Analysis of microtubules and F-actin structures in hyphae and conidia development of the opportunistic human pathogenic black yeast *Aureobasidium pullulans*

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Organization of the cytoskeleton was studied in the ascomycetous black yeast *Aureobasidium pullulans*, an opportunistic human pathogen, in an effort to present it as a potential target of antifungal therapy. Long cytoplasmic microtubules, extending along the hyphae from the base to the growing apex, were the dominant structures in multinucleate interphase cells. Before mitosis these microtubules disappeared and were replaced by intranuclear spindles. This reorganization of microtubules occurred along the whole length of hypha before synchronous division of the nuclei. Actin cytokinetic rings were rarely seen. Cortical actin in the form of patches accumulated in areas of cell wall growth, i.e. in the hyphal apex and near the occasionally formed septum. Actin cables were not seen. During synchronous conidiogenesis, the cytoplasmic microtubules extended along developing conidia, and actin patches lined their subcortical areas. Actin rings were formed regularly at the base of uninuclear conidia. Microtubule inhibitor methyl benzimidazol-2-ylcarbamate disintegrated the microtubules, and inhibited nuclear division, development of hyphae and conidiogenesis. Actin inhibitor Cytochalasin D induced swelling of hyphal apexes and developing conidia. This inhibitory activity ceased after 5 to 12 h when the occasional septa appeared and conidiogenesis was completed. The lack of unicellular organization in multinucleate hyphae of *A. pullulans* seems be related to a rarity of F-actin structures: i.e. absence of actin cables, the lack of actin cytokinetic rings in particular, resulting in the uncoupling of the nuclear division from cytokinesis; the association of both processes is, however, retained during conidiogenesis.

INTRODUCTION

*Aureobasidium pullulans* is an anamorphic fungal species of the euascomycetous phylogeny, a representative species of the black yeasts (De Hoog, 1998), which was first described by De Bary (1866) as a common saprophyte in wood and other plant material (Rippon, 1982, and references therein) and as an opportunistic plant pathogen (Laskin & Lechevalier, 1978). Recently, *A. pullulans* was identified as an opportunistic human pathogen that caused chromoblastomycosis in immunosuppressed patients after liver transplantation (Redondo-Bellon et al., 1997). In other immunocompromised patients *A. pullulans* caused sepsis (Giradi et al., 1993), splenic abscesses (Nelken et al., 1987), infections of cornea and jaws (Koppans et al., 1991), hypersensitivity pneumonitis, and accidental implantation dermatitis as a result of introduction into the skin of wood contaminated with *A. pullulans* (Mehregan & Rudner, 1980; Coskey et al., 1983; Kwon-Chung & Bennett, 1992). However, a specific therapy for *A. pullulans* infections has not been defined (Redondo-Bellon et al., 1997; Georgopapadakou & Walsh, 1994).

At the microscopic level, *A. pullulans* reveals an interesting morphogenesis. In contrast to classical model systems of uniform cells of ascomycetous budding or fission yeasts, a culture of *A. pullulans* contains cells of different shapes and sizes with variable numbers of nuclei. Some of these resemble uninucleate cells of budding or fission yeasts, while others reveal multinucleated hyphae (Takeo & de Hoog, 1991; Kopecká et al., 1998) with differentiation of conidia (de Hoog, 1998). In spite of this unique and interesting

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Received 17 September 2002
Revised 12 December 2002
Accepted 2 January 2003
polymorphism, the cytoskeleton has not been studied in *A. pullulans*. The relationship of the actin cytoskeleton to cell polarity and generation of cell shape has really only been studied in fungi. Mutations in genes for actin and actin-associated proteins result in morphological changes in yeasts (Novick & Botstein, 1985; Gabriel & Kopecká, 1992, 1995; Ishiguro & Kobayashi, 1996; Winsor & Schiebel, 1997; Botstein et al., 1997; Ishiguro, 1998; Kopecká & Gabriel, 1998; May & Hyams, 1998; Le Goff et al., 1999; Pruyne & Bretscher, 2000a, b). A similar role for microtubules in cylindrical and hyphal forms of cells is known (Heath, 1994; Hagan, 1998; Brunner & Nurse, 2000; Nakaseko & Yanagida, 2001; Geitmann & Emons, 2000). Thus, changes in the cytoskeleton are implicated by cell polymorphism, which is a general feature of pathogenic fungi. Knowledge of the cytoskeleton and its dynamics during conidiogenesis in fungi would be interesting not only as general knowledge of conidiogenesis, but also as a target structure for antifungal agents for conidia, the most resistant fungal forms.

