# Natamycin and the germinating spore

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**Natamycine en de ontkiemende spore**

(met een samenvatting in het Nederlands)

**Proefschrift**

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*voor mijn familie*

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Chapter 1

# General Introduction

## The fungal kingdom

Fungi exhibit a bewildering variation in morphology and species. The Kingdom *Fungi* consists of five phyla: *Chytridiomycota*, *Glomeromycota, Zygomycota*, *Ascomycota* and *Basidiomycota* (Carlile *et al.*, 2001; Schüßler *et al.*, 2001). The latter three are designated as the *Eumycota*. The three fungal phyla within the *Eumycota* are recognized on basis of their sexual stage also known as the teleomorph or the perfect stage. They form zygospores, ascospores and basidiospores, respectively. The three fungal divisions also form an anamorph or imperfect stage which can form a diversity of asexual spores. Many fungi have no known sexual or teleomorphic stage and are generally referred to as *Fungi imperfecti,* or Deuteromycetes. This group includes important food- and airborne contaminants. The comparison of DNA sequences has related many Deuteromycetes with teleomorphs and recently a number of important fungi have revealed their teleomorphic state (Houbraken *et al.*, 2008; O'Gorman *et al.*, 2008).

The fungal genera *Aspergillus* and *Penicillium* are probably the most well known Deuteromycetes and comprise approximately 300 species each. They belong to the order Eurotiales, within the phylum of *Ascomycota* (Frisvad and Samson, 2004; Geiser *et al.*, 2007). The fungi are able to form numerous single-celled asexual spores, conidia, on structures that are called conidiophores. *Aspergillus* forms conidiophores that are characterized by a large thick walled stipe with a swollen apex, termed the vesicle (Fig. 1A). Conidia are formed from flask-shaped phialides that are in many species placed on short branches (metulae) that develop from the vesicle. The conidia are produced in chains by basipetal succession: the youngest conidium is formed at the base, pressing the older conidia further away from the phialide. Conidiophores of *Penicillium* also consist of a single stipe. This stipe terminates into a whorl of conidia-producing phialides (monoverticilate, e.g. *P. glabrum*), or more generally, forms a penicillus (Fig. 1). The penicillus consists of metulae bearing the phialides that originate from, one-stage branched (biverticilate), two-staged branched (terverticilate), or three-staged branched (quaterverticilate) aerial hypha (Fig. 1B). *Aspergilli* and *Penicilli* are saprophytic and natural habitants of soil and decaying organic matter. They are extremely common as spoilage fungi in foods and in stored commodities such as grains, nuts and spices. Because of their global distribution, species of *Aspergillus* and *Penicillium* are competitors in soil and decaying matter, as well as in foods and food products (Hocking, 2006). The average growth rate of *Aspergilli* is usually higher than that of *Penicilli*. However, they generally take longer to sporulate and produce spores which are often more resistant to light and chemical compounds (Hocking, 2006). Moreover, *Aspergilli* generally grow at higher temperatures and withstand lower water activities than *Penicilli*. This enables *Aspergillus* species to dominate spoilage in the tropics, whereas in temperate zones, *Penicillium* species are more common (Hocking, 2006; Pitt, 2006).

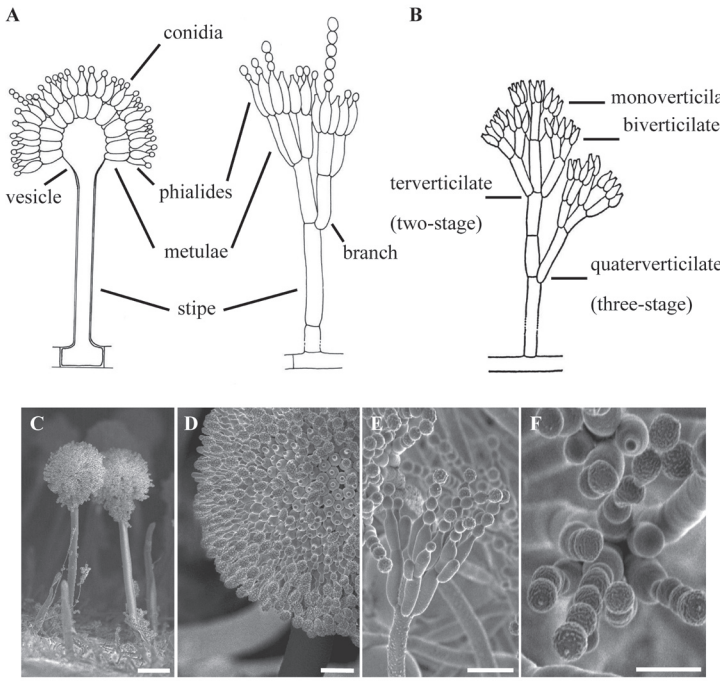


**Figure 1.** Formation of conidia by *Aspergillus* and *Penicillium*. **(A)** *Aspergillus* (left graph) and *Penicillium* (right graph) form distinct conidiophores. **(B)** Morphological features of the *Penicillium* conidiophore used in species determination. Scanning Electron Microscopy (SEM) micrographs of conidiophores of *A. niger* **(C)** and *P. discolor* **(E)** with the conidia producing phialides located on the vesicle **(D)** or within the penicillus **(F)**, respectively. Bar represents 10 μm.

## Fungal spoilage

Fungi are specialists in the degradation of complex polymers of organic origin such as lignin, cellulose and hemicellulose. To this end, fungi secrete a wide variety of enzymes. Many of these enzymes, such as cellulases, laccases, amylases and pectic esterases are applied in the food and feed industry to modify texture, colour, flavor and taste (Wösten *et al.*, 2007). *Aspergillus niger* is widely used in biotechnology and has a long tradition of safe use in the production of enzymes, organic acids and heterologous proteins. The metabolic versatility of fungi is also exploited to produce and ferment food products. Important bio-transformers are *Saccharamomyces cerevisiae* (bread, wine and beer), *Aspergillus oryzae* (soy sauce), *Rhizopus oligosporus* (tempeh and black oncom) and *Neurospora sitophila* (red oncom) (Nout, 2007). A side effect of the fungal lifestyle is bio-deterioration, generally referred to as spoilage of food, feed and crops (e.g. discoloration, colony formation, loss of structure,

change of texture, off-odour, off-flavour and increase in acidity). Renowned post-harvesting spoilers are species that belong to *Alternaria*, *Colletotrichum*, *Fusarium* and *Botrytis* that penetrate crops by appressoria. Other species like *Rhizopus*, *Aspergillus* and *Penicillium* invade a natural wound or wounds caused by chilling, high temperature, or handling (Prusky and Kolattukudy, 2007).

The annual losses of post harvest fungal spoilage are enormous and it constitutes a substantial financial burden. As the result of poor handling during harvesting, processing, storage and transport, losses from post harvest spoilage can be as high as 50% (Prusky and Kolattukudy, 2007). In general, a surprisingly small number of fungal species are found on a food product or crop. In other words, each substrate is related to a fungal community consisting of a few species only (Frisvad *et al*., 2007a). Certain fungal species such as *A. niger*, *P. brevicompactum* and *P. citrinum* can be considered as generalists and are found on a wide range of crops and plants. However, there are clear quantitative differences. For example, on grapes *A. niger* and *A. carbonarius* are more common than species such as *A. ochraceus* and *A. westerdijkiae*, while the opposite is observed on green coffee beans (Frisvad *et al*., 2007a). Other fungi are associated with a relatively narrow host range. For instance, *P. expansum* can be found on pomaceous and stone fruits and on nuts, whereas *P. italicum*, *P. digitatum* and *P. ulaiense* are found on citrus fruits only. Interestingly, also processed foods and food products can be related to specific associated mycobiota. Rye bread, for example, holds a specific mycobiota dominated by *P. roqueforti*, *Monascus ruber* and *Paecilomyces variotii*, while soft and hard cheeses are prone to be spoiled by *A. versicolor*, *P. commune, P. discolor* and *P. palitans*. Some fungi are adapted to relatively extreme conditions with respect to pH, water activity or temperature. These adaptations enable fungi like *Eurotium*, *Wallemia* and *Xeromyces* to grow on various foods and food products with low water activity resulting from the addition of salt or sugar (Frisvad *et al*., 2007a).

Fungi generally produce extrolites (Bohra and Purohit, 2003). An extrolite is a secreted compound and can be anything like an enzyme, a volatile or non-volatile secondary metabolite, or an organic acid (Frisvad *et al.*, 1998). Because some extrolites are bioactive and toxic, correct identification of the spoilage fungus is crucial for health concern. Such toxic extrolites, called mycotoxins, are often carcinogenic. They can be the causative agent of acute and chronically mycotoxicosis (Frisvad *et al.*, 2007b). Well-known mycotoxin producers are *Aspergillus flavus* (aflatoxin), *Byssochlamys nivea* (mycophenolic acid), *Penicillium expansum* (patulin), *Alternaria citri* (tenuazonic acid), *Penicillium citrinum* (citrinin), *Aspergillus ochraceus* (ochratoxin A) and *Fusarium anthophilum* (fumonisins) (Frisvad *et al.*, 2007b). In *Aspergillus* and *Penicillium* it has been shown that extrolite production is dependent on the composition of the growth medium and that it is surprisingly stable *per* species. Identification of fungi based on HPLC-profiles of extracts strongly correlated with identification based on morphological and DNA sequence data (Samson and Frisvad, 2004).

Strategies to prevent fungal spoilage include manipulation of so-called extrinsic and intrinsic parameters. Extrinsic parameters are substrate independent and consist of physical properties of the storage environment. An important extrinsic parameter is temperature. Lowering of the temperature results in low metabolism and slow growth and also affects mycotoxin production. On the other hand, heat treatment or pasteurization before storage or packaging inactivates fungal hyphae, conidia and other survival structures. Fungal growth can also be decreased by lowering the oxygen tension or increasing the carbon dioxide levels. Intrinsic parameters include the use of specific additives or manipulation of the physical state of the food itself. An important intrinsic parameter is lowering of pH. Undissociated organic acids like acetate, citrate, malate, propionate and sorbate can affect fungal growth. Fungal growth can also be diminished by reducing available water by the addition of salts and sugars to the food product. Microbial antagonism can also be used. In this case, micro-organisms compete for nutrients or inhibit growth of micro-organisms directly by the production of anti-fungals. However, a number of fungi are able to grow despite these barriers. For instance, acquired resistance has been reported for two major food preservatives (sorbate/benzoate) in various fungi, including species of *Penicillium* and *Zygosaccharomyces* (Kinderlerer and Hatton, 1990; Golden and Beuchat, 1992).

## Fungal spores and germination

Spores are the prominent fungal structures for dispersal and for survival of unfavorable conditions. Some fungal species produce different types of spores that are tailored for dispersion and/or for survival. Spores that easily disperse by wind, water, or insects are generally produced in large numbers directly from the vegetative mycelium or from specialized aerial structures such as conidiophores and sporangiophores. Spores that are dispersed by air are exposed to oxygen, sunlight and desiccation. This makes the air a hostile environment, especially when the large surface to volume ratio of spores is taken into account. Species like *Aspergillus* and *Penicillium* produce large numbers of small pigmented, hydrophobic and thick-walled spores that are well adapted for widespread dispersal and long term survival in the air. In contrast, water dispersed spores such as those from *Fusarium* and *Verticillium* are thin-walled, colorless and hydrophilic. Such spores are well adapted for dispersal from an infected host to a healthy plant nearby.

Spores that are able to survive for prolonged time like chlamydospores may not be separated from the vegetative mycelium. These asexual thick walled spores are produced within hyphae and have no dispersal mechanism. Since chlamydospores are unlikely to be dispersed, production of vast numbers is less important than for dispersal spores. Yet other survival spores like the ascospores of the plant pathogen *Mycosphaerella* are successfully

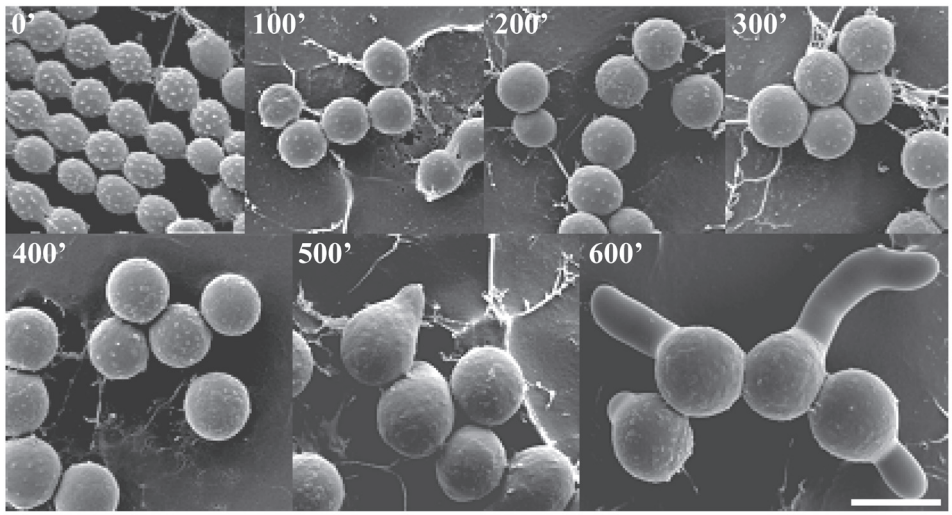


**Figure 2.** SEM micrographs of germinating conidia of *Penicillium discolor* in a 600 min time frame. Bar represents 10 μm.

dispersed to the next host by rain splashes. These spores are produced in *asci* that are enclosed in an aggregation of hyphae, the *ascoma*. These ascomata exist as a completely closed cleistothecium, a flask-like perithecium or an open cup-like apothecium. Spores destined for survival are extremely stress resistant and can be dormant for prolonged time. High levels of compatible solutes like trehalose, mannitol and glycerol ensure dormancy and protect the cellular membrane and proteins (Dijksterhuis and Teunissen, 2004). These solutes enable survival spores even to endure pasteurization and high-pressure treatment. Ascospores of *Talaromyces macrosporus* can stay dormant for at least 17 years and are able to withstand temperatures up to 85°C for 100 min (Nagtzaam and Bollen, 1994; Dijksterhuis and Teunissen, 2004).

The settling of spores on an appropriate substrate is the first event in fungal colonization. How fast propagules are cleared form the air to settle at a surface is mainly determined by the size and shape of the spores: if the spore is too large they will not efficiently be transported through the air, if the size is too small the chance of landing on a surface is minimal (McCartney and West, 2007). After settling, dormancy is broken in the presence of nutrients. Isotropic growth, commonly referred as swelling, is the first morphological change in spore germination (Fig. 2). This is accompanied by a twofold or more increase in spore diameter. It generally involves water uptake and is characterized by the dispersion of materials and machinery to the cell cortex for the addition of new plasma membrane and cell wall (Momany, 2002). At the same time, isotropic growth is concomitant with the resumption of numerous metabolic activities including respiration, DNA, RNA and protein

synthesis (Mirkes, 1974; Osherov and May, 2001), as well as trehalose breakdown (d'Enfert, 1997). At some stage during germination, there will be a transition from isotropic expansion to polarized growth, the defining feature of filamentous fungi. To initiate polarized growth, a spot is established at the cell cortex for the emergence of the germ tube in a process called 'polarity establishment'. In this process, the plasma membrane and cell wall components and machinery are redirected to this established area. The process 'polarity maintenance' ensures that materials and machinery for making new plasma membrane and cell wall are continued to be directed to the spot of the apically protruding germ tube (Fig. 2). As polar growth continues, crosswalls or septa are formed, partitioning the main extending hypha into compartments. After a period of tip growth, filamentous fungi establish new subapical axes of polarity. As a consequence, hyphal branches emerge from the main hypha. The formation of hyphal branches and the process of hyphal fusion results in a network of hyphae called the mycelium.

## Macrolide Polyene Antibiotics

Antifungal compounds are used to prevent or treat infections of plants, animals and humans. These compounds interfere with cellular functions such as cell wall synthesis (echinocandins), ergosterol synthesis (imidazoles, triazoles) and plasma membrane integrity

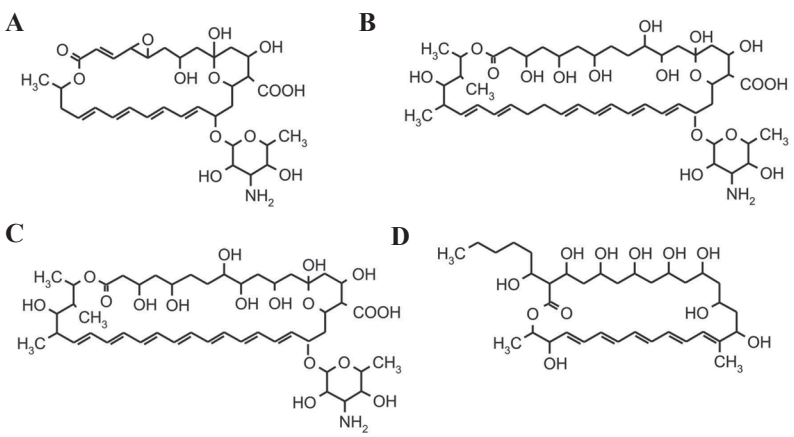


**Figure 3.** Molecular structure of the most common polyene antibiotics with the distinctive macrolide backbone comprising a hydroxyl bearing side and a side with double bonds. Note the mycosamine sugar of natamycin **(A),** nystatin **(B)** and amphotericin B (**C**) and the hydrophobic tail of filipin **(D)**.

