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Pulsed growth of fungal hyphal tips

(cell growth/secretion/*Spitzenkörper*/vesicles)

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ABSTRACT **Somatic fungal hyphae are generally assumed to elongate at steady linear rates when grown under constant environmental conditions with ample nutrients. However, patterns of pulsed hyphal elongation were detected during apparent steady growth of hyphal tips in fungi from several major taxonomic groups (Oomycetes, *Pythium aphanidermatum* and *Saprolegnia ferax*; Zygomycetes, *Gilbertella persicaria*; Deuteromycetes, *Trichoderma viride*; Ascomycetes, *Neurospora crassa* and *Fusarium culmorum*; Basidiomycetes, *Rhizoctonia solani*). Growing hyphal tips were recorded with video-enhanced phase-contrast microscopy at high magnification, and digital images were measured at very short time intervals (1–5 s). In all fungi tested, the hyphal elongation rate was never perfectly steady but fluctuated continuously with alternating periods of fast and slow growth at more or less regular intervals. Pulsed growth was observed in fungi differing in cell diameter, overall growth rate, taxonomic position, and presence and pattern of *Spitzenkörper* organization, suggesting that this is a general phenomenon. Frequency and amplitude of the pulses varied among the test organisms. *T. viride* and *N. crassa* showed the most frequent pulses (average of 13–14 per min), and *F. culmorum* the least frequent (2.7 per min). Average pulse amplitude varied from 0.012 μm/s for *F. culmorum* to 0.068 μm/s for *G. persicaria*. In *F. culmorum* and *T. viride*, the fast phase of the growth pulses was correlated with the merger of satellite *Spitzenkörper* with the main *Spitzenkörper*. These findings are consistent with a causal relationship between fluctuations in the overall rate of secretory vesicle delivery/discharge at the hyphal apex and the fluctuations in hyphal elongation rate.**

Somatic fungal hyphae elongate at their apices, presumably by localized deposition of new plasma membrane and cell wall via exocytosis of secretory vesicles (1–9). Growing hyphae characteristically contain a population of secretory vesicles in the apical dome (3, 9). In the higher fungi, these vesicles and other small cell components are organized into a visible assemblage called the *Spitzenkörper* (apical "body") (3, 4, 9, 10).

Established hyphae are assumed to extend at a constant linear rate (1, 11–18) when nutrients are not limiting and other environmental factors are stable. When detailed measurements of hyphal growth were made on seven taxonomically diverse fungi, we discovered that the seemingly constant elongation rates actually oscillated continuously. Video-enhanced microscopy imaging allowed us to measure increments of cell elongation as small as 0.03 μm at very short intervals (1–5 s) and thus made it possible to detect the growth pulses.

MATERIALS AND METHODS

Organisms. Fungi used were *Fusarium culmorum* (W. G. Smith) Sacc. (no. 1964), *Gilbertella persicaria* (Eddy) Hesseltine (no. 1133), *Pythium aphanidermatum* (Edson) Fitzp. (no. 2096), and *Rhizoctonia solani* Kuhn [imperfect stage of *Thanatephorus cucumeris* (Frank) Donk] (no. 283) (all from E. E. Butler, University of California, Davis); *Neurospora crassa* Shear and Dodge, wild type (FGSC 988, from Fungal Genetics Stock Center, Arcata, CA); *Trichoderma viride* Pers.: Fr. (no. 2001, from J. F. Tuite, Purdue University); and *Saprolegnia ferax* (Gruith.) Thuret (MV-1, from M. J. Powell, Miami University).

Culture Method. All fungi were maintained at 22 ± 2°C in Petri plates on permeable cellulose xanthate membranes (DuPont) (ordinary dialysis membrane works equally well) overlying potato dextrose agar (Difco) at pH 5.5. A sterile slide culture chamber with hinged coverslip (10) was coated with potato dextrose/gelatin [3.9% potato dextrose (Difco) in 12–15% gelatin (Difco)]. Mycelium was transferred to the slide chamber and incubated in a humid chamber at room temperature. After 24–36 hr, the slide chamber with the new growth was handled carefully for microscopic observation (10). Measurements were made with mycelium that had grown at least 2 cm in the slide chamber before microscopic observation. During observation of *G. persicaria* hyphae, it was necessary to perfuse the slide chamber with oxygenated 2.9% potato dextrose broth (10) because this fungus is sensitive to oxygen deprivation.

