Landmarks in the early duplication cycles of *Aspergillus fumigatus* and *Aspergillus nidulans*: polarity, germ tube emergence and septation

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When the spores of filamentous fungi break dormancy, nuclear division is accompanied by a series of ordered morphological events including the switch from isotropic to polar growth, the emergence of a second germ tube from the conidium and septation. Correlation of these morphological events with nuclear number allows them to serve as duplication cycle landmarks. Early duplication cycle landmarks have been characterized in *Aspergillus nidulans*, but not in other filamentous fungi. To learn more about duplication cycle control in filamentous fungi, a study was undertaken to compare the timing of landmarks in *Aspergillus fumigatus* and *A. nidulans*. Nuclear duplication took approximately 45 min in *A. fumigatus*, with mitosis occupying roughly 5% of this period. Under the same conditions, nuclear duplication in *A. nidulans* took approximately 60 min, with mitosis occupying roughly 4% of this period. In *A. fumigatus* the isotropic to polar switch preceded the first mitosis in 22% of cells, while in *A. nidulans* the isotropic to polar switch did not occur until after the first mitosis. In both *A. fumigatus* and *A. nidulans* the earliest emergence of a second germ tube from the conidium occurred after the third mitotic division. However, by the fifth mitosis only 19% of *A. fumigatus* conidia had a second germ tube, compared to 98% of *A. nidulans* conidia. In both *A. fumigatus* and *A. nidulans*, formation of the first septum occurred after the fourth mitotic division. In all experiments a few cells lagged behind the others in nuclear number. In this delayed group, it was common to see landmark events at an earlier mitotic division. Differences in nuclear number when identical landmarks occur in *A. fumigatus* versus *A. nidulans*, and uncoupling of mitotic division and landmarks in delayed cells suggest that nuclear division and morphogenesis lie in parallel pathways, perhaps coordinated by checkpoints.

**Keywords:** cell cycle, mitosis, morphogenesis

**INTRODUCTION**

The realization that ordered morphological landmarks correlate with mitotic state in *Saccharomyces cerevisiae* provided the framework for understanding the cell cycle of this yeast (reviewed by Pringle & Hartwell, 1981; Lew et al., 1997). During the G1 phase the unbudded mother cell grows isotropically, adding new material uniformly in all directions. Late in G1 the bud emerges and grows in a polar manner, adding new material at the tip of the bud. This polar growth continues during S phase until sometime in G2, when the bud switches to isotropic growth. As M phase ends, septation occurs, separating the mother and daughter cells. Many biochemical, genetic and ultrastructural landmarks have been added to this simple morphological framework, giving a view of relationships among the pathways required to replicate and divide the components of the yeast cell.

Morphological landmarks correlate with mitotic state in filamentous fungi as well. Because the cells of filamen-
tous fungi remain attached after septation, these landmarks also correlate with mitotic history. A cell containing two nuclei has undergone one mitotic division. A cell containing four nuclei has undergone two divisions, and so on. The mitotic cycle in filamentous fungi is often called the duplication cycle to distinguish it from cell cycles where daughters completely separate (Fiddy & Trinci, 1976).

The Aspergillus nidulans duplication cycle is the best characterized among filamentous fungi (reviewed by Harris, 1997). After breaking dormancy, the uninucleate asexual spores of A. nidulans grow isotropically until the first mitosis. After this first nuclear division, an axis of polarity is established and the germ tube begins to emerge (Momany et al., 1999; Harris et al., 1999; Harris, 1999). The axis of polarity is maintained as the germling elongates by tip growth. The first nuclear division after the germling reaches a predetermined size triggers synthesis of the first septum at the base of the germ tube (Harris et al., 1994; Wolkow et al., 1996). This can occur as early as the third mitosis. Subsequent septa are laid down at regular distances along the hypha after each round of mitosis. The nuclei in the subapical compartments are arrested in interphase, while the nuclei in the apical compartment undergo synchronous mitosis. When a second germ tube emerges from the spore, it is most often at 180° to the first, in a bipolar arrangement (Harris et al., 1999; Momany et al., 1999).