(polyene antifungals). Polyene antibiotics are part of the macrolides that are produced by *Streptomyces*, *Streptoverticillium* and *Chainia* spp. (Hamilton-Miller, 1973). They show little or no antibacterial activity, but are potent antifungal compounds. However, problems associated with solubility, stability, absorption and toxicity have made only a few of these compounds clinically useful. The polyene antibiotics amphotericin B and nystatin are the most widely used polyene antibiotics against systemic and non-systemic mycosis (Kobayashi and Medoff, 1977). Nystatin has been used successfully in the treatment of *Candida* infections of the skin, nails and mucosal surfaces. Orally administered, nystatin rapidly clears candidosis of the alimentary tract. Because the antibiotic is little, if at all, absorbed from the gut, oral nystatin therapy is ineffective in the treatment of systemic mycosis. Amphotericin B has been the primary polyene antifungal to treat deep-seated systemic mycosis, such as blastomycosis, candidosis, coccidioidomycosis and cryptococcal meningitis (Chapman *et al*., 2008).

Polyene antibiotics, like amphotericin B, nystatin and filipin consist of a large macrocyclic ring of carbon atoms closed by the formation of an internal ester, generally known as a lactone (Fig. 3; Hamilton-Miller, 1973). Synthesis of the macrocyclic ring involves polyketide synthases (PKS), present in large biosynthetic gene clusters, which transform acetate and propionate into the antibiotic backbone (Aparicio *et al.*, 2003). In addition, polyene antibiotics contain a series of functional hydroxyl groups on one side of the ring and conjugated unsaturations, or double bonds, on the other side, resulting in an amphipatic nature (Fig. 3). These unsaturations involve relatively loosely bound electrons causing absorption of UV-light of longer wavelengths. Based on the UV absorption spectra, polyene antibiotics are divided into five groups: the trienes, tetraenes, pentaenes, hexaenes and heptaenes, representing 3, 4, 5, 6 and 7 double bonds, respectively. The double bonds in the lactone act as a strong chromophore. Trienes are generally colorless or pale yellow; tetraenes, such as nystatin and natamycin (pimaricin), are cream or pale yellow; pentaenes, such as filipin, are yellow; hexaenes, such as, candihexin, are yellow-orange; heptaenes, such as amphotericin B, appear orange. Another characteristic of most macrolide polyene antibiotics is the presence of either an aromatic moiety, or more common, the hexosamine sugar mycosamine (Fig. 3). Mycosamine confers an alkaline character on the molecule rendering polyene antibiotics as nystatin, amphotericin B and candicidin amphoteric, possessing an equal number of alkaline (mycosamine or aromatic side chain) and acidic (carboxyl) groups. In contrast, filipin does not possess ionizable groups as carboxyls and aromatic side chains and is therefore non-polar (Hammond, 1977). Polyene antibiotics are relatively stable and potency will not be lost when stored pure and dry in a dark and low temperature environment. However, light with a large wave length causes photo-deterioration of the polyenes, resulting in epoxides. The most damaging radiations are between 380 and 410 nm, corresponding to the absorption maxima of the polyene chromophore (Hamilton-Miller, 1973). Exciting the filipin chromophore at 360 nm produces a strong fluorescent signal with an emission maximum at

480 nm (Drabikowski *et al*., 1973). This property is commonly exploited as a fluorescent signal for sterol localization (Robinson and Karnovsky, 1980; Behnke *et al.*, 1983).

## Biological Activity of Macrolide Polyene Antibiotics

Antifungal compounds that are fungicidal cause irreversible damage to the fungal cell and as such eradicate the fungal population on a product or in an infected organism. Fungistatic compounds do not kill the fungus but prevent its growth. Although the fungistatic compound is able to prevent fungal spoilage, the cells can be regarded as 'time bombs' as growth is able to resume at the moment the compound disappears from the environment. Polyene antibiotics can act as fungistatic or fungicidal compounds. Polyene antibiotics are classified into group I and II based on their activity to cause leakage of potassium and cell death of cells (Kotler-Brajtburg *et al.*, 1979). Members of group I include filipin, etruscomycin and natamycin. They induce $K^+$ leakage and cell death in *S. cerevisiae* cells and erythrocytes at similar concentrations of added polyene. This class affects cells in an all-or-none effect, destroying the cell membrane without noticeable prior damage (Kotler-Brajtburg *et al.*, 1979). In contrast, members of group II, containing the polyene antibiotics candicidin, nystatin and amphotericin B induce $K^+$ leakage at much lower concentrations than those inducing cell death or lysis. As a result, the concentrations of the polyene to induce fungistatic effects are clearly distinguishable from concentrations that induce fungicidal effects (Kotler-Brajtburg

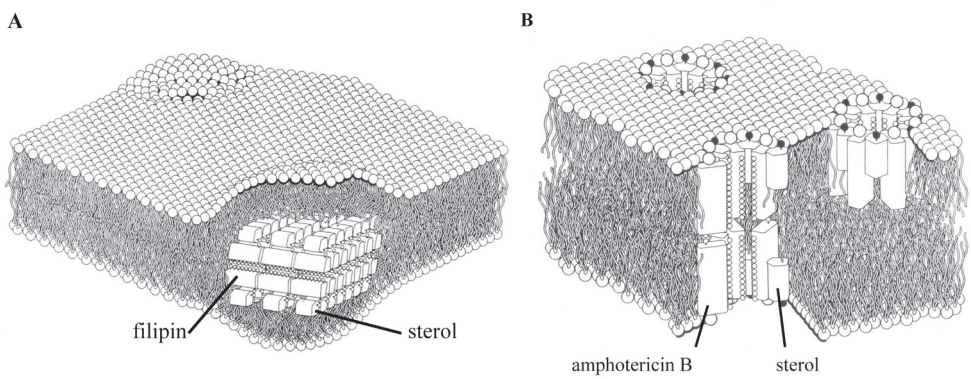


**Figure 4.** Mode of action of filipin and amphotericin B as proposed by De Kruijff and Demel (1974). **(A)** Filipin forms planar complexes with sterol. Two of these complexes associate into a double layer -sandwich like- aggregate, able to disrupt membrane integrity. **(B)** In contrast, amphotericin B forms half pores within the membrane leaflet. Two of those half pores combine into a membrane spanning channel increasing membrane permeability.

*et al.*, 1979). It should be noted that the developmental stage of the fungus also determines whether the polyene is fungistatic or fungicidal. Russell *et al*. (1975, 1977) observed that dormant conidia of the opportunistic pathogenic fungus *Aspergillus fumigatus* are relatively tolerant to amphotericin B methyl ester (AME), a chemically improved polyene antibiotic. However, during the early swelling stage of germination, conidia become sensitive which is maintained throughout the emergence of the germ tube. This phenomenon may well have implications for the use of antifungals in food and clinical applications, since conidia could well survive the antifungal treatment.

Polyene antibiotics exert their effect on the plasma membrane. Specific changes in membrane permeability and integrity are observed after treatment with polyene antibiotics in model membrane systems as well as in sterol-containing cells (Bolard *et al.*, 1986). Although various polyene antibiotics have common features like the prevalence for membranes containing ergosterol, the main sterol in fungi, their mode of action varies. In contact with sterol, amphotericin B and nystatin form a half pore within the membrane leaflet, containing 8 units of an antibiotic and sterol molecule (De Kruijff and Demel, 1974; Marty and Finkelstein, 1975; Baginski *et al.*, 1997). Two half pores build up to combine a membrane spanning conducting channel perpendicular to the plane of the bilayer (Fig. 4A). The hydrophobic channel of such a pore has a diameter of about 7-10 Å (De Kruijff and Demel, 1974; Khutorsky 1996). Etruscomycin is smaller than amphotericin B and nystatin, but possesses a four-carbon hydrophobic tail attached to the macrolide ring. Analogous to amphotericin B or nystatin, half pores can be formed from etruscomycin and sterol. Natamycin has no effect on membrane permeability. Yet it is identical to the structure of etruscomycin with the exception of the hydrophobic tail (De Kruijff and Demel, 1974). Filipin on the other hand does not form conducting pores as a result of its shorter length and the absence of the charged carboxyl group and the bulky mycosamine. Instead, it withdraws sterols from its association with phospholipids to form a planar complex (Fig. 4B). Two such planar aggregates associate, resulting in double layer -sandwich like- aggregates located in the hydrophobic core of the plasma membrane. The filipin-sterol complexes are visualized as large supramolecular aggregates of 190-250 Å in diameter and 4-20 Å in height (Santos *et al.*, 1998).

Polyene antibiotics have been tested on liposomes, large unilamellar vesicles (LUV), spherules, the yeast *Saccharomyces cerevisiae* and the mycoplasma *Acholeplasma laidlawii*. Cells from *A. laidlawii* can be considered a modelsystem to study membranes. Their sterol composition can be modified by altering the composition of the culture medium. De Kruijff *et al.* (1974) studied the membrane perturbing properties of polyene antibiotics on egg lecithin liposomes and *A. laidlawii* cells. In the absence of sterols amphotericin B, filipin, nystatin and natamycin had no effect upon membrane permeability. In the presence of sterols however, different permeability changes are observed (Table 1). Disruption of the membrane by filipin results in a non-specific efflux of cytoplasmic components, including

**Table 1.** Studies on polyene antibiotic inducement of permeability

| Polyene Antibiotic | Model System | Approx. sterol conc. (%) | Released Compound | Release | Reference |
|---|---|---|---|---|---|
| **Filipin** | Liposomes | 15.7 | $K^+$, Glucose | Yes | De Kruijff |
| | *Acholeplasma laidlawii* | 10 | $K^+$, G6PD, G6P, $NADP^+$ | Yes | De Kruijff |
| | LUV | 10 | $H^+$, Carboxyfluorescein | Yes | Te Welscher |
| | Liposomes | 30 | Dextran | Yes | Teerlink |
| | Spherules | 10 | $CrO_4^{2-}$, $H_2PO_4^-$ | Yes | Weissmann |
| **Amphotericin B** | Liposomes | 15.7 | $K^+$ | Yes | De Kruijff |
| | Liposomes | 15.7 | Glucose | Slowly | De Kruijff |
| | Liposomes | 20-40 | $K^+$ | Yes | Teerlink |
| | Liposomes | 30 | Dextran | No | Teerlink |
| | *Acholeplasma laidlawii* | 10 | Glucose | Slowly | De Kruijff |
| | *Acholeplasma laidlawii* | 10 | $K^+$, $Li^+$, $Na^+$, $Rb^+$ | Yes | De Kruijff |
| | *Acholeplasma laidlawii* | 10 | $Ca^{2+}$, $Mg^{2+}$, G6PD, G6P, $NADP^+$ | No | De Kruijff |
| | *Acholeplasma laidlawii* | 10 | Ribose, Xylose, Erythritol, Urea | Yes | De Kruijff |
| | Spherules | 10 | $CrO_4^{2-}$, $H_2PO_4^-$ | Yes | Weissmann |
| **Nystatin** | Liposomes | 15.7 | $K^+$ | Yes | De Kruijff |
| | Liposomes | 15.7 | Glucose | No | De Kruijff |
| | *Acholeplasma laidlawii* | 10 | $K^+$, $Li^+$, $Na^+$, $Rb^+$ | Yes | De Kruijff |
| | *Acholeplasma laidlawii* | 10 | $Ca^{2+}$, $Mg^{2+}$, G6PD, G6P, $NADP^+$, Glucose | No | De Kruijff |
| | *Acholeplasma laidlawii* | 10 | Ribose, Xylose, Erythritol, Urea | Yes | De Kruijff |
| | *Saccharomyces cerevisiae* | 20 (*) | $H^+$ | Yes | Te Welscher |
| | LUV | 10 | $H^+$ | Yes | Te Welscher |
| | LUV | 10 | Carboxyfluorescein | No | Te Welscher |
| | Spherules | 10 | $CrO_4^{2-}$, $H_2PO_4^-$ | Yes | Weissmann |
| **Etruscomycin** | *Acholeplasma laidlawii* | 10 | $K^+$ | Yes | De Kruijff |
| | *Acholeplasma laidlawii* | 10 | G6PD, G6P, $NADP^+$, Glucose | No | De Kruijff |
| | *Acholeplasma laidlawii* | 10 | Ribose, Xylose, Erythritol, Urea | Yes | De Kruijff |
| | Liposomes | 20-50 | $K^+$ | Yes | Teerlink |
| | Liposomes | 30 | Dextran | No | Teerlink |
| | Spherules | 10 | $CrO_4^{2-}$ | yes | Weissmann |
| **Natamycin** | Liposomes | 15.7 | $K^+$ | No | De Kruijff |
| | *Acholeplasma laidlawii* | 10 | $K^+$ | No | De Kruijff |
| | Liposomes | 20-50 | $K^+$ | Yes | Teerlink |
| | Liposomes | 30 | Dextran | No | Teerlink |
| | Spherules | 10 | $CrO_4^{2-}$ | No | Weissmann |

* Adapted from Zinser *et al.*, 1993

small molecules like $K^+$, $CrO_4^{2-}$, $H_2PO_4^-$ and large molecules like glucose, dextran, glucose-6-phosphate (G6P) and nicotinamide adenine dinucleotide phosphate ($NADP^+$) (Weissmann and Sessa, 1967; De Kruijff *et al.*, 1974; Teerlink *et al.*, 1980). Even enzymes such as glucose-6-phosphate dehydrogenase (G6PD) are released from filipin disrupted membranes (De Kruijff *et al.*, 1974). Recently, it was demonstrated by Te Welscher *et al.* (2008) that filipin could also release trapped carboxyfluorescein and $H^+$ from large unilamellar vesicles containing sterol. Amphotericin B, etruscomycin and nystatin alter the membrane permeability in a more specific manner (Table 1). Enzymes and large molecules like G6PD, G6P, $NADP^+$, dextran and divalent ions like $Mg^{2+}$ and $Ca^{2+}$ are unable to permeate through the antibiotic disrupted membrane (Weissmann and Sessa, 1967; De Kruijff *et al.*, 1974; Teerlink *et al.*, 1980). In contrast, the monovalent ions, $K^+$, $Na^+$, $Rb^+$ and $Li^+$ and the small molecules $CrO_4^{2-}$, $H_2PO_4^-$, ribose, xylose, erythritol and urea do permeate with a rate dependent on the size of the molecule (Weissmann and Sessa, 1967; De Kruijff *et al*., 1974). Interestingly, pores formed by nystatin and etruscomycin must be of smaller size compared to those of amphotericin B, as the latter, though slowly, is able to permeate glucose (De Kruijff *et al*., 1974). Polyene antibiotics

interact with sterols in a hydrophobic manner and require certain structural characteristics (Norman *et al.*, 1972). Incorporation of structurally different sterols in *A. laidlawii* and liposomal membranes showed relative membrane permeability in the order cholesterol > ergosterol > epicholesterol > stigmasterol and cholesterol = ergosterol > stigmasterol > epicholesterol for filipin and amphotericin B, respectively. Membrane permeability was only observed with sterols containing a 3β-OH group, a planar sterol nucleus and an intact side chain at $C_{17}$ (De Kruijff *et al.*, 1974).

## Natamycin as an antifungal compound

Natamycin (pimaricin) has been used for decades to suppress fungal development on surfaces of cheeses and sausages and in human and veterinary medicine for the topical treatment of fungal infections of skin and eye. Natamycin has a very low toxicity towards mammals. Absorption from the human intestinal tract after oral administration of doses from up to 500 mg per day during a 7 days period has not been observed. Natamycin and/or sorbates are in most countries the only approved antifungal agents for the topical treatments of food products like cheese and dry sausages. Compared with natamycin, relatively high concentrations of sorbate are needed to prevent fungal growth and some common spoilage moulds have

**Table 2. Sensitivity of fungi to natamycin**

| Micro-organism | MIC (ppm) | | MIC (ppm) |
|---|---|---|---|
| **Filamentous fungi** | | **Yeast** | |
| *Aspergillus penicillioides* | 10-20 | *Candida zeylandoides* | < 5 |
| *Aspergillus flavus* | 10-20 | *Candida krusei* | < 5 |
| *Aspergillus parasiticus* | 10-20 | *Candida pseudotropicalis* | < 5 |
| *Aspergillus versicolor* | 5-10 | *Candida guilliermondii* | < 10 |
| *Cladosporium cladosporioides* | < 5 | *Candida kefyr* | < 10 |
| *Eurotium herborariorum* | < 10 | *Debaryomyces hansenii* | < 5 |
| *Geotrichum candidum* | < 10 | *Dekkera bruxellensis* | < 5 |
| *Penicillium discolor* | 20-30 | *Saccharomyces bayanus* | < 5 |
| *Penicillium glabrum* | 5-10 | *Saccharomyces carlsbergensis* | < 5 |
| *Penicillium commune* | < 5 | *Saccharomyces cerevisiae* | < 5 |
| *Penicillium chrysogenum* | < 5 | *Saccharomyces exiguus* | < 5 |
| *Penicillium nalgiovense* | < 5 | *Hanseniasporum uvarum* | < 5 |
| *Penicillium verrucosum* | < 5 | *Hansenula polymorpha* | < 5 |
| *Penicillium brevicompactum* | < 5 | *Pichia membranaefaciens* | < 5 |
| *Penicillium roqueforti* | < 5 | *Torulopsis candida* | < 5 |
| *Penicillium camemberti* | < 5 | *Rhodotorula mucilaginosa* | < 5 |
| *Penicillium corylophilum* | < 5 | *Rhodotorula gracilis* | < 10 |
| *Penicillium solitum* | < 5 | *Kloeckera apiculata* | < 10 |
| *Phoma glomerata* | < 5 | *Kluyveromyces lactis* | < 10 |
| *Fusarium solani* | < 5 | *Zygosaccharomyces barkerii* | < 10 |
| *Wallemia sebi* | < 10 | *Torulaspora rosei* | < 10 |

MIC = Minimal Inhibitory Concentration
Sources: Delves-Broughton *et al.*, 2005 and Stark, 2007

developed resistance towards this acid. Moreover, sorbate diffuses from the surface to the interior of the product, resulting in reduced effectiveness. The use of sorbate may also affect the flavor and the color of the product (Stark, 2007). The major advantages of natamycin are its broad-spectrum activity and the fact that development of natural resistance has not been reported (Table 2). Other advances of natamycin are: its long history of safe use, the prolonged working time and the relative high activity at low, neutral and high pH (Stark, 2007). Moreover, it is chemically stable and has no colour, odour, taste, or any negative effects on the product quality. Due to its low water solubility, natamycin will mainly be present in the solid state as crystals. As natamycin slowly dissolves from the crystals, it become active and diffuses over the surface, compensating natamycin eliminated by interaction with fungal cells, hydrolyses, oxidative inactivation or light. This slow release of natamycin guarantees a prolonged activity over time. Natamycin is effective at very low concentrations (Delves-Broughton *et al.*, 2005; Stark, 2007). Important species that are inhibited are food-related fungi such as *Aspergillus flavus* (the producer of the mycotoxin aflatoxin A), *Wallemia sebi* (a spoiler of bread, chocolate and cakes) and *Debaryomyces hansenii* (important salt-tolerant spoilage yeast) (Table 2).