Video Microscopy. Hyphae growing in the slide chambers were observed with an Olympus Vanox microscope equipped with a phase-contrast ×100 oil-immersion-objective lens (n.a. 1.25). The light source was a 12-V, 100-W halogen bulb with a green (550-nm) filter and an IRS-1 IR heat shield (Scientific Supply, Schiller Park, IL) in the light path. Growing hyphal tips were imaged with a Hamamatsu C2400-1 video camera connected to an Argus-10 digital contrast enhancer (Hamamatsu Photonic Systems, Bridgewater, NJ) for real-time image processing. Images of growing hyphal tips were recorded on VHS videotapes and displayed on a 12-inch monitor at ×12,000 (electronically zoomed ×2).

Measurements. Growth rates in closed slide chambers during microscopic observation were measured directly on the video screen with the digital measuring function of the Argus-10. Hyphal elongation was measured under three different conditions to compare the impact of the conditions imposed during microscopic observation vs. cultivation on Petri plates (Table 1). Mycelial extension rates were measured on Petri plates and open slide chambers approximately every 12 hr with a millimeter ruler.

Only robust hyphae elongating at an overall linear rate were measured. Pseudocolored, videotaped images of growing cells were displayed at ×12,000 on a 12-inch RGB monitor for growth measurements. The boundaries between gray levels at the cell surface were made distinct by the pseudocoloring. More than 100 hyphae per fungus were studied, and at

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Table 1. Comparison of hyphal diameters and overall growth rates for fungi growing under different conditions

Fungus	Hyphal diameter,* μm	Hyphal elongation rate, μm/s: Petri plate†	Open slide chamber‡	During observation§
Nc	11.5	0.70	0.42	0.19
Gp	10.5	0.37	0.32	0.24
Pa	9.7	0.30	0.27	0.20
Tv	8.0	0.29	0.29	0.20
Sf	8.1	0.29	0.21	0.13
Fc	7.0	0.13	0.11	0.06
Rs	6.7	0.11	0.09	0.04

Nc, *N. crassa*; Gp, *G. persicaria*; Pa, *P. aphanidermatum*; Tv, *T. viride*; Sf, *S. ferax*; Fc, *F. culmorum*; Rs, *R. solani*.

*Average of at least 10 hyphae for each species, measured where the lateral walls of the cells were essentially parallel to the long axis.

†Inoculated at the edge of 9-cm Petri plates containing potato dextrose/agar overlaid with cellulose xanthate membrane, then measured at the colony edge every 12 hr until the mycelial mat covered the entire plate.

‡Measured at the edge of advancing hyphal mats every 12 hr for at least 48 hr, with coverslip raised.

§Average of at least 10 hyphae elongating at an overall steady rate, coverslip in place, illuminated under normal observation conditions on the microscope stage.

least 10 sequences were analyzed. In developing and refining the procedure for making measurements at this scale, we repeatedly measured individual sequences to confirm the reproducibility of the measurement technique.

Hyphal length was measured at time intervals depending on the elongation rate of each fungus. Faster growing fungi were measured at 1-s intervals (every 30 frames); for slower fungi, at 2-s or 5-s intervals (every 60 or 150 frames, respectively). These intervals were chosen so that hyphae would extend by at least 2 pixels on the video screen—i.e., above the accuracy threshold of the technique, which we estimated to be within ±1 pixel on the video screen (equivalent to 0.03 μm). Running averages were calculated for every two neighboring data points to smooth out small measurement errors.

RESULTS

Hyphal Growth Pulses. At the magnification used to display and analyze images on the video monitor, individual living hyphae 6–12 μm in diameter appeared 8.5–17 cm wide, and hyphal elongation was visible to the unaided eye. Under these conditions, hyphal elongation for each fungus at first appeared linear, but subtle variations in growth rates of some fungi could be perceived by eye directly on the video screen. Further, when the cells were measured at regular intervals of 1–5 s, plots of hyphal length vs. time showed an overall linear trend but were not perfectly straight (Fig. 1). When data were plotted to display growth rates (Fig. 2), it became obvious that the hyphae were elongating in alternating pulses of faster and slower growth. For the faster growing fungi, measurements disclosed pulses when data points were plotted at intervals of 1 s (Fig. 2). For slower growing fungi (*R. solani* and *F. culmorum*) measurements at longer intervals (2 and 5 s, respectively) were sufficient to disclose the pulses (Fig. 2).

