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Fungal surface remodelling visualized by atomic force microscopy

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ABSTRACT

Most fungal growth is localized to the tips of hyphae, however, early stages of spore germination and the growth of certain morphological mutant strains exhibit non-polarized expansion. We used atomic force microscopy (AFM) to document changes in *Aspergillus nidulans* wall surfaces during non-polarized growth: spore germination, and growth in a strain containing the *hypA1* temperature sensitive morphogenesis defect. We compared wall surface structures of both wild-type and mutant *A. nidulans* following growth at 28 ° and 42 °C, the latter being the restrictive temperature for *hypA1*. There was no appreciable difference in surface ultrastructure between wild-type and *hypA1* spores, or hyphal walls grown at 28 °C. When dry mature *A. nidulans* conidia were wetted they lost their hydrophobin coat, indicating an intermediate stage between dormancy and swelling. The surface structure of *hypA1* germlings grown at 42 °C was less organized than wild-type hyphae grown under the same conditions, and had a larger range of subunit sizes. AFM images of hyphal wall surface changes following a shift in growth temperature from restrictive (42 °C) to permissive (28 °C), showed a gradient of sizes for wall surface features similar to the trend observed for wild-type cells at branch points. Changes associated with the hyphal wall structure for *A. nidulans hypA1* offer insight into the events associated with fungal germination, and wall remodelling.

Introduction

Aspergillus species affect humans in a variety of ways ranging from biotechnological applications to human disease. Fungal activities are mediated through their cell walls, for which a more complete structural understanding may offer insight into function. Fungi grow by polarized extension and form mycelia by branching. Atomic force microscopy (AFM) and force spectroscopy (FS) of growing *A. nidulans* hyphae have provided direct evidence for the enhanced compliance of growing hyphal tips and forming branches compared with mature regions, accompanied by changes in cell wall surface architecture (Ma *et al.* 2005). In addition to tip growth, germinating spores swell before germ tube extension, and some morphological mutants swell rather than, or in addition to, tip extension. Thus, understanding fungal wall remodelling will give insight into fungal development, and will aid in the evaluation of mutant strains.

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Fungal aerial hyphae and air-dispersed spores have a hydrophobin rodlet coat that enables them to emerge from damp substrates, and that protects the spore contents during dormancy (Wosten et al. 1994). The vegetative cycle of *Aspergillus* begins with spore wetting, water imbibing, and germination (d'Enfert 1997). Germination encompasses two stages, non-polarized swelling and polarized germ tube growth (Momany 2002). Hydrophobins can be mechanically dislodged from the conidial surface of *A. fumigatus* by sonication (Paris et al. 2003), but clearly other mechanisms function in nature.

Fungal hyphal walls are composed of (1→3)- and (1→6)-β-glucans reinforced by chitin microfibrils (Beckett et al. 1974; Guest & Momany 2000; Lipke & Ovalle 1998; Vermeulen & Wessels 1986). The surface architecture of spores and cells has been imaged at high resolution using electron microscopy (EM), but EM preparation requires fixed or frozen specimens. In contrast, AFM can generate images of living and/or fixed cells with nanometer scale resolution, and force spectroscopy (FS) can probe their physical parameters (Dufrêne 2004; Ma et al. 2005). Recently, we used AFM and FS to examine growing *A. nidulans* hyphae, and our results support fungal tip growth models (Ma et al. 2005).

Mutant strain analysis is a powerful and widely used tool to study cell function. For example, *hypA* encodes a novel (Kaminskyj & Hamer 1998) cytoplasmic protein involved in coordinating aspects of cell growth (Shi et al. 2004, Kaminskyj & Boire 2004). *A. nidulans* strains containing the *hypA1* allele have a wild-type phenotype when grown at 28 °C, but abnormal morphology when grown at 42 °C, characterized by hyphal swelling (Kaminskyj & Hamer 1998; Kaminskyj & Boire 2004; Shi et al. 2004). We have compared the spore and hyphal surfaces of *A. nidulans* wild-type and *hypA1* strains at 28 ° and 42 °C, focusing particularly on changes associated with fungal wall remodelling during early stages of germination, and in *hypA1* germlings grown at 42 °C.

