Filipin is a reliable in situ marker of ergosterol in the plasma membrane of germinating conidia (spores) of *Penicillium discolor* and stains intensively at the site of germ tube formation

M.R. Van Leeuwen a, W. Smant b, W. de Boer b, J. Dijksterhuis a,⁎

a Applied and Industrial Mycology, CBS/Fungal Biodiversity Centre, Uppsalalaan 8, 3584 CT, Utrecht, The Netherlands

b Department of Terrestrial Microbial Ecology, NIOO/Centre for Terrestrial Ecology, Boterhoeksestraat 48, 6666 ZG Heteren, The Netherlands

A B S T R A C T

Filipin, a widely used fluorescent sterol marker is also a potent antibiotic. In this study we address the reliability of filipin as a monitor of ergosterol in fungal cells. A revised staining protocol was developed to minimize any biological effect of the compound. Germinating conidia of *Penicillium discolor* stained with filipin, displayed a fluorescent cap at the location of germ tube appearance and formation. During germ tube emergence, the fluorescent intensity of the cap increased. This was confirmed by HPLC as an increase of the net cellular ergosterol content. Filipin staining is absent during early germination, while FM dyes, similar molecules, stain the plasma membrane after 1 h. This indicates that the conidial cell wall is no barrier for filipin. To evaluate if filipin does bind ergosterol in situ, nystatin, more specific to ergosterol, was added before filipin staining. This resulted in a marked decrease in fluorescence indicating high ergosterol levels. This was characterized further in ergΔ1-mutant cells of *Saccharomyces cerevisiae* containing altered sterols. Here ergosterol containing cells showed a high fluorescence decrease. Taken together, these data suggest that filipin monitors an ergosterol-enriched cap in germinating conidia at the site of germ tube formation. Furthermore, the sterol-rich cap decreases and reappears after a period of actin disruption. Myriocin that affects sphingolipid synthesis results in an increase of cellular ergosterol and overall filipin fluorescence, but not at the ergosterol cap, where fluorescence is significantly lowered. In conclusion, in this work we have demonstrated an effective revised method for ergosterol staining with filipin and demonstrated its specificity in both *Penicillium* and *Saccharomyces*.

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1. Introduction

Filipin, like amphotericin B, nystatin, and natamycin (pimaricin) belongs to the macrolide polyene antibiotics, which are characterized by the possession of a macrocyclic ring closed by lactonization (Hamilton-Miller, 1973). In addition, polyene antibiotics contain a series of conjugated double bonds at one side of the ring and functional hydroxyl groups on the other side, resulting in the amphipathic nature of the molecule. Specific changes in membrane permeability and integrity are observed when polyene antibiotics are added to cells or used in membrane model systems that contain sterols (Bolard, 1986), which are the main target of polyene antibiotics. Sterols are characterized by a 3β-OH group, a planar sterol nucleus, and an intact side chain at C-17 (Norman et al., 1972).

In contact with sterol, amphotericin B and nystatin form a half pore within the membrane leaflet, containing 8 units derived of a single antibiotic and sterol molecule (De Kruijff and Demel, 1974a). Two half pores build up to combine a membrane-spanning conducting channel, perpendicular to the plane of the bilayer. This results in leakage of monovalent ions, particularly K+, and other small cellular molecules such as urea and glycerol (Baginski et al., 1997; Holz and Finkelstein, 1970; Marty and Finkelstein, 1975). Filipin on the other hand is unlikely to form conducting pores as a result of its shorter length and to the absence of the charged carboxyl group as well as a bulky mycosamine group. Instead, filipin is thought to withdraw sterols from their association with phospholipids to form a planar complex. Two such planar aggregates associate in a double layer ‘sandwich’ like complex, located in the hydrophobic core of the plasma membrane. These complexes will lead to membrane disruptions and cellular leakage of monovalent ions and even small enzymes e.g. glucose-6-phosphate dehydrogenase (De Kruijff et al., 1974b). Apart from sterol molecules, the hydrophobic environment of phospholipids is a necessary prerequisite for the action of filipin (Drabikowski et al., 1973). At higher concentrations (>200 μM), exceeding that of cellular staining, binding of filipin with phospholipids also takes place in competition with its specific binding with sterol (Milhaud et al., 1996). In membranes of large unilamellar vesicles (LUV) and high filipin/

*Corresponding author. Tel.: +31 30 2122654; fax: +31 30 2512097.
E-mail address: j.dijksterhuis@CBS.knaw.nl (J. Dijksterhuis).
sterol ratios (>1) an interaction between filipin and phospholipids, identical to the one with sterol-free LUV, was observed (Milhaud et al., 1989; Milhaud, 1992).