Natamycin is produced by *Streptomyces natalensis*, which was isolated for the first time in 1955 from a soil sample of Pietermaritzburg, a town in the province of Natal, South Africa (Struyk *et al.*, 1957-58). A few years later, Burns *et al.* (1959) isolated natamycin from a culture of *S. chattanoogensis*, obtained from a soil sample from Chattanooga, Tennessee. The production of natamycin is performed on an industrial scale with *S. natalensis*. Because most of the natamycin is bound to the mycelium it is isolated either by whole broth extraction or by extraction of the mycelium, using lower alcohols. The crude compound is precipitated by pH regulation or by evaporative concentration (Brik, 1981). Natamycin is a white to cream-colored, crystalline powder. The amphoteric character of natamycin is responsible for its insolubility in non-polar solvents. The solubility in certain polar solvents like dimethylsulphoxide (DMSO) can be greatly enhanced by adding water. This enables to solve 6 mg/ml natamycin in 85% (v/v) DMSO-water (Brik, 1981). Natamycin as a powder is a stable compound. When protected from light and moisture it will withstand temperatures up to 120°C for 1 h. Aqueous natamycin suspensions with pH values between 5 and 9 and stored in the dark are nearly as stable as the dry powder and can be boiled for a short time before activity is lost. At extreme pH values, natamycin is rapidly inactivated. At a low pH the aminosugar mycosamine will split off. The resulting sugarless molecule, also called aglycone, is instable and reacts with either another aglycone or with an intact molecule of natamycin. At higher pH values, especially at pH 12, the lactone ring of the natamycin molecule is hydrolyzed (saponified) with the formation of the microbiologically inactive natamycoic acid and several reaction products such as acetaldehyde, acetone and ammonia (Brik, 1981). Oxidative degradation of natamycin by peroxides or oxygen proceeds at the fastest rate in

solution or suspension and is promoted by several metal ions, especially Fe(III), Ni(II) and Cr(III). Addition of oxygen on the conjugated double bonds, which takes place at one of the ends of the unsaturated chain, results in either an epoxy group or a hydroperoxide. Oxidative inactivation can be prevented by antioxidants like chlorophyll and ascorbic acid and by metal complexing agents like EDTA and polyphosphates (Brik, 1981).

Surprisingly, despite the long use of natamycin, its mode of action is an enigma. There are indications that the integrity of sterol containing membranes is not altered under the influence of natamycin. But these data have been acquired with artificial constructed membranes (Table 1). However, in one study natamycin was able to induce increased membrane permeability, but this was likely the result of liposomes containing abnormal high concentrations of sterols, on a molar basis, up to 50% (Teerlink *et al.*, 1980).

## Sterol-rich plasma membrane domains in fungi

Polyenes bind firmly to ergosterol which is a constituent of fungal membranes. Yet, in yeast mitochondrial outer membranes do not contain ergosterol, whereas vacuolar membranes have rather low levels with an ergosterol to phospholipid ratio (mol/mol) of 0.18 (Zinser *et al.*, 1991). The plasma membrane on the other hand contains high amounts of the sterol with a ratio of 3.31 (Zinser *et al.*, 1991). Sterols have long been regarded as mediators of membrane permeability or rigidity and fluidity. Sterol molecules can reduce membrane fluidity at high temperatures and are able to prevent membrane gelling at low temperatures (Haines, 2001). The fluid mosaic model of Singer and Nicholson (1972) has long provided the concept for understanding the organization of cellular membrane structures. It proposes that the lipid bilayer functions as a neutral two dimensional solvent with a mosaic pattern of proteins discontinuously embedded in or attached to the fluid lipid bilayer. However, novel insights in the structure of the membrane of the eukaryotic cell suggest that the plasma membrane is separated into different functional areas. Compared to intracellular membranes, the plasma membrane contains a high fraction of sterols and sphingolipids. The latter are only distributed in the outer leaflet whereas ethanolamine and phosphatidylserine are exclusively present in the inner leaflet (Kobayashi *et al.*, 1998). It is thought that sterols with their four-ring nucleus and aliphatic tail are able to cluster with sphingolipids, molecules that contain a ceramide and long saturated acyl chains. The resulting sterol-rich domains (SRDs) are in a liquid-ordered state forming so-called lipid rafts. Glycerophospholipids contain unsaturated acyl chains with a *cis* double bond, preventing tight packing with lipid rafts resulting in a liquid-disordered state in which lipid rafts can move (Brown and London, 2000). In yeast and filamentous fungi, SRDs have been observed that are much larger than lipid rafts are assumed to be (Alvarez *et al.*, 2007). Fungal SRDs range from 3 to 15 $\mu m^2$ while lipid rafts have recently

been estimated about 75 nm$^2$ (Edidin, 2003; Alvarez *et al.*, 2007). Staining with the sterol marker filipin showed SRDs at sites of mating projections in *Saccharomyces cerevisiae* and hyphae of *Candida albicans* (Proszynski *et al.*, 2006; Martin and Konopka, 2004; Bagnat and Simons, 2002). In rod-shaped cells of the fission yeast *Schizosaccharomyces pombe* a SRD was observed in a cell-cycle regulated manner in the medial zone at the future site of cell division (Wachtler *et al.*, 2003). SRDs were also detected at sites of polarized growth and septa in *Cryptococcus neoformans* and *Aspergillus nidulans* (Nichols *et al.*, 2004; Pearson *et al.*, 2004). The role of SRDs seems very divergent in fungi. In pathogenic fungi they could play a role in pathogenesis by mediating the presentation of GPI (glycosylphosphatidylinositol) anchored virulence factors and by altering the physical properties of the plasma membrane (Alvarez *et al.*, 2007). Furthermore, SRDs are essential for determining growth directionality in filamentous fungi by localizing cell end markers like TeaA and TeaR to the hyphal tip (Takeshita *et al.*, 2008). The role of SRDs in localizing proteins is still under debate. Valdez-Taubas and Pelham (2003) suggest efficient polarization of proteins destined to the tip by localized exocytosis and endocytic recycling. In this process, polarized membrane-bound proteins diffuse to equilibrium losing their polarized location. This is slowed down by the property of the sterol enriched plasma membrane. Subsequently, the membrane proteins are repolarized by endocytic uptake and polarized exocytosis at the tip (Valdez-Taubas and Pelham, 2003).

Over the past years it has become evident that in addition to proteinaceous factors, lipids, especially sterols, play an essential role in membrane trafficking processes including endocytosis (Kobayashi *et al.*, 1998). The importance of the structure of the sterols in the endocytic process is reflected in *S. cerevisiae*. Yeast *erg* mutants, deleted for enzymes in the ergosterol biosynthetic pathway are unable to synthesize ergosterol, but display distinct sets of other sterols in the membranes. In early steps of endocytosis the *erg2Δ* and *erg6Δ* single mutants and the *erg2Δerg6Δ* double mutant displayed inhibited internalization of the radiolabeled mating pheromone α-factor. The occurrence of particular sterols in each *erg* mutant and the desaturation of the B-ring of sterol molecules, especially at location C-5, 6 and C-7, 8 or at C-5, 6 and C-8, 9 were of importance for internalization during endocytosis (Munn *et al.*, 1999). Although sterols seem of importance for the endocytic process, it is not known if there is any relationship between endocytosis and SRDs.

## Aim and outline of this thesis

In this thesis the effect of the macrolide polyene antibiotic natamycin was studied in germinating conidia of the food spoilage fungi *A. niger* and *P. discolor*. Its effects were compared with two other polyene antibiotics nystatin and filipin.

In **Chapter 2**, the reliability of filipin as an ergosterol marker was confirmed. A revised method was developed to stain germinating conidia of *P. discolor*. This method was based on short filipin incubation time and cooling of the sample. Dormant conidia as well as spores during early germination did not stain with filipin. During late germination, however, a fluorescent cap at the presumptive location of germ tube appearance was observed. The intensity increased as the germ tube emerged. Staining with filipin correlated with the increase in ergosterol content in germinating conidia as was assessed with HPLC. Taken together, these results suggest an important role for ergosterol during the selection of the site of polarization.

In **Chapter 3** endocytosis was shown to occur in germinating conidia using confocal microscopy with the endocytic marker FM4-64. Natamycin inhibited early endocytosis of FM4-64. In contrast, nystatin and filipin disrupted the plasma membrane of germinating conidia as was shown by viability staining using TOTO-1 and electron spin resonance spectroscopy (ESR). These data suggest that natamycin unlike other polyenes does not form membrane-disrupting complexes with ergosterol, but acts as an inhibitor of ergosterol function by interfering with for instance membrane trafficking.

In **Chapter 4** the effect of natamycin was compared on waterborne and airborne conidia. Compared with air-dispersed conidia, conidia dispersed by water stained remarkably early with filipin. This coincided with a higher sensitivity for natamycin. Moreover, it was shown that waterborne spores have a lower intracellular viscosity as was measured with ESR. These data imply that dormant waterborne spores are in a more "germinated state" than dormant airborne spores. Data also show that natamycin is not a fungistatic polyene in case of all fungal survival structures.

The germination of conidia of *A. niger* in absence and presence of natamycin was studied by whole genome expression analysis (**Chapter 5**). This required a novel protocol for RNA extraction. The yield of total RNA was low in dormant conidia (8 μg/$10^8$ conidia) compared to later stages of germination (> 30 μg/$10^8$ conidia), but with approximately 35% of the genes present, the complexity of the mRNA was higher than at all the other stages. A high degree of 3341 differentially expressed genes was observed after 2 h of germination. Between 2 and 4 h the expression profile changed less intense to 692 differentially expressed genes which were dominated (15.6%) with genes that play a role in mitosis. Hardly any change (203 genes) in expression was observed between 4 and 6 h, whereas after 8 h novel transcripts related to transcription and protein synthesis were observed. Expression analysis of conidia kept at low and high concentrations of natamycin for 2 and 8 h showed that the polyene affects functions of the cell including sterol and lipid biosynthesis.

The data are summarized and discussed in **Chapter 6**.

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Chapter 2

# 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

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# ABSTRACT

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*, natamycin, 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 *ergΔ*-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*.

# 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 amphipatic 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/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 absorpts u.v. 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 (Drabikowski *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 *Arabidopsis* (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 (Bagnat and Simons, 2002). The dimorphic fungus *Candida albicans* can grow isotropically, but switches to polarised growth after induction. Filipin staining is primarily localized at the tips of germ tubes and mature hyphae and at the location of septum formation, but less intensive at yeast-like cells, buds or pseudohyphae (Martin and Konopka, 2004). In the filamentous fungus *Aspergillus nidulans*, the hyphal apex is stained by filipin as is the plasma membrane near forming septa (Li *et al.*, 2006). The hyphal apex is a highly dynamic area where different organelles act cooperatively to ensure polar growth. These include highly polar exocytosis (Seiler *et al.*, 1997) and sub-apical endocytosis (Fig. 5 from Fischer-Parton *et al.*, 2000). At the site of the hyphal dome large numbers of vesicles are fusing with the plasma membrane to deliver cell wall building blocks and excrete enzymes into the environment (Bartnicki-Garcia *et al.*, 1989).

The fluorescent patterns observed at growing zones and sites of cytokinesis are reported as evidence for the existence of membrane rafts. Lipid rafts are compositionally distinct structures within membranes. They are highly enriched in sterols and sphingolipids that create a biochemical microenvironment that might be capable of incorporating and excluding proteins (e.g. GPI-anchored proteins). Furthermore, several authors hypothesize that lipid rafts play an important role in vesicle transport, endocytosis and exocytosis (Parton and Richards, 2003; Saläun *et al.*, 2004). However, in yeast cells the existence of rafts is debated strongly. Bagnat and Simons (Bagnat and Simons, 2002) suggest that polarized

localization of proteins uses lipid rafts as a platform for membrane segregation. In contrast, Valdez-Taubas and Pelham (2003) state that slow lateral diffusion in the fungal membrane in combination with localized exocytosis and endocytic recycling can result in effects that otherwise can be attributed to rafts.

In this study we evaluate filipin staining after the use of a modified staining procedure of fungal cells. The model system used in this study are germinating conidia of the fungus *Penicillium discolor*. 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.

# MATERIALS AND METHODS

***Organisms and growth conditions***

*Penicillium 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 *Saccharomyces cerevisiae* strains that were defective 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 (*erg3Δerg6Δ*), and RH5228 (*erg2erg3Δ*) 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.

**Table 1.** *Saccharomyces cerevisiae* strains used in this study

| Strain | Genotype | Source |
|---|---|---|
| **RH448** | *MATa leu2 ura3 his4 lys2 bar1* | Heese-Peck *et al.* (2002) |
| **RH4213** | *MATa erg3Δ::LEU2 leu2 ura3 his4 lys2 bar1* | Heese-Peck *et al.* (2002) |
| **RH5225** | *MATa erg3Δ::LEU2 erg6Δ::LEU2 leu2 ura3 his4 lys2 bar1* | Heese-Peck *et al.* (2002) |
| **RH5228** | *MATa erg2Δ(end11)-1Δ::URA3 erg3Δ::LEU2 leu2 ura3 his4 lys2 bar1* | Heese-Peck *et al.* (2002) |

***Antimycotic susceptibility test***

Conidia of 10-12 days old cultures were harvested in 10 ml cold (4°C) ACES-buffer (10mM 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 were used to inoculate 96-well microtiter plates containing malt extract broth with filipin (Sigma) or natamycin (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 $10^4$ spores to a final volume of 100 µl. After 5 days at 25°C the minimal inhibitory concentration (MIC) was visually determined.

***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), 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.

***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 63x/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).

***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 sec 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 vital, fluorescent membrane dyes, conidia were incubated at RT with either 5 µM FM4-64 (Molecular Probes) or 5 µM FM1-43 (Molecular Probes) in ACES buffer for 1 min or 2 min, respectively. In 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 1 minute 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 100x/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 filterset #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 sec, for the FM dyes this was 300 msec). 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.

***Alkaline ergosterol extraction***

Conidia were harvested as described above and three independent liquid cultures in Erlenmeyers containing 200 ml MEB were inoculated with $10^7$ conidia/ml and incubated at 25°C (125 rpm). At hourly intervals between 0-9 h, 15 ml was collected from each of the three Erlenmeyers. The pooled suspensions were centrifuged (1100 x 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 (Bransonic, 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 sec, and centrifuged at 3200 x g (MSE, Mistral 400) for 10 min. The top (n-hexane) fraction was removed and the water fraction was shaken with 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 $C_{18}$ 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.

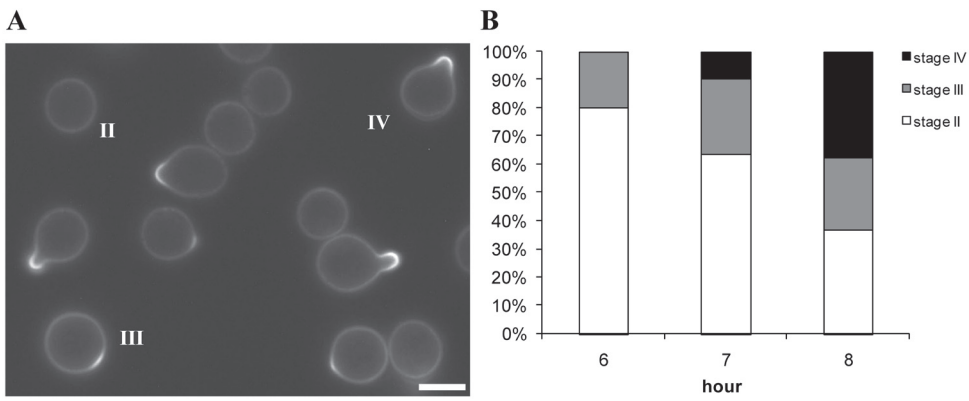



**Figure 1.** Polarization of membrane ergosterol at different stages during germination of conidia of *P. discolor*. **(A)** Conidia were stained with 15 µM filipin for 30 sec and then analyzed by fluorescence microscopy. After 8 h of germination stages II, III and IV can be discerned. Stage II; uniform 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 represents 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.