Methods

Biological material and growth conditions

Aspergillus nidulans strains A28 (*pabaA6*, *biA1*, *veA1*) and ASK30 (*hypA1*, *wA3*, *pyroA4*; *veA1*; www.fgsc.net) were maintained as described in Kaminskyj (2001). Conidia were inoculated onto dialysis membranes overlying solid complete medium as described by Ma et al. (2005). Freshly harvested conidia, and conidia harvested from a water suspension (Kaminskyj 2001) were examined directly. To examine living hyphae, conidia were germinated on dialysis tubing overlying agar medium (Ma et al. 2005), and grown at 28 ° or 42 °C for 16 h before imaging. To study the effect of growth temperature shifts on hyphal wall surface ultrastructure, ASK30 spores were grown at 42 °C for 16 h and then shifted to 28 °C for 4 h growth (Kaminskyj & Hamer 1998; Kaminskyj 2000).

Preparation of fixed cells for (cryo) scanning EM (SEM) and AFM

Hyphae and conidia were prepared for imaging by AFM and SEM as previously described (Ma et al. 2005). Briefly, fixed hyphae for AFM imaging were grown on dialysis tubing overlying agar medium, fixed by vapour diffusion [aqueous osmium tetroxide, 4 % (v/v)], frozen (anhydrous acetone; 60 ml, −80 °C), and dehydrated (≥4 h at –80 °). Samples were warmed slowly (2 h at −20 °C, 2 h at 4 °C, 2 h at room temperature) to avoid condensation and critical point dried to circumvent surface tension artefacts. Samples were mounted on aluminum stubs (for SEM) or on glass coverslips (for AFM) using double-sided tape, and gold coated for 3 min (Edwards S150B sputter coater). Gold coating facilitated fast sample imaging and results were comparable to samples without gold coating (Ma et al. 2005).

Samples for imaging by cryoSEM were grown on dialysis tubing overlying CM agar, frozen by plunging into nitrogen slush, sublimated at −60 °C, gold coated, and examined at −80 ° using a 1630C field emission SEM (JEOL, Peabody, MA) equipped with digital image capture.

AFM topography and lateral force images were collected in contact mode using Si_3N_4 probe tips [Model #1520-00 TM Microscopes (now Veeco), Sunnyvale, CA; $k = 0.032$ nN/nm; $\nu_{nominal} = 17$ kHz] and processed as previously described (Ma et al. 2005). Topography images report on surface height changes (nm). Lateral force images are a convolution between frictional forces and height information, and as a result of the 'edge effect' for tip-sample friction, this mode often provides greater contrast for very convoluted samples (Ma et al. 2005). Image resolution is 6.25–12.5 lines μm^{-1} (low), 62.5–170 lines μm^{-1} (medium), and 660–2500 lines μm^{-1} (high). Images were processed using horizontal levelling for images with a large z range and two-dimensional (2D) levelling for samples with a small z range, with the maximal height adjusted for optimum contrast (SPMLab v. 6.0 software; Veeco, Sunnyvale, CA). As images of wall components are convoluted by the shape of the AFM probe, widths were measured at the full width at half maximum (FWHM) of the peak height in topography images, and both widths and heights were measured manually using the line measurement option. AFM probes used for this study were capable of resolving 5 nm diameter colloidal gold particles (Xu & Arnsdorf 1994), within experimental error (Ma et al. 2005). In some cases, images have been presented enlarged for greater visual clarity. Data are presented as mean ± standard deviation. Differences in the mean spore and hyphal cell wall subunit sizes for wild-type and *hypA1* were assessed by a one-way analysis of variance (ANOVA) test using the program InStat 3 (GraphPad, San Diego, CA).

Results

Wild-type and hypA1 *mutant phenotypes*

Aspergillus nidulans strain A28 is morphologically wild-type at 28 ° and 42 °C. ASK30 contains the temperature-sensitive allele, *hypA1*, derived by mutagenesis of A28 (Kaminskyj & Hamer 1998; Shi et al. 2004). ASK30 was grown at 28 ° (Fig 1A) or 39 °C (Fig 1B) before cryoSEM imaging. ASK30 hyphae grown at 28 °C are wild-type (diam ~3 μm) with lateral branches extending from subapical regions. In contrast, ASK30 conidia grown at or above 37 ° produce wide, uneven hyphae that branch from the apex as well as laterally.

