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Subapical Wall Synthesis and Wall Thickening Induced by Cycloheximide in Hyphae of *Aspergillus nidulans*

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Hyphae of *Aspergillus nidulans* continued to synthesize all the major polysaccharide components of the cell wall when cycloheximide was added to cultures. In the presence of cycloheximide, hyphae did not elongate, but electron microscopy showed that the walls became thicker around the cell. The conclusion that cycloheximide changed wall synthesis from extension at the apex to subapical thickening was supported by grain distributions on radioautograms of mutant hyphae labeled with galactose. These findings are discussed in relation to the control of hyphal wall synthesis and are compared with the effects of protein synthesis inhibitors on wall formation in gram-positive bacteria.

Several lines of evidence have shown that hyphae of filamentous fungi grow by inserting new wall at the apex (1, 11, 14). However, cycloheximide, an inhibitor of protein synthesis, produces a change in the sites where the wall polymer chitin is incorporated (12). In the presence of cycloheximide, *Aspergillus nidulans* hyphae continue to synthesize chitin, but insert the polymer along the whole length of the hypha. On removing the inhibitor, chitin incorporation again becomes apical.

Little is known about the controls determining the sites of wall growth in filamentous fungi or about the effects of cycloheximide on the synthesis of various wall polymers. It is thus not clear whether complete *A. nidulans* walls are formed subapically in the presence of cycloheximide or whether chitin alone is deposited. In yeasts, with a different type of wall, cycloheximide inhibits the synthesis of mannans but not of glucans (5). Nor is it known whether polymers inserted subapically lead to a thickening of the envelope, as happens in bacteria treated with inhibitors of protein synthesis (18), to abnormal cell growth such as may be occurring in the giant cells induced by cycloheximide in yeasts (9), or to a normal lengthening of the hyphae. In the present work we have attempted to clarify these points, which have a bearing on the mechanism of hyphal extension, in strains of *A. nidulans*.

MATERIALS AND METHODS

Organisms, media, and growth conditions. The two strains of *A. nidulans* used were R21, with yellow conidia and requiring *p*-aminobenzoic acid (genotype *pabaI y*), and 212 with white conidia and requiring biotin and unable to utilize galactose as sole carbon source (genotype *biI, galS; wB*). The preparation of conidial suspensions, basal media composition, and conditions of cultivation were as previously described (10), except that all incubations were carried out at 30 C. For strain R21, 0.5% (wt/vol) glucose was added to the basal medium as carbon source, and for strain 212 arabinose and galactose were added as previously described (11, 12). Cycloheximide was added from freshly prepared filter-sterilized solutions to give a final concentration of 50 μg/ml.

To label strain R21 hyphae and walls with ^{14}C-glucose, 24-h cultures were filtered onto membrane filters (0.45-μm pore size, Millipore Corp.), washed with basal medium containing 0.1% (wt/vol) glucose, and suspended in this latter medium. After incubation for 15 min, uniformly labeled ^{14}C-glucose (specific activity 2 to 4 mCi/mmol, Radiochemical Centre, Amersham, England) was added to give a final radioactivity of 2 μCi/ml. Labeling of strain 212 walls with D-galactose-*1*-^{3}H was carried out as previously described (11).

Fractionation, chemical analysis, and radioautography of labeled hyphae. After labeling, hyphae of strain R21 were broken in a Nossal disintegrator, and the wall fraction was purified as previously described (10, 11). Purified walls were fractionated into their constituent polymers by the methods of Mahadevan and Tatum (13) and Bull (2). The contents of neu-

tral and aminosugars in the walls and in fractions derived from them was determined after acid hydrolysis (10), and radioactivity was determined by liquid scintillation counting (10, 11). The preparation of radioautograms from strain 212 hyphae after labeling with galactose has been described (10), as has the quantitative determination of grain distributions in labeled hyphae (12).

Measurement of hyphal extension by light microscopy. Conidia were germinated on cellophane squares placed on basal medium containing 0.5% (wt/vol) glucose and 2% (wt/vol) agar. Cellophane squares were transferred to the agar medium and spread on sterile slides with and without cycloheximide. The preparations were covered with Teflon membranes permeable to oxygen, and the slides were incubated in a water-saturated atmosphere. Individual hyphae were located by using microscope stage micrometer calibrations, measured with a calibrated eyepiece scale, reincubated, and remeasured at intervals.