The objectives of this work were (i) to study the life cycle of *A. pullulans*, (ii) to identify F-actin structures and microtubules in hyphae of *A. pullulans*, (iii) to find out whether polymorphism of *A. pullulans* was related to its cytoskeleton organization, (iv) to study the organization of the actin and microtubule cytoskeleton during conidiogenesis of *A. pullulans*, and (v) to find out whether the classical inhibitors of cytoskeleton function would result in inhibition of the typical morphology or the lethality of *A. pullulans*.

**METHODS**

**Yeast strains.** Three strains of *A. pullulans*, IFM 40212, IFM 41140 (var. *pullulans*) and IFM 41111 (var. *melanigenum*), from the Culture Collection of the Research Center for Pathogenic Fungi and Microbial Toxocoses, Chiba University, Japan (Nishimura et al., 1998), were used in this study. *A. pullulans* CCY 27-1-111, from the Culture Collection of Yeasts of the Chemical Institute of the Slovak Academy of Sciences, Bratislava, Slovakia (kindly provided by Ing. E. Breierová, CSc), was also used. *Saccharomyces cerevisiae* DBY 1690 with the standard actin allele *ACT1* (Novick & Botstein, 1985) (kindly provided by Professor D. Botstein, MIT, Cambridge, MA, USA) was used for comparison of the actin and microtubule cytoskeleton of 'standard' budding yeast to *A. pullulans*. *Schizosaccharomyces japonicus* var. *versatilis* CC-250 (Gabriel et al., 1998) (kindly provided by Professor H. J. Phaff, University of California, FL, USA) was used for comparison of the actin and microtubule cytoskeleton of ‘standard’ fission yeast to *A. pullulans*.

**Media and cell cultivation.** *A. pullulans* was cultivated in YPG medium [1 % (w/v) yeast extract, 1 % (w/v) peptone and 1 % (w/v) glucose] (Takeo & de Hoog, 1991) on a shaker overnight at 22 °C (used usually after about 16 h cultivation) or on solid YPD medium containing 2 % (w/v) agar. *Saccharomyces cerevisiae* was cultivated in YEPD medium [1 % (w/v) yeast extract, 2 % (w/v) bactopeptone and 2 % (w/v) glucose] (Novick & Botstein, 1985) on a shaker overnight at 27 °C. The fission yeast *Schizosaccharomyces japonicus* var. *versatilis* was cultivated in malt extract or in YED medium [0-5 % (w/v) yeast extract and 3 % (w/v) glucose; J. Ishiguro, personal communication] on a shaker overnight at 27 °C.

**Cytochalasin D (CD) treatment.** A stock solution of CD (Sigma), prepared by dissolving 5 mg CD in 1 ml DMSO, was kept at −20 °C. It was added to YPG medium containing about 10⁶ cells ml⁻¹ to give final concentrations of 20, 50 and 100 µg CD ml⁻¹.

**Methyl benzinimidazol-2-y carbamate (BCM) treatment.** BCM (Chinoin Pharmaceutical Company, Budapest, Hungary; kind gift of Professor L. Ferenczy) stock solution was prepared by dissolving 5 mg BCM in 1 ml DMSO. It was kept at −20 °C and added to YPG medium containing 10⁶ cells ml⁻¹ to give final concentrations of 20, 50 and 100 µg ml⁻¹.

**Fixation.** Exponential growth phase cultures of *A. pullulans* were fixed using 5 % (w/v) paraformaldehyde in phosphate buffer (pH 6-9) containing 1-25 M ethylene glycol-bis-(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) and 1-25 mM MgCl₂ for 10 or 90 min. The cells were then washed three times with buffer without fixative.

**Calcium white staining.** Calcofluor white was added to exponential growth phase cultures for 15–20 min at a final concentration of 0-005 % (w/v). Stained cells were then washed twice with nutrient medium without Calcofluor, and immediately fixed and washed as described above.

**Microtubule visualization.** Walls of fixed and washed hyphae and conidia of *A. pullulans* were digested using lysing enzymes of *Trichoderma harzianum* (Sigma) (2 mg ml⁻¹) for 40 min, in contrast to budding and fission yeasts for which 1 mg ml⁻¹ for 20 min was sufficient. Objects were permeabilized with 0-3 % (v/v) Triton X-100 in PBS for 5 min. BSA (2 %, w/v) in PBS was applied for 30 min at 37 °C, followed by incubation with mAb TAT1 (Woods et al., 1989) for 2 h at 37 °C, triple washing with PBS at 5 min intervals, and treatment with the secondary antibody Sw-AM-FITC (Institute of Sera and Vaccines, Prague, Czech Republic) for 1-5 h at 37 °C (Svoboda et al., 1995). mAb TAT1 was kindly provided by Professor K. Gull (Manchester University, UK).