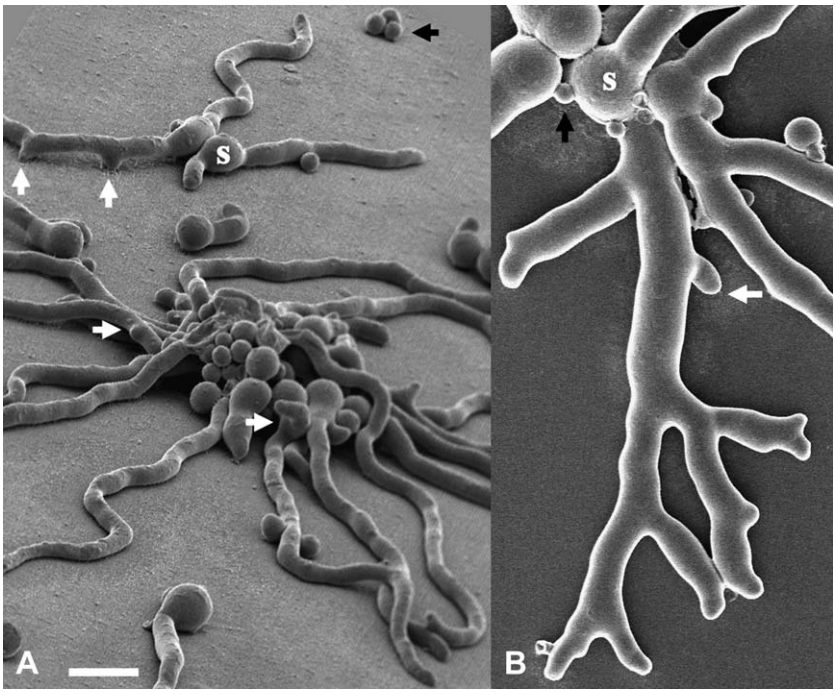

Fig 1 – *Aspergillus nidulans* hyphae imaged with cryo-scanning electron microscopy. ASK30 containing the temperature-sensitive *hypA1* allele were grown for 16 h at (A) 28 ° or (B) 39 °C. At 28 °C ASK30 show the wild-type phenotype, cylindrical hyphae that branch laterally from subapical sites, whereas above 39 °C ASK30 has variable width hyphae that branch apically and laterally. Germinated spores are labelled 's', white arrows indicate branch buds and black arrows show dormant spores. The spore wall is rougher than the hyphal wall particularly noticeable at 39 °C. Bar for both images = 10 μm.

Spore surface architecture and wall properties

Mature *Aspergillus nidulans* conidia are spherical with a convoluted surface (Fig 2). Dry harvested strain A28 (top panel, Fig 2A–E) and ASK30 (bottom panel, Fig 2G–I) conidia have similar diameters (3.2 ± 0.5 μm *versus* 3.3 ± 0.4 μm, respectively, both $n = 60$) and surface features (154 ± 27 nm *versus* 149 ± 38 nm in *x* and *y*, respectively, each $n = 30$). High-resolution AFM images of dry ASK30 conidia shaken from an agar plate have a hydrophobin rodlet layer (rodlets were 11 nm ± 2 wide, $n = 60$, and >100 nm long; Fig 2G inset) indistinguishable from A28 rodlets (Ma *et al.* 2005). Rodlet 'strands' (~3 nm width, Zhao *et al.* 2005) were resolved in line scans of the rodlets (data not shown). A28 (Fig 2F) and ASK30 (Fig 2J) conidia harvested from water suspensions (Kaminskyj 2001) lacked this rodlet layer, but share the same dimensions (3.4 ± 0.4 μm *versus* 3.2 ± 0.5 μm, respectively, each $n = 60$) and convoluted surface structure (153 ± 29 nm in *x* and *y*; $n = 30$) as dry spores (Fig 2A–E, G–I).

A. *nidulans* conidia swell from 3 μm to 4–8 μm diameter, up to a 20-fold increase in surface area, before germ tube extension. Swollen spores (Fig 3A) had large convoluted features (Fig 3B–F) similar in shape to those before swelling, but two to fourfold smaller in relative height. The larger feature size (in *x* and *y*) and shallower profile (~100–200 nm *z* range) contribute to swollen spores appearing smoother than dry or wetted conidia.