Electron microscopy. Mycelia were collected by filtration on membrane filters (0.45-μm pore size) and washed several times with 0.075 M phosphate buffer, pH 7.2. The hyphae were fixed in 4% (vol/vol) glutaraldehyde in 0.075 M phosphate buffer, pH 7.2, for 4.5 h at 5 C and then were dialyzed for 15 h against phosphate buffer at 5 C. The hyphae were collected on membrane filters, washed several times with 0.14 M sodium barbital, pH 7.4, and suspended in 1% (wt/vol) OsO_4 in sodium barbital for 4.5 h at 5 C. Finally, the mycelia were collected on membrane filters and washed several times with the sodium barbital buffer.

For embedding, the hyphae were enrobed in warm (45 C) 0.5% (wt/vol) Noble agar. The agar blocks were cut into 1-mm squares, dehydrated through ethanol and propylene oxide, and embedded in Epon 812.

Ultrathin sections were cut with a diamond knife on an LKB Ultratome III. Sections were stained with 1% (wt/vol) uranyl acetate in absolute ethyl alcohol and with Reynold's lead citrate (16). They were examined in a Phillips EM300 electron microscope at 60 kV and with a 50-μm aperture. The magnification was calibrated by the use of a diffraction grating replica of 54,864 lines per cm (Polaron Equipment Ltd., England).

Wall thickness was measured on electron micrographs of sections which had been cut perpendicularly, or almost perpendicularly, to the long axis of the hypha and which were circular, or almost circular, in outline (Fig. 2). The wall thickness was measured at two different points of each sectioned hypha, and the measurements were averaged. Where sections appeared oval, measurements were made at the two points lying on the shortest diameter of the oval, where wall width would not be distorted by the obliqueness of the cut.

RESULTS

Synthesis of wall polymers in the presence of cycloheximide. To determine which wall polymers were synthesized in cycloheximide-treated hyphae, samples from a 24-h culture were labeled for 1 h with ^{14}C-glucose in the presence and absence of cycloheximide. Walls from these hyphae were then extracted to yield four fractions whose chemical nature has been described by Bull (2). This technique does not yield pure polymers, but does separate the major components. Our results (not shown) on the chemical nature of the fractions agree with the findings of Bull, and their composition, together with the distribution of counts from a typical experiment, are shown in Table 1.

Total counts per mg of purified wall were similar in cycloheximide-treated and in control hyphae (Table 1). Because portions of the same culture were labeled, this indicates that cycloheximide did not reduce the rate of glucose incorporation into wall polymers. The distribution of counts between the fractions varied by up to 20% in different experiments (results not shown). In all cases, however, all the major polysaccharide components were synthesized in the presence of cycloheximide, and the radioactivity in the glucan fractions of the cycloheximide-treated hyphae was 20 to 40% lower than that of the controls (Table 1).

Effect of cycloheximide on the incorporation sites of galactose- and glucose-containing wall polymers. The sites where galactose- and glucose-containing polymers are incorporated in the hyphal wall can be determined by pulse labeling an *A. nidulans* mutant lacking galactose phosphate uridylyl transferase with ^{3}H-galactose and by radioautography of the hyphae (11). When labeled in this way with no cycloheximide, hyphae had a high grain density at the apex (Fig. 1). Labeling in the presence of cycloheximide, on the other hand, gave an even distribution of grains (Fig. 1). The total number of grains (sum of grains on the apical, 77-μm region of 30 hyphae) for the hyphae shown in Fig. 1 was 1,220 for the cycloheximide-treated hyphae and 1,410 for the controls. Cycloheximide thus prevented apical incorporation of galactose- and glucose-containing polymers without, however, causing an obvious inhibition of total incorporation.

Morphology of hyphae treated with cycloheximide. Deposition of new wall polymers could produce either an increase in hyphal length or wall thickening, or both of these effects. To determine whether hyphae elongated in the presence of cycloheximide, we measured 20 hyphae with an initial length of 50 to 150 μm. During a 5-h incubation none of these elongated by more than 3 μm, whereas similar hyphae in the absence of cycloheximide elongated by 100 to 300 μm, depending on the initial length.

TABLE 1. *Radioactivity in wall polymers of hyphae labeled in the presence and absence of cycloheximide*

Wall fraction		Lyophilized wall (counts per min per mg)	
No.	Main components[a]	+ Cyclo-heximide	No cyclo-heximide
1	Glucose [α-1,3-glucan], and galactose	15,187	25,430
2	Glucose, mannose, galactose, arabinose, and amino acids	31,168	20,515
3	Glucose [β-1,3-glucan]	32,912	44,144
4	Glucosamine [chitin]	46,166	32,912
	Total counts	125,433	129,438

[a] The monomer constituents obtained by acid hydrolysis of the extracted polymers. Where polymer structure is known, this is given in brackets.