**Actin visualization.** For F-actin staining of budding and fission yeasts rhodamine–phalloidin (Molecular Probes) was applied (Pringle et al., 1989) in amount of 10 µl to 100 µl of fixed and washed cells (Gabriel et al., 1998; Kopecká et al., 2001), while for *A. pullulans* rhodamine–phalloidin was applied in amount of 20 µl to 100 µl of fixed and washed cells. Fifty microlitres of 1 % (w/v) Triton X-100 in PBS were added to the samples, and the objects were stained for 70 min at 37 °C. The objects were then washed with PBS. For actin visualization by indirect immunofluorescence, the anti-actin mouse mAb N 350 (Amersham) was applied to the samples, followed by the secondary antibody Sw-AM-FITC, by the procedure described for microtubules.

**Nuclear staining.** Nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) dissolved at a final concentration of 1 µg ml⁻¹ in Vectashield mounting medium (Vector Laboratories).

**Phase-contrast and fluorescence microscopy.** Fluorescence microscopes from Olympus (BH2-RPCA), Leica Laboluxor S Leitz and Jenalumar were used with standard filter blocks for violet (355–425 nm), blue (450–490 nm) and green (515–560 nm) excitation light, with a Plan Phaco 3100/1-25 objective and with equipment for phase-contrast microscopy. The primary magnifications were 412.5 ×, 375 × and 320 × with the Olympus, Laboluxor and Jenalumar microscopes, respectively. Preparations were photographed on Kodak Tri-X-pam 400, Provia Fujichrome 400, Ilford 400 and Kodak Ektachrome 400 films.

**Ultra-thin sections for transmission electron microscopy.** Objects were fixed with 3 % glutaraldehyde in 200 mM cacodylate buffer (pH 7-4) for 2 h, post-fixed in 1 % osmium tetroxide in 100 mM cacodylate buffer for 1 h, dehydrated by a graded alcohol
series, embedded in LR White resin, and polymerized for 2 days at 60°C. Ultra-thin sections were stained with 2.5 % uranyl acetate for 30 min and lead citrate for 6 min. Ultra-thin sections were observed with a Tesla BS 500 transmission electron microscope at 90 kV.

Freeze-substitution for transmission electron microscopy. Objects were collected by centrifugation and then sandwiched between two copper disks. They were cryo-fixed by plunging into a propane slush kept in liquid nitrogen. The specimens were freeze-substituted in 2% osmium tetroxide/acetone at −80°C for 2 days and embedded in epoxy resin (Yamaguchi et al., 1994). Ultra-thin sections were stained with uranyl acetate and lead citrate, and observed with a JEM-1200EX transmission electron microscope (JEOL, Tokyo, Japan).

RESULTS

Microtubules in hyphae of A. pullulans

Under optimal nutrient and aeration conditions, A. pullulans grew as filamentous, multinucleated, unseptated hyphae from conidia. The centre of hyphae was occupied by more or less regularly spaced nuclei, which stained well with DAPI (Fig. 1a, b, c, d). In hyphae, synchronous nuclear divisions (Fig. 1c, d) were observed. Extremely long and abundant cytoplasmic microtubules (Fig. 1a, b, d) extended along the longitudinal axis of hyphae from the basal region to the growing apex. The microtubules were organized into several parallel thick bundles that were located subcortically near the lateral wall (Fig. 1a, b, d). When compared to budding and fission yeasts (Fig. 1e, f and g, h, respectively), the microtubule bundles in hyphae of A. pullulans were clearly longer (Fig. 1a, b, d).

In contrast to narrow hyphae in the wider compartments separated by septa, the position of the microtubules looked irregular (Fig. 1b, d). Around the nuclei, ‘baskets’ of microtubules were observed (Fig. 1b, d). Similar to the fission yeast at mitosis, cytoplasmic microtubules disappeared in A. pullulans and the fluorescent signal of the anti-tubulin antibody was detected in the nuclei in which intranuclear spindles appeared synchronously (Fig. 1c). During synchronous mitoses, the mitotic spindles elongated (Fig. 1d). In multinucleate hyphae, the nuclei in anaphase kept a remarkable relative position in their ‘imaginary compartments’ (Fig. 1d). Astral microtubules (Fig. 1d) appeared on both ends of the spindle and resembled the pattern at mitosis in the budding yeast (Fig 1e). Microtubular spindles normally seen in the cylindrical cells of fission yeasts during mitosis (Fig. 1i) and the microtubule ring usually visible after mitosis of fission yeast cells (Fig. 1j) were not observed in the hyphae of A. pullulans.

