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2 February 1969; revised 12 March 1969 ■

Fungal Morphogenesis: Cell Wall Construction in Mucor rouxii

Abstract. *An autoradiographic study revealed two different patterns of cell wall construction associated with two types of morphogenesis in* Mucor rouxii. *In hyphae, the cell wall was preferentially synthesized in the apical region; these cylindrical walls seemed to be generated by a sharply descending gradient of wall synthesis radiating from the apex. In spherical cells (germinating spores, yeast cells), wall formation occurred largely, if not entirely, in uniformly dispersed fashion over the entire cell periphery.*

The vegetative morphology of *Mucor rouxii* is highly dependent on the environment. By suitable adjustment of the concentrations of carbon dioxide, oxygen, hexoses, and other factors (*1–3*), the fungus may be grown in a variety of forms ranging from slender hyphae to spherical budding yeast cells. This well-defined dimorphic capacity makes *M. rouxii* a convenient subject for exploring the molecular basis of vegetative morphogenesis in fungi.

Our previous studies on *M. rouxii*, when considered with the observations of others on the apical growth of fungi (*4, 5*), led to the hypothesis that fungal dimorphism might result from two different modes of cell wall construction: (i) apically localized in hyphae and (ii) uniformly dispersed in budding yeast cells (*6*). The present study was undertaken to test this proposal.

We obtained cylindrical and spherical cells by germinating spores of *M. rouxii*, IM-80, in yeast extract-peptone-glucose (YPG) medium (*1*)—hyphae were grown in medium containing 0.01 percent glucose and incubated under purified nitrogen; yeast cells were formed in medium with 0.1 percent glucose incubated under 30 percent CO_2 and 70 percent purified nitrogen (*3*). Prior to emergence of hyphae or yeast buds, spores undergo an obligatory phase of spherical growth (*7*); these spherical cells ("swollen" spores) were also examined.

To reveal the pattern of cell wall construction, we labeled growing cells with *N*-acetyl-D-[GL-^{3}H]glucosamine (*8*). Spores of *M. rouxii* (2×10^4 per milliliter) were inoculated in 20 ml of YPG medium as specified above, supplemented with 0.01 percent *N*-acetylglucosamine, and incubated in specially designed culture tubes (*3*). Cultures were chilled and centrifuged, and most of the supernatant fluid was discarded, leaving the cells suspended in about 2 ml. Suspensions of chilled unlabeled cells were flushed with the gas under which they were grown to eliminate any air introduced during centrifugation, and the temperature was again brought to 28°C. Cell suspensions were then added to 200 μc of tritiated *N*-acetylglucosamine and incubated anaerobically for 1 or 5 minutes. Cells were immediately killed by addition of an equal volume of 2*M* HCl. After being washed with water, the cells were extracted with 3 ml of alkaline ethanol (aqueous 1*M* NaOH : 95 percent ethanol, 1 : 2, by volume) in a boiling water bath for 5 minutes. This procedure removed all cytoplasmic radioactivity without destroying the original shape of the cell. The resulting cell ghosts were stained with congo red, heat-fixed onto a microscope slide, and coated with Kodak nuclear emulsion NTB-3 by the dipping method. Slides were exposed for several days and then developed. Silver grains on the autoradiograph correspond largely, if not entirely, to glucosamine molecules incorporated into two main cell wall polymers—chitin and chitosan (*9*).

The observed patterns of cell wall construction conformed to those previously postulated for cylindrical and spherical cells (Fig. 1). The hyphae exhibited a highly localized apical pattern of wall formation (Fig. 1E). Cell wall synthesis occurred mainly in the apical dome, an approximately hemispherical surface of about 4 to 6 μ in diameter. Rates of wall synthesis along the hyphae were measured by quantitative autoradiography (Fig. 2). A sharp gradient of cell wall synthesis exists in the apical region; the highest rate occurred at, or within 1 μ of, the zenith of the apical dome. Within 3 μ from

