Arg-1 × Arg-1	0
Arg-3 × Arg-3	0
Leu-1 × Leu-1	0
Arg-1 × Leu-1	1.0
Arg-3 × Leu-1	2.0
Arg-1 × Arg-3	1.0
Ade-1 × Leu-1	6.0

The fusion of auxotrophic spheroplasts allows the complementation of genetically dissimilar nuclei within the coenocytic *Mucor* mycelium. Each heterokaryon formed has a unique nuclear composition (8), probably based on the proportion of each nuclear type in the original fusion product. Sporangiospore formation generally segregates cells of parental phenotype. The small proportion of spores which are prototrophic are probably binucleate and also segregate parental phenotypes at a high frequency when resporulated, indicating that the parental nuclei are independent and have not fused (8).

Chitinase and chitosanase as lytic enzymes for *Mucor* have other potential uses. For instance, we have used the enzymes to counterselect against germinated spores to enrich a mutagenized spore population for auxotrophs (unpublished data). This technique takes advantage of the fact that spore walls are insensitive to the enzymes, since they are devoid of chitin and chitosan (2). In addition, it may be possible to utilize *Mucor* spheroplasts for genetic transformation, as in *Saccharomyces* (10).

By optimizing the conditions for spheroplast formation, cell fusion, and wall regeneration the frequency of heterokaryon formation was increased from 10^{-5} (8) to about 5×10^{-2}. This high frequency is necessary to detect complementation between developmental mutants for which there are no selective conditions.

ACKNOWLEDGMENT

This work was supported by Public Health Service grant 5 RO1 GM25176 from the National Institute of General Medical Sciences.

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