F-actin in hyphae of A. pullulans

For comparison with A. pullulans, the rhodamine–phalloidin staining of F-actin (Fig. 2) was applied to the model systems of budding and fission yeasts (Fig. 2a, c, respectively). It revealed the regular occurrence of brightly fluorescing actin patches that were located mainly at the growing regions of the yeasts. Actin cables extended longitudinally towards the developing daughter cell. After nuclear divisions (Fig. 2b, d, respectively), an F-actin cytokinetic ring was formed (Fig. 2a, c) that initiated the septum formation in both yeast species. Then F-actin patches accumulated in the area of septum development (Fig. 2c). In contrast to the budding and fission yeasts, the application of the rhodamine–phalloidin staining and indirect immunofluorescence methods to the cells/hyphae from a young culture of A. pullulans revealed no actin cables (Fig. 2e, f, g, h, j). In multinuclear hyphae of A. pullulans (Fig. 2i) only a few actin cytokinetic rings (Fig. 2e, f, h, j) were detected, giving rise to the formation of multinuclear compartments separated by few septa, i.e. they originated from multicellular hyphae (Figs 1c, d and 2g, j). Except for conidia-producing compartments (discussed later), F-actin patches were scattered along the hyphae, and accumulated at the growing apex of hyphae (Fig. 2e, f, h, j) and in the developing single septa (Fig. 2g).

Conidiogenesis in A. pullulans: development, morphology, nuclei and cytoskeleton

During prolonged cultivation, growth of A. pullulans became slow and hyphae became thicker and septated. Asexual conidia originated on the surface of the thick cylindrical compartments (Fig. 3). Conidia occurred frequently in groups and originated mainly on the apex or near the septa. Actin patches appeared to be concentrated at sites where future conidia would originate (Fig. 3a), while in other areas of these compartments actin patches almost disappeared (Fig. 3a). Originating conidia grew out of these predilection sites as small buds (Fig. 3a). During this period, the orientation of the cytoplasmic microtubules was irregular in these barrel-like cells (Fig. 3d, e). These compartments contained several nuclei (Fig. 3f). Interestingly, while nuclei were in the ‘mother’ compartment (Fig. 3f) the cytoplasmic microtubules (Fig. 3e, f) moved into the originating conidium before the movement to the daughter nucleus (Fig. 3g). Several fork-like microtubule bundles copied the cylindrical shape of the conidium (Fig. 3e, f), resembling the longitudinal cytoplasmic microtubules in the cylindrical fission yeast cells (Fig. 1g, h). Nuclei started to divide, spindles appeared synchronously (Fig. 3d) and the groups of conidia originated synchronously. Microtubular spindles were in irregular positions (Fig. 3d, e) and astral microtubules were seen radiating from the spindle pole body (Fig. 3d), while, surprisingly, cytoplasmic microtubules (Fig. 3d) were still present among the spindles (Fig. 3d). We do not know whether one or two nuclear divisions preceded conidiogenesis or whether all the nuclei gave rise to conidia. At late anaphase, one of the daughter nuclei appeared in the budding conidium (Fig. 3g). At this time the cytoplasmic microtubules were not detected. The dividing nuclei and synchronous development of conidia were limited to certain barrel-like compartments. During all stages of conidiogenesis, the strong fluorescent signals of actin patches under the surface of conidia were detected.
**Fig. 1.** Microtubules (yellow-green) visualized by indirect immunofluorescence (TAT1 anti-tubulin mAb), and nuclei (blue-green) stained by DAPI. (a, b) Microtubules (m) and nuclei (n) in hyphae of *A. pullulans* 40212. (c) Cytoplasmic microtubules disappeared before mitosis and spindles (s) were seen inside the nuclei of *A. pullulans* 40212. (d) Microtubules and nuclei in hyphae of *A. pullulans* 40212. Synchronous division of nuclei was seen in one hypha. s, Spindles; b, microtubular 'basket' around nuclei; i, irregular microtubules; a, astral microtubules; m, cytoplasmic microtubules. (e) Cytoplasmic and spindle microtubules
(Fig. 3a, b, c). When a conidium matured (Fig. 3b, c) and had a nucleus (Fig. 3g), an actin ring-like structure appeared at the base of the conidium (Fig. 3b, c) for a brief interval and then a septum was formed (see Fig. 4a). Development of the conidium, the actin patches (Fig. 3a, b) and the actin ring-like structure at the base of conidia in *A. pullulans* (Fig. 3b, c) resembled the development of the bud and the actin cytokinetic ring of the budding yeast (Fig. 2c).

