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Model for Branch Initiation in *Aspergillus nidulans* Based on Measurements of Growth Parameters

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The rate of hyphal elongation and the number of branches per hypha were measured on short sporelings of *Aspergillus nidulans* growing at different rates. The rate of elongation was proportional to total length in unbranched and branched hyphae. At each growth rate, the number of branches per hypha increased with increasing length and gave approximately straight-line graphs when plotted against length. The average number of branches per unit of hyphal length was quite different for the various growth rates and increased in direct proportion to the growth rate. The results are interpreted to mean that (i) growing tips have a maximum rate at which they can elongate and which is reached at hyphal lengths characteristic of the particular growth rate and (ii) a new branch is formed when the capacity of the hypha to elongate exceeds that of the existing tips.

Studies of branch formation in filamentous fungi have shown that mutations (2, 8, 13), various chemicals (3, 6, 14), alterations in wall composition (7), and changes in physical conditions (10) can markedly affect the frequency of branching. The data obtained have clarified features of this complex process but have given little information on whether branching is linked to normal hyphal growth and, if so, how it is linked. Short coenocytic hyphae, however, increase exponentially in mass (4, 12, 17) while they elongate only at the apex (10). Since branching is the way new growing apices are generated, a relationship between the rate of mass increase and branch initiation would not be unexpected.

If such a relationship existed, it should lead to different branch densities at different growth rates. We have measured the numbers of branches on hyphae of the ascomycete *Aspergillus nidulans* growing on media supporting various doubling times. The results indicate that a simple relationship between the elongation rate at the apex and branch formation does indeed exist. When the apex or apices reach a characteristic maximum rate of extension, which is achieved at different hyphal lengths with different growth rates, a new branch is initiated.

MATERIALS AND METHODS

Strains, media, and growth conditions. Strain R 21 of *A. nidulans* had the genotype *paba 1 y* and strain ts6 the genotype *ad 15 bi 1; w; cnx ts6 glc NAc*$^-$ (for gene symbols, see reference 1; for description of strain ts6, see reference 5).

The medium for strain ts6 contained salts, glucose, *N*-acetylglucosamine and sodium acetate, as described previously (5). Cultures of ts6 were grown at 41 C to block endogenous amino sugar synthesis. Three different media were used for R21: (i) 20 g of malt extract (Difco), 1 g of peptone (Difco), and 20 g of glucose per liter of water; (ii) a defined basal medium (5) plus 1 g of sodium acetate and 1 mg of *p*-aminobenzoic acid per liter; (iii) the above basal medium lacking $(NH_4)_2SO_4$ and containing 5 g of glucose, 2 g of l-tryptophan (as nitrogen source) and 1 mg of *p*-aminobenzoic acid per liter. Preparation of conidial suspensions for inoculation was as previously described (5). Cultures were grown on a rotary shaker in baffled 250-ml Erlenmeyer flasks containing 30 ml of medium; incubation temperature for R21 was 30 C. Growth rates were determined by inoculating flasks with identical numbers of conidia and taking the whole contents of duplicate flasks at intervals as samples for dry weight estimations by weighing.

Microscope measurements of elongation, length, width, and branching. To determine the rate of elongation of individual hyphae, conidia of R21 were germinated on cellophane sheets placed on

agar on sterile slides. 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 a calibrated eyepiece scale, reincubated, and remeasured at intervals. The volume of cytoplasm in the spore was added to the measured length of the hypha.

To measure length, width, and branching frequency, cultures in liquid media were filtered, and the mycelium was washed and resuspended for 5 min at 20 C in 0.5 N NaOH to separate hyphal clumps. After the mycelium was placed on gelatin-coated slides, hyphal length and the number of branches were measured with phase-contrast optics by using a calibrated eyepiece. The volume of the spore cytoplasm was added to the length. Hyphal widths and germinated spore diameters were measured from photographs of unstained material taken under phase- or interference-contrast optics or after staining for cell walls (16). The measurements from the different methods agreed to within 15%.

Radioautography. Labeling of ts6 walls with *N*-[*acetyl*-^3H] glucosamine was carried out as previously described (5) with the following modifications: labeling medium contained 30 μg of cold *N*-acetylglucosamine per ml and cultures were incubated for 15 min before 15 μCi of *N*-[*acetyl*-^3H] glucosamine per ml was added.

RESULTS

Rate of extension at the apex in relation to hyphal length. The strictly apical incorporation of *N*-[^3H-*acetyl*]glucosamine by strain ts6 at 41 C (5) produces labeled hyphal ends whose lengths can be measured in radioautograms and compared with the length of the hypha at the start of labeling. When short, unbranched or branched hyphae were labeled for 20 (Fig. 1) or 30 min (results not shown), plots of the initial length against the radioactive tip or tips gave straight-line graphs. The rate of extension was thus proportional to the length. A similar result was obtained for hyphae of R21 growing on agar and measured at intervals under a microscope (Fig. 1). In these and in later experiments, we used strain R21 as well as ts6 to ensure that we were not measuring effects due to the *N*-acetylglucosamine requirement of ts6.