Because of the conjugated double bonds, filipin act as a chromophore that absorbs UV light (360 nm) and emits visible blue light (480 nm) (Hamilton-Miller, 1973). The fluorescent signal is enhanced markedly by the presence of sterols in a phospholipid environment (Drahkikowski et al., 1973). Filipin is used in varying plant and animal cells including mammals (Flesch et al., 2001), Drosophila (Huang et al., 2005), Caenorhabditis (Grigorenko et al., 2004), and Anabidopsis (Grebe et al., 2003). In fungi, different studies (Bagnat and Simons, 2002; Li et al., 2006; Martin and Konopka, 2004; Pearson et al., 2004; Takeda et al., 2004; Valdez-Taubas and Pelham, 2003; Wachtler et al., 2003) use filipin as a reporter for ergosterol localization.

Filipin is also a potent antibiotic compound and effects on the cell cannot be ruled out when it is used as a fluorescent sterol marker. The concentration of filipin used in the studies ranges from 11.9–13.3 μM and staining times are 15 min or longer at room temperature (see for instance Bagnat and Simons, 2002; Valdez-Taubas and Pelham, 2003).

Evaluation of freeze-fracture techniques and thin-section electron microscopy showed differences in the distribution of filipin–sterol complexes in fixed as well as unfixed cells. Treatment of unfixed cells with filipin resulted in aggregation of intra-membrane particles into regions of the plasma membrane that were previously devoid of filipin–sterol complexes (Robinson and Karnovsky, 1980). Since virtually all studies that use filipin as a sterol marker have been on unfixed cells these effects may occur and result in artifacts that can be interpreted incorrectly.

In a number of studies with fungi, filipin fluorescence is predominantly observed at growing zones and sites of cytokinesis like septa formation. In the case of fission yeast Schizosaccharomyces pombe this occurs at the growing end of the cell and as a ring around the middle of the cell where the septum is to be formed (Wachtler et al., 2003). In the budding yeast Saccharomyces cerevisiae this is at tips of mating projections (shmoo’s) of pheromone stimulated cells (Robinson et al., 2006; Martin and Konopka, 2004; Pearson et al., 2004; Takeda et al., 2004; Valdez-Taubas and Pelham, 2003; Wachtler et al., 2003) use filipin as a reporter for ergosterol localization.

2. Materials and methods

2.1. Organisms and growth conditions

P. discolor CBS 271.97 and CBS 112557 (Frisvad et al., 1997) were grown on malt extract agar (Oxoid, Hampshire, UK) for 10–12 days at 25 °C. Stocks of conidial suspensions in 17% glycerol were kept at −20 °C. The S. cerevisiae strains that were effective in ergosterol synthesis were kindly provided by Prof. Howard Riezman (Department of Biochemistry, University of Geneva, Switzerland) and summarized in Table 1. The strains RH448 (wildtype), RH4213 (erg3Δ), RH5225 (erg3Δerm6Δ), and RH5228 (erm2erg3Δ) were grown overnight at 25 °C and 150 rpm in YPUATD medium (Munn et al., 1995), which was inoculated directly from YPUATD agar surfaces that were inoculated for less than 3 weeks.

2.2. Antimycotic susceptibility test

Conidia of 10–12 days old cultures were harvested in 10 ml cold (4 °C) ACES buffer (10 mM ACES (Sigma), 0.02% Tween-80, pH 6.8). The entire surface of the plate was gently rubbed with a sterile spreader and the conidial solution was filtered through sterile glass wool. Cells were washed twice in ACES buffer and resuspended in Malt Extract Broth (MEB) (Oxoid, Hampshire, UK). The conidia was used to inoculate 96-well microtiter plates containing malt extract broth with filipin (Sigma) or natacymcin (DSM, the Netherlands) from a 10 mM stock in DMSO or a 10 mM stock in 85% DMSO (Brik, 1997), respectively. Concentrations ranged from 0.059 μM to 100 μM. Each well was inoculated with 10⁴ spores to a final volume of 100 μl. After 5 days at 25 °C the minimal inhibitory concentration (MIC) was visually determined.

2.3. Conidial immobilization

Conidia were immobilized to glass surfaces by poly-L-lysine (Sigma). This allowed us to change media quickly and minimize unnecessary incubation with filipin. For coating with poly-L-lysine, microscope glass cover slides were washed with detergent (5 min), is germinating conidia of the fungus Penicillium disolor. These are dormant asexual survival vehicles that are moderately drought and thermo-resistant (see for a review Chitarra and Dijksterhuis, 2007). After breakage of dormancy, the cells grows isotropically (also designated as “swelling”), followed by the initiation of a site for polarized growth, the onset of germ tube emergence. Once polarity is established, its continuance is dependent on the sustained localization of the morphogenetic machinery at the tips of the extending germ tube (Harris and Momany, 2004). We observed that the onset of germ tube formation was accompanied with the appearance of a stained cap at that location of the cell. In addition, a number of control experiments suggest strongly that the filipin staining does report ergosterol in the model system and that the stained cap is not an artifact, but an ergosterol-enriched and dynamic structure.

### Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>RH448</td>
<td>MATa leu2 ura3 his4 lys2 bar1</td>
<td>Heese-Peck et al. (2002)</td>
</tr>
<tr>
<td>RH4213</td>
<td>MATa erg3Δ::LEU2 leu2 ura3 his4 lys2 bar1</td>
<td>Heese-Peck et al. (2002)</td>
</tr>
<tr>
<td>RH5225</td>
<td>MATa erg3Δ::LEU2 erg6Δ::LEU2 leu2 ura3 his4 lys2 bar1</td>
<td>Heese-Peck et al. (2002)</td>
</tr>
<tr>
<td>RH5228</td>
<td>MATa erg2Δ(end11)–1Δ::URA3 erg3Δ::LEU2 leu2 ura3 his4 lys2 bar1</td>
<td>Heese-Peck et al. (2002)</td>
</tr>
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HCl (1 M, 5 min) and ethanol (96%, 5 min). The cleaned glass slides were subsequently coated with 0.01% (w/v) poly-l-lysine in distilled water for 5 min. Finally, the slides were rinsed with distilled water and air dried. In addition, glass surfaces were coated with silanization solution II (Sigma) by washing with HCl (1 M, 1 h), ethanol (96%, 1 h) and air dried at 40 °C. After drying, slides were incubated with silanization solution II for 1 h and dried overnight at 40 °C followed by rinsing with distilled water. Conidia were harvested as described above and 100 μl was dropped on the coated glass cover slips and allowed to germinate at 25 °C in a humid environment.

2.4. Light-microscopy

The development of germinating conidia in MEB was followed in small Erlenmeyer flask containing 50 ml broth at 150 rpm and 25 °C. Samples were taken at hourly intervals from the liquid cultures and cells were analyzed with a Zeiss AxioSkop 2 plus microscope equipped with a 63×/1.25 oil Plan-NeoFluar objective. Pictures were captured with a Nikon Digital Sight DS-5M camera (Nikon instruments, Badhoevedorp, The Netherlands) and analyzed using Eclipsenet (Laboratory Imaging, Prague, Czech Republic).

2.5. Fluorescent staining

For direct observation of developing conidia, small examination chambers were made. Coated glass cover slides with immobilized conidia were mounted on object glasses by means of two-sided adhesive acrylic foam (3M, 4905F, Leiden, The Netherlands). Conidia of P. discolor CBS 112557 were stained with 15 μM filipin in ACES buffer for 30 s at RT and washed twice with ice cold ACES buffer. We used this staining procedure at RT to obtain fluorescent filipin–sterol complexes followed by cooling down to lower the diffusion coefficient. Yeast strains were incubated with 15 μM filipin for 1 min. For staining with filipin and FM4-64 was performed under conditions described above. In all staining studies, filipin, FM4-64, and FM1-43 were freshly made from a 10 mM DMSO stock. Latrunculin A (Molecular Probes) in ACES buffer for 1 min or 2 min, respectively. Dual staining with filipin and FM4-64 was performed under conditions described above. For all staining studies, filipin, FM4-64, and FM1-43 were freshly made from a 10 mM DMSO stock. Latrunculin A (Molecular Probes) was added directly to the cells from a 1 mM DMSO stock at a final concentration of 30 μM and incubated at RT. Myriocin treated conidia were germinated in 200 μM myriocin (Sigma) dissolved in MEB. For the natamycin competition study, 15 μM natamycin was added after a wash step with ACES buffer. After a 1 min incubation at room temperature, cells were washed twice with ACES buffer and stained with filipin as described above. After staining with filipin, micrographs of all conidia within an experiment were made within 10 min. Images were acquired with a Zeiss Axioplan II microscope equipped with a Plan-Apochromat 100×/1.4 oil objective, using a Zeiss AxioCam MRc digital camera run by Zeiss AxioVision 4 software. To prevent damage to the spores, illumination (through the Zeiss filter set #02 for filipin and #14 for the FM dyes) was only during acquisition of the micrograph, using a filter wheel installation (Ludl Electronic Products Ltd., Hawthorne, NY, USA). For filipin, spores were exposed for 10 s, for the FM dyes this was 300 ms. Then, images were converted to grayscale with Adobe Photoshop CS2. From each stage 30–50 cells were selected and the pixel intensity was analyzed using the public available ImageJ software written at NIH (http://rsb.info.nih.gov/nih-image). The contrast of the micrographs of the selected conidia was not digitally changed and the exposure times were the same. Fluorescence of the conidia was measured as the pixel intensities over line selections across the membrane. Per conidium, 4 locations were selected at the positions A–D and the background-fluorescence was subtracted. The obtained results indicate fluorescence emission in gray values (0 = black, 256 = white) and the least significant difference (l.s.d.) was analyzed by means of analysis of variance (ANOVA). For the yeast strains the pixel intensity of the total plasma membrane was measured (n=85) and the data was analyzed using a multiple comparison procedure, the Tukey-test.