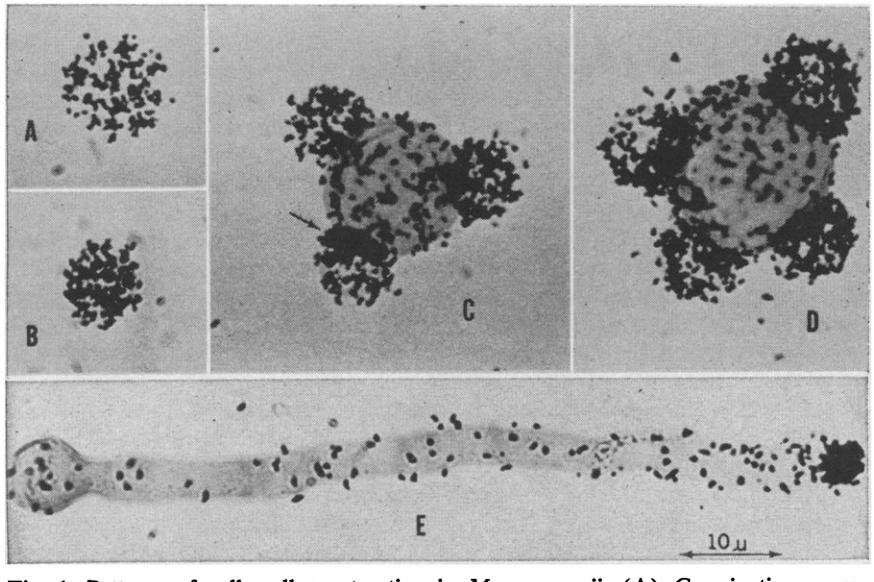

Fig. 1. Patterns of cell wall construction in *Mucor rouxii*. (A) Germinating spores prior to budding; (B) germinating spores prior to hyphal tube emergence; (C) and (D) yeast cells with three and four buds; and (E) hypha. Cells are 3 hours old in A and B and 12 hours old in C to E. All cells were exposed to tritiated *N*-acetyl-D-glucosamine for 5 minutes.

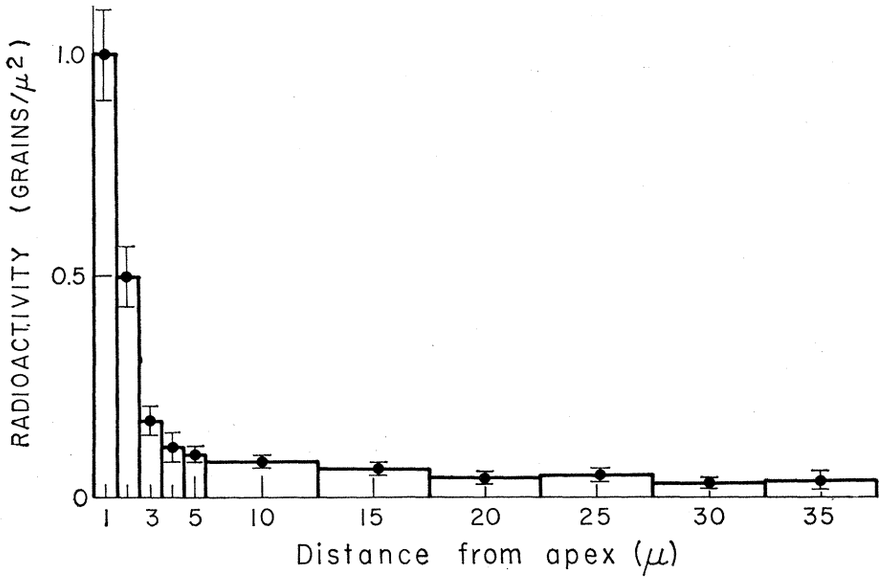

Fig. 2. Rates of wall synthesis in a young hypha. Rates were measured on 8-hour-old hyphae incubated with tritiated *N*-acetyl-D-glucosamine for 1 minute; grains were counted in segments 1 μ long at the tip, and 10 μ long elsewhere. The surface area of each segment was measured gravimetrically by cutting out the outline of the cells copied on paper from projected Kodachrome slides. Points are mean values with standard error markers for $n = 25$. The elongation rate of the hyphae at harvest time was about 0.6 μ/min.

the zenith, the rate of synthesis decreased abruptly by about 50 percent per linear micron. In the tubular portion of the hyphae, the rate of wall synthesis had dropped to only a few percent of that at the apex. Here, too, the rate of synthesis of cell wall declined toward the older portions of the tube, but the gradient was much less pronounced than the apical one. Seemingly, the hyphal morphology is generated by the continuous and progressive conversion of the quasi-hemispherical wall of the apical dome into the quasi-cylindrical wall of the hyphal tube. Such a topologic transition appears to be accomplished by the establishment of the steep descending gradient of wall synthesis found in the apical dome. This is most likely a radial gradient of cell wall growth centered at or very near the zenith of the apical dome.