The doubling times (t_D) calculated from these microscope measurements were 127 min (20-min label) and 123 min (30-min label) for ts6 and 130 min for R21. These values agreed well with t_D of 129 min for ts6 and 117 min for R21 obtained by weighing the mycelium in samples from liquid cultures grown under the same conditions.

Frequency of branching at different growth rates. R21 was grown in liquid cultures at t_D of 1.9 hr (malt extract medium), 5.1 hr (acetate), and 6.4 hr (glucose-tryptophan). Hyphal diameters changed with the growth rate and were (average of 20 measurements) 1.8 μm at t_D 1.9 hr, 1.6 μm at t_D 5.1 hr, and 1.1 μm at t_D 6.4 hr. At each growth rate, the number of branches increased with increasing hyphal length (Fig. 2) and was roughly proportional to the length as regression lines with correlation coefficients of 0.9 could be fitted to the graphs. Particularly at the slower growth rates, suggestions of a stepwise increase in branch numbers can be seen (Fig. 2).

At the three growth rates, the average number of branches per hypha was quite different for hyphae of equivalent lengths (Fig. 2). When, however, the number of branches per hypha was plotted against the linear rate of extension ($dL/dt = L \times \ln 2/t_D$, where L = length), points from all the three growth rates approximated to the same curve (Fig. 3). Branching frequency was thus proportional to the growth rate. From the same data, we further calculated the average rate of extension per growing tip. This was similar at the 3 growth rates for equivalent rates of extension and rose from a low value at low extension rates to a maximum as the extension rate increased (Fig. 4).

DISCUSSION

The relationships observed between growth rate, frequency of branching, and rate of extension per tip can be explained by the following simple model for hyphal extension and branch initiation. (i) The rate of extension at the growing points of short hyphae is proportional to both the hyphal length and the specific growth rate; (ii) each growing point can extend at a rate between a low value and a maximum, characteristic of the organism and independent of the growth rate, and (iii) a new growing point is formed when the capacity of the hypha to extend exceeds that of the existing growing points.

Some evidence has been published (15, 17) that protoplasm in regions distant from the apex can contribute to synthesis at the tip. Our results confirm and extend these findings, and transport of precursors to the growing points is implied. Although the factors involved in intrahyphal transport are largely unknown, local variation in precursor concentrations are likely to occur. A scatter in the extension rates of individual tips and of branch initiation in hyphae of the same length may thus be expected. However, in the above model, changes in both parameters will be self-correcting. For example, a hypha which has

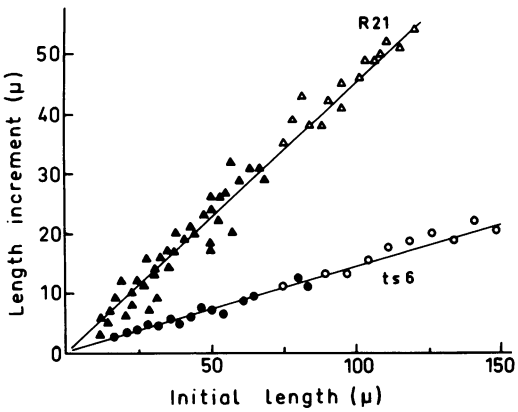

FIG. 1. *Rate of hyphal elongation in relationship to initial length. Hyphae of ts6 were labeled with ^{3}H-N-acetylglucosamine for 20 min, and the labeled tip and unlabeled initial length were measured on 97 unbranched (●) and 70 branched (○) hyphae from radioautograms. In branched hyphae, tips were summed. Hyphae with initial lengths within 3.7 μm (unbranched) and 7.4 μm (branched) of each other were averaged, and the averages were plotted. Number of samples in averaged groups varied from 3 to 14. R21 hyphae growing on agar were measured at 60-min intervals under the microscope. Data for individual hyphae plotted: ▲, unbranched; △, branched.*

formed a branch prematurely because of local concentration gradients will be more likely to initiate the next branch with a delay.

In our model and depending on the length, the extension rate per growing point can vary from a low value to the postulated maximum in hyphae with a single tip, from half-maximal to maximal in hyphae with two growing points, from two-thirds maximal in hyphae with three growing points, and so on. Averaging the measurements for hyphae of different lengths should thus show an increasing rate per tip which eventually reaches a maximum value (Fig. 4).