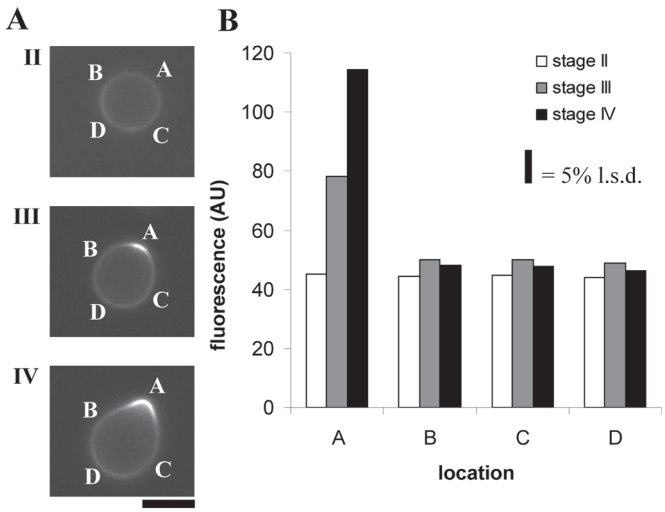



**Figure 2.** Filipin staining at different stages of germination of conidia. **(A)** Pixel intensities over line selections perpendicular to the membrane were determined at locations A-D at conidia during stage II-IV. Bar represents 5 µm. **(B)** Representation of the maximal fluorescence of the line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

# RESULTS

***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.

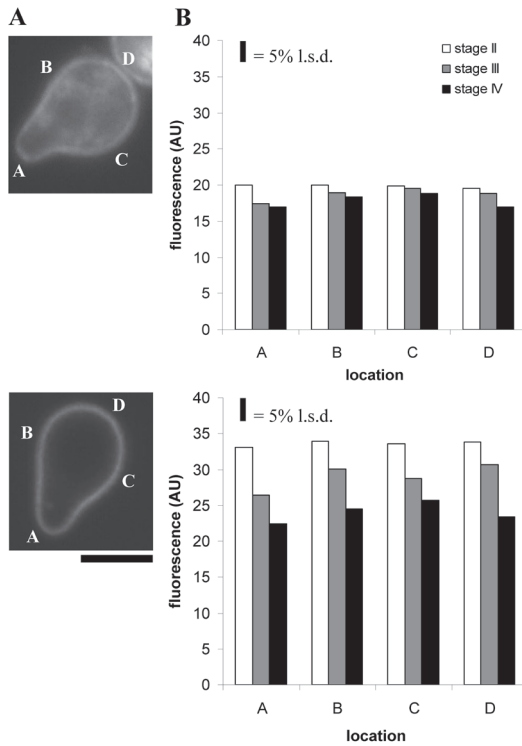



**Figure 3.** Staining of germinating conidia with the styryl dyes FM1-43 and FM4-64. Pixel intensities over line selections perpendicular to the membrane were determined at locations A-D. **(A)** Stage IV conidia stained with FM1-43 (top) and FM4-64 (bottom) for 1 and 2 min respectively. Both cells exhibit uniform membrane staining, while FM1-43 also stains the internal cell. Bar represents 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 represents 5% l.s.d., n = 20.

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.

***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 styryl 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

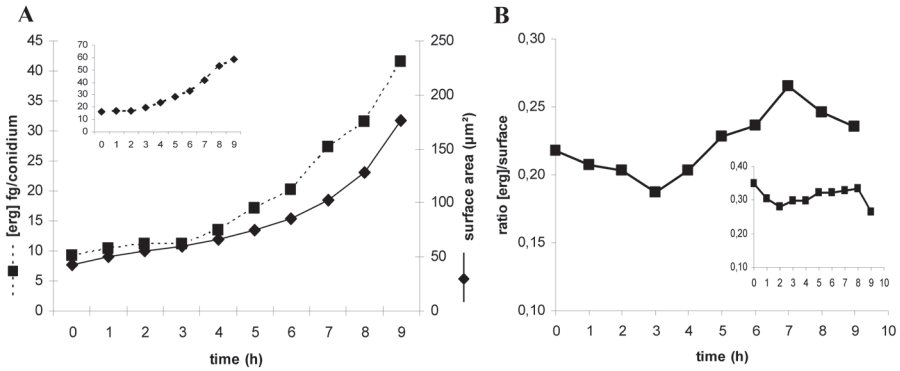



**Figure 4.** Ergosterol levels of conidia during germination of *P. discolor* CBS 112557. **(A)** Measurement of the amount of ergosterol present in conidia during germination plotted together with the surface area after several time intervals. The insert shows the ergosterol concentration of the related strain *P. discolor* CBS 271.97. **(B)** The ratio of the total ergosterol content and the surface area during germination. The insert show the ratio of ergosterol content and surface area of the related strain *P. discolor* CBS 271.97.

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, 3B). FM4-64 is regarded as a marker of endocytosis while it binds only to the outer leaflet of the plasma membrane (Dijksterhuis, 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).

***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 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 fourfold 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

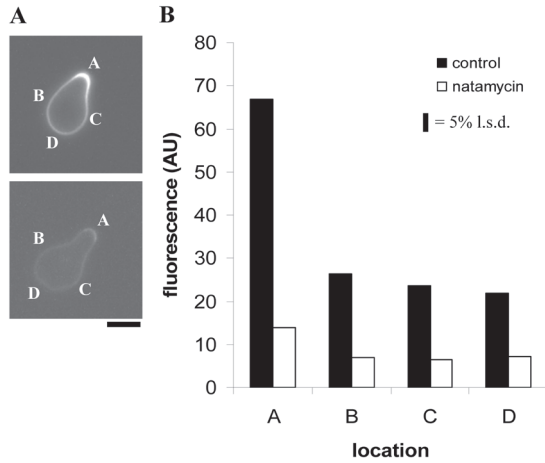


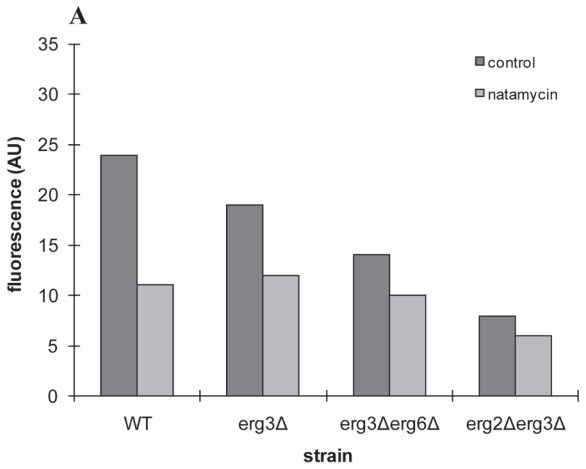

**Figure 5.** Pre-treatment of conidia with the antibiotic natamycin before filipin staining of the cells. Pixel intensities over line selections perpendicular to the membrane were determined at locations A-D. **(A)** Conidia during stage IV stained with filipin without (top) or with a pre-treatment with natamycin (bottom). Bar represents 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 represents 5% l.s.d., n = 30.

of the spore. After 7 h of incubation a decrease in the ratio of approx. 10% was observed, correlating with the appearance and outgrowth of germ tubes. The closely related *P. discolor* strain CBS 271.97, show a similar pattern as strain CBS 112557 suggesting a net increase of ergosterol over the conidial surface extension. To summarize, ergosterol levels correlate with filipin staining and indicate that this compound can be used to monitor increasing ergosterol levels in germinating conidia during stage II to stage IV.

***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 pretreatment 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 pretreatment 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




**B**

| strain | decrease (%) | q - q $_{\alpha, v, k}$ |
|---|---|---|
| WT | 54 | 21.9 |
| erg3Δ | 37 | 9.1 |
| erg3Δerg6Δ | 29 | 2.7 |
| erg2Δerg3Δ | 25 | -1.6 |

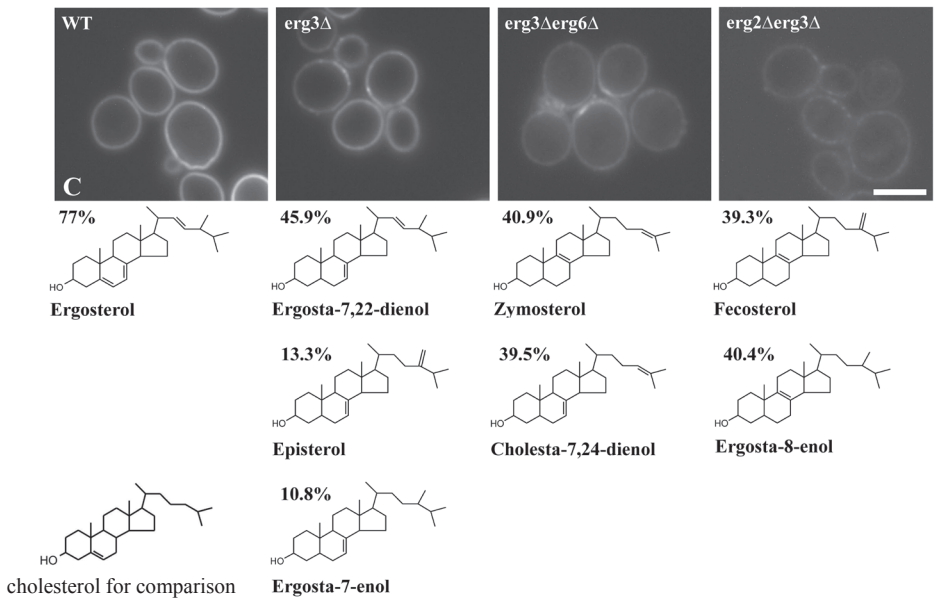



**Figure 6.** Pre-treatment of different yeast *erg* deletion mutants natamycin before filipin staining of the cells. **(A)** Fluorescence intensity of filipin stained yeast cells with (light bars) or without (dark bars) natamycin pre-treatment. **(B)** Statistical analysis by means of a Tukey-test (n = 85) shows significance of the decrease of fluorescence in wildtype and two mutant strains, except for *erg2Δerg3Δ*. Note the decline in fluorescence decreases among the mutants. **(C)** Sterol structure and filipin fluorescence of the plasma membrane of the *Saccharomyces cerevisiae* wildtype as well as three mutant strains RH448 (wildtype), RH4213 (*erg3Δ*), RH5225 (*erg3Δerg6Δ*), and RH5228 (*erg2Δerg3Δ*). Bar represents 5 µm.

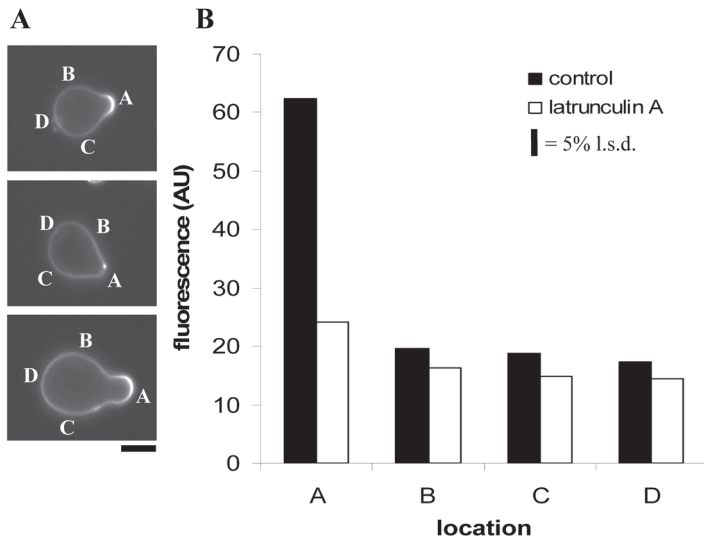



**Figure 7.** Effect of the actin-disrupting agent latrunculin A on filipin staining of germinating spores. Pixel intensities over line selections perpendicular to the membrane were determined at locations A-D. **(A)** Staining of conidia (stage IV) without (top) and after minimally 60 min incubation in latrunculin A containing medium (middle). Note the disappearance of the fluorescent cap. After 150 min recovery in MEB, the cap reappeared (bottom). Bar represents 5 µm. **(B)** Maximal fluorescence intensity of the plasma membrane at different locations. Note the significant decrease of cap fluorescence while the remainder of the membrane shows no decrease of staining intensity. Bar represents 5% l.s.d., n = 30.

of *Saccharomyces 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 intercellular 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 pretreated 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 wild type cells RH448 shows a 54% decrease of fluorescence. The *erg3Δ* mutant RH4213 and the *erg3Δerg6Δ* 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

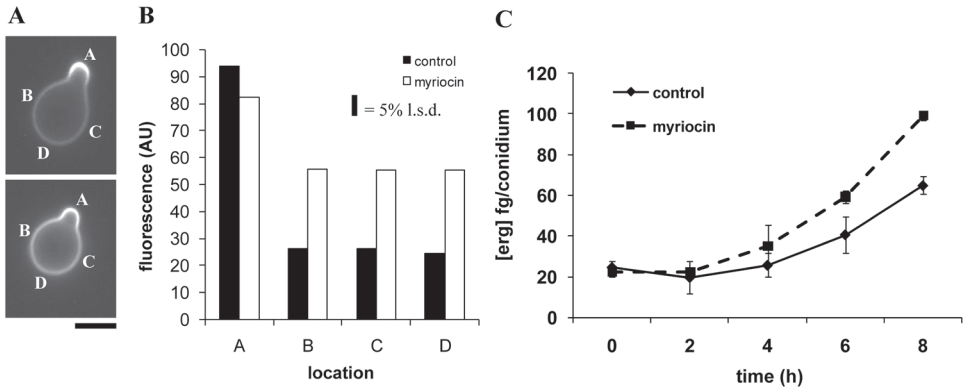



**Figure 8.** Changes in the fluorescence pattern of filipin-staining under influence of myriocin. Pixel intensities over line selections perpendicular to the membrane were determined at locations A-D. **(A)** Filipin staining of a conidium that has germinated in a myriocin-containing medium (bottom) compared to an untreated conidium. Bar represents 5 µm. **(B)** Maximal fluorescence at the plasma membrane. Note a significant decrease of the fluorescence of the cap while the rest of the membrane exhibits a strong increase of fluorescence. Bar represents 5% l.s.d., n = 50. **(C)** Ergosterol content assessed by HPLC of conidia germinated in the presence of myriocin or not. Note the higher ergosterol levels in treated cells. Error bars indicate standard deviations.

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 pretreatment 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.

***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 localisation 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). Figure 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 h 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.

# 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). Russell *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) has 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 levels 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 subapical 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. roqueforti, A. niger, Trichoderma harzianum*) took up FITC-dextran molecules as large as 150 kDa (Brul *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 ($^{14}C$) 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 wild type *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 > RH4213 > RH5225 > RH5228. Natamycin sensitivity was highest in case of the wild type 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 effectivity 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 *in situ* is the $sp^2$ 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 $\mu m^2$. 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 $g/cm^2$ (in *Penicillium chrysogenum*; Hillenga *et al.*, 1994). This would lead to a weight of the plasma membrane of one cell of approx. 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 $\mu m^2$, Fig. 4.) was observed that takes 21/5 = 4.3 fg ergosterol/ cell while the rest of the ergosterol (approx. 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 $2\pi$h(of cap)r(of the basis of the cap area]. The weight of such an area in a cell with a total surface of 100 $\mu m^2$ is approx. 53 fg (see 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 ($L_o$). These lipid associations are laterally mobile in the liquid-disordered ($L_d$) "solvent" of largely unsaturated phospholipids. This phase segregation results in the formation of micro-domains 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 also visible

in Fig. 4b of the study of Martin and Konopka (2004) in case of filamentous growth of the yeast *Candida albicans*. It is tantalizing to observe that the germinating spore seems to compensate the lack of domain formation by overproduction of ergosterol in the plasma membrane and that this is still resulting in functional cap region and germ tube formation.

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Chapter 3

# The polyene antibiotics nystatin and filipin disrupt the plasma membrane, whereas natamycin inhibits endocytosis in germinating conidia of *Penicillium discolor*

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# ABSTRACT

The differences in membrane permeability and the effect on endocytosis of the polyene antibiotics nystatin, filipin and natamycin on germinating fungal conidia were investigated. The model system was *Penicillium discolor*, a food spoilage fungus. Filipin resulted in permeabilization of germinating conidia for the fluorescent probes TOTO-1 and FM4-64, but not for ferricyanide ions. Nystatin caused influx of all these compounds while natamycin did not. Untreated germinating conidia internalize the endocytic marker FM4-64. Pre-treatment of germinating conidia with natamycin showed a dose and time dependent inhibition of endocytosis as judged by the lack of formation of early endosomal compartments. The results obtained from this study indicated that, unlike nystatin and filipin, natamycin is unable to permeabilize germinating conidia but interferes with endocytosis in a dose and time dependent manner. Natamycin acts via a different mode of action than other polyene antibiotics. These results offer useful information for new strategies to prevent fungal spoilage on food products and infection on agricultural crops. For laboratory use, natamycin can be used as a specific inhibitor of early endocytosis in fungal cells.