Hyphal surface architecture

After imbibing, *Aspergillus nidulans* spores establish a site for germ tube extension (Momany 2002). Germ tube walls (Fig 4) were smoother than swollen spores, with smaller and more uniform wall features. As expected, germ tube and hyphal surfaces had similar patterns of subunit maturation. Wall concavities (100– 200 nm wide and up to 50 nm deep) were found on both fixed (Fig 4A, B) and live (Fig 4C, D) germling walls, particularly at the spore–germ tube junction.

Walls of the wild-type strain A28 were similar following growth at 28 ° and 42 °C (Fig 5C), like ASK30 grown at 28 °C, whereas walls of the *hypA1* strain ASK30 grown at 42 °C had gross and fine morphological abnormalities (Fig 5D–I). Mature walls of strain A28 grown at 42 °C (Fig 5C) had surface subunits 26 ± 4 nm ($n = 60$; range 18.2–38.3 nm) in diameter, similar to those of ASK30 grown at 28 ° (25 ± 6 nm, $n = 60$) and A28 grown at 28 °C (Ma *et al.* 2005). In contrast, ASK30 germlings grown at 42 °C (Fig 5D–I) had larger surface subunits, 56 ± 11 nm ($n = 60$).

Fungal cell surface remodelling after growth temperature changes

ASK30 germlings established at 42 °C and shifted to 28 °C produce wild-type branches (Kaminskyj & Hamer 1998; Kaminskyj & Boire 2004). These changes in growth morphology were relatively abrupt (Fig 6A, B). The gradient of surface