Ultrathin sections of hyphae grown with and without cycloheximide are shown in Fig. 2 and 3. Five hours of incubation with cyloheximide induced a marked increase in hyphal wall thickness. The average wall thickness in control hyphae was 84 nm as measured (Fig. 2) on 47 transverse hyphal sections (standard error ± 21 nm). In 38 hyphae treated with cycloheximide, the average wall thickness was 165 nm (standard error ± 61 nm). Longitudinal sections showed that the wall was thickened evenly along the length of the hypha (Fig. 3). Another change induced by cycloheximide was in the general appearance of the hyphal cytoplasm which, after fixation and staining, seemed less electron dense (Fig. 2 and 3).

DISCUSSION

All of the major wall polymers were synthesized in the presence of cycloheximide, and although the proportion of glucans was lower in the wall of cycloheximide-treated hyphae, it was never less than 60% of that in the controls. In addition, the walls along the length of the hyphae underwent thickening when cycloheximide was added. Together, these findings strongly suggest that something approaching complete wall is deposited subapically when protein synthesis and cell elongation are inhibited. This conclusion is supported by the experiments with the galactose-labeled hyphae. It could have been argued that, because there is direct evidence for subapical incorporation of chitin in the presence of cycloheximide (12), subapical wall thickening is due to chitin alone. The other wall polymers would then continue to be deposited at or near the apex. However, no accumulation of galactose- or glucose-containing polymers was seen at the apex in the radioautograms.

In changing wall synthesis from extension at specific growing points to thickening around the cell, the effect of cycloheximide resembles that of protein synthesis inhibitors and of amino-acid starvation in gram-positive bacteria (18). It may be pointed out that hyphae resume growth after cycloheximide treatment when removed to an antibiotic-free medium (12). This similarity of action becomes rather remarkable when the differences in wall composition and structure between these organisms are considered. No effect which appeared analogous to the induction by cycloheximide of giant yeast cells (9) was observed in *A. nidulans*.

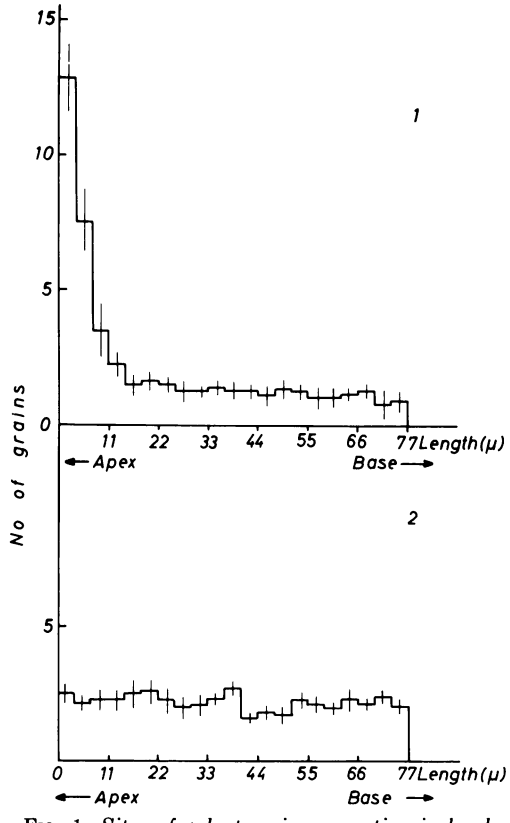

FIG. 1. *Sites of galactose incorporation in hyphae lacking galactose phosphate-uridylyl transferase. After labeling with galactose-1-^{3}H, radioautograms were developed, and the grains in segments (3.67-μm long) of the hypha were counted by starting at the tip. Each column represents the average grain count from corresponding segments of 30 different hyphae, and the lines show the standard error of the mean. 1, No cycloheximide; 2, labeled in the presence of 50 μg of cycloheximide per ml.*