By contrast, spherical cells of *M. rouxii* (both germinating spores and yeast buds) exhibited a scattered pattern of wall synthesis (Fig. 1, A–D). In yeast buds there was no indication of higher activity at the bud "apex," that is, the pole opposite the budding site [in contrast with claim of "tip growth" in *Saccharomyces cerevisiae* buds (*10*)]. In fact, there was often slightly higher incorporation of tritium at the base of the bud. In some cases, a narrow band of wall synthesis was seen between bud and mother cell (Fig. 1C, arrow). The basal hemisphere incorporated an average of 1.23 times more radioactivity than the distal hemisphere. In the largest buds the ratio was 1.45. This higher basal activity, especially of larger (older) buds, is probably related to localized formation of septum walls separating bud from mother cell, rather than an indication of anisotropy in the growth of the bud itself. Our findings, which pertain to skeletal polymers of the wall (*9*), suggest that spherical yeast buds of *M. rouxii* grow uniformly around their entire periphery, with only a minor tendency, if any, for greater wall synthesis in the basal hemisphere [in contrast with claims for basal growth in buds of *S. cerevisiae* (*11*)].

Our findings may contribute to the understanding of the biochemical and subcellular basis of apical growth of fungi. Long ago, Reinhardt (*4*) and Castle *et al.* (*5*) had shown, by using external surface markers, that hyphae or young sporangiophores of fungi elongate by tip growth; the same conclusion was recently reached by using fluorescent antibodies (*12*). None of these reports, however, clearly demonstrated that the structural polymers of the cell wall fabric were apically deposited. We have now established this and also confirmed the predicted gradients in the rate of wall formation of apically growing cells (*4, 13*). Also, our findings on the position, smallness, and steepness of gradient in the active region of synthesis of hyphal walls suggest that the subcellular organization responsible for apical growth is probably a discrete, minute, apical organelle ($\ll 1$ μ). Among the organelles found in hyphal tips of fungi, two plausible candidates for such morphogenetic function are the Spitzenkörper discovered by Brunswik in growing hyphae of higher fungi (*14*), and the apical corpuscle recently seen in germ tubes of *M. rouxii* (*7*).

Current speculations on the mechanism of apical wall construction include the participation of lytic (plasticizing, softening) as well as synthetic activities (*15, 16*); their interaction is invoked to account for the orderly expansion of an otherwise rigid cell wall, which grows in surface area without a significant change in thickness. Accordingly, one may postulate an apical gradient of wall lysis paralleling the synthesis gradient found in *M. rouxii*. Such a postulation is consistent with observations on the plasticity and autolytic tendencies of hyphal tips of fungi (*15, 17*), including those of *M. rouxii* (*18*). The relative importance of cell wall synthesis and lysis in governing apical growth remains to be determined.

In conclusion, this study supports the contention that the mechanism of cell wall construction plays a decisive role in fungal morphogenesis. Specifically, the mold-yeast dimorphism of *M. rouxii* resides, respectively, in the ability or failure of the fungus to establish radial gradients of wall formation at points on the cell surface which would thus become hyphal apices. In the absence of such polarization, we contend, the cell wall grows uniformly, giving rise to spherical cells.

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(1954)] by acetylation of D-[GL-^{3}H]glucosamine (specific activity, 3.7 c/mmole) purchased from the International Chemical and Nuclear Corporation, Irvine, California.
9. A minimum of 66 percent of the radioactivity incorporated into the ghosts was recovered as glucosamine after hydrolysis with 6*N* HCl and chromatographic separation on a Dowex-50 column eluted with a gradient of from 0 to 0.05*N* HCl. Most of the fraction unaccounted for was probably also glucosamine, part destroyed during acid hydrolysis and part incompletely hydrolyzed chains retained by the column. The labeled polymers are most likely chitosan and chitin in a ratio 6.6 : 1 as determined by the relative proportion of hot 1*N* HCl soluble and insoluble radioactive material. This ratio approximates that previously found in nonlabeled walls by S. Bartnicki-Garcia and W. J. Nickerson [*Biochim. Biophys. Acta* **58**, 102 (1962)].
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17 February 1969

Virus-like Antigen, Antibody, and Antigen-Antibody Complexes in Hepatitis Measured by Complement Fixation

Abstract. *Complement fixation techniques are described for measuring a virus-like antigen associated with viral hepatitis. Antigen was found in the blood of 98 percent of 130 patients, with the serum form of hepatitis, from whom multiple samples were obtained. Antibodies arising during hepatitis are usually combined with antigen and cause anticomplementary activity in the serum, which is reversible with excess antigen or antibody. Tests for antigen and specific anticomplementary activity can be used diagnostically and to screen blood donors for hepatitis carriers.*