In all the fungi we examined, hyphal elongation rate was never perfectly steady but fluctuated at more or less regular intervals. In every instance, cell elongation proceeded throughout the measured sequences. We never detected a

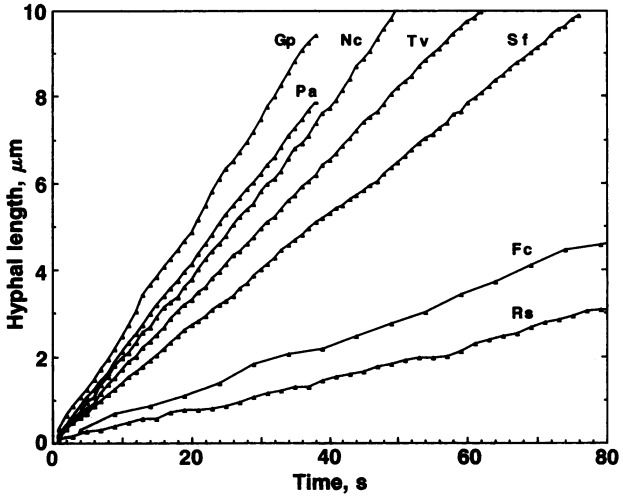

FIG. 1. Increase in hyphal length for individual hyphae of seven fungi (abbreviations as in Table 1). The overall trends of cell length show long-term linear rates. Small fluctuations in the plot lines suggest variations in growth rates at short time intervals. For *R. solani* and *F. culmorum*, data were recorded every 2 s and 5 s, respectively. For the other fungi, data were taken and plotted every second.

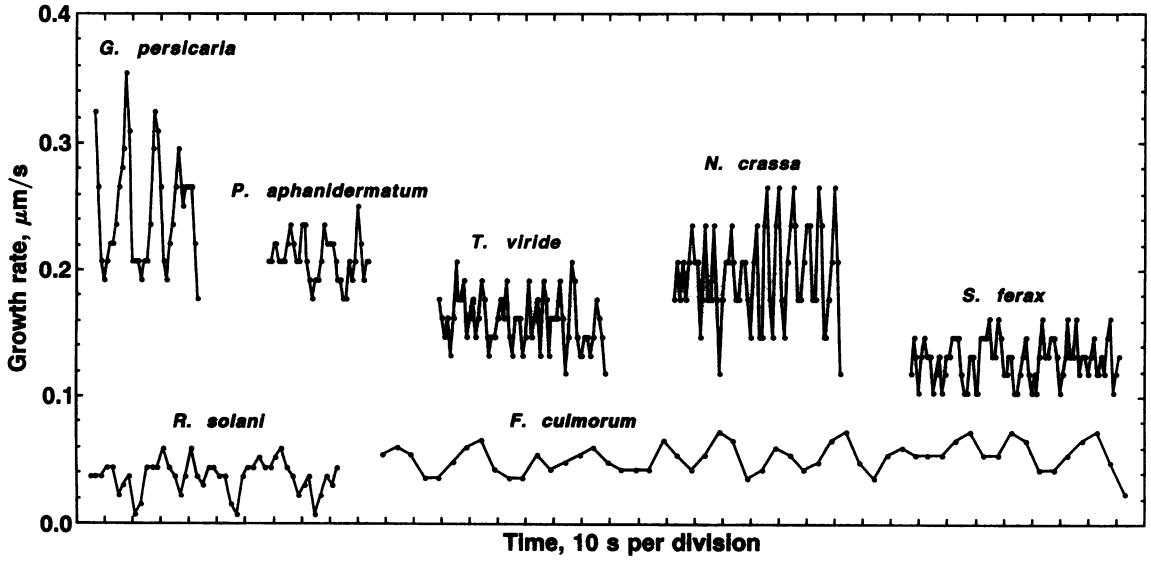

FIG. 2. Comparisons of growth rates for seven fungi. The plots reveal pulses of slow and fast hyphal elongation at differing frequencies and amplitudes for the various fungi.