**Ultrastructure of *A. pullulans***

Ultra-thin sections of *A. pullulans*, observed by transmission electron microscopy, showed the cell walls of the conidia to be thin (Fig. 4a), while the cell walls of the hyphae were unusually thick (Fig. 4a). In chemically fixed objects, the inner-most layer of the cell wall formed outgrowths while appearing to fill the plasma membrane invaginations (Fig. 4a, d, e). The few electron-transparent septa (Fig. 4e), separated few hyphal compartments. Freeze-substituted objects revealed the very fine ultrastructure of *A. pullulans* (Fig. 4b, c). Nucleus (Fig. 4b, c) with nucleolus, nuclear envelope with ribosomes, nuclear pores and the spindle pole body (Fig. 4c) were observed. Mitochondria, vacuoles, cisternae of the endoplasmic reticulum, ribosomes and the plasma membrane were also visible. Single microtubules were seen in the cytoplasm (Fig. 4c). Several nuclei were seen in one compartment of hyphae, similar to Fig. 4(c), which shows two nuclei in one growing conidium. At magnifications of 30,000 to 100,000 x, the filamentous network in the cytoplasm was detected, inside of which ribosomes, mitochondria and other organelles were located. The filamentous network in the cytoplasm appeared to join the plasma membrane (Fig. 4d, e). Measurement of the filaments showed them to be 7 nm in width, which may correspond to single microfilaments of F-actin, which are known to have a width of 7 nm.

**Effect of cytoskeletal inhibitors on *A. pullulans***

In the presence of a cytoskeletal inhibitor (BCM) aberrant morphogenesis of *A. pullulans* was induced, as shown by our preliminary data (Fig. 5b, c). The control culture grew in the form of long, narrow, vacuolated hyphae that formed conidia in 1% (w/v) YPG medium containing 2% DMSO after 24 h cultivation at 22 ºC (Fig. 5a).

BCM was applied to the cultures at three different concentrations (20, 50 and 100 µg BCM ml⁻¹ dissolved in DMSO). The highest concentration was the most effective. BCM efficiently disintegrated microtubules, inhibited nuclear division and completely blocked hyphal development. Conidiogenesis was also completely inhibited by BCM. When the inhibitor was applied to young conidia, the development of hyphae from conidia was fully inhibited (Fig. 5b). Only ovoidal or spherical cells originated from conidia that did not die; some conidia lysed, releasing their cytoplasmic contents. Conidiogenesis was also completely inhibited by BCM at 100 µg ml⁻¹.

CD-treated hyphae revealed swelling of hyphal apexes (Fig. 5c). While the lower concentrations of CD (20 and 50 µg CD ml⁻¹) had only weak effects, 100 µg CD ml⁻¹ was the most effective concentration for inducing aberrant hyphal morphogenesis. However, decrease of activity of CD after 5–12 h resulted in the appearance of septa in hyphae (Fig. 5c), development of ovoid or spherical compartments and conidiogenesis (Fig. 5c). When CD was applied to young conidia, malformed isodiametric or ovoid formations originated, which were vacuolized and overcrowded by many nuclei and contained a dense microtubular cytoskeleton. This state was maintained for several hours while CD was repeatedly added. When no new CD was applied, septa originated in these formations after 5–12 h, conidiogenesis was completed and further budding of conidia occurred among dying hyphae. CD had no effect on actin patches, while during the first hours of CD inhibition no actin rings were detected.

Results presented in this study on the cytoskeleton in hyphae and conidia of *A. pullulans* were repeatedly observed in different experiments and in all strains of *A. pullulans* tested.