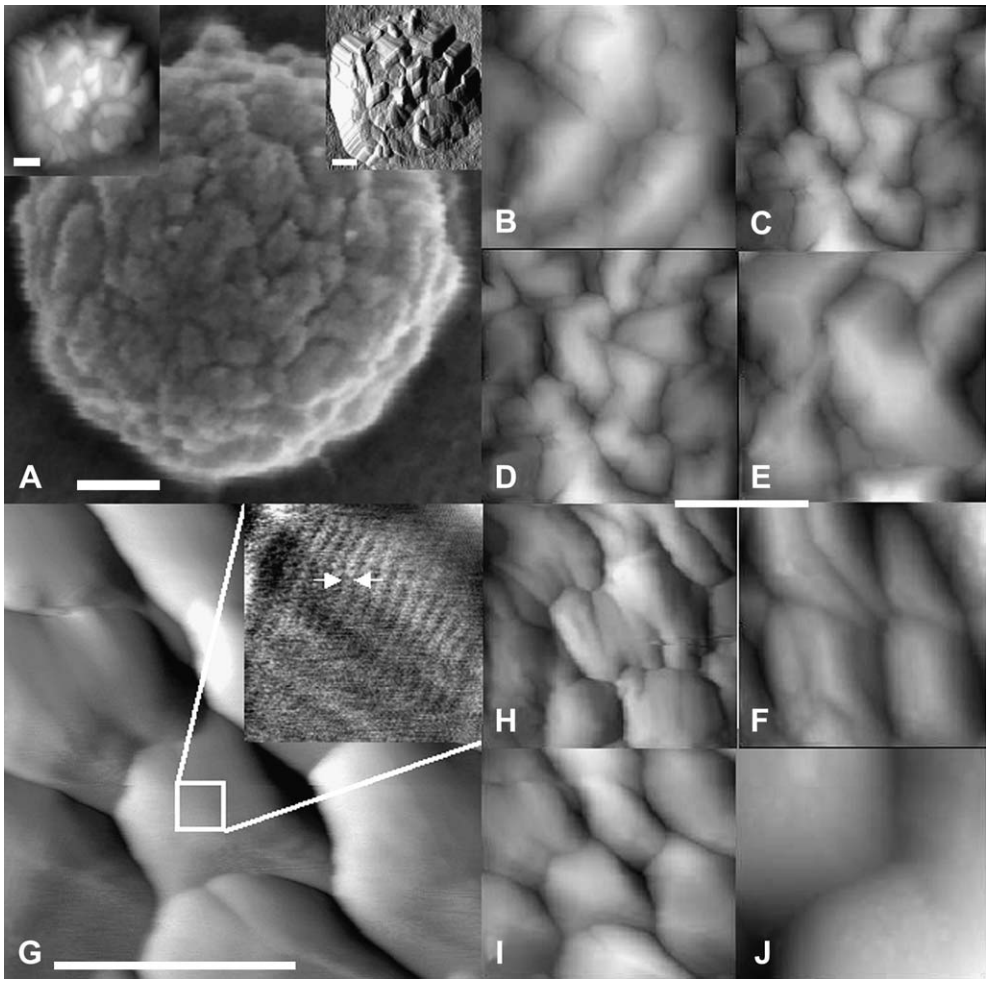

Fig 2 – *Aspergillus nidulans* conidia imaged with SEM and AFM. Spore of wild-type strain A28 harvested from a dry culture (A; 3 μm × 3 μm) imaged with conventional SEM and AFM (3 μm × 3 μm each, top left inset topography and top right inset lateral force). Dry (B–E) and wet-harvested (F; see experimental procedures) A28 spores topography imaged by contact mode AFM (each 1 μm × 1 μm) show similar height surface convolutions (200–400 nm z range). Medium and high-resolution AFM topography image of a dry ASK30 spore (G; 1 μm × 1 μm) showing the hydrophobin rodlet layer (inset 0.25 μm × 0.25 μm). Surface topography of dry (H–I) and wetted (J) ASK30 *A. nidulans* conidia imaged by AFM (each image 1 μm × 1 μm). Bar on all images = 0.5 μm.

subunit size distribution at these junctions corresponds to a range of subunit sizes (Fig 6B) at and between those observed for single growth temperatures.

Discussion

Hyphal tip growth uses polarized insertion of wall matrix materials and wall fibril synthesizing enzymes that together generate an elastic tension-bearing wall upon maturation (reviewed in Bartnicki-García 2002). These processes can be imaged by AFM in growing cells as well as fixed ones (Ma *et al.* 2005). Although it is the predominant growth mode for fungi, tip growth is not ubiquitous: spore germination and certain morphological mutants exhibit non-polarized growth. We have characterized changes associated with fungal wall remodelling during wild-type (spore swelling) and morphogenetic mutant (*hypA1*) growth.

Dry-harvested *Aspergillus nidulans* conidia have a surface overlay of hydrophobin rodlets. Germination requires water imbibing (d'Enfert 1997; Osherov & May 2000), which is difficult to reconcile with a hydrophobin barrier. AFM images of *A. nidulans* spores harvested as a water suspension had a gross morphology similar to dry spores but lacked visible rodlets, implying an intermediate stage between spore dormancy and swelling that includes rodlet loss or rearrangement. Germination requires the spore to detect a metabolizable carbon source (d'Enfert 1997), although the early stages of germination do not appear to require nutrient uptake (Shi *et al.* 2004). Dry *A. nidulans* spores retain high viability at 4 °C, whereas spores stored in distilled water at 4 °C lose viability after a few weeks (Kaminskyj 2001). Thus, the rodlet layer could be important for maintaining full dormancy and protecting spore metabolite reserves.

Rodlets have been imaged by AFM on the spore surface of *Phanerochaete chrysosrium* (Dufrêne *et al.* 1999), *A. oryzae*