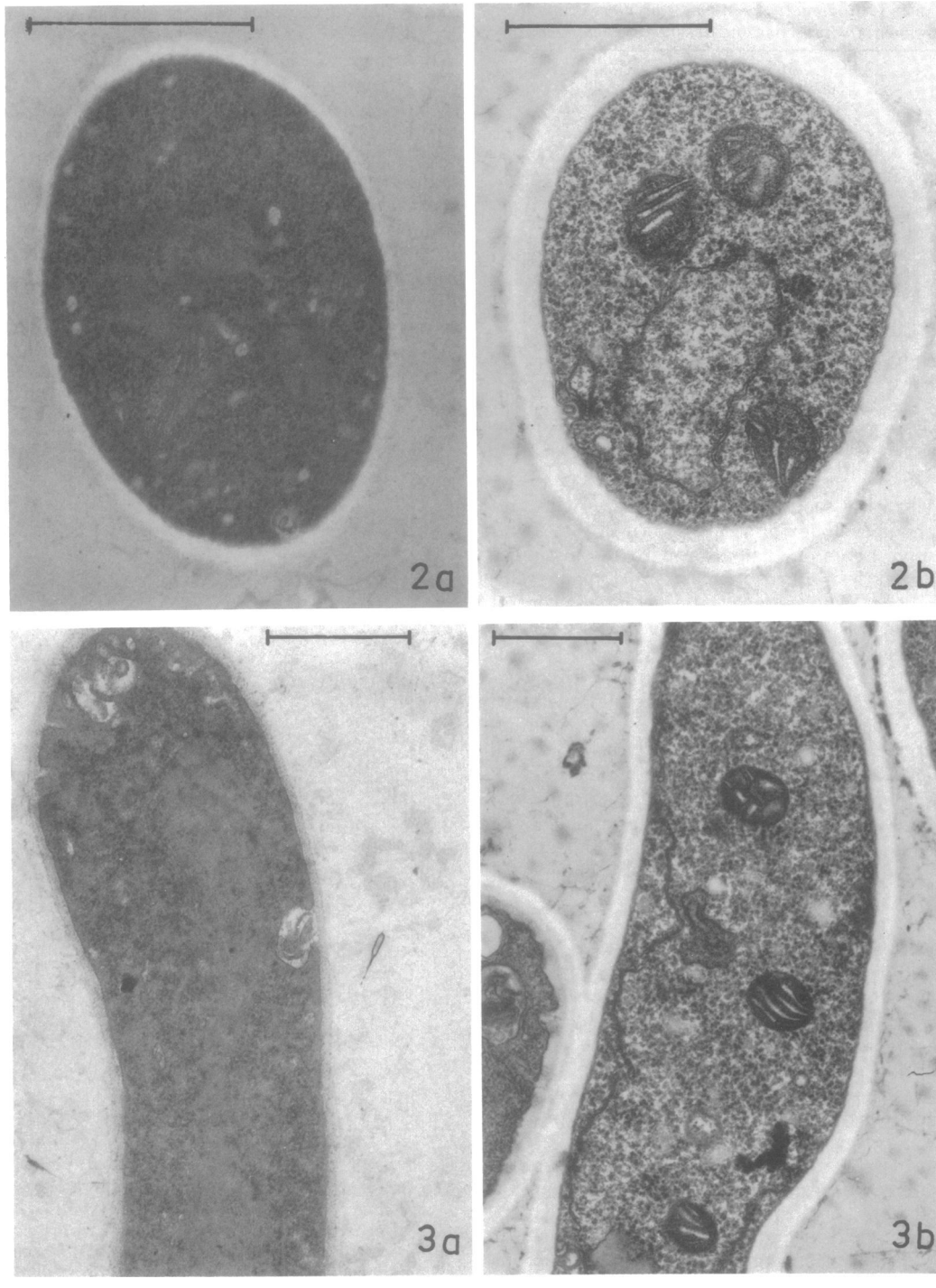

FIG. 2 and 3. *Electron micrographs of Aspergillus nidulans hyphae grown without cycloheximide and after 5 h of incubation with cycloheximide. 2a, 3a, No cycloheximide; 2b, 3b, 5 h with cycloheximide. The bar represents 1 μm.*

Inhibition of protein synthesis conceivably could change wall deposition by a derepression of dormant, subapical sites, a dispersal of synthetic sites from the growing point, a change in the availability of precursors at different points in the hypha, or by a combination of these factors. Although activation and inhibition of a wall lysin (15) and of a chitin synthase (3) are known to exist, there are at present no data which allow a distinction between the above possibilities. However, a particular feature of wall growth in filamentous fungi, which may be absent in bacteria and yeasts, is the length of hypha supplying precursors to the tip, and this deserves consideration.

Measurements of the rates of dry weight increase and of hyphal extension have shown that in filamentous fungi a long length of protoplasm must supply precursors to the synthesising tip (19, 20). In *A. nidulans*, the length of hypha contributing to apical synthesis has been calculated to be 460 to 950 μm (19), depending on the particular strain. Thus, any explanation of the changed mode of wall synthesis will have to take into account the existence of a cytoplasmic transport system which efficiently channels precursors to the apex. If subapical wall thickening is due to derepression of previously dormant sites, these must acquire both the ability to carry out the necessary enzymatic reactions and to capture precursors. There is evidence that cytoplasmic vesicles are involved in wall synthesis and transport of precursors (4, 6–8), and the intrahyphal distribution and enzymatic contents of such vesicles would appear to be important points in further investigations of the effect of cycloheximide on wall synthesis.

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