Table 2. Analysis of properties of growth pulses for the cells depicted in Figs. 1 and 2

Fungus	Overall elongation rate,* μm/s (mean ± SE)	Pulse period range,† s	Pulse frequency,‡ pulses per min	Pulse amplitude,§ μm/s (mean ± SD)	Relative pulse intensity¶
G. persicaria	0.248 ± 0.00113	8–13	5.8	0.068 ± 0.008	0.275
N. crassa	0.201 ± 0.00071	3–6	12.9	0.046 ± 0.010	0.228
P. aphanidermatum	0.207 ± 0.00050	4–12	8.6	0.022 ± 0.006	0.107
F. culmorum	0.057 ± 0.00064	12–30	2.7	0.013 ± 0.004	0.220
T. viride	0.161 ± 0.00027	4–6	14.4	0.024 ± 0.008	0.149
R. solani	0.038 ± 0.00040	7.4–11.6	5.9	0.014 ± 0.006	0.368
S. ferax	0.129 ± 0.00026	7.3–9.1	11.8	0.018 ± 0.005	0.137

*Regression values of measured hyphal elongation rates from data in Fig. 1.
†Range of pulse duration from data in Fig. 2.
‡Calculated from the mean values for pulse periods.
§Calculated as half the difference between the mean values for the fastest and slowest elongation rates within the pulse periods in Fig. 2.
¶Ratio of pulse amplitude to overall hyphal elongation rates.

time when growth stopped or when a hyphal apex retracted its position even momentarily.

Frequency, Amplitude, and Relative Intensity of the Pulses. The pulses occurred continuously during growth of the hyphae but varied in frequency and amplitude from fungus to fungus as well as within each fungus (Fig. 2; Table 2). Pulse amplitude was calculated as half the difference between fastest and slowest elongation rates within each pulse period, averaged over many pulses. For example, the growth pulses of *T. viride* averaged 4–6 s each, during which the overall elongation rate (0.161 μm/s) varied with an average amplitude of 0.024 μm/s. *T. viride* and *N. crassa* showed the most frequent pulses (average of 13–14 per min), and *F. culmorum* the least frequent (2.7 per min). Average pulse amplitude varied from 0.013 μm/s for *F. culmorum* to 0.068 μm/s for *G. persicaria*. However, when the ratio of pulse amplitude to overall elongation rate was considered, a value for relative pulse "intensity" was obtained (Table 2). In this context, the most intense pulses belonged to *R. solani*, where the growth rate increased and decreased by as much as 37% of the overall rate, and the least intense pulses were found in *P. aphanidermatum*, which showed fluctuations only about 11% above and below the overall elongation rate.

Correlation of Pulses with Other Phenomena. In *F. culmorum* and *T. viride*, the fast phase of a pulse was preceded by the incorporation of one or more satellite *Spitzenkörper* into the main *Spitzenkörper*. The satellites are small but visible clusters of apical vesicles and microvesicles which first appear near the cell surface at the base of the apical crown in growing hyphal tips. They increase in size, migrate toward the apical pole, and then fuse with the main *Spitzenkörper* (Fig. 3), contributing their vesicles to the total vesicle population of the *Spitzenkörper* (see ref. 19). Satellites were obvious in three of the fungi—*F. culmorum*, *T. viride*, and *N. crassa*—and were present but less obvious in *R. solani*. Fig. 4 shows a correlation between the merger of satellites and the onset of the fast phase of growth pulses. However, pulses also occurred when no satellite *Spitzenkörper* were visible in these fungi. *G. persicaria*, *S. ferax*, and *P. aphanidermatum* have no visible *Spitzenkörper* although they have abundant populations of apical vesicles in their hyphal tips (3, 20).