Aspergillus fumigatus, an important pathogen of humans, is an increasing problem in immunocompromised patients (Kwon-Chung & Bennett, 1992). Because it lacks a sexual cycle, A. fumigatus is often studied in conjunction with the related saprobe A. nidulans (Tang et al., 1994; Borgia et al., 1994; Guest & Momany, 2000). To better understand the biology of A. fumigatus we have undertaken studies comparing its duplication cycle with that of A. nidulans. We have uncovered differences in the isotropic to polar switch, emergence of the second germ tube and septation. Our results suggest that nuclear duplication and morphogenesis lie in parallel pathways in filamentous fungi.

**METHODS**

**Strains and growth conditions.** A. fumigatus strain 237 was a clinical isolate provided by Dr David Holden, Department of Infectious Disease and Bacteriology, Royal Postgraduate Medical School, Hammersmith Hospital, London W12 ONN, UK. Aspergillus nidulans strain A28 (pabaA6; biaA1) was received from the Fungal Genetics Stock Center, Dept of Microbiology, University of Kansas Medical Center, Kansas City, KS, USA. All incubations were at 37 °C in complete medium (1% glucose, 0.2% peptone, 0.1% yeast extract, 0.1% Casamino acids, nitrate salts, trace elements and 0.01% vitamins, pH 6.5). Trace elements, vitamins, nitrate salts and amino acid supplements are described in the appendix to Kafer (1977).

**Staining and microscopy.** The protocol of Harris et al. (1994) was used for growth and staining of A. fumigatus and A. nidulans as follows. Ten millilitres of complete liquid medium were inoculated with 1–5 x 10⁴ conidia ml⁻¹, poured into a Petri dish containing a glass coverslip and incubated at 37 °C for the time indicated in each experiment. Coverslips with adhering germlings were fixed in 3.7% formaldehyde, 50 mM phosphate buffer (pH 7.0) and 0.2% Triton X-100 for 30–60 min. Coverslips were then washed with water, incubated for 5 min with 10 µg calcofluor white ml⁻¹ (Bayer) and 100 ng Hoechst 33258 ml⁻¹ (Sigma), washed again and mounted on a microscope slide for viewing. Germlings were photographed using a Zeiss Axiosplan microscope and Zeiss MC100 microscope camera system with Kodak Tmax 100 film.

Because germinating conidia are not perfectly synchronous, mitotic division number in Fig. 1 is based on the earliest time point when > 50% of the population shows the appropriate nuclear number. Morphological landmarks shown in Fig. 4 were scored at the time points represented in Fig. 1, i.e. the earliest time point when > 50% of the population had completed the indicated mitotic division. Only those germlings that had completed the appropriate division were used to determine the percentage showing a landmark. For example, 11% of A. nidulans cells that contained two nuclei (had completed the first mitotic division) were polar. A. fumigatus experiments were repeated at least three times and A. nidulans experiments were repeated twice, with essentially identical results. Typical data sets are shown.

**RESULTS AND DISCUSSION**

**Length of the nuclear cycle**

When the uninucleate, asexual spores (conidia) of A. fumigatus or A. nidulans are inoculated into rich medium they break dormancy and remain roughly synchronized through the first several mitotic divisions. We took advantage of this synchrony to monitor the length of time between nuclear doublings (Fig. 1).

![Fig. 1. Nuclear division in A. fumigatus and A. nidulans. Conidia were inoculated into rich media and samples were taken every 15 min. Germlings were fixed and stained with Hoechst 33258 and calcofluor white to visualize nuclei and chitin, respectively. The number of nuclei per germling was counted. The earliest time point when at least 50% of the population had completed each mitotic division is shown for A. fumigatus (black triangles with solid line) and A. nidulans (white squares with broken line).](https://www.microbiologyresearch.org/content/3280/Fig_1.png)
Conidia were inoculated into rich medium, incubated, fixed at 15 min time points and stained with Hoechst 33258 and calcofluor white to visualize nuclei and chitin, respectively. Cells treated in this way are easily scored for nuclear number and morphological events (Fig. 2). In *A. fumigatus* the first nuclear division occurred after a lag of 4 h 15 min with subsequent nuclear divisions every 45 min. In *A. nidulans* the initial lag was 3 h 45 min with subsequent divisions every 60 min. This is somewhat faster than previously reported nuclear cycle lengths of 90–120 min for *A. nidulans* (Robinow & Canten, 1969; Bergen & Morris, 1983), but agrees with doubling times observed under similar conditions (Harris et al., 1994).