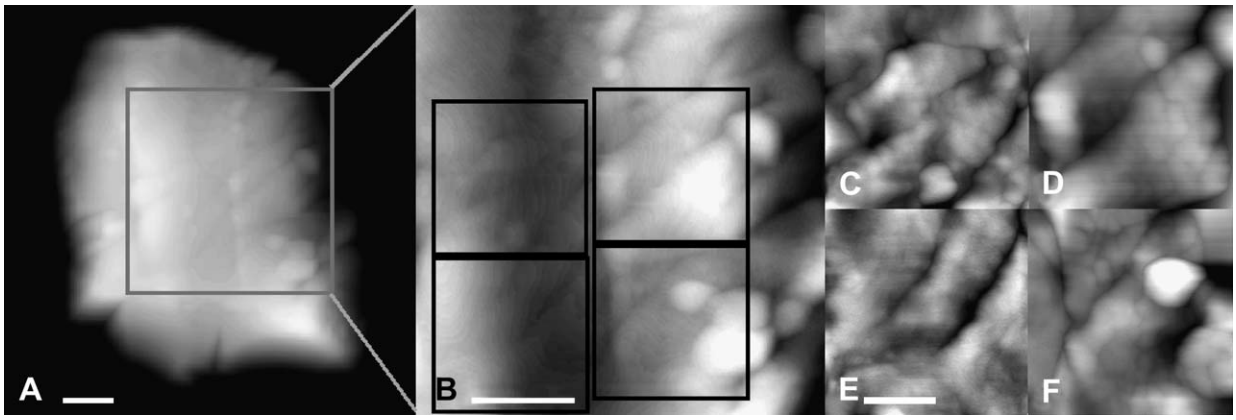

Fig 3 – A wild-type *Aspergillus nidulans* A28 conidium was swollen at 28 °C, and its topography imaged by AFM. The intact conidium (A; 4 μm × 4 μm) and its surface structure in a small region (B; 2 μm × 2 μm; 100 nm z range) are shown at medium resolution. The four quadrants outlined in B are show in C–F (0.75 μm × 0.75 μm each) at high resolution. Bar for A, B = 0.5 μm, bar for E = 0.25 μm refers to C–F.

(Van der Aa *et al.* 1994), *A. fumigatus* (Paris *et al.* 2003) and *A. nidulans* (Zhao *et al.* 2005). The latter two groups showed that rodlets were removed by ultrasonication in water, a more vigorous method than rubbing with a glass rod (this study). This discrepancy may reflect disparate hydrophobin properties. In *Schizophyllum*, hydrophobin is released from spores exposed to water (Wessels 1994), consistent with our results. The experimental evidence is consistent with hydrodynamic shear being able to dislodge the rodlets (Zhao *et al.* 2005). Since the hydrophobin rodlet layer appears to be incompatible with imbibing, and rodlets are not observed post-germination, there must be a mechanism for their reorganization or loss *in vivo*.

After swelling, *A. nidulans* spore surfaces were relatively smooth although features from the dry mature spore were still visible. Remnants of the original spore wall likely remain, consistent with *Phanerochaete chrysosporium* (Dufrêne *et al.* 1999; Dufrêne 2000). *A. nidulans* germ tube walls (data not

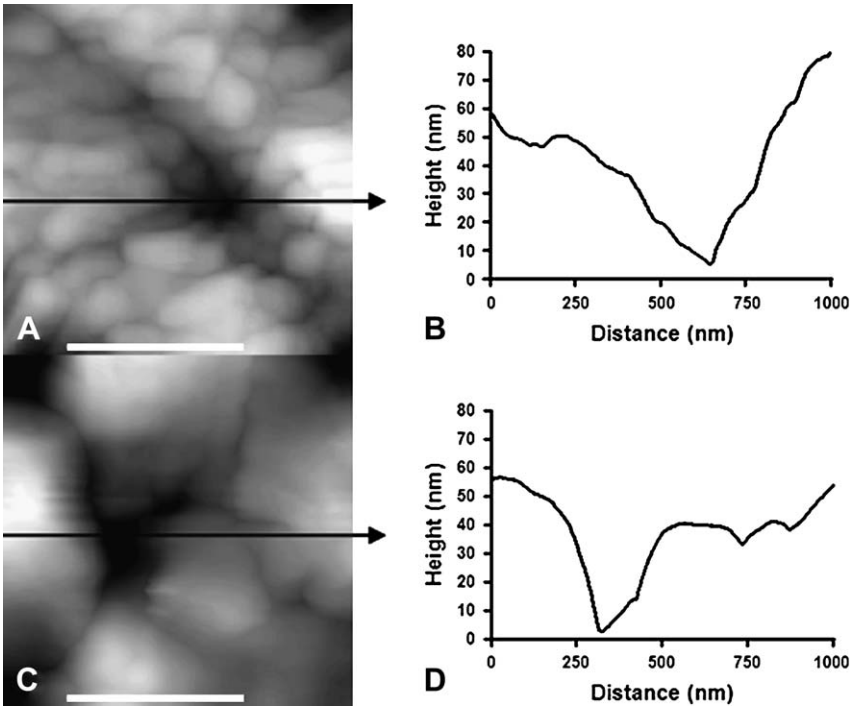

Fig 4 – AFM topography images (1 μm × 1 μm) of concave discontinuities on the cell surface of fixed (A) and live (C) *Aspergillus nidulans* germlings at high resolution. Depth (nm) is indicated for each by a height line scan (B, D) of images A and C, respectively. The regions the arrows transect in the images (A, C) are shown in the graphs (B, D). Bars on all images = 0.5 μm.