Australia antigen (*1*) is present in the blood of a high proportion of patients with serum (posttransfusion) hepatitis, but it is found less frequently (*1, 2*) or not at all (*3*) in patients with infectious hepatitis. The antigen has morphologic and physical characteristics suggesting that it is hepatitis virus itself (*2, 4, 5*). Agar-gel precipitation has been the method for demonstrating this antigen and the human antibodies used have arisen in patients who had multiple exposures to blood potentially containing hepatitis virus. This report concerns measurement of Australia antigen by complement fixation (CF) techniques that are much more sensitive than agar-gel precipitation.

The antibodies we used to measure antigen were found in serums from 7 of 21 hemophiliacs, in 3 of 8 patients with refractory anemias, all but one of whom had been treated with multiple transfusions over a period of years, and in serum of 2 of 8 chimpanzees that had been transfused repeatedly with chimpanzee blood. The two chimpanzees and half of the patients who developed antibodies had no history of hepatitis. One patient developed an antibody within 10 days after his first transfusion and subsequently had anicteric hepatitis. Eleven antibodies were in the second protein peak (>60,000 and <200,000 molecular weight) of serum fractionated on Sephadex G-200 (*6*) and one was equally distributed in the first (>200,000 molecular weight) and second peaks. All of these antiserums precipitated antigen in agar gel.

Our quantitative CF technique was developed for measuring cellular antigens (*7*). The incubation mixture (0.4 ml) contained appropriate dilutions of antigen and antibody and 10 or more hemolytic (50 percent effective) units of complement (C). Complement fixation was 85 percent complete within 15 minutes at 37°C, 95 percent complete at 30 minutes, and not significantly increased by additional incubation at 5°C. Human and guinea pig C were fixed equally. We also used a standard microtiter CF system (see *8*).

When tested at a constant concentration of antigen, the highest titered antibodies were from hemophiliacs who had received more transfusions over longer periods than had other patients or the chimpanzees (Fig. 1). Three antibodies that precipitated allotypic human beta lipoproteins in agar gel (*9*) were used as controls and did not fix C with reactive beta lipoproteins or with hepatitis antigen.

The same relative concentrations of eight different antigens produced similar CF (Fig. 2). Antigen stored as long as 15 years at −20°C or heated at 56°C for 1 hour gave results essentially the same as antigen in fresh serum. Partially purified virus-like particles, which could be separated at a density of 1.28 on cesium chloride gradients from all antigenic serums after lipid extraction (*5*), reacted in CF quantitatively like whole serum from which the particles were obtained. The similar reactions of antigens from patients with serum or infectious hepatitis or from normal gibbons, chimpanzees, and orangutans with each of 12 antibodies suggests that a single immunologic specificity is involved. The same 12 antibodies also gave agar-gel precipitation reactions of identity with these antigens, although a rabbit antiserum has been reported to differenti-

Table 1. Sensitivity of techniques for detecting hepatitis-related antigen. Aggregates of virus-like particles were prepared for electron microscopy (EM) by concentration on cesium chloride gradients (*5*). Quantitative complement fixation (CF) was performed with an amount of antibody that produced maximum fixation at each antigen dilution. In the box microtitration, 0.025-ml samples of twofold dilutions of antibody and antigen were mixed with 2 units of C in a total volume of 0.075 ml and incubated at 4°C for 18 hours before 0.025 ml of a 1 percent suspension of sensitized cells was added. Degree of complement fixation is indicated by (−) for complete hemolysis, to 4+ for no hemolysis.

Antigen dilution	Optimum antibody			CF box titration at antibody dilution				
	Agar gel	EM	CF units	30	60	120	240	480
1	3+	4+	>10	—	—	—	—	—
2	1+	3+	>10	—	—	—	—	—
4	—	2+	>10	2+	—	—	—	—
8		1+	>10	3+	1+	—	—	—
16		±	>10	4+	3+	—	—	—
32			>10	4+	4+	1+	—	—
64			>10	4+	4+	4+	±	—
128			8	4+	4+	4+	4+	—
256			4.8	4+	4+	4+	4+	—
512			2.5	4+	4+	4+	4+	±
1024			<1	4+	4+	4+	4+	±
2048			0	3+	4+	4+	2+	—
4096				±	±	—	—	—