As illustrated in Fig. 2, after staining, mitotic nuclei are small and bright, while interphase nuclei are ellipsoidal with dark spots (compare Fig. 2f and g). The distinctive appearance of mitotic versus interphase nuclei allowed us to determine that approximately 5% of *A. fumigatus*...
Table 1. Nuclear state in germlings

Spores were incubated at 37 °C for the indicated time, fixed and stained with Hoechst 33258 to stain nuclei. Germlings containing small, bright nuclei were counted as mitotic. Germlings containing larger nuclei with dark spots were counted as interphase.

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<th>7 h</th>
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<sup>a</sup> Cell mitotic index = percentage of germlings in mitosis.

Fig. 3. Polar growth can begin before mitosis in *A. fumigatus*. Germlings were grown and stained as described for Fig. 1. Both cells contain one nucleus. The cell on the left is polar; the cell on the right is nonpolar. Right panel, fluorescent image. Left panel, phase-contrast image. Bar, 5 µm.

Fig. 4. Timing of morphological landmarks. Germlings were grown and stained as described for Fig. 1. *A. fumigatus* (black symbols, solid line) and *A. nidulans* (white symbols, broken line) cells were monitored for polarization (circles), emergence of the second germ tube (squares) and septum formation (triangles).

cells are in mitosis at any time (Table 1). Thus mitosis occupies about 5% of the 45 min duplication cycle, or 2:25 min, in *A. fumigatus*. Under the same conditions approximately 4% of *A. nidulans* cells are in mitosis so that mitosis occupies about 2:4 min. This agrees with the previously reported value of 3–4% mitotic cells in *A. nidulans* (Robinow & Canten, 1969; Bergen & Morris, 1983).

Polarity establishment

The conidia of both *A. fumigatus* and *A. nidulans* are initially uninucleate and spherical. After a growth period, but before the first nuclear division, 22% of *A. fumigatus* spores took on a pear shape (Figs 3, 4), indicating that an axis of polarity was established. After the first mitosis, greater than 90% of *A. fumigatus* cells were polar. In *A. nidulans*, however, no cells were polar before the first mitosis, 12% were polar after the first mitosis, and greater than 90% were polar after the second mitosis.

Our results are consistent with inhibitor studies that suggest a mitotic division is normally needed for polarity establishment in *A. nidulans* in rich medium (Harris, 1999). Interestingly, *A. nidulans* spores grown in poor medium do not require completion of mitosis for polarization (Harris, 1999). A size-control mechanism that monitors nuclear to cytoplasmic ratio for optimal hyphal growth has been previously suggested (Trinci, 1978). Harris (1999) postulates that the ability of *A. nidulans* to polarize without mitosis in poor medium may reflect a lowering of this size threshold when nutrients are scarce. When we grew *A. fumigatus* in minimal medium rather than complete medium, we observed no difference in polarization (data not shown). If a nuclear to cytoplasmic ratio must be achieved for polarity establishment, it appears that the size threshold is not lowered by nutrient scarcity in *A. fumigatus*.

Emergence of the second germ tube

We observed the earliest conidia with second germ tubes after the third mitosis in both *A. fumigatus* and *A. nidulans* (Fig. 4). However, by the fifth mitosis just 19% of *A. fumigatus* cells possessed a second germ tube, while 98% of *A. nidulans* cells possessed a second germ tube. Three patterns of germ tube emergence were observed. The second germ tube was either 180° from the first (bipolar), 90° from the first (quartepolar) or at some other angle (random). For both *A. fumigatus* and *A. nidulans*, the bipolar pattern was seen in just over half of germlings, the quartepolar pattern was seen in just over a third of germlings and the random pattern was seen in the remaining tenth. Though our observed percentages are different, the relative frequency of each pattern is consistent with previously published reports in *A. nidulans* which showed bipolar 84% of the time, quartepolar 16% of the time and random 1:5% of the time (Harris et al., 1999).
Emergence of the second germ tube is analogous to the emergence of a second bud from the mother cell in *S. cerevisiae*. In yeast, the site of bud emergence is controlled by actin, septins and Bud proteins (Pringle et al., 1995). It seems likely that a similar marker system acts to establish polarity in both *A. fumigatus* and *A. nidulans*. Our data suggest however, that polarity markers may be regulated differently in these two fungi, with emergence of the second germ tube either suppressed in *A. fumigatus* or enhanced in *A. nidulans*. Perhaps by directing growth primarily in one direction, the lower percentage of second germ tubes allows *A. fumigatus* to scavenge nutrients more efficiently in the host.