# INTRODUCTION



Fungi are a cause of enormous food losses due to food spoilage or crop infection. Furthermore, fungi pose a significant threat to immune-compromised individuals. A number of antifungal compounds are in use in food and medical applications to prevent fungal development. These compounds include weak-organic acids, azole derivatives, fluorocytes, allylamines and polyenes (Brul and Coote, 1999; Ghannoum and Rice, 1999). Polyene antibiotics like amphotericin B, nystatin, filipin and natamycin are characterized by the possession of a closed macrocyclic ring that contains a series of conjugated double bonds on one side of the molecule and a number of hydroxyls on the opposite side. Often, a mycosamine group is present in combination with a carboxyl moiety, conferring amphoteric properties to these molecules (Hamilton-Miller 1973; Bolard 1986). In the past, convincing evidence has been presented that the effect of polyenes is as a result of the binding of these compounds to sterol molecules in the cellular membranes (De Kruijff and Demel 1974a). Large polyenes like amphotericin B and nystatin interact in a hydrophobic manner with sterols to form a half pore (Baginski *et al*., 1997). The combination of two half-pores builds up a membrane spanning aqueous pore that alters the selective permeability of the lipid bilayer. Filipin, a fluorescent dye of sterols, is unlikely to form membrane spanning pores due to its short length, the absence of a charged carboxyl group and a bulky mycosamine. Instead, filipin seems to form a planar complex with sterols (De Kruijff and Demel 1974a) and two of these planar aggregates associate with their hydrophilic sides to form a double 'sandwich' like structure. The entire complex is thought to be embedded within the hydrophobic core of the lipid bilayer, which results in membrane fragmentation and cellular leakage (De Kruijff and Demel 1974a; De Kruijff *et al*., 1974b). Natamycin (pimaricin) is a polyene produced by the filamentous bacterium *Streptomyces natalensis* (Struyk *et al.,* 1957-1958). It has been used for the treatment of superficial fungal infection in medical practice and to prevent fungal spoilage of food products (Stark, 2007). Surprisingly, the mode of action of this antifungal agent is not known and was only recently addressed (Te Welscher *et al.,* 2008). Te Welscher *et al*. (2008) studied model membrane systems and yeast cells and concluded that natamycin binds specifically to ergosterol. In contrast to nystatin and filipin, natamycin did not change the permeability of the yeast plasma membrane nor did it change the permeability of the lipid bilayer from ergosterol-containing model membranes. These results indicate that natamycin acts via a novel mode of action and blocks fungal growth by binding specifically to ergosterol.

Polyene antibiotics have been studied extensively by making use of model-membrane systems and yeast cells (Kotler-Brajtburg *et al*., 1979; Teerlink *et al*., 1980; Te Welscher *et al*., 2008). We addressed for the first time the effect of natamycin in a filamentous fungus by employing *Penicillium discolor* as a model system (Frisvad *et al*., 1997). *Penicillium*

*discolor* is a well-known contaminant of cheese where it forms numerous mildly stress-resistant, dormant conidia that colonize the surface of the product (Stark 2007). Conidial germination is characterized by an initial stage of "swelling" otherwise known as isotropic growth (Momany, 2002). Subsequently, one area of the spore membrane and wall is selected for cell wall extension and outgrowth of the germ tube (Chapter 2). This represents the shift from isotropic to polarized growth. Interestingly, dormant conidia of *Aspergillus fumigatus* are insensitive to the polyene amphotericin, but rapidly become sensitive during the initial stages of germination (Russell *et al*., 1975). We used germinating conidia of *Penicillium discolor* as a model system to determine the effect of polyene antibiotics on membrane permeability and the early stages of endocytosis. Our results indicate permeabilization of the membrane by nystatin and filipin. However, no membrane permeabilization was observed when conidia were treated with natamycin. Instead, natamycin was found to inhibit endocytosis, the active uptake of membrane vesicles into the cell.

# MATERIALS AND METHODS

***Organisms and growth conditions***

*Penicillium discolor* CBS112557 (Frisvad *et al*., 1997) was maintained as a conidial suspension in 17% glycerol at -20°C. Conidia were inoculated onto Malt Extract Agar (MEA; Oxoid, Hampshire, UK) and grown for 10-12 days at 25°C and subsequently harvested in 10 ml ice-cold ACES-buffer [10mM ACES (Sigma), 0.02% Tween-80, pH 6.8]. The colony surface was gently rubbed with a sterile Drigalski spatula and the conidial suspension was filtered through sterile glass wool. Conidia were washed in cold ACES-buffer and resuspended in Malt Extract Broth (MEB; Oxoid, Hampshire, UK) and kept on ice before further processing on the same day.

***Minimal inhibitory concentration assay***

The Minimal Inhibitory Concentration (MIC) of polyene antibiotics was assessed at 25°C. Filipin (Sigma), nystatin (Sigma) and natamycin (DSM, Delft, The Netherlands) were added to MEB in 96-well microtiter plates. Filipin and nystatin were dissolved in 100% DMSO, and natamycin in 85% DMSO (Brik, 1981). A 10 mM stock solution of each antibiotic was used for stepwise dilution. Therefore, 8 independent dilutions ranging from 30–100 μM (well A1 contained 30 μM and well B1 40 μM etc.) were added to the first column of the 96-well microtiter plate. Following this, a 1:1 dilution series was prepared and final concentrations ranged from 0.059–100 μM. Conidia were harvested and resuspended in MEB. Each well was inoculated with $10^4$ spores to a final volume of 100 μl. After 5 days at 25°C the minimal inhibitory concentration (MIC) was determined visually. The MIC measurements

were performed in duplicate or triplicate. Both freshly harvested conidia and cells that had germinated for 6 h were assayed.

***Conidial immobilization***

Conidia were immobilized on glass coverslips coated with poly-L-lysine (Sigma) for optimal microscopic resolution and for the possibility to remove solutions from around the cells. For coating with poly-L-lysine, microscope coverslips were washed with commercially available detergent, followed by two wash-steps with 1 M HCl, and 96% ethanol, for 5 min respectively. Subsequently, coverslips were coated with 0.01% (w/v) poly-L-lysine in distilled water for 5 min. The coverslips were rinsed with distilled water and air dried with filtered compressed air. Conidia (≈ 1.5 x $10^7$ $ml^{-1}$) were inoculated on the coated coverslips and allowed to germinate at 25°C in a humid enclosed environment. Following 6 h of germination, conidia were treated with polyene antibiotic containing solutions for 10, 30 and 60 min at concentrations of 2x, 5x and 10x MIC.



***Microscopy and fluorescent staining***

*Fluorescent Microscopy:* Germinating immobilized conidia were treated with polyene antibiotics and subsequently with 10 µM of FM4-64 (Molecular Probes, Breda, The Netherlands) for 2 min in 100 µl MEB, followed by a wash step with MEB. Conidia were subsequently studied with standard fluorescence microscopy (FM) or confocal laser scanning microscopy (CLSM). Viability staining (Thanh *et al*., 2007) was undertaken with 2 µM TOTO-1 (Molecular Probes, Breda, The Netherlands) in 100 µl MEB at 25ºC for 30 min. Following dye removal, glass coverslips with the immobilized conidia were placed upside-down onto a thin (< 0.5 mm) layer of 2% agar. Any remaining liquid was removed with filter paper. Images were taken with a Zeiss Axioplan II microscope (Zeiss, Sliedrecht, The Netherlands), Zeiss Plan-ApoChromat 100x/1.4 oil objective, a green BP510-560 (FT580, LP590) excitation filter for FM4-64, and a blue BP450-490 (FT510, LP520) excitation filter for TOTO-1. Images were captured with a Zeiss AxioCam MRc digital camera run by Zeiss AxioVision 4 (Zeiss, Sliedrecht, The Netherlands) and a shutter system (Ludl Electronic Products Ltd., Hawthorne, NY, USA).

*Confocal Laser Scanning Microscopy*: For staining of conidia with FM4-64, 40 µl of MEB with 10 µM FM4-64 was added to the top of a thin layer of 2% agar, placed on a glass microscope slide. Immobilized conidia on coated coverslips were mounted, upside-down, on top of this layer of agar. Excess MEB containing FM4-64 was removed with filter paper. Conidia were scanned immediately after staining using a Leica TCS NT (Leica, Mannheim, Germany). FM4-64 was excited using the 488/568 nm laser line of the Ar/Kr laser and a LP590 emission filter setting. Laser intensity was kept to a minimum to reduce photobleaching. Projections were obtained from a z-stack of 16 sections, each one an average

of 2 images. Images of 512 x 512 pixels were captured at a 63x magnification using an electronic zoom of 4 times. Conidial fluorescence was measured as the pixel intensities over the selections as indicated in the figures. Images were converted to grayscale with Adobe Photoshop CS2 and analyzed using the public available ImageJ software (http://rsb.info.nih.gov/nih-image). The results indicated 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).

*Inhibitor treatments*: Twenty µl of sodium azide (Sigma-Aldrich) was added to the 100 µl inoculum on glass coverslips, at a final concentration of 10 mM or 55 mM. After three or 15 min, the medium was replaced by FM4-64 containing the matching concentration of sodium azide.

***ESR spectroscopy***

Spin label electron spin resonance spectroscopy (ESR) can be utilized as an approach to measure cytoplasmic volume and permeability of plasma membranes (Miller and Barran 1977; Miller 1978). The (amphipathic) spin-label TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) (Sigma, St Louis, USA) can enter germinating conidia and reside in both lipid (membranes and lipid droplets inside the spore) and aqueous environments and can be measured by an ESR spectrometer. Potassium ferricyanide $K_3Fe(CN)_6$ cannot penetrate intact membranes and removes ("broadens") the signal of TEMPO outside the spores. Conidia were allowed to germinate in MEB at a density of 5 x $10^7$ spores $ml^{-1}$ in Eppendorf tubes and heated at 25ºC in a 16500 Thermolyne dri-bath (Beun de Ronde, Abcoude, The Netherlands). After 6 h, polyene antibiotics were added to the suspension to obtain a 5x MIC concentration (10.0, 18.0, and 7.0 µM for natamycin, nystatin or filipin, respectively) and were used for ESR measurements after time intervals of 10, 30, or 60 min. Samples were centrifuged at 8000 rpm for 2 min and the conidia were resuspended in 25 µl TEMPO-FC mixture (1 mM and 120 mM, respectively) and transferred to a glass capillary (outer diameter: 2 mm) with a

**Table 1.** Minimal Inhibitory Concentrations (MIC) of natamycin, nystatin, and filipin under various conditions.

| Conidial condition | T (°C) | Natamycin (µM) | Nystatin (µM) | Filipin (µM) |
|---|---|---|---|---|
| Conidia directly from plate | 25 | 2.0 ± 0.8 | 3.6 ± 0.8 | 1.4 ± 0.7 |
| Conidia after 6h of germination | 25 | 2.7 ± 0.6 | 4.1 ± 0.9 | 0.7 ± 0.1 |

Hamilton syringe. Conidia in the capillary were centrifuged at 4000 rpm for 2 min, and the supernatant removed. Subsequently, the capillary was placed in the quartz tube for spectra recording. ESR spectra were recorded at room temperature in an X-band ESR spectrometer 300E (Bruker Analytik, Rheinstetten, Germany). The modulation amplitude was 0.5 gauss, the microwave power was 5 mW, and the sweep range was 100 G.



# RESULTS

***The effect of polyene antibiotics on the plasma membrane of germinating conidia***

The biological activity of the different polyene antibiotics natamycin, nystatin, and filipin against conidia was assessed by means of measuring the minimal inhibitory concentration (MIC). The MIC of dormant conidia, harvested directly from agar plates was within the micromolar region being 2.0 ± 0.8, 3.6 ± 0.8 and 1.4 ± 0.7 μM, for natamycin, nystatin and filipin respectively. After 6 h of germination, the activity of polyenes was within a similar range (Table 1). An important characteristic of cell death is the loss of plasma membrane integrity and the fluorescent marker TOTO-1 has been used as a reliable viability marker of fungal spores (Chitarra *et al*., 2005; Thanh *et al*., 2007). TOTO-1 is a plasma membrane impermeable cyanine dimer with an affinity to nucleic acids including the DNA of the nucleus and RNA. As such, the dye reports membrane disruption and killed cells exhibit bright internal staining that includes the nuclei. Conidia incubated with 10x the MIC for 60 min show 87% and 88% stained cells after nystatin and filipin treatment respectively. However, only 2.6% of the natamycin-incubated conidia were stained and no fluorescence was observed with untreated cells. The effect of filipin and nystatin showed dose and time dependent characteristics as judged from the number of stained cells (Fig. 1B). Nystatin reaches high killing rates especially after longer treatment. Filipin shows more concentration-dependent effects where the number of killed cells increases with concentration (Fig. 1B). Accurate measurements of conidial size during treatment are shown in figure 1C. Control spores exhibited an increase in size (swelling) of 17% during 1 h of measurement. All polyene-treatments showed effects on spore size, but the 2x MIC treatment with natamycin did not prevent swelling of the spore within 30 min while after 60 min the surface area of the spores was 91% of the size of the control spores. The other natamycin treatments resulted in smaller spores with respect to dose and duration of treatment. At 10x MIC for 60 min, conidia were 60% of the size of the control. The effects of filipin and nystatin were more dramatic. Compared to the size of the control spores, filipin and nystatin treated cells were 58% and 54% after 60 min at 2x MIC, and 61% and 52% after 60 min at 10x MIC respectively. With respect to the size of the spores, natamycin had a more dose dependent effect while filipin and nystatin reached maximal effects at lower concentrations.

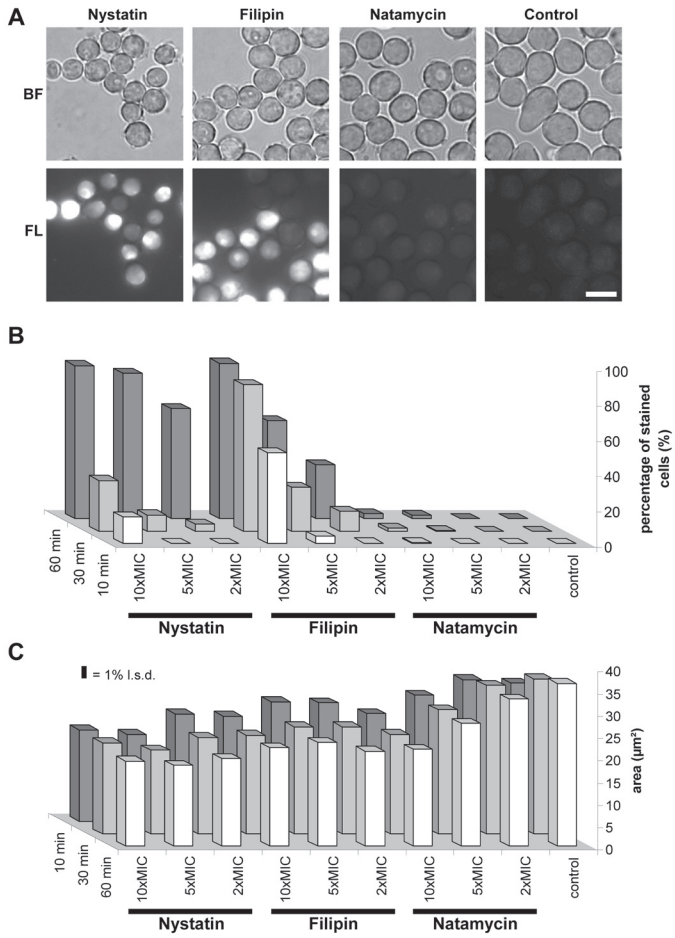


**Figure 1.** TOTO-1 staining of conidia of *P. discolor* incubated with nystatin, filipin, and natamycin. **(A)** Fluorescence microscopy of conidia treated for 60 min with 5x MIC nystatin, filipin, and natamycin. Bar represents 5 µm. **(B)** The percentage of TOTO-1 positive stained conidia after treatment with 2x, 5x, and 10x MIC of nystatin, filipin or natamycin for 10, 30, and 60 min. n = 100. **(C)** Size of germinating conidia after polyene treatment. Bar represents 1% l.s.d., n = 100.

The nitroxide TEMPO was utilized as a spin probe to determine membrane permeability. The spectrum of TEMPO in water is isotropic and consists of three equidistant narrow resonance lines, typical for freely rotating nitroxide radicals. Addition of paramagnetic ions $Fe(CN)_6^{-3}$ (FC) causes removal (broadening) of the TEMPO spectrum, due to spin-spin interactions between spin probe and paramagnetic ions. TEMPO easily penetrates cells due to its amphiphilic nature and relatively small size, while the charged FC ions cannot penetrate through intact membranes. ESR spectrum of conidia consists of broad and narrow components.