2.6. Alkaline ergosterol extraction

Conidia were harvested as described above and three independent liquid cultures in Erlenmeyers containing 200 ml MEB were inoculated with 107 conidia and incubated at 25 °C (125 rpm). At hourly intervals between 0 and 9 h, 15 ml was collected from each of the three Erlenmeyers. The pooled suspensions were centrifuged (1100 × g, 5 min, 5 °C), washed in ACES buffer, and the pellet was stored at −20 °C.

The alkaline ergosterol extraction was performed the following day as described by Bååth (2001) with some minor alterations. In short, conidia were broken in a 50 ml polypropylene screw-cap centrifuge tube (Greiner, Frickenhausen, Germany) containing 4 ml 10% KOH in methanol (Sigma, Chromasolv® grade) and an equal amount of 0.5 mm glass beads. The mixture was thoroughly vortexed for 5 min and sonicated (Branson, Branson 2510) for 15 min. Then the preparation was heated for 90 min at 70 °C in a water bath. For extraction, 1 ml distilled water and 2 ml n-hexane (Sigma, Chromasolv® grade) were added at room temperature, vortexed for 30 s, and centrifuged at 3200 × g (MSE, Mistrall 400) for 10 min. The top (n-hexane) fraction was removed and the water fraction was shaken with

![Fig. 1. Polarization of membrane ergosterol at different stages during germination of conidia of P. discolor (CBS 112557). (A) Conidia were stained with 15 μM filipin for 30 s and then analyzed by fluorescence microscopy. After 8 h of germination stages II, III and IV can be discerned. Stage II; uniform membrane staining. Stage III; cap structure at presumptive site of germ tube formation. Stage IV; polarized fluorescence at the apex of the germ tube. Bar = 5 μm. (B) Distribution of the frequency of different germination stages among the population of spores during 6, 7 and 8 h after the start of incubation, n=250.](image-url)
of the conidial cell wall at the site of the emerging germ tube.

fresh n-hexane for an additional extraction. The pooled n-hexane fractions were evaporated over night in a water bath at 45 °C. After evaporation the precipitates were dissolved in 1 ml methanol by vortexing and sonication for 3 min. The samples were filtered through an Acrodisc® 0.2 μm PTFE syringe filter (Sigma-Aldrich, Zwijndrecht, The Netherlands) and loaded for HPLC analysis. Ergosterol was measured with an Allsphere ODS-2 C18 column (Alltech, Ridderkerk, The Netherlands). The mobile phase consisted of methanol with a flow rate of 1.5 ml min⁻¹ and ergosterol was detected at 282 nm.

3. Results

3.1. Filipin stains a cap on germinating conidia

Immobilized conidia showed no or very faint staining during early stages of germination (designated as stage I cells). After 8 h of germination different staining patterns could be discerned, which are correlated with different stages of germination (Fig. 1). Stage II was characterized by uniformly distributed low fluorescence around the spore membrane. Stage III exhibited an intensive staining at a restricted site on the plasma membrane representing a “cap”, at the presumptive site of germination. The staining of the remainder of the membrane was comparable with stage II. Stage IV cells showed a distinct germ tube possessing an intensively stained cap at the apex. A population of conidia exhibited different staining patterns at a certain time, but the proportion of these patterns varied in time. Fig. 1B shows that the proportion of stage IV cells increased, and that stage I and II cells decreased in number between 6 and 8 h of germination. Stage III cells remained approximately constant in number indicating that germination proceeds at a fixed speed when conidia enter this stage. During stage II, equal values of fluorescence intensity (40±AU) were measured at 4 positions as indicated in the figure (Fig. 2B). During stage III, position A was set at the filipin stained “cap” and with 80±AU fluorescence was significantly higher than the positions B–D. During stage IV, the fluorescent intensity had increased further significantly to 115±AU at the tip of the germ tube, while the remainder of the membrane did not show significant changes in intensity from stage II on.

3.2. Does the cell wall block uptake of fluorescent dyes?

Localized staining of filipin may be a result of a higher permeability of the conidial cell wall at the site of the emerging germ tube.