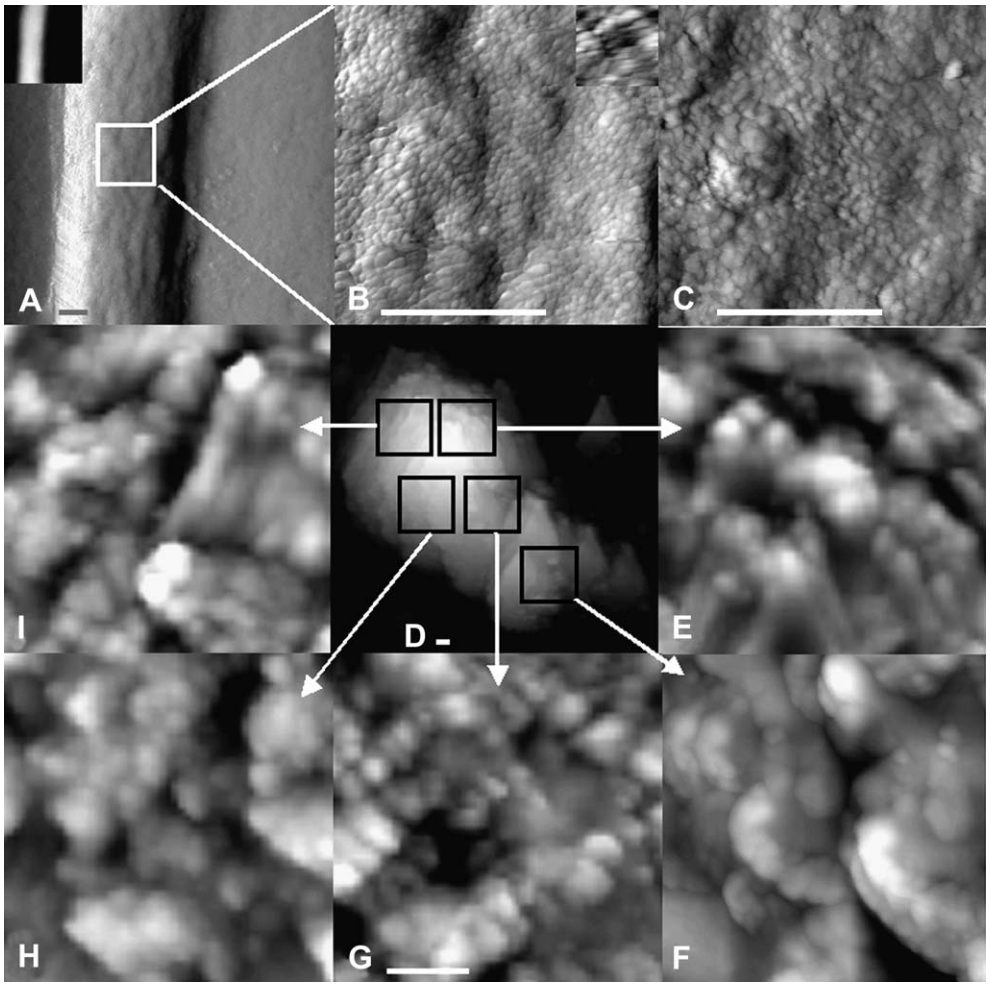

Fig 5 – AFM images of fixed *Aspergillus nidulans* hyphae for the ASK30 strain grown at 28 °C (A, B), and ASK28 (C) and ASK30 (D–I) grown at 42 °C. Lateral force images (A, B) show a region ~20 μm back from a hyphal tip (A: 6 μm × 6 μm; topography inset ¼ scale) and mature hyphal wall structure (1 μm × 1 μm; topography inset, 8 nm z range), respectively, for ASK30 grown at 28 °C. Image C is the analogous region of an A28 hypha grown at 42 °C (C: 1 μm × 1 μm). Topography image D shows a whole ASK30 hypha (12 μm × 12 μm) grown at 42 °C, with boxes corresponding to cell wall surface regions shown in images E–I (2 μm × 2 μm; z ranges ~ 100–200 nm). Bar on all images = 0.5 μm.