In the higher fungi (Ascomycetes, Basidiomycetes, Deuteromycetes), cytoplasm flowed acropetally through the septal pores behind the hyphal apex. In the subapical compartments of hyphae elongating at overall steady rates, the cytoplasm did not flow constantly but in recurrent bursts that carried the cytoplasm into the more apical compartments. For example, in three hyphal tips of *R. solani* (total observation time, 5 min), the cytoplasmic bursts occurred every 15–20 s. It was not possible to observe a growing hyphal tip and cytoplasmic flow through the septal pore simultaneously at high magnification, because the field of view was <50 μm.

DISCUSSION

Pulsed elongation appears to be a feature of hyphal tips that has been overlooked in numerous previous studies (3, 11, 14, 16, 21–23). A study of hyphal tip organization and apical dynamics among >30 fungi showed unexpected diversity of *Spitzenkörper* patterns (10) in septate fungi, and we discovered small satellite *Spitzenkörper* which arose behind the apex and migrated toward the apical pole where they merged with the main *Spitzenkörper* (19). The evidence presented here challenges the assumption that the linear extension rate of somatic hyphae is truly constant. Our investigation of the seven fungi here indicates that this pulsed pattern of hyphal tip elongation is widespread. The pulses occurred regardless of cell diameter, overall growth rate, taxonomic position, or pattern of *Spitzenkörper* organization. Since we did not detect stoppage or retraction of the hyphal apices during

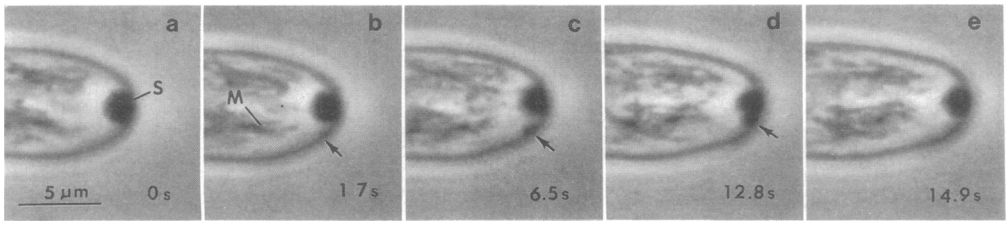

FIG. 3. Video-enhanced phase-contrast micrographs of a growing hyphal tip of *T. viride*, illustrating the development and fate of a satellite *Spitzenkörper*. (*a*) Hyphal apex with main *Spitzenkörper* (S) but no satellite. (*b*) A satellite (arrow) has begun to form and is migrating toward the apical pole of the cell. (*c*) The satellite has increased in size. (*d*) The satellite has contacted the main *Spitzenkörper* and is merging with it to form a larger *Spitzenkörper*. (*e*) Very soon after the merger, the enlarged *Spitzenkörper* has decreased in size so that it is smaller than in *a*. S, *Spitzenkörper*; M, mitochondrion.

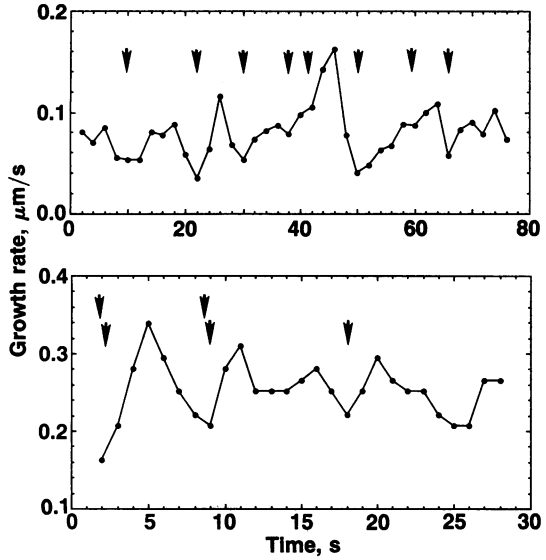

FIG. 4. Correlation of fluctuations of growth rates in individual hyphae of *F. culmorum* (*Upper*) and *T. viride* (*Lower*) and occurrence of satellite *Spitzenkörper*. Each arrowhead indicates the time when a satellite *Spitzenkörper* merged with the main *Spitzenkörper* and contributed its vesicles to the total population of vesicles in the organized apical cluster.

these observations, we conclude that the pulses are intrinsic oscillations in the overall growth rate of fungal hyphae.