The frequency of branching was proportional to the linear rate of hyphal extension, and it is this parameter that is postulated to reach a maximum per tip, presumably because of saturation of the enzymes involved in membrane and wall synthesis. No conclusions can be drawn at present on how saturation of wall and membrane synthesis sites might initiate branching, although accumulation of some precursor in the cytoplasm would be a possible trigger. One prediction of our model is that partial inhibition of the rate of synthesis at the growing point should increase the frequency of branching. On the assumption that other filamentous, coenocytic fungi behave in the same way, this appears to be the case. In *Fusarium* species and *Neurospora* species, mechanical damage, osmotic shock, or the addition of sorbose slows elongation at the tips and leads to markedly increased branching (11). In *A. nidulans*, osmotic shock and cycloheximide inhibit elongation and induce chitin incorporation and eventually branching along the length of the hypha (5). It may be relevant that the point mutations which change colonial morphology and increase branching frequency in *Neurospora* species are in the structural genes of enzymes of hexose metabolism such as phosphoglucomutase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconic acid dehydrogenase (2, 8, 13). If these mutations decrease the rate of synthesis of some wall precursor more sharply than that of overall cell metabo-

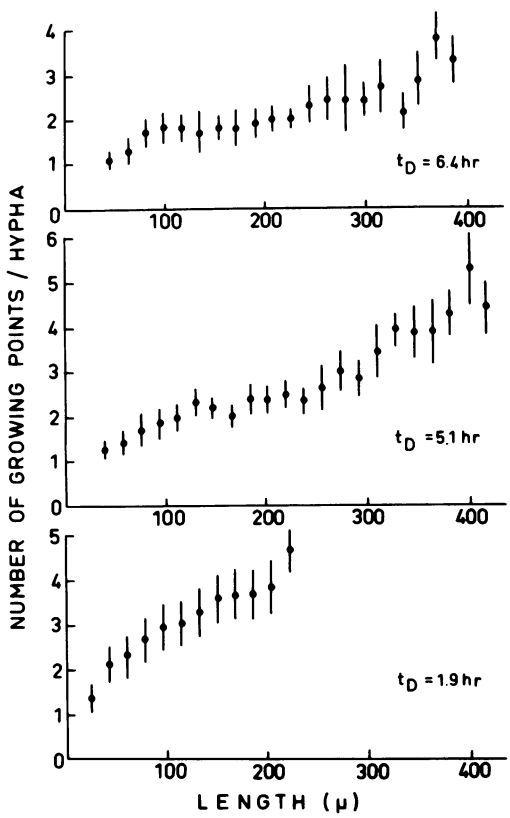

FIG. 2. *Number of growing points on R21 hyphae growing at different rates; 291 hyphae from malt extract (t_D 1.9 hr), 596 hyphae from acetate (t_D 5.1 hr), and 534 hyphae from tryptophan medium (t_D 6.4 hr) were measured. Hyphae with lengths within 18.5 μm of each other were grouped, and the number of growing points were averaged. Vertical lines show ± standard error of the mean. Number of samples in averaged groups varied from 6 to 50.*

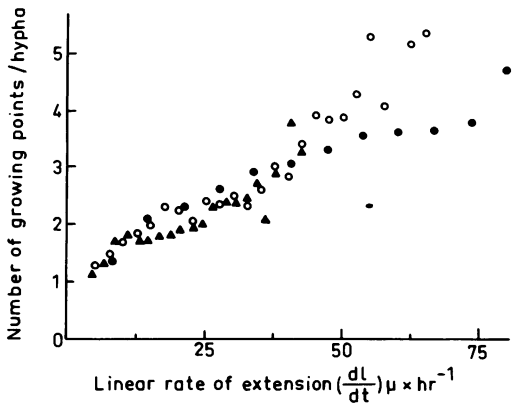

FIG. 3. *Number of growing points per hypha in relationship to the rate of hyphal extension. The average number of growing points on the hyphae described in Fig. 2 is plotted against the rate of hyphal extension $dL/dt = L \ln 2/t_D$, where L = length). Symbols: ●, malt extract; ○, acetate; ▲, tryptophan.*

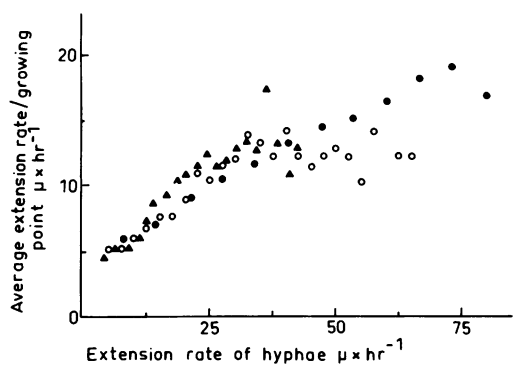

FIG. 4. *Average rate of extension per tip ($L \times ln\ 2/t_D \times$ number of growing tips) as a function of the rate of hyphal extension ($L \times ln\ 2/t_D$). Data from hyphae described in Fig. 2. Symbols: ●, malt extract; ○, acetate; ▲, tryptophan.*

lism and do not affect the mechanism for branch initiation, our model would predict more highly branched hyphae. Such a situation could be analogous to that of deoxyribonucleic acid synthesis in thymine-requiring strains of *Escherichia coli* growing with low thymine concentrations (9). There, an increased number of synthesis sites working at reduced rates because of lowered precursor concentrations still maintain a wild-type rate of total synthesis.

In nature, fungal growth will be slow in the presence of a poor food source, and the ability to reach a more nutritious environment would have survival value. For a given amount of dry weight synthesis, the hypha with the least branches will cover the largest distances. Selection for a mechanism which reduces the frequency of branching at slow growth rates thus may have occurred during evolution.

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