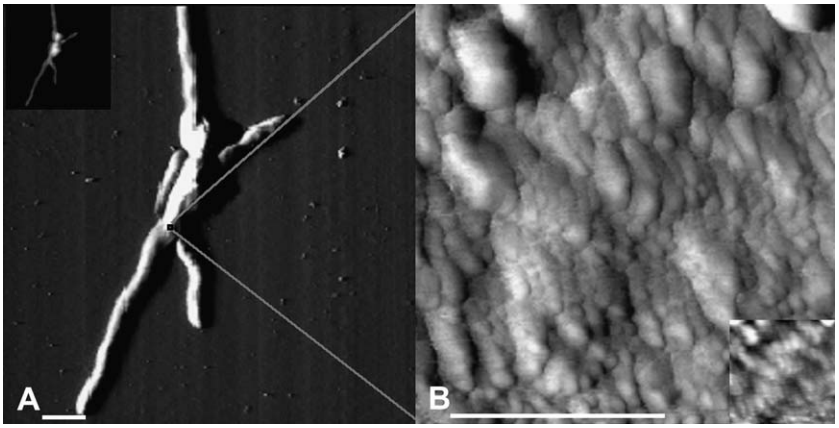

Fig 6 – AFM images of fixed ASK30 *Aspergillus nidulans* hyphae grown at grown at 42 °C then shifted to 28 °C (A, B). Shown are lateral force images (topography insets ¼ scale) of the whole hypha (A; 100 μm × 100 μm), and wall ultrastructure (B: 1 μm × 1 μm) of the transition region following a 42 ° to 28 °C growth temperature change. Bar for A = 10 μm, bar for B = 0.5 μm.

shown) resembled apical hyphal walls (Ma *et al.* 2005), both of which were smoother than those of swollen spores having been deposited during tip growth.

Some AFM images showed discontinuities in wall surface subunit organization at the spore–germ tube junction, distinct from the shallower undulations at the surface of mature hyphal walls (this report; Ma *et al.* 2005). Although originally noticed on fixed hyphae, where they could be ascribed to a preparation artefact, they were also found on the surface of living hyphae (Ma *et al.* 2005) and germlings (this report), respectively. Wall discontinuities appear to be novel features of unknown function. Clearly the tensile fibrillar meshwork of the wall must remain intact to prevent cell rupture. Discontinuities of similar size have been detected on the surface *Saccharomyces cerevisiae* cells (Pereira *et al.* 1996; Pereira & Geibel 1999) and are suggested to enlarge under oxidative stress to allow an increased influx of organic molecules (Pereira & Geibel 1999). We have not yet investigated this possibility for *A. nidulans*.

At 28 °C, the wild-type *hypA* gene product promotes tip growth, while suppressing basal cell expansion (Kaminskyj & Hamer 1998; Kaminskyj 2000; Kaminskyj & Boire 2004). The morphology and surface ultrastructure of wild-type A28 hyphae grown at 28 ° and 42 °C, and ASK30 hyphae grown at 28 °C were indistinguishable. In contrast, ASK30 cells grown at 42 °C had abnormal morphology and wall surface ultrastructure, possibly relating to abnormal wall deposition and/or maturation. The *A. nidulans hypA1* mutation provides an unusual opportunity to examine the dynamics of hyphal remodelling following changes in growth temperature. The *hypA1* hyphal wall surface features in regions corresponding to temperature shifts were abrupt, consistent with branch point junctions (Ma *et al.* 2005), which also require local wall remodelling but on a much smaller scale.

In summary, we have provided direct evidence of changes during fungal wall restructuring. We have identified a stage prior to imbibing during which hydrophobin rodlets on conidial surfaces are lost or rearranged in response to wetting. Changes in spore surface architecture during swelling prior to germination, and during morphologically abnormal growth of *hypA1* mutantstrains were similar. Although there is a significant difference in the cell wall surface ultrastructure of a *hypA1* strain grown at restrictive and permissive temperatures, walls corresponding to growth temperature transitions change relatively abruptly, consistent with changes at hyphal branch points and cell wall remodelling.

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