Why Pulses Have Not Been Observed Previously. Pulses in hyphal elongation have not been described before probably because the time intervals of earlier observations were too long or the measurements were too coarse, or both (Table 3). Under favorable growth conditions and over times measured in minutes or hours, hyphae elongate at seemingly constant rates (14, 16, 30–32), but we were able to measure hyphal elongation at intervals at least 2 orders of magnitude shorter than most of those reported previously. Jackson and Heath (21) measured video images at roughly 10-s intervals but did not detect growth pulses. Even at the fastest elongation rates, conventional techniques used to measure growth from still pictures or directly through a microscope could not be expected to reveal the changes in elongation rate that occur within 30 s. Furthermore, minute variations in growth rate probably have been disregarded previously as measurement error. Yamada *et al.* (23) were uncertain whether growth pulsations of several minutes each were intrinsic or caused by mechanical perturbations.

Table 3. Growth rates and measurement intervals for several fungi reported by others

Fungus (ref.)	Rate, μm/s	Interval	Method
Aspergillus nidulans (11)	0.09*	1 min	Photo
A. nidulans (25)	0.04*	2 min	Photo
A. nidulans (15)	0.03*	15 min	Photo
Aspergillus niger (23)	0.09*	30 sec	Video
Candida albicans (26)	0.012	Daily	Photo
Geotrichum candidum (15)	0.04*	15 min	Photo
Geotrichum lactis (25)	0.02*	15 min	Photo
N. crassa (27)	0.27*	Daily	Not specified
N. crassa spco-9 (17)	0.36	15 min	Photo
N. crassa spco-1 (15)	0.19	15 min	Photo
N. crassa spco-1 (15)	0.24*	15 min	Photo
N. crassa cot-3 (17)	0.21	15 min	Photo
Podospora anserina (28)	0.09*	Daily	Direct
Polystictus versicolor (1)	0.08*	1 min	Direct, cinema
S. ferax (29)	0.13*	10 min	Video
S. ferax (21)	Not given	30–60 s	Video

*Growth rates in μm/s calculated from original published data.

The use of video-enhanced microscopy and digital imaging permitted precise measurements on images at high magnification. This technique is increasingly used to measure fine organelle movements in living cells (31, 33–35). Thus, we could measure changes in cell elongation as small as 0.03 μm during intervals as short as 1 s. The convenience of using videotapes made it possible to examine individual sequences of growing hyphal tips repeatedly and make digital measurements on selected frames. Extensive testing of measurement protocols early in this study eliminated measurement error as an artifactual source of the observed shifts in growth rates and ruled out the sorts of problems caused by digital measurements of slow-moving video images described by Hammad *et al.* (36).

The selection of time intervals for plotting data was important for revealing the growth pulses. Measurements at 1-s intervals disclosed pulses in the faster growing fungi (*G. persicaria, P. aphanidermatum, N. crassa, T. viride, S. ferax*) of 3–13 s. For the slower growing fungi (*R. solani* and *F. culmorum*), measurements at 2-s and 5-s intervals revealed respective pulses of 7.4–11.6 s and 12–30 s. Measurements at shorter intervals were not practical because the growth increments were too small to be resolved accurately, and plots of such measurements revealed excessive noise relative to actual growth increments. Measurements over longer periods tended to conceal the pulses so that they disappeared if intervals approached or exceeded the duration of the pulses. In selecting time intervals for measurements, we chose a compromise between the two competing variables—space and time—in order to strike an acceptable balance between spatial and temporal resolution.

Other Variations in Hyphal Growth Rates. Intrinsic variations in hyphal extension rates have been recorded previously and correlated with such developmental events as mitosis and septum formation (36, 37), the "cell cycle" (11, 16, 35), rhythmic colony growth and sporulation (28, 37), and dichotomous branching (25, 28). Erratic variation in growth rate was reported for *S. ferax* (25, 29). However, all of these fluctuations were measured on a much longer time scale and thus cannot be directly compared, nor do they contribute to an explanation of the rapid elongation pulses we measured.