**DISCUSSION**

**Microtubules and actin cytoskeletons in hyphae of *A. pullulans***

**Microtubules in hyphae.** Long cytoplasmic microtubules in young hyphae are directed to the apex. This arrangement is a characteristic for hyphae of fungi (Heath, 1994; Geitmann & Emons, 2000). It functions in transport and signal transduction during rapid apical growth of hyphae. The presumed Spitzenkörper (MTOC) in the apexes of hyphae, typical for some fungi (Bracker, 1967; Groe & Bracker, 1970; Heath, 1994; Oakley, 2000; Geitmann & Emons, 2000), was not observed in *A. pullulans*. When polar growth ceased, the hyphae of *A. pullulans* became thick. This was reflected in old hyphae by the irregular orientations of cytoplasmic microtubules. This arrangement of cytoplasmic microtubules precedes conidiogenesis. It remains unclear as to whether these cytoplasmic microtubules participate in the determination of the localization of future conidia. Shortly before mitosis, a diffuse fluorescent signal of tubulin is seen in the nucleus. It may
Fig. 2. Actin cytoskeleton stained with rhodamine–phalloidin (a, c, e, f, g, h, j), and nuclei stained by DAPI (b, d, i). (a) Actin in *Schizosaccharomyces japonicus* var. *versatilis*. (b) Nuclei in *Schizosaccharomyces japonicus* var. *versatilis*. (c) Actin in *Saccharomyces cerevisiae*. (d) Nuclei in *Saccharomyces cerevisiae*. (e–h, j) Actin in hyphae of *A. pullulans*. (e, f) Strain 41410; (g) strain 40212; (h) strain 41410; (j) strain 40212. Arrows show the actin cytokinetic rings. (i) Strain 41411. n, Nuclei. Bars, 10 μm.
represent a pool of tubulin preceding the formation of mitotic spindles. Similar localization of a tubulin signal in the nucleus was observed in Cryptococcus neoformans (Kopecká et al., 2001), and in fission yeast before mitosis (Svoboda et al., 1995). During this period cytoplasmic microtubules are not visible. In dividing nuclei, short astral microtubules are present in synchronous spindles.

Although mitosis is observed as synchronous, deviations in the number of nuclei from the number of multiplied dividing nuclei are found. Also, single (non-dividing) nuclei are found during synchronous nuclear divisions. Whether this is a time delay in synchronous mitoses, a dormant nucleus or the result of randomly septating separating nuclei is not known.

**Actin in hyphae.** Actin patches in *A. pullulans* correspond to localization common for fungi, i.e. they are present at sites of cell wall (septum) synthesis (Heath, 1990, 2000). Although they were localized mainly at the apex, the actin patches were widely scattered in lateral cell walls of both young and old hyphae, as well as signalling the synthesis or restructuring of wall material in these areas. The corresponding findings for the thick cell walls were detected at the ultrastructural level.

**Actin cables.** These are typical for yeasts and for some hyphal fungi (Botstein et al., 1997; May & Hyams, 1998; Pruyne & Bretscher, 2000a, b; Heath, 1990, 2000; Heath et al., 2000; Geitmann & Emons, 2000). Surprisingly, in *A. pullulans* no actin cables were visualized with either the rhodamine–phalloidin or the anti-actin mAb. Although not detected, we cannot exclude the presence of actin cables in *A. pullulans*. The finding, in ultra-thin sections, of fine single filaments with a width of about 7 nm

![Fig. 3. Conidiogenesis of *A. pullulans* strains 41410 (a, b, d, e) and 40212 (c, f, g). (a, b, c) Actin in conidiogenesis of *A. pullulans* after rhodamine–phalloidin staining. Arrowheads, actin patches in conidia; triangle, accumulation of actin patches; arrow, area of disappearance of actin patches; r, actin cytokinetic ring. (d, e) Microtubules after indirect immunofluorescence. (d) m, Microtubules in the ‘mother’ compartment; s, spindles; c, cytoplasmic microtubules; sp, spindle pole body. (e) m, Microtubules in conidia; s, spindle in ‘mother’ compartment. (f, g) Double fluorescence: microtubules after indirect immunofluorescence and nuclei after DAPI staining. (f) m, Microtubules in conidia; n, nuclei. (g) n, Nuclei in conidia.](http://mic.sgmjournals.org)
(immunolabelling was not used) and the observed change in morphology after the effect of CD (inhibitor of actin polymerization) suggest the presence of single actin microfilaments in *A. pullulans*, and their important role in the cylindrical development of hyphae and conidia.