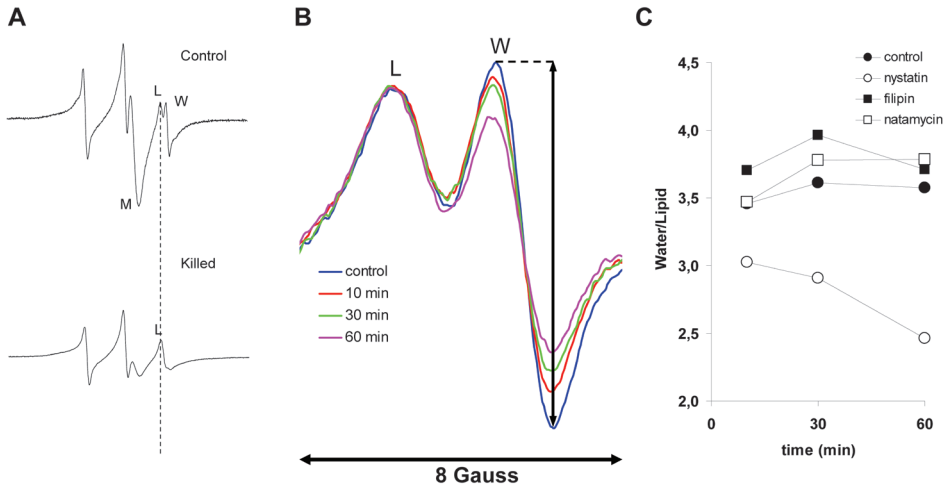


**Figure 2.** Electron Spin Resonance (ESR) on conidia of *P. discolor*. **(A)** Conidia of *P. discolor* contain melanin, which is paramagnetic and therefore has its own ESR signal (M-peak). The L-peak and W-peak, originates from TEMPO residing in a lipid or aqueous environment, respectively. Killed cells results in membrane disruption and the disappearance of the W-peak. **(B)** The high-field region of TEMPO spectra from conidia incubated with 5x MIC nystatin for 0, 10, 30, and 60 min. The spectra were fitted in peak amplitude and peak position of the lipid component (L). The decrease of the amplitude of the W-peak as duration of the treatment proceeds indicates the gradual loss of membrane integrity. **(C)** The ratio of the W- and L-peak gives an indication of membrane disruption. Nystatin-treated conidia show gradual membrane disruption; while the control, natamycin and filipin treated conidia do not show signs of membrane integrity loss.

The broad component, which is almost invisible, originates from TEMPO molecules outside the cell. The narrow components have an intracellular origin and are the superposition of two triplets. One triplet originates from TEMPO molecules in the aqueous cytoplasm (W-peak), while the second triplet represents the hydrophobic lipid bodies and membranes (L-peak). Different splitting constants (the distance between lines) and different g-value (position of central line) for hydrophobic and hydrophilic environments allow the resolution of the two triplets in the high-field (right-hand) region of the spectrum. In case of membrane disruption FC ions can penetrate cells and broaden the intracellular signal originating from TEMPO in the aqueous cytoplasm. The signal of TEMPO in the hydrophobic environment will not be broadened because of inability of charged FC ions to partition into the lipid phase.

*Penicillium* spore cell walls contain melanin, which is paramagnetic and thus has its own ESR signal (singlet with g-factor of 2.004; Dijksterhuis *et al*., 2007), designated as the M-peak. Within the ESR spectrum the L-peak and W-peak can be discerned, originating from TEMPO residing in a lipid or aqueous microenvironment, respectively (Fig. 2A). When cells are killed by heating (70°C, 5 min), gross disruption of the membrane occurs and ferricyanide enters the cell and broadens the aqueous signal (visible as the disappearance of

the W-peak), but not the lipid signal, which is clearly visible in the spectrum as the L-peak that remains narrow (Fig. 2A). Because the spectrum of the sample is the superposition of the spectra originated from all individual spores, the proportion between lipid (L) and water (W) peaks can be used to estimate the extent of membrane damage in spores due to different treatments. In this case, the lipid signal is proportional to the total number of spores, while the water signal is proportional to the total number of spores with FC impermeant membranes, a measure of membrane integrity. During treatment with nystatin, the W-peak was consistently lowered (Fig. 2B). These effects did not occur in case of filipin, natamycin and control cells (Fig. 2C).

Taken together, these data indicate that the immediate effects of the three polyene antibiotics on germinating conidia differ. Filipin and nystatin disrupt the membrane of germinating conidia to a different extent whereas natamycin does not.

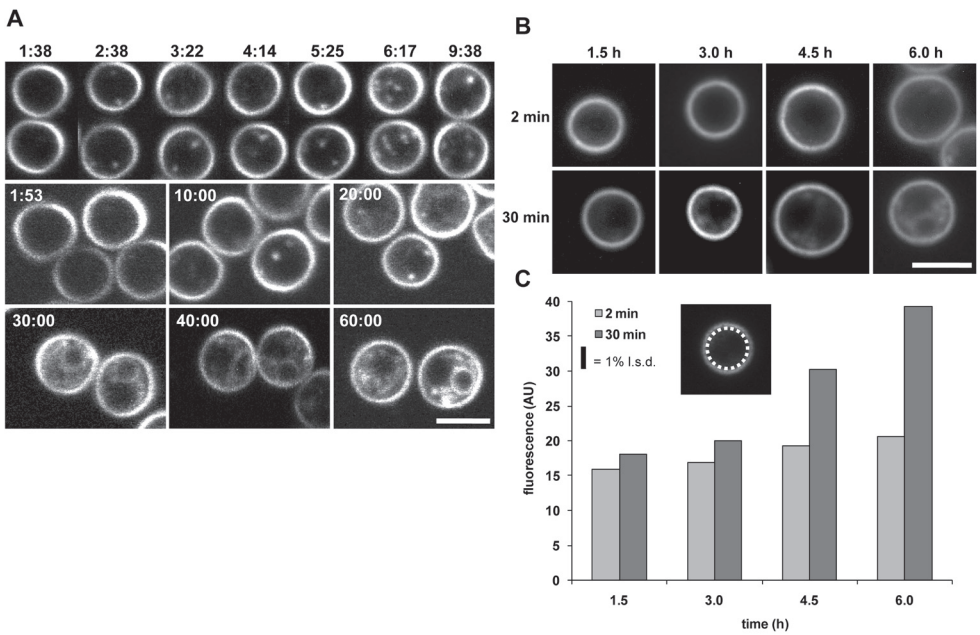


**Figure 3.** Characterization of endocytosis during conidial germination of *P. discolor*. **(A)** Distribution of FM4-64 was followed with Confocal Laser Scanning Microscopy (CLSM) and clearly showed the presence of endocytosis. Top panel indicates the formation of putative endosomal compartments during the first 10 min of staining. Bar represents 5 µm. Bottom panel illustrates the endocytotic route of germinating conidia. The membranes of vacuoles are visible after longer staining times. Bar represents 5 µm. **(B)** Fluorescence Microscopy (FM) micrographs of FM4-64 stained conidia at different stages of germination. Pictures were taken after 2 and 30 min post-staining and show an increase of internal fluorescence. Bar represents 5 µm. **(C)** Histogram represents the intracellular fluorescence of germinating conidia, 2 and 30 min after FM4-64 staining. Bar represents 1% l.s.d., n = 40.

***Natamycin affects endocytosis during isotropic growth of conidia***

Natamycin does not show any sign of disruption of the plasma membrane, but has a biological activity similar to nystatin and filipin at micromolar concentrations. In published literature on yeast biology it has been suggested that ergosterol plays a role in endocytosis (Munn *et al.*, 1999; Heese-Peck *et al.*, 2002). Therefore we evaluated to which extent endocytosis occurs in germinating conidia and assessed the effect of polyene antibiotics. Figure 3A shows conidia during the stage of isotropic expansion after 6 h of germination, stained with FM4-64 as visualized by CLSM. The staining pattern indicates that endocytosis occurs in these cells up to the staining of vacuolar membranes as is observed in fungal hyphae and spores of *Magnaporthe grisea* (Fischer-Parton *et al.*, 2000; Atkinson *et al.*, 2002). Immediately after addition of FM4-64, bright fluorescence was associated with the conidial membrane. Within 2 min, faint fluorescent punctuate structures (0.4 ± 0.03 μm; n = 5) were observed in the cytoplasm. Larger ovoid-to-spherical compartments, 0.6 ± 0.08 μm (n = 27), were discernable after 4-7 min throughout the cytoplasm (Fig. 3A). Stained vacuolar membranes (2.0 ± 0.14 μm; n = 39) could be discerned as early as 25-30 min following dye application. Notably, these vacuoles tended to be in contact, or in close proximity, with the cellular plasma membrane. Figure 3B shows staining of isotropically growing conidia during different stages of germination. This was quantified by the measurement of the internal fluorescent intensity of the conidium after 2 and 30 min of staining (Fig. 3C, inset). There was no significant increase in fluorescence inside early germinating conidia (1.5 and 3 h), but after 4.5 and 6 h a large increase was seen. The ratio between 30 and 2 min after FM4-64 addition was 1.14, 1.19, 1.57, and 1.91 for the 1.5, 3.0, 4.5, and 6.0 h stages, respectively. This indicates that conidia start with endocytosis after 3 h of germination.

To determine if the endocytic process is affected by polyene antibiotics, conidia that had germinated for 6 h were incubated for 10, 30, and 60 min in the presence of filipin, nystatin or natamycin using concentrations of 2x, 5x, and 10x the MIC. Subsequently the conidia were stained with FM4-64 and images were taken after 10 min of staining. After a 2x or 5x MIC treatment with nystatin, some endosomal compartments were visible. At higher concentrations or longer treatments, conidia stained intensively over and above the staining of the initial cells (Fig. 4A). The staining patterns after filipin incubation had very similar features as nystatin-treated cells, albeit visible at shorter treatment times (Fig. 4B). The intense staining indicates that FM4-64 had freely entered the cell and that the permeability of the plasma membranes was lost. Thus, quantification of the internalization of the dye and thus endocytosis was not possible.

Natamycin-treated conidia did not exhibit intracellular staining, but showed a dose and time dependent decrease in the number of endosomal compartments after 10 min of staining with FM4-64. A treatment of 10x MIC for 30 and 60 min and with 2x and 5x MIC for 60 min showed no, or very low numbers of endosomal compartments (Fig. 4C).

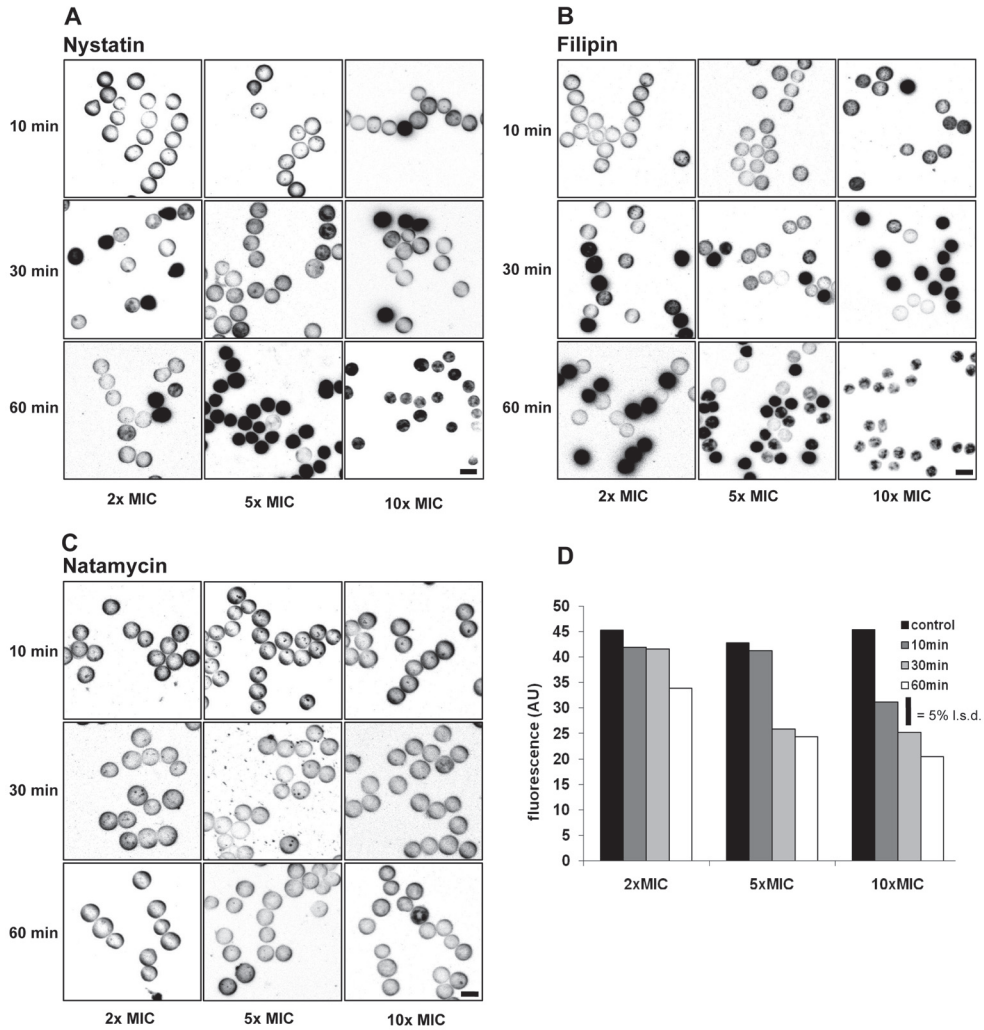


**Figure 4.** Confocal images of conidia incubated for 10, 30, and 60 min with 2x, 5x, and 10x MIC nystatin, filipin or natamycin. **(A)** Conidia incubated with nystatin were stained with FM4-64. Pictures were taken 10 min post staining. The last micrograph is underexposed and shows conidia that would normally be visible as fully fluorescent spheres, as seen with the former micrographs. Bar represents 5 µm. **(B)** Similar to (A) but incubated with filipin. Bar represents 5 µm. **(C)** Conidia incubated with natamycin and stained with FM4-64. Bar represents 5 µm. **(D)** Fluorescent micrographs of conidia stained for 10, 30, and 60 min with 2x, 5x, and 10x MIC natamycin were analyzed. The histogram represents the intracellular fluorescence of the conidia under influence of natamycin. Bar represents 5% l.s.d., n = 50.

The fluorescence intensity inside the conidia was quantified and a significant decrease was observed after 60 min treatment at 2x MIC, a 30 and 60 min treatment at 5x MIC and at all 10x MIC treatments (Fig. 4D). Taken together, these data show that the early stage of endocytosis in germinating conidia is inhibited in a dose and time dependent manner.



# DISCUSSION

The mode of action of natamycin has only recently addressed by Te Welscher *et al.* (2008) using membrane modelsystems and yeast cells. In this study, it became clear that natamycin binds very specifically to ergosterol, but different techniques provide evidence that membranes do not become permeable in contrast to other polyenes, filipin and nystatin, that cause leakage. We show for the first time that filipin and nystatin disrupt the conidial plasma membrane of the filamentous fungus *Penicillium discolor*, whereas natamycin does not permeabilize germinating conidia, but interferes with endocytosis in a dose and time dependent manner. Natamycin binds to ergosterol and has a biological activity in the micromolar range like filipin and nystatin. A pre-treatment with natamycin can effectively decrease the fluorescence of filipin binding to germinating conidia of *P. discolor*. This indicates that both compounds bind to ergosterol in these cells (Chapter 2). Russell *et al.*, (1975; 1977) have shown that freshly harvested conidia of *Aspergillus fumigatus* were insensitive to amphotericin B, but became sensitive during the early swelling stage of germination and later (Russell *et al*., 1975). Sensitivity towards polyenes is manifested by the inhibition of conidial swelling, phase darkening and germ tube formation. In our study, filipin and nystatin disrupt the plasma membrane of germinating spores during swelling, as judged by TOTO-1 staining, influx of FM4-64 and FC-ions. Natamycin does not show any evidence for permeabilization of the membrane with these techniques.

Polyenes have been tested on liposomes, LUV (Large unilamellar vesicles), spherules, the mycoplasma *Acholeplasma laidlawii* and the yeast *Saccharomyces cerevisiae* (De Kruijff *et al.*, 1974a, 1974b; Te Welscher *et al.*, 2008). These studies showed that different ions ($K^+$, $CrO_4^{2-}$, $H_2PO_4^-$) and other molecules ($NADP^+$, glucose-6-phosphate) were released after filipin treatment. Amphotericin B and nystatin did not cause release of divalent ions ($Ca^{2+}$, $Mg^{2+}$), but displayed a quicker $K^+$ leakage than filipin (De Kruijff *et al*., 1974b). Monovalent ions permeate the altered membrane in a rate dependent on the size of the molecule in the case of amphotericin B and nystatin (De Kruijff *et al*., 1974b). In conidia of *P. discolor,* filipin is not able to cause FC leakage into conidia within 1 h, while nystatin does. As these ions are bulky and charged this indicates a difference between the disruptions caused by filipin compared to the pores that nystatin forms with ergosterol. All data suggest that filipin and nystatin (and the related amphotericin B) bind to ergosterol and form a membrane disrupting

complex. Natamycin does not form such complexes, but inhibits the functioning of ergosterol in the cell.