Therefore conidial membranes were stained with the steryl dyes FM4-64 and FM1-43, which are of similar size and characteristics as filipin. FM4-64 and FM1-43 are amphiphilic molecules with strongly enhanced fluorescent properties in a hydrophobic environment (Betz et al., 1996). Both dyes stained the membranes at a much earlier stage compared to filipin staining, namely after 1 h (data not shown). There was no increased fluorescence at the site of the emerging germ tube, which indicates that there is no higher permeability of the cell wall or accumulation of plasma membrane (which may be an alternative cause of, enhanced filipin staining) (Fig. 3A,B). FM4-64 is regarded as a marker of endocytosis while it binds only to the outer leaflet of the plasma membrane (Dijkstra, 2003; Fischer-Parton et al., 2000). Stage IV cells showed lower membrane fluorescence compared to stage II cells after 10 min of removal of the dye (Fig. 3B). This indicates that these cells exhibit more intense endocytosis (in a period of 10 min). FM1-43 that crosses membrane leaflets and enters the spores did not show this difference between stage II and IV but showed equal fluorescence intensity (Fig. 3A).

3.3. Ergosterol levels in germinating conidia

The lack of early conidial staining might be reflected by low sterol contents of the plasma membrane during early germination. Therefore the correlation of fluorescent patterns with the total ergosterol levels at conidia during stages II–IV. Bar=5 μm. (B) Representation of the maximal fluorescence of the line selections at locations A–D during stage II to stage IV. Bar=5% l.s.d. (least significant difference), n=40.

Fig. 2. Filipin staining at different stages of germination of conidia. (A) Pixel intensities over line selections perpendicular to the membrane were determined at locations A–D at conidia during stages II–IV. Bar=5 μm. (B) Representation of the maximal fluorescence of the line selections at locations A–D during stage II to stage IV. Bar=5% l.s.d. (least significant difference), n=40.

Fig. 3. Staining of germinating conidia with the steryl dyes FM1-43 and FM4-64. Pixel intensities over line selections perpendicular to the membrane were determined at locations A–D. (A) Stage IV conidia stained with FM1-43 (top) and FM4-64 (bottom) for 1 and 2 min respectively. Both cells exhibit uniform membrane staining, while FM1-43 also stains the internal cell. Bar=5 μm. (B) Fluorescence intensity of FM1-43 over the conidial membrane remains constant during germination. On the contrary, fluorescent intensity of FM4-64 decreases. Bar=5% l.s.d., n=20.
content (as measured by HPLC) was studied during germination. To minimize internal experimental fluctuations, conidia of three independent experiments were pooled to determine the total ergosterol content. Dormant, freshly harvested conidia of *P. discolor* CBS 112557 contained 9–16 fg ergosterol per spore. The amount of ergosterol per spore was constant during the first initial 3 h of germination and increased more than four-fold between 3 and 9 h to reach 40–70 fg/spore (Fig. 4A). The ratio of the ergosterol content to the surface area of conidia showed a slight decrease during the first 2–3 h, which could be interpreted as conidial swelling without synthesis of ergosterol (Fig. 4B). As swelling proceeds, the ratio increased, indicating an increase of ergosterol content over the growth of the spore. After 7 h of incubation a decrease in the ratio of approximately 10% was observed, correlating with the appearance and outgrowth of germ tubes. The closely related *P. discolor* strain CBS 271.97, shows a similar pattern as strain CBS 112557 suggesting a net increase of ergosterol over the conidial surface extension. To summarize, ergosterol levels correlate closely related increase of ergosterol content over the growth of the spore. After 7 h of germination, the ratio of ergosterol content and surface area of the related strain CBS 271.97. (A) Conidia during stage IV stained with filipin without (top) or with a pre-treatment with natamycin (bottom). Bar=5 μm. (B) Representation of the maximal fluorescence at the plasma membrane at different locations at the cell. Note that fluorescence has strongly decreased at both the cap as the remainder of the membrane. Bar=5% l.s.d., n=30.

3.4. Binding specificity of filipin

In order to determine if filipin is binding to ergosterol in the germinating spore, a competitive inhibition experiment with natamycin was performed. Natamycin is another polyene antibiotic that more specifically binds to ergosterol compared to filipin (Hammond, 1977; Teerlink et al., 1980; Te Welscher et al., 2008). Compared to untreated conidia a significant decrease of filipin fluorescence was observed, after pre-treatment with natamycin at the same concentration as filipin (Fig. 5). This decrease was observed at both the area of intensive staining as the remainder of the plasma membrane, but the basic staining pattern of stage IV was not affected. After pre-treatment with natamycin, values of fluorescence in the cap dropped by 78%, compared to 72% in the remainder of the plasma membrane.