**Actin rings.** These occur randomly in older hyphae of *A. pullulans*. Irregular actin ring formation and cytokinesis suggest that the mechanism linking mitosis and cytokinesis in hyphae of *A. pullulans* (if any) is different from that in unicellular model yeasts. Two pathways, SIN (Septum Initiating Network) in fission yeast and MEN (Mitosis Exit Network) in budding yeast, have already been described in detail (Balasubramanian et al., 2000; McCollum & Gould, 2001; Cid et al., 2002). Preliminary observations showed an increased septation in *A. pullulans* that was induced by unfavourable conditions (not shown).

**Mitosis without cytokinesis**

Mitosis without cytokinesis is not unique to *A. pullulans*. In this regard, *A. pullulans* resembles some other fungi, e.g. the oomycetes *Saprolegnia* and *Achlya* (Heath & Harold, 1992), and the chitridiomycetes *Neocallimastix* and *Orphinomyces* (Li & Heath, 1994). For these organisms, a multinuclear stage was followed by uninuclear zoospore formation in the zoosporangium, which also involved actin. Then mature zoospores migrated outside of the zoosporangium by means...
of flagella. Interestingly, findings of three synchronous mitoses have been presented in *Neozygites* sp. and two synchronously dividing nuclei in *Orphinomyces* (Butt & Heath, 1988), in contrast to other fungi (Fuller, 1976; Fisher, 1999). The exception was the fission yeast *Schizosaccharomyces japonicus* var. *versatilis*, in which time-lapse microcinematography showed that mitosis without cytokinesis could be induced experimentally by continual degradation of the cell wall by hydrolytic enzymes. The uninuclear protoplasts divided synchronously resulting in \(2 \rightarrow 4 \rightarrow 8 \rightarrow 16 \rightarrow 32 \rightarrow 64\) up to 128 nuclei (Gabriel, 1983, 1984; M. Gabriel, unpublished data).

In *Drosophila melanogaster* embryos, nuclear divisions also proceed synchronously without cytoplasmic division to create a large syncytium. Most of the nuclei then migrate to the cortex and the plasma membrane extends inwards and pinches off to surround each nucleus, to form individual cells in a process called ‘cellularization’ in which actin is involved (for review see Tram et al., 2001, and references therein). Surprisingly, in *A. pullulans*, nuclei also migrated to the cortex for ‘cellularization’ by conidiogenesis. However, during conidiogenesis outward membrane extension proceeded, resembling yeast budding. This external ‘cellularization’ by conidiogenesis resulted in the release of single conidial cells, in contrast to *Drosophila*. Also, some types of mammalian cells (osteoclasts, throphoblasts, hepatocytes, heart muscle cells, etc.) that become multinucleated (Alberts et al., 2002) demonstrate common mechanisms of mitosis that are dissociated from cytokinesis conserved in fungal and human cells. These data provide support for a common link in the evolution of fungi and metazoa, which has been suggested from molecular taxonomic data (Wainright et al., 1993; Cavalier-Smith, 1993, 1998; Alberts et al., 2002). We propose that the multinuclear stage of higher fungi and metazoa might be a relic of an ancient stage in the evolution of multicellular eukaryotes from unicellular cells. Multinuclear fungi may represent an ancient stage in the evolution common to metazoa.

Multinuclear organisms, such as chytridiomycetes and zygomycetes, may represent an intermediate stage in the evolution of multicellular organisms from unicellular eukaryotes along the hypothetical pathway (i) unicellular eukaryotes, to (ii) multinuclear eukaryotes, to (iii) multicellular eukaryotes. The multinuclear stages of *A. pullulans* or *Drosophila* may be conserved relics of such an evolutionary pathway. *A. pullulans* shows in one culture all these ‘three stages’ – uninuclear conidia, multinuclear hyphae and multicellular hyphae.

**Microtubules and actin cytoskeletons in conidiogenesis of *A. pullulans***

**Microtubules in conidiogenesis.** Undoubtedly, cytoplasmic microtubules have a transport function during growth of conidia before nuclear division. Mitotic spindle shows an intensive fluorescent signal during mitosis. It is not known whether cytoplasmic microtubules disappear during anaphase (Fig. 3d) but the intensity of their fluorescent signal is decreased at this time. From our
observations, we deduce that during synchronous nuclear divisions in the thick hyphae (Fig. 3d), cytoplasmic microtubules could form a network joining microtubular spindles in a given compartment.