Over the past several years evidence has accumulated which indicates ergosterol has multiple functions in fungal cells, including endocytosis, vacuole fusion, polarity, and morphogenesis (Kato and Wickner 2001; Heese-Peck *et al*., 2002; Mysyakina and Funtikova 2007; Takeshita *et al*., 2008). The *S. cerevisiae*, *erg* mutants are unable to synthesize ergosterol and display distinct sets of different sterols in the cell. Sterols structurally different from ergosterol are unable to support internalization at the initial stages of endocytosis (D'Hondt *et al*., 2000). Depending on double bonds within the sterol structure, internalization of the α-factor mating pheromone during endocytosis was markedly disrupted (Munn *et al*., 1999; Heese-Peck *et al*., 2002). Neither hyperphosphorylation nor ubiquitination of the α-factor receptor Ste2p was observed in these *ergΔ* cells, which is a prerequisite for internalization. Moreover, fluid phase uptake was strongly reduced in yet another combination of mutants (Heese-Peck *et al*., 2002). In our study the kinetics of staining of components of the endomembrane system of germinating conidia of *P. discolor* was similar to conidia of *Magnaporthe grisea* (Atkinson *et al*., 2002). Upon addition of the dye, diffuse fluorescence was visible in the cytoplasm and beneath the plasma membrane followed by brightly stained ovoid-to-spherical structures that were mobile in the cell. These structures corresponded in size to the putative endosomes visualized by FM4-64 staining in *Saccharomyces cerevisiae*, *Neurospora crassa*, and *Ustilago maydis* (Vida and Emr 1995; Wedlich-Söldner *et al*., 1995; Fisher-Parton *et al*., 2000; Fuchs *et al*., 2006). Higuchi *et al.* (2006) labeled similar structures with the fusion protein uric acid-xanthine permease and enhanced green fluorescent protein (eGFP). Endocytosis occurred in conidia after 4.5 h of germination, which coincides very well with the increase of total cellular ergosterol in germinating conidia of *P. discolor* (see Fig. 5; Chapter 2) and confirms its role in endocytosis. Endocytosis is only inhibited in germinating conidia at high levels of the metabolic inhibitor azide (over 50 mM), but was disturbed by micromoles of natamycin (data not shown). No endosomal compartments were observed after natamycin incubation. This indicates that natamycin is a very specific novel inhibitor of endocytosis, which does not cause extensive cell damage. Filipin and nystatin also inhibit endocytosis, but very quickly disrupt the membrane at higher levels.

Growth in filamentous fungi occurs at the extreme hyphal apex and sterol-rich membranes at this location may play an important role in the positioning of marker proteins that help to maintain the exocytotic machinery at that site (Takeshita *et al*., 2008). Endocytosis and exocytosis are believed to play a combined role in the establishment of polar growth in fungi (Araujo-Bazán *et al*., 2008; Taheri-Talesh *et al*., 2008; Upadhyay and Shaw 2008) and both are linked to ergosterol functioning. A precise analysis of the effect of natamycin will provide novel data on the establishment of these processes in germinating conidia and will deepen the insight in the role of ergosterol in the fungal cell.

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Chapter 4

# Water and air-distributed conidia exhibit differences in sterol content and cytoplasmic microviscosity

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# ABSTRACT

Colonization of food commodities by fungi often starts with dispersal vehicles that enter the product by air, by water or by other vectors. These vehicles often consist of asexual spores, dubbed conidia. In this study, microviscosity and ergosterol content were compared of airborne conidia of *Aspergillus niger* and *Penicillium discolor* and waterborne spores of *Fusarium oxysporum* and *Verticillium fungicola*. *Aspergillus niger* and *P. discolor* conidia showed a higher cytoplasmic microviscosity (3-3.8 cP) than the those of *F. oxysporum* and *V. fungicola* (1.7-2.1 cP), indicative of a higher stress resistance of the airborne spores. Filipin staining showed the presence of ergosterol in dormant waterborne conidia but not in dormant airborne conidia. In the latter case, filipin staining was only observed in germinating spores during and after the process of swelling. The absence of ergosterol in dormant conidia of *A. niger* and *P. discolor w*as confirmed by the finding that these spores did not display sensitivity to the polyene antibiotic natamycin that binds ergosterol. In contrast, the waterborne conidia exhibited partial cell death as judged by viable counts and fluorescent live-dead staining. Taken together, these data suggest that the mode of spore-dispersal is reflected in a distinct cellular composition that has a bearing on the capability of antibiotics to eradicate fungal propagules.

# INTRODUCTION

A gigantic loss of available food in the world is caused by fungal spoilage. The contamination of food products by fungi often starts with dispersal vehicles that include air- and waterborne spores. The fungal genera *Aspergillus* and *Penicillium* have the capability to produce numerous airborne spores called conidia making them dominant fungi in air samples (Mullins, 2001). The spore-forming structures of *Penicillium* and *Aspergillus*, called conidiophores, lift long chains of interconnected spores just above the level of laminar air flow where they are released into the air (McCartney and West, 2007). Spore dispersal is facilitated by a hydrophobic lining of hydrophobin proteins that make up the rodlet layer (Beever and Dempsey, 1978; Wösten, 2001). Spores that are dispersed through the air are subjected to dry conditions, and might even collapse during traveling (Deising *et al.*, 1992). They display moderate resistance to heat and drought resulting from the presence of compatible solutes like mannitol and trehalose (D'Enfert *et al.*, 1999; Ruijter *et al.*, 2002; Doehlemann *et al.*, 2006). These compounds increase the viscosity of the cytoplasm. Melanin and other pigments in the cell wall are also protective agents. They protect conidia against solar UV radiation (Brags *et al.*, 2005).



Conidia of fungi belonging to genera like *Fusarium, Colletotrichum,* and *Verticillium*, are not dispersed by air, but by means of other vectors including water splashes (aerosols) or droplets. *Fusarium* forms compact spherical clusters of spores around the tip of short tubular extensions that are not sticking out into air currents. Asexual water-dispersed spores can be surrounded by mucilage that prevents dislodging by air even more. The mucilage also protects against desiccation and loss of viability during drought. Upon wetting, the mucilage dissolves to leave a suspension of spores on the surface of the substratum (Fitt *et al.*, 1989). In contrast to airborne spores, these spores are easily wetable.

The aim of this study was to assess whether air- and waterborne spores are not only different with respect to surface wetability, but also have a distinct membrane and cytoplasmic composition. To this end, microviscosity and the presence of ergosterol in the plasma membrane was determined. Ergosterol is the target of many antifungals and its presence or absence will affect the sensitivity to such antifungals including natamycin (pimaricin). Natamycin belongs to the family of macrolide polyene antibiotics (Bolard, 1986). It is used in the cheese and sausage industry to prevent growth of fungi on the product surface (Stark, 2007). Natamycin is considered a fungistatic antibiotic. It binds to ergosterol, but is not able to disrupt the plasma membrane (Te Welscher *et al.*, 2008; Chapter 3). In this study, conidia of *Penicillium discolor* and *Aspergillus niger* (air borne) and *Fusarium oxysporum* and *Verticillium fungicola* (waterborne) were used. All species are relevant in applied situations. *Penicillium discolor* causes spoilage problems on cheese surfaces during storage (Stark, 2007) and *A. niger* causes -among other problems- post-harvest diseases

on fruit and flowerbulbs (Perrone *et al.*, 2007). *Fusarium oxysporum* is a widespread plant pathogen. The isolate used in this study causes a devastating dry rot of tulip bulbs during storage. *Verticillium fungicola* is the causative agent of dry bubble disease, the most common fungal disease of the commercial mushroom *Agaricus bisporus* (Collopy *et al.*, 2001).

# MATERIALS AND METHODS

***Fungal strains and harvesting of conidia***

*Aspergillus niger* N402 and *P. discolor* CBS112557 were grown on Malt Extract Agar (MEA, Samson *et al.*, 2004) at 25˚C. *Fusarium oxysporum* CBS116593 and *V. fungicola* MES12712 were grown on Oatmeal Agar (OA, Samson *et al.*, 2004) at 25˚C. Conidia of 10-12 days old cultures were harvested in cold ACES-buffer (10 mM ACES, 0.02% Tween-80, pH 6.8). To this end, the surface of the plate was gently rubbed with a sterile Drigalski spatula and the conidial solution was filtered through sterile glass wool. Spore suspensions were washed twice by centrifugation at 3000 rpm for 5 min in ice-cold buffer and stored on ice until experimental handling on the same day.

***Viability measurements***

Freshly made 10 mM natamycin (DSM, the Netherlands) in 85% DMSO was used as a stock. 45 µM natamycin was added to $10^7$ spores in 10 ml ACES buffer (see above) in 15 ml tubes (Greiner, cat# 188271). Spores were shaken for 20 h in a GFL 3020 orbital shaker (Progen Scientific, Merton, UK) at 125 rpm and 25˚C. The 15 ml tubes were placed sideways to prevent settling of the conidia. Viability experiments were done in triplo on MEA plates. Solutions were diluted to 1000 spores/ml and plated out in quadruple. The CFU (colony forming units) were counted after 2 days of growth at 25˚C.

***Fluorescence microscopy***

Spores were diluted in Malt Extract Broth (MEB; Samson *et al.*, 2004) to $10^7$ spores/ml and supplemented with the appropriate natamycin concentration. 150 µl of the suspension was placed on poly-L-lysine (Sigma) coated glass coverslips (Chapter 2) and spores were incubated for 20 h. Viability of conidia was assessed by staining with 2 µM TOTO-1 (Molecular Probes) in 100 µl ACES for 30 min at RT (Thanh *et al.*, 2007). Staining for sterol was performed with 15 µM filipin (Sigma) in ACES buffer for 30-60 sec at RT, followed by two washes with cold ACES buffer (Chapter 2). After removal of the filipin or TOTO-1 dye, the glass cover slips with the immobilized conidia were put upside-down on top of a thin (< 0.5 mm) layer of 2% agar. Liquid excess was removed with filter paper. Images were made with a Zeiss Axioplan II microscope using a Zeiss Plan-ApoChromat 100x/1.4 oil objective, a blue BP450-490

(FT510, LP520) excitation filter for TOTO-1, and an UV G365 (FT396, LP420) excitation filter for filipin. Images were captured with a Zeiss AxioCam MRc digital camera run by Zeiss AxioVision 4 and a shutter system (Ludl Electronic Products Ltd., USA).

***Low-temperature scanning electron microscopy (LTSEM)***

*Aspergillus niger* and P. discolor were grown on MEA and *F. oxysporum* and *V. fungicola* on OA for 10-12 days. Small cubes (3 x 3 mm) were excised from the colony with a surgical blade and transferred to a copper cup for snap-freezing in nitrogen slush. Agar cubes were fixed with KP-Cryoblock (Klinipath, Duiven, Netherlands) supplemented with 1 part colloidal graphite (Agar Scientific, Stansted, U.K.). Samples were examined, without a gold coating, in a JEOL 5600LV scanning electron microscope (JEOL, Tokyo, Japan) equipped with an Oxford CT1500 Cryostation for LTSEM. Micrographs were acquired at 3 kV acceleration voltage resulting from 30 averaged fast scans (SCAN 2 mode).



***ESR spectroscopy***

Spin label electron spin resonance spectroscopy (ESR) was used to measure cytoplasmic microviscosity of conidia. The spin label TEMPONE (4-oxo-2,2,6,6-tetramethylpiperidine-*N*-oxy [Sigma, St. Louis, USA]), easily penetrates the cell and is particularly suitable to study rotational mobility in the cytoplasm (Dijksterhuis *et al.*, 2007). Potassium ferricyanide $K_3Fe(CN)_6$ (FC) is unable to diffuse through the conidial membrane and 'broadens' (removes) the signal of TEMPONE outside the spores. Conidia were harvested and washed in cold ACES buffer and stored on ice overnight. Conidia ($10^8$ $ml^{-1}$) were centrifuged at 8000 rpm for 2 min in a tabletop centrifuge and resuspended in 25 µl TEMPO-FC mixture (1 mM and 120 mM, respectively) and transferred to a 2-mm-diameter glass capillary. Subsequently, conidia were centrifuged in the capillary at 4000 rpm for 2 min. The excess of supernatant was carefully removed and the capillary was placed in a quartz ESR tube for the recording of ESR-spectra at room temperature in an X-band ESR spectrometer 300E (Bruker Analytik, Rheinstetten, Germany). The modulation amplitude was 0.25 G, the microwave power was limited to 2-5 mW. To record the entire spectrum, the field scan width was set at 100 G.

***Calculation of cytoplasmic viscosity and anisotropy***

Molecular rotation is characterized by the rotational correlation time $\tau_R$. According to the Stokes-Einstein-Debye theory of liquid state, the size of the molecule and the bulk viscosity of the solvent influence the rotational correlation time. If the TEMPONE molecule is approximated with a radius of 3 Å in a medium of viscosity $\eta$, then the isotropic rotational correlation time $\tau_R = 4\pi(3)^3\eta/3kT$ (Stokes-Einstein relationship), where $k$ is the Bolzmann constant and $T$ the absolute temperature in Kelvin. If the size of the probe is larger than that of the solvent molecule and the rotational correlation time is known, the viscosity $\eta$ of the

cytoplasm can be calculated according to the equation. The rotational correlation time can be calculated from the ESR spectra following the equation proposed by Keith and Snipes (1974): $\tau_R = 6.5 \times 10^{-10} \Delta W_0(\sqrt{h_0/h_{-1}} -1)$, if $\tau < 10^{-9}$ s, where $\Delta W_0$ represents the peak-to-peak width of the central line, and $h_0$ and $h_{-1}$ are the line heights of the central line and high-field lines, respectively. In our study, we found that the central line was broadened by interfering spectra of other compounds and as such the $\Delta W_0$ and $h_0$ parameters were influenced. Therefore, in this study the $\Delta W_{-1}(\sqrt{h_{+1}/h_{-1}} -1)$ equation was used, where $\Delta W_{-1}$ represents the peak-to-peak width of the high-field line, and $h_{+1}$ and $h_{-1}$ are the line heights of the low-field line and high-field lines, respectively.

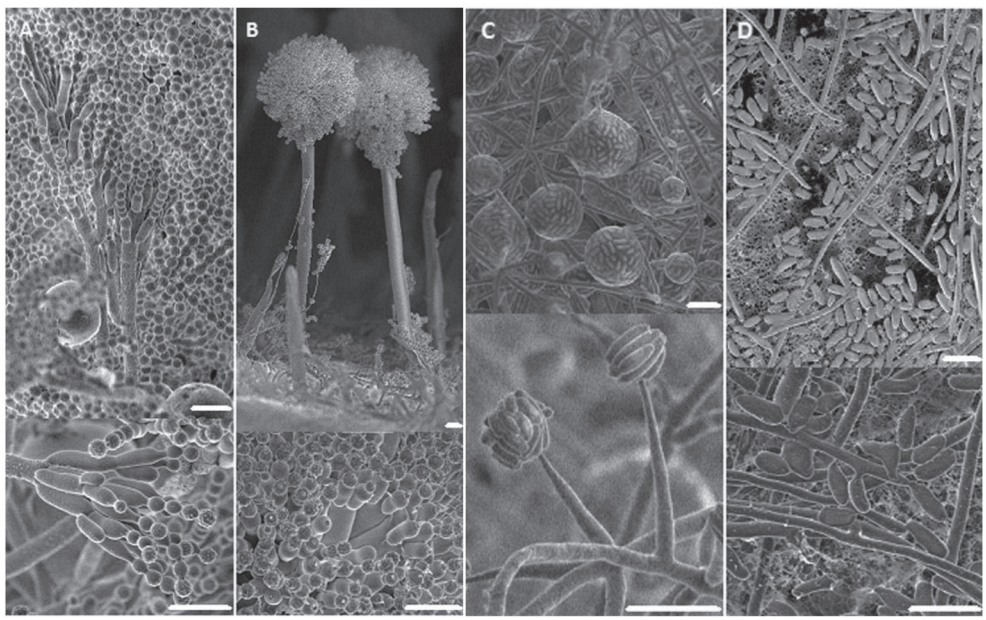


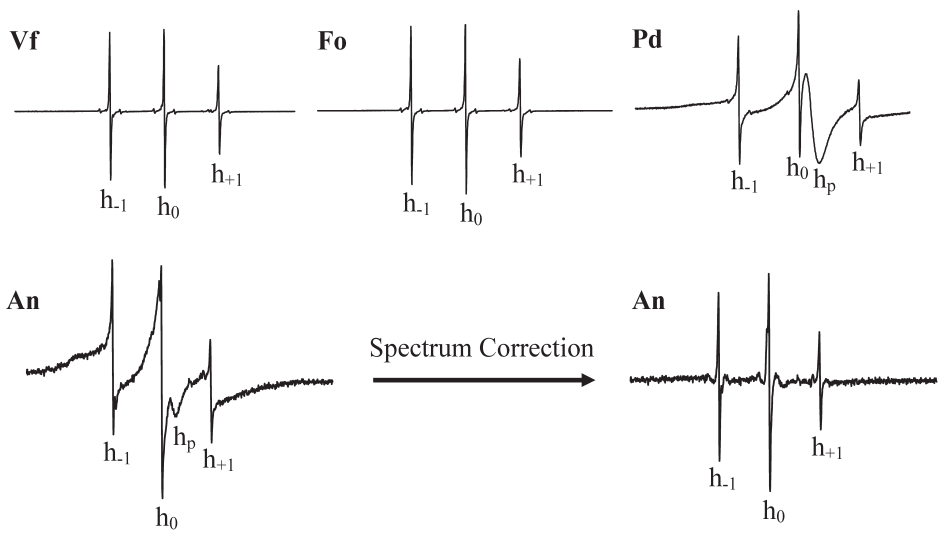

**Figure 1.** Formation of conidia by *P. discolor, A. niger, V. fungicola*, and *F. oxysporum* as observed by cryo SEM. **(A)** Numerous conidia of *P. discolor* are formed on conidiophores that arise nearly 100 µm above the substrate (top). The detailed view (bottom) shows that the ornamented conidia are formed as chains on individual phialids. **(B)** Conidia of *A. niger* are also formed on conidiophores. The spores are formed more than 150 µm above the substratum (top). The phialides that form the ornamented conidia are placed on secondary structures called metulae (bottom). **(C)** Conidia of *V. fungicola* are formed in large clusters that coalesce to form large aggregates of spores inside the mycelium (top). In detail, long, slender phialides form a cluster of conidia (bottom). **(D)** Conidia of *F. oxysporum* are formed within the mycelium (top). A detailed view shows the formation of these microconidia in short structures along hyphae and at the hyphal tips (bottom). Bar represents 10 µm.