Subsequently, we studied the fluorescent patterns of filipin staining of *ergΔ* mutants of *S. cerevisiae*. As a result of deletion of specific enzymes of the ergosterol biosynthetic pathway, each mutant displays a distinct set of sterols (Heese-Peck et al., 2002). This allowed us to determine the binding-specificity of filipin by means of competition experiments. In this way we obtained evidence that filipin binds to ergosterol in situ and not to other membrane compounds. The intensity of filipin staining in the deletion mutants decreased significantly following the order RH448 (wildtype) > RH4213 > RH5225 > RH5228 (Fig. 6A). These four strains showed sterol binding of filipin exclusively in the plasma membrane and not in inter-cellular structures as other *ergΔ* mutants did (data not shown). The fluorescent pattern of the mutants displayed, unlike the wildtype, a more discrete appearance (Fig. 6C). Wildtype and mutant cells were pre-treated with natamycin, and showed significant differences with respect to inhibition of filipin fluorescence (Fig. 6A). The wildtype showed the strongest decrease of fluorescence confirming that ergosterol is an important compound for filipin binding in the plasma membrane. With 77% ergosterol the wildtype cells RH448 shows a 54% decrease of fluorescence. The *ergΔ* mutant RH4213 and the *erg2Δerg6Δ* mutant RH5225 have a different composition of sterols (Fig. 6C) and the decrease of filipin staining was 37% and 29% respectively. These are statistically significant differences as judged by the Tukey-test (a thorough multiple range test). The *erg2Δerg3Δ* mutant RH5228 showed a non-significant decrease of filipin staining.
of 25% (Fig. 6B). Comparing the sterol structures of the mutant strains, the structural feature that causes the loss of fluorescent intensity seems to be the double bonds located in the B-ring (Fig. 6C). Conidia of P. discolor showed an even stronger reduction of fluorescence after natamycin pre-treatment compared to the yeast wildtype (see above), which is interpreted as a characteristic of membranes that contain high levels of ergosterol. This indicates that filipin predominantly binds to ergosterol in the plasma membrane of germinating conidia.

3.5. Modulation of the sphingolipid biosynthesis and actin polymerization strongly influence ergosterol cap formation

In order to study if actin fibers play a role in the establishment of the ergosterol cap, stage IV conidia were treated with latrunculin A that reversibly prevents assembly of actin fibers (Ayscough et al., 1997). After 15 min, filipin staining became clustered, followed by disappearance after 60 min (Fig. 7A, middle picture). Quantification of fluorescence intensity showed severe reduction, over 60% after 1 h latrunculin A treatment (Fig. 7B), while the rest of the plasma membrane showed no reduction of fluorescence. When the germlings were washed and allowed to recover, polarized fluorescence gradually returned after 150 min (Fig. 7A, lower picture). The localization pattern of filipin fluorescence under influence of latrunculin A and the recovery suggests an active role for actin in the stabilization of ergosterol rich membrane domains.

The sphingolipid synthesis inhibitor myriocin (Miyake et al., 1995) had a different effect on the filipin staining pattern of stage IV conidia. This included a somewhat slower formation of germ tubes, a decrease...
in fluorescence intensity of the cap, and an increase in the fluorescence of the remainder of the plasma membrane (Fig. 8). Fig. 8B shows that the decrease in the fluorescence of the cap was restricted, but significant among the measured cells. The increase in fluorescence in the remainder of the plasma membrane however was very notable (from values round 25 AU to 55 AU). Consequently, the ratio between cap and other plasma membrane fluorescence dropped from nearly 4 to 1.3. As compared with the control, 4 hour-old conidia germinated in medium supplemented with myriocin, displayed an increase in total fluorescence and was correlated to an increase in ergosterol levels as measured by HPLC (Fig. 8C).

Combined, these data suggest that a hampered sphingolipid function results in a higher amount of ergosterol in the conidia and that filipin accurately monitors this change.

4. Discussion

The first objective of this study was to evaluate if filipin staining of the fungal membrane is a reliable marker for the presence of ergosterol. Our aim was to prevent the formation of artifacts by means of a modified staining protocol. It is stated that the time for filipin to enter the membrane and associate with sterol to form visible filipin–sterol complexes lies in the order of minutes. During this process, sterols can be relocated by diffusion if longer incubations are used, inducing potential artifacts (Miller, 1984; Robinson and Karnovsky, 1980). Temperatures below 12 °C decrease the binding of filipin to sterols (De Kruijff et al., 1974c; Miller, 1984). Indeed, we had very poor fluorescence when cells were incubated on ice (results not shown). We used a very short staining period (see also Takeda et al., 2004) and lowering of the temperature in combination with quick acquisition of the micrographs.