Cause of the Growth Pulses. Our incomplete understanding of the mechanism of hyphal tip growth makes it difficult to explain the cause of the fluctuations in elongation rate. Nevertheless, some known features of fungal cytology and physiology allow us to offer preliminary explanations. Because the cell wall delimits the boundaries of the cell, it is logical to regard the fluctuations in elongation rate as periodic fluctuations in the rate of cell wall expansion. The generally held view, that the cell wall is synthesized largely from materials and enzymes discharged exocytotically by secretory vesicles, leads to the speculation that periodic surges in the overall rate of vesicle discharge could bring about pulsed growth.

Still unanswered is whether the pulses represent periodic increases in the mass of the cell wall or whether the cell wall increases its surface area momentarily during the fast phase of a growth pulse without concomitant increase in wall synthesis. If changes in the rate of the overall secretory process are not the immediate cause of the pulses, other factors that could cause transient changes in the physical properties of the cell wall must be considered, such as brief periods of greater and lesser wall plasticity. It remains to be seen if the pulses represent synchronized fluctuations in the rates of wall synthesis and wall loosening, or if they are produced by a certain asynchrony between these two processes.

In two fungi with small but visible aggregates of vesicles in the form of the *Spitzenkörper* satellites (Fig. 3), the behavior of those aggregates was correlated with the growth pulses. Significantly, the fast phase of a growth pulse was often detected after a satellite *Spitzenkörper* merged with the main *Spitzenkörper* (ref. 19 and Fig. 4). The sudden and periodic delivery of populations of vesicles to the main *Spitzenkörper* via satellites would not in itself explain how elongation rate accelerates momentarily unless it elicited a corresponding burst of secretory activity in the zone of cell expansion. The reduction in the size of the *Spitzenkörper* after a satellite was incorporated (19) suggests that such a secretory burst did occur. If a sudden output of vesicles from the *Spitzenkörper* to the cell surface is correlated with corresponding delivery of new vesicles to the *Spitzenkörper*, a critical mass relationship would be implied in which the number of vesicles entering the territory of the *Spitzenkörper* influences the number of vesicles that subsequently leave the apparatus and embark on their short final journey to the cell surface.

However, not all the pulses could be accounted for by observable satellites. Granted it was not possible to see all the satellites that merged with the main *Spitzenkörper* in *F. culmorum* or *T. viride* because the depth of field of the microscope was significantly shallower (1.2–1.5 μm) (19) than the hyphal diameters. Moreover, fungi lacking a visible *Spitzenkörper* (*P. aphanidermatum, S. ferax, G. persicaria*) but containing apical vesicles (3, 20) also displayed pulsed growth. In fungi without a visible *Spitzenkörper*, the organization and regulation of secretory activity may occur in ways that parallel the activity of the *Spitzenkörper*, as suggested earlier (29, 38). In other words, the concept of a functional "*Spitzenkörper*" in the absence of a visible organized apical accumulation remains a distinct possibility.

Clues pointing to a probable cause for fluctuating secretory rates await further study, but an attractive possibility would be to consider oscillations in the spatial distribution or concentration of molecules that would either regulate vesicle traffic [e.g., actin (38, 39)] or alter targeting of specific sites for vesicle docking and fusion [e.g., vesicle-associated proteins and plasma membrane receptors (39, 40)]. Additionally, some other phenomena which undergo pulsation might have an impact on vesicle dynamics and cell expansion. These include influx of cytoplasm through septal pores into the apical compartment, oscillations in ion concentrations and electrical phenomena, and changes in turgor and/or cytoskeletal activities (40–42), which may be harmonically combined with the delivery of vesicles to the cell surface to accomplish polarized growth in pulses.

Although its cause remains uncertain, pulsed growth is a clear manifestation of periodic physiological changes during tip growth and demonstrates that discontinuous wall growth, first observed by Castle (24) during sporangiophore elongation in *Phycomyces blakesleeanus*, applies to apically as well as nonapically growing fungal cells. Thus, considerations of the mechanism of hyphal tip growth would benefit from information that will help explain how and why hyphal elongation occurs in pulses rather than at truly constant rates.

Note. Two recent unpublished examples of pulsed growth in hyphae of *Aspergillus niger* (C. G. Reynaga-Peña and S.B.-G., unpublished work) and *N. crassa* (G. D. Robson and A. P. J. Trinci, personal communication) reinforce the idea that pulsed elongation of hyphae is widespread among fungi.

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