**Nuclear division.** Numbers of nuclei differ in individual hyphal compartments before conidiogenesis. Therefore, it is very difficult to find out the exact relationship of the number of nuclei to the number of conidia. Some findings signal two subsequent nuclear divisions before conidiogenesis (meiosis?). Due to different findings, the exact mechanism of nuclear division before conidiogenesis is not known and will be the subject of further studies. The finding of both cytoplasmic microtubules and spindles in one compartment during conidiogenesis is unusual and differs strongly from the behaviour of microtubules before nuclear division in growing hyphae (Fig. 1c), in which cytoplasmic microtubules disappeared completely before nuclear division. This indicates differences in nuclear division in hyphal growth and conidiogenesis (mitosis/meiosis?).

**Actin in conidiogenesis.** Actin patches (Heath, 1990, 2000) before conidiogenesis in *A. pullulans* accumulate at hyphal apexes or at septa and disappear in old hyphae. However, whether mutual dependence exists between cessation of wall synthesis and the origin of conidia is questionable. The accumulation of actin patches in areas of future origin of conidia is not clear. Actin cables were not detected in *A. pullulans*. Actin ring structures at the base of conidia could be better termed actin ring-like structures instead of actin rings. Due to their small size, typical circles were not observed. However, their occurrence in hyphae was a regular finding.

In contrast to the rarity of F-actin cytoskeleton in *A. pullulans*, there is sufficient actin present for reproduction by conidiogenesis. Special behaviour of the multinuclear hyphae of *A. pullulans*, in which mitosis is dissociated from cytokinesis, is reminiscent of the special cell cycles of embryonic cells; however, conidiogenesis is reminiscent of the cell cycles of typical somatic cells in which mitosis is coupled with cytokinesis.

A remarkable observation was that the conidia of *A. pullulans* appear in polarity-favoured sites, such as those adjacent to septa or on hyphal tips, but never in random positions along the hyphae. This might mean that conidiogenesis in *A. pullulans* follows the polarity rules and molecular mechanisms known to exist in budding yeast.

**Consequences of cytoskeleton inhibition in *A. pullulans***

The response of *A. pullulans* to CD corresponded to that of several other fungi to Cytochalasins, e.g. *Candida albicans* (Yokoyama *et al.*, 1990), *Schizosaccharomyces japonicus* (Gabriel *et al.*, 1998) or *Saprolegnia ferax* (Heath, 1990, 2000; Heath *et al.*, 2000). Apical swelling of *A. pullulans* in the presence of CD suggests that CD was an effective inhibitor of actin polymerization and indicates that F-actin filaments are necessary for normal polar growth of hyphae and conidia. When CD was applied to originating conidia, the conidia developed wide bases. No actin rings were observed and conidia did not separate from hyphae. Repeated application of CD showed that it is inactivated after a period of time. Surprisingly, septum formation after 5–12 h in the presence of CD may be caused by the decrease of activity of CD during its long presence in the culture medium. This possibility may also play a role in initiating conidiogenesis during the long exposure to CD.

In contrast, BCM inhibited nuclear division and hyphal growth. In *A. pullulans*, microtubules are essential for hyphal morphogenesis. Actin was considered to be more important for hyphal morphogenesis than microtubules in some fungi (e.g. *Saprolegnia* and *Neurospora*) (Heath *et al.*, 2000). The fact that after the BCM effect no conidia could be formed signifies the new leading function of microtubules in conidiogenesis.

In the cytoskeleton of *A. pullulans*, microtubules appear to be a key cytoskeletal target for the inhibition of conidiogenesis in this organism. This will prompt us to extend our study of *A. pullulans* to those cytostatics inhibiting microtubules that are used for the treatment of human cancers.

**ACKNOWLEDGEMENTS**

Financial support was provided by the Japanese Ministry of Education, Science and Culture for Marie Kopecká as a Guest Professor at Chiba University, Japan, between 1998 and 1999. The research was supported by grants nos 310/00/0391 and 310/03/1195 from the Grant Agency of the Czech Republic. We would like to thank our students Bhambini Patel and Samrath Chema, from the UK, and Savvas Rossides, from Cyprus, all of whom study medicine at the Faculty of Medicine, Masaryk University, Brno, for their kind correction of the English text. Thanks are also expressed to Professor K. Gull (Manchester University, UK) for his kind gift of the mAb TAT1, to Professor L. Ferenczy (Attila Jozsef University, Szeged, Hungary) for his kind gift of BCM and for technical work to Vladimir Ramiková, Dobromila Klemová and Premysl Hnilíčka (Brno) and to Misako Ohkusu, and especially to Dr Soichi Yoshida (Chiba). Special thanks are expressed to the Microbiology Editor who dealt with our paper, Professor M. Molina, and the two anonymous referees, as they provided highly professional and competent suggestions that substantially improved our paper.

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