With this staining technique, we identified a change in the pattern of ergosterol distribution in conidia of P. discolor from a relatively weak general staining of the membrane after 5 h of germination to an intensive staining of a fluorescent cap at the presumptive site of germ tube emergence at 6 h and later. The fluorescent intensity of the cap increased significantly during early stages of germ tube formation indicating an increase of the sterol concentration. Measurements of extracted total ergosterol by HPLC confirm a link between filipin staining and ergosterol content. We observed a net increase over and above the surface extension of the plasma membrane between 3 and 7 h of germination. When conidia are broken directly after harvesting, there is an ergosterol content of 9–16 fg/spore which increases to 40–70 fg in 9 h (data taken from all different experiments). Russel et al. (1975 and 1977) observed that conidia of the fungus Aspergillus fumigatus showed similar characteristics. The amount of sterol they measured per spore is of the same order of magnitude as our measurements and they observed a two-fold increase in total sterol content at germ tube emergence (between 3 and 6 h). Both ergosterol and ergosterol esters are present inside the germinating conidia. In yeasts, the sterol esters reside in lipid bodies with several enzymes of the ergosterol synthesis pathway (Leber et al., 1994; Sorger et al., 2004). Morozova et al. (2002) have studied germinating conidia of A. niger and found that sterol levels roughly doubled during 8 h of germination and that sterol esters made up 50% of the total sterols at the beginning of germination; after 8 h the level of free sterols was 80%.

Now, the question remains if the filipin cap itself is a staining artifact as a result of the state of the fungal cell wall at the site of germ tube formation. At the apices of hyphae, precursor wall polymers are continuously delivered to the outside of the apical membrane by exocytosis (Wessels, 1989). During extension of the hyphal apex the
cell wall “flows” to sub-apical regions while the membrane extends through the fusion of exocytotic vesicles (Bartnicki-Garcia et al., 2000). Cross-linking of the cell wall polymers is relatively slow resulting in low rigidity and a more porous state of the cell wall at hyphal apices (Sietsma and Wessels, 1994; Wessels, 1990). This more permeable cell wall could account for a polarized fluorescent pattern of filipin. However, a study on fluorescent probes for wall porosity concluded that germinating conidia do not form an exclusion barrier for fluorescent probes. Common spoilage moulds (e.g. P. poaeuorti, A. niger, Trichoderma harzianum) took up FITC-dextran molecules as large as 150 kDa (Bruel et al., 1997). In our study, FM dyes, molecules of similar size and characteristics as filipin stained the membrane of germinating spores at an earlier stage than filipin. Interestingly, staining with FUN-1, a metabolic activity and viability marker, showed that conidia were already metabolically active after 1 h (M. R. van Leeuwen, unpublished results). This is also confirmed by Russel et al. (1977) by the uptake of radioactive (14C) acetate in the conidia of A. fumigatus. Taken together, our observations suggest that young conidia are active cells with a plasma membrane that contains only very low levels of ergosterol.

Filipin staining of conidia showed much lower values after a pre-treatment with natamycin. We interpret this as evidence that filipin binds to ergosterol in the plasma membrane of the spore. Natamycin is a polyene antibiotic like filipin but has a higher specificity for ergosterol compared to filipin (Behnke et al., 1984; Hammond, 1977; Teerlink et al., 1980; Te Welscher et al., 2008). Conidia of P. discolor showed even stronger natamycin-dependent inhibition of staining than cells of the wildtype S. cerevisiae (with 77% ergosterol of total sterol; Heese-Peck et al., 2002) indicating a major ergosterol fraction in conidia at that stage of germination. Te Welscher et al. (2008). studied the same erg-mutants of S. cerevisiae as we did and observed that the Minimal Inhibitory Concentration (MIC) of both filipin and natamycin increases (thus the sensitivity drops) among the deletion mutants in the following order: RH448 > RH2413 > RH5225 > RH5228. Natamycin sensitivity was highest in case of the wildtype and its MIC value dropped 3.4, 10.6 and 27.1-fold in the mutants RH4213, RH5225 and RH 5228, respectively. Filipin showed a reduction in the MIC value from 2.1, 4.3 and 5.7-fold. So, there is an absolute reduction in the effectiveness of filipin that correlates with the measured fluorescence of filipin. However, the effectivity of natamycin is dropping much faster among the mutants and also this corresponds very well with our data. Thus, the sensitivity of the mutants for natamycin drops similarly as the decrease in fluorescence of filipin staining. Different other studies highlight the association between polyene susceptibility and the presence of modified sterols in the membrane (Hapala et al., 2005; Safe et al., 1977; Sanglard et al., 2003; Young et al., 2003). Important for filipin staining is the sp² hybridization of C-7 of the B-ring of ergosterol. The packing of the polyene antibiotic molecule with ergosterol is very likely dependent on the saturation of the B-ring (Te Welscher et al., 2008). Taken together, data concerning the in situ effectivity (MIC-value) as the in situ staining (fluorescence) favor the hypothesis that filipin staining is representing ergosterol location in germinating conidia of P. discolor.