**Septation**

We observed the first septum in 21% of *A. fumigatus* germlings that had undergone four rounds of mitosis (Fig. 4). The first septum was found anywhere within about 20 μm of the base of the germ tube (Fig. 5). In *A. nidulans* we observed the first septum in 55% of germlings that had undergone four rounds of mitosis. This is in contrast to previous reports that the first septum in *A. nidulans* is usually laid down after the third nuclear division (Harris et al., 1994). This difference in reported nuclear number at first septation probably arose from differences in incubation temperature and the methods used to define nuclear state of the population. Harris et al. (1994) grew cells at 28 °C, took hourly time points and separately counted nuclear number in the population and the presence of the first septum. They then correlated the two events, i.e. the majority of the population had 8 nuclei when the first septa appeared. We, on the other hand, grew cells at 37 °C, took time points every 15 min and counted septa in germlings with 8 nuclei separately from those with 16 nuclei. The data diagrammed in Fig. 4 reflect the earliest time points when > 50% of the population had completed the appropriate division and only that part of the population with the indicated nuclear number. A few *A. nidulans* cells did lag behind the others in mitotic divisions, having only eight nuclei when the majority of the population had 16 or even 32 nuclei. In these delayed cells it was common to see septation after the third mitosis (results not shown). The lower incubation temperature, less frequent time points, separate scoring of mitotic number and septation, and the presence of lagging cells probably account for the difference in timing observed by Harris et al. (1994). We never observed septa forming in *A. nidulans* germlings with fewer than eight nuclei or in *A. fumigatus* germlings with fewer than 16 nuclei.

Using *A. nidulans* conditional mutants that do not go through mitosis, but still grow, Wolkow et al. (1996) established that the formation of the first septum is triggered by a mitotic division after the germling reaches a critical size. They also showed that the position of the first septum is determined by the position of mitotic nuclei. Previous work (Fiddy & Trinci, 1976) had established that mitosis proceeds in a parasympathetic wave starting at the tip of the hypha and moving back toward the conidium. Wolkow et al. (1996) postulated that an inhibitor of septation with a tip-high gradient is diluted out by cell growth before mitosis triggers septation. During apical extension, the inhibitor would be diluted below the inhibition threshold first at the base of the germ tube. When the mitotic wave intersects this inhibition-free zone at the base of the germ tube, septation would be triggered nearby. They further postulated that formation of a septum suppresses formation of septa at adjacent nuclei. Their model is supported by the observation that delays in mitosis cause *A. nidulans* to misplace septa away from the base of the germ tube. By giving the hypha more time to extend before mitosis, the zone of cytoplasm below the critical inhibitor threshold is enlarged. The slightly later septation of *A. fumigatus* may result from some difference in size control. Alternately, because *A. fumigatus* hyphae are about half the diameter of *A. nidulans* hyphae, perhaps they must extend further to dilute the inhibitor enough to allow septum formation.

**Uncoupling of mitosis and morphogenesis**

In our experiments a few cells (< 1%) always lagged behind the others in mitotic divisions. For example, some germlings had only a single nucleus when the majority of the population had 16 nuclei. In delayed *A. nidulans*...
Aspergillus nidulans cells it was common to see polarization with a single nucleus, emergence of the second germ tube after only two mitotic divisions and septation at the third mitotic division (data not shown). This uncoupling of nuclear division from morphological landmarks suggests that these events are not inter-dependent. Rather, nuclear division and morphogenesis appear to lie in parallel pathways in filamentous fungi. The delay in mitosis seen along with the delay in polarization further suggests that these pathways may be coordinated by checkpoints in much the same way that the nuclear division and bud emergence pathways are coordinated by the morphogenesis checkpoint in S. cerevisiae (Pringle & Hartwell, 1981; Lew et al., 1997).

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REFERENCES


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