Alvarez et al. (2007) reviewed sterol-rich plasma membrane domains in fungi and recognized some of the problems of filipin staining that are addressed in this paper. They also state that there might be a limit to the ergosterol level of the ergosterol domains. Values of ergosterol contents in fungal plasma membranes are up to 20% in the yeasts (Zinser et al., 1993) and 22% in membranes of the filamentous fungus Neurospora crassa (Bowman et al., 1987). The surface area of freshly harvested conidia is approximately 50 μm². The thickness of the plasma membrane is 7.5 nm (Van Der Rest et al., 1995) and its buoyant density is on average 1.185 g/cm³ (in Penicilium chrysogenum; Hillenga et al., 1994). This would lead to a weight of the plasma membrane of one cell of approximately 350 fg which would mean that the ergosterol level would be 70 fg per spore if it was 20% of the membrane. We measured 10–20 fg ergosterol per spore that mostly resides not in the plasma membrane, but as a sterol ester (Morozova et al., 2002). The question remains how much of the actual ergosterol is extracted by the procedure we used, but the absence of filipin staining in the plasma membrane of conidia during the first hours of germination indicates that the ergosterol level of the plasma membrane of conidia is indeed very low compared to the reports of growing fungal cells.

Our data combined to that of the literature may provide an estimation of the amount of ergosterol inside the cap areas. Staining with filipin shows an increase to 40 AU in stage II cells. Subsequently, all the increase of staining is confined to a restricted area of the membrane (see Fig. 2) and to the novel membrane added to the spore due to isotropic growth. We measured ergosterol levels in cells of 5.5 h and 6.5 h in triplo and found levels of 44 and 73 fg/spore, respectively. The number of caps at 5.5 h of germination was 5% (18 caps in 364 spores) and 28% in case of 6.5 h cells (as extrapolated from Fig. 1). It is stated that 80% of the sterols is free in conidia (of A. niger) that have germinated for 6–7 h (Morozova et al., 2002). There are also estimations that 60% of the free ergosterol might be present in the plasma membrane of yeasts (Sullivan et al., 2006). Then, 48% of the ergosterol might be located in the plasma membrane. For the conidia at 5.5 and 6.5 h this would mean an ergosterol content of the plasma membrane of 21 and 35 fg/spore, respectively. In this time interval a 20% increase in size (from 80 to 100 μm², Fig. 4) was observed that takes 20/5=4.3 fg ergosterol/cell while the rest of the ergosterol (approximately 9 fg) is confined to the caps. At 6.5 h of germination only part of the caps is formed, thus 9 fg is meant for 28% of the caps. If all the cells had caps this would lead to an amount of 33 fg of ergosterol for a cap. The size of a cap is roughly 20% of the circumference of a spore (as indicated from several micrographs) and that is approximately 6% of the surface of the cell [according to 2πr²/3 of the cap area], The weight of such an area in a cell with a total surface of 100 μm² is approximately 53 fg (as seen above). According to the 48% estimation, 33 fg of this would be ergosterol inside the cap, that is 62% ergosterol, which seems to us a biologically impossible high amount of ergosterol. Another estimation (G. Daum, personal communication) states that roughly 25% of the ergosterol may reside in the plasma membrane of growing cells, which leads to ergosterol levels in the cap of 31%. The non-cap area has levels of ergosterol between 5 and 10%. This means that the cap is 3.3 to 12 times more enriched in ergosterol than the remainder of the plasma membrane. It is clear that the cap has high levels of ergosterol above the levels reported in plasma membranes of growing fungi, but we have to conclude that the remainder of the plasma membrane is rather ergosterol poor compared to the figures of literature.

The process of cap formation and ergosterol location is influenced by compounds that modulate actin cable formation and sphingolipid synthesis. Incubation with latrunculin A resulted in reversible disappearance of the tip localized ergosterol cap. Consistently, the group of Harris also provided evidence for a role of the cytoskeleton in membrane organization in the filamentous fungus A. nidulans (Pearson et al., 2004). Actin cables have a role in the delivery of vesicles at the apex of the germ tube. These might deliver high levels of ergosterol to the site of growth.

Sphingolipids and phospholipids with saturated acyl chains associate with sterols into a liquid ordered phase (L0). These lipid associations are laterally mobile in the liquid-disordered (Ld) “solvent” of largely unsaturated phospholipids. This phase segregation results in the formation of microdomains or lipid rafts (Maxfield, 2002; Pike, 2004; Simons and Ikonen, 1997). The question rises if the micrometer-large sterol-rich cap (SRC) has to be interpreted as a giant lipid raft. The role of sphingolipids within the formation of the SRC is illustrated by the effect of myriocin, an inhibitor of serine palmitoyltransferase, which catalyzes the first step in sphingolipid biosynthesis (Miyake et al., 1995). We observed a significant decrease of the fluorescence...
intensity at the apices of germ tubes of germinating conidia while the fluorescence of the conidial membrane had increased. This was
induced by the polyene antibiotics. Biochim. Biophys. Acta 339, 30–70.