# RESULTS

***Formation of air- and waterborne spores***

Spore formation was visualized by cryo SEM (Schubert *et al.*, 2007). This technique does not disrupt the very delicate conidiophores. *Aspergillus niger* and *P. discolor* (Fig. 1A, B) form chains of the round single-celled conidia on phialidic (flask-shaped) cells. *Verticillium fungicola* and *F. oxysporum* (Fig. 1C, D) form spores in large (spherical) clusters and on the surface of the colony, respectively. Markedly, the spore masses are present amidst the hyphae of the colony and not present as a chain.






| Species | $h_{-1}/h_{+1}$ | $\Delta W_{-1}$ | $\tau_R 10^{-10}$ s | η cP |
|---|---|---|---|---|
| *Fusarium oxysporum* | 1.515 | 0.29 | 0.45 | 1.65 |
| *Verticillium fungicola* | 1.663 | 0.29 | 0.57 | 2.07 |
| *Aspergillus niger* | 1.722 | 0.39 | 0.82 | 3.00 |
| *Penicillium discolor* | 1.955 | 0.39 | 1.04 | 3.83 |

**Figure 2.** Electron Spin Resonance (ESR) with the amphipatic spin probe TEMPONE on dormant conidia of *V. fungicola* (Vf), *F. oxysporum* (Fo), *P. discolor* (Pd) and *A. niger* (An). ESR spectra of the conidia contain a central line, flanked by a low-field and high-field line ($h_0$, $h_{-1}$, and $h_{+1}$, respectively) after subtraction of the signal derived from the cell wall associated probe. Spectra of *A. niger* and *P. discolor* contain also a superimposed singlet ($h_p$), originating from the paramagnetic melanin present in the conidial cell wall. Because the spectrum of *A. niger* was broadened and influenced by multiple components, an additional spectrum correction was performed. The ratio of the line heights of $h_{-1}$, and $h_{+1}$ ($h_{-1}/h_{+1}$), the peak-to-peak width of the high-field line ($\Delta W_{-1}$), the isotropic rotational correlation time ($\tau_R$), and the effective viscosity (η) are given in the lower panel.

***Cytoplasmic viscosity of air- and waterborne spores***

The viscosity of the interior of the cell has been correlated to the dormancy and stress-resistance of fungal spores (Dijksterhuis *et al.*, 2007). Microviscosity of the selected fungal spores was determined by ESR using the spin probe TEMPONE (Fig. 2). The spectra contained a narrow and a broad component. The narrow component, originating from the cytoplasmic signal was detectable after subtraction of the broad component originating from the cell wall associated TEMPONE. The narrow spectrum showed a central line, flanked by a low-field and high-field line (designated as $h_0$, $h_{-1}$, and $h_{+1}$, respectively). The spectra of *P. discolor* and *A. niger* also possessed a superimposed singlet ($h_p$) that was not present in conidia of *V. fungicola* and *F. oxysporum*. This $h_p$ signal originates from the melanin in the conidial cell wall (Dijksterhuis *et al.*, 2007). The ratio of the $h_{+1}$ and $h_{-1}$ lines and the peak-to-peak width of the high-field line $\Delta W_{-1}$ were used to calculate the rotation correlation time for TEMPONE in the micro-environment, which is indicative for the microviscosity. The rotation correlation times for *F.*

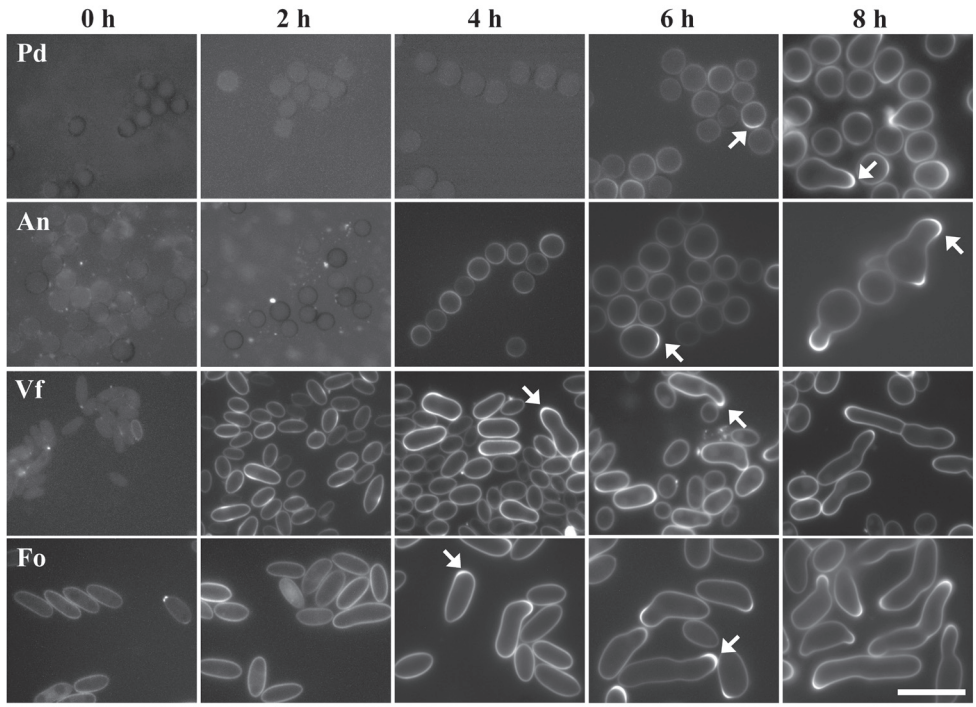



**Figure 3.** Filipin staining of membrane localized sterol in conidia of *P. discolor* (Pd), *A. niger* (An), *V. fungicola* (Vf), and *F. oxysporum* (Fo) after 0, 2, 4, 6 and 8h of germination. *Aspergillus niger* and *P. discolor* conidia stain later than those of *V. fungicola* and *F. oxysporum* (4-6h against 0-2h, respectively). Arrows point to local increased fluorescence prior to and after the formation of germ tubes. Bar represents 10 μm.

*oxysporum* and *V. fungicola* spores were $0.45x10^{-10}$ and $0.57x10^{-10}$ s, respectively, and for *A. niger* and *P. discolor* $0.82x10^{-10}$ and $1.04x10^{-10}$ s. This indicates that TEMPONE molecules in the cytoplasm of water-distributed conidia experience isotropic rotation in an environment with a lower viscosity compared to air-distributed conidia. The effective viscosity (η) of conidia of *F. oxysporum* and *V. fungicola* was 1.65 and 2.07 cP, respectively and for *A. niger* and *P. discolor* 3.00 and 3.83 cP, respectively.

***Ergosterol content in air- and waterborne spores***



The sterol distribution of the conidial plasma membrane was assessed in freshly harvested conidia and during germination of the spores using the polyene antibiotic filipin (Chapter 2). Freshly harvested spores of *V. fungicola* and *F. oxysporum* showed uniform membrane staining with filipin between 0-2 h of germination. In contrast, freshly harvested spores of *A. niger* and *P. discolor* showed no membrane fluorescence (Fig. 3). Uniform fluorescence of the plasma membrane was only observed after 4 and 6 h of germination for conidia of *A. niger* and *P. discolor*, respectively. At these time points, the spores were in the stage of swelling. After 4 to 6 h all species showed intensive staining at the presumed site of germ tube formation. Similar results were obtained when staining was performed in mixtures of airborne and waterborne conidia, showing that small variations in the staining method were not responsible for the observed differences (data not shown). Taken together, these data show that freshly harvested waterborne conidia but not airborne conidia show early staining of the plasma membrane.

Freshly harvested conidia of *A. niger*, *P. discolor*, *V. fungicola* and *F. oxysporum* were incubated in ACES buffer supplemented with 45 μM natamycin for 20 h. This concentration of the antifungal is 10 times or more than that of the Minimal Inhibitory Concentration (MIC) for the conidial suspensions (Chapter 3). After natamycin was removed by washing, spores were inoculated on MEA plates and grown for 2 days. No reduction in conidial viability was observed for *A. niger* and *P. discolor* (Fig. 4B). However, the viability of *V. fungicola* and *F. oxysporum* conidia showed a decrease in viability of 37% and 46%, respectively. In a second approach, conidia were incubated for 20 h in MEB supplemented with 45 μM natamycin. TOTO-1 was used as a fluorescent viability marker of fungal spores (Chitarra *et al.*, 2005; Thanh *et al.*, 2007). This dye cannot cross the plasma membrane of viable cells, but does so after loss of plasma membrane integrity. As a result, the cytoplasm and nuclei are stained. Conidia of *A. niger* and *P. discolor* did not show staining with TOTO-1. In contrast, cytoplasm and/or nuclei of *V. fungicola* and *F. oxysporum* conidia did stain, indicating a decrease of viability and membrane integrity (Fig. 4A). The viability of *V. fungicola* and *F. oxysporum* conidia was reduced with 80% and 62%, respectively (Fig. 4B). Thus, part of the waterborne conidia of *V. fungicola* and *F. oxysporum* show permanent damage after treatment with natamycin which contrasts the air-distributed conidia of *P. discolor* and *A. niger*.

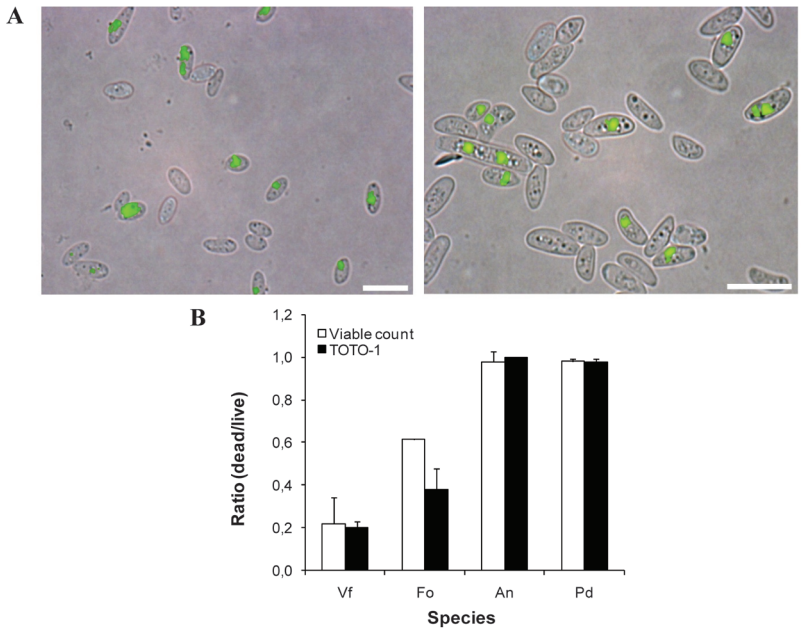



**Figure 4.** Overnight treatment of conidia of *V. fungicola* (Vf) , *F. oxysporum* (Fo), *A. niger* (An) and *P. discolor* (Pd) with 45 µM natamycin. **(A)** Micrographs of viability staining with TOTO-1 of *V. fungicola* (left panel) and *F. oxysporum* (right panel). The dye can enter the cell when cells have lost their membrane integrity resulting in staining of cytosol and nuclei. Bar represents 10 µm. **(B)** Ratio's of dead/live cells as measured by a viable count measurement and after viability staining. Error bars indicate standard deviations.

# DISCUSSION

Airborne spores of *A. niger* and *P. discolor* are water repellent and contain pigments. In contrast, waterborne spores of *F. oxysporum* and *V. fungicola* are easily wettable and are devoid of pigments. This study shows that sterol content of the plasma membrane and microviscosity of the cytoplasm also differs between water- and airborne spores. These differences have implications for their dormancy and stress resistance as well as their ability to survive treatment with antifungals that target ergosterol.

Freshly harvested airborne conidia were viable after treatment with natamycin, but 37 – 80% of the waterborne conidia were killed as judged by viable counts and fluorescent staining. Similar results were obtained by Brothers and Wyatt (2000). They observed a decrease of viable counts after natamycin treatment of waterborne conidia of *Fusarium moniliforme,* but no or hardly any effect was observed for the airborne spores of *Penicillium rubrum* and *Aspergillus parasiticus*. These observations can be explained by the presence of ergosterol

in the plasma membrane of freshly harvested water borne spores. The waterborne conidia showed immediate or very early staining with filipin during germination. In contrast, the plasma membrane of freshly harvested airborne conidia did not stain with filipin, which is in agreement with the fact that these spores contain very low amounts of (ergo)sterol (Chapter 2). Absence of ergosterol explains why global gene expression was hardly affected when freshly harvested conidia of *A. niger* were exposed to 0, 3 and 10 μM natamycin for 2 h (Chapter 5). The airborne conidia did stain with filipin during and after the process of swelling. In agreement, HPLC showed a fourfold increase in ergosterol content in *P. discolor* spores between 3 and 9 h of germination (Chapter 2) Russell *et al.* (1975, 1977) observed that the potassium leakage of germinating conidia of *Aspergillus fumigatus* caused by the polyene amphotericin B methyl ester (AME) also correlated with an increased sterol content during germination.



Freshly harvested conidia of airborne spores displayed a higher microviscosity compared to the waterborne conidia. Microviscosity of the conidia of *F. oxysporum* and *V. fungicola* were 1.65 and 2.07 cP, respectively and for *A. niger* and *P. discolor* 3.00 and 3.83 cP. A much higher viscosity (namely > 10 cP) has been observed in ultra-stress resistant ascospores of *T. macrosporus* (Dijksterhuis *et al.*, 2007) and bacterial spores (De Vries, 2006). This and the fact that spores of *A. niger* and *P. discolor* display moderate stress resistance indicate that the ability of the spore to survive adverse conditions as drought and heat is correlated with a condensed state of the cytoplasm.

Based on the low viscosity and the presence of ergosterol we postulate that freshly harvested conidia of *F. oxysporum* and *V. fungicola* are in a more "germinated" status than airborne conidia. Indeed, during germination the onset of germ tube formation (Fig. 2) is faster in the case of *F. oxysporum* and *V. fungicola*. The fact that airborne conidia are in a more dormant state helps them to survive adverse conditions in the air. This strategy also influences the efficacy of antifungals that target ergosterol. In the case of *F. oxysporum* and *V. fungicola* both hyphae and conidia are killed, while only germinating spores and hyphae are affected in the case of *A. niger* and *P. discolor.*

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Chapter 5

# The major changes in the transcriptome during germination occur before isotropic growth and are not affected by the antifungal compound natamycin

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# ABSTRACT

The transcriptome of *Aspergillus niger* was analysed during germination of conidia in the absence or presence of the antifungal compound natamycin. Most changes in the transcriptome were observed during the first 2 h after inoculation. During this time the complexity of the transcriptome was decreased by 23%, while the abundance of the transcripts increased with about 35%. A number of 3341 genes showed a difference in expression of at least 2-fold. This was accompanied by a reduction in cellular microviscosity and initiation of carbon metabolism. The RNA complexity increased gradually with 36% between 2 and 8 h but the majority of the genes with a 2-fold change or more showed down regulation. During this time period spores grew initially isotropically, which was followed by polarized growth, one cycle of mitosis and formation of germ tubes. Germination was arrested at the stage of mitosis and germ tube formation in the presence of 3 μM natamycin. In contrast, at 10 μM natamycin conidia even did not swell. Interestingly, the transcriptome of natamycin-treated conidia was identical to untreated conidia after 2 h of incubation. However, natamycin-treated conidia showed severely affected gene expression after 8 h. Taken together, these data show that changes in gene expression are most prominent during the first 2 h of germination, which is not accompanied by any morphological change. During this period the transcriptome is not affected by natamycin.

# INTRODUCTION

Asexual spores called conidia are the main vehicles of distribution for fungi (Navarro-Bordonaba and Adams, 1996). Conidia are characterized by a dormant state and are transported via different media such as water and air. Spores that travel via the air possess moderate resistance towards low water activity conditions and high temperature. The dormancy of these cells is broken upon contact with a moist substrate such as the surface of a food product. Effective new prevention strategies of fungal contamination in agriculture and the food industry are needed due to the development of fungal resistance against preservatives (Samson, 1989) and the increase of knowledge of toxic metabolites produced by food spoilage fungi (Frisvad *et al.*, 2007). Studying germination of spores should reveal targets for control of fungal growth.



In general, conidial germination can be divided into several stages. First, dormancy is broken when conidia are in the presence of one or more of the following parameters: water, air, inorganic salts, amino acids and fermentable sugars (Osherov and May, 2001, Thanh *et al.*, 2007). Some species such as *Neurospora crassa* only require water and air for germination (Schmit and Brody, 1976). The environmental signal is recognized by a receptor(s) and is transmitted through Ras/MAPK and cAMP/PKA signal-transduction pathways (Osherov and May, 2000; Liebmann *et al.*, 2004; Reyes *et al.*, 2006; Zhao *et al.*, 2006). Upon activation of germination, the disaccharide trehalose and/or the polyol mannitol are often degraded (Witteveen and Visser, 1995; Thevelein, 1996; d'Enfert *et al.*, 1999; Dijksterhuis *et al.*, 2007; Fillinger *et al.*, 2001; Ruijter *et al.*, 2003). As a consequence, glycerol is formed, which is indicative for an active sugar metabolism (d'Enfert, 1997).

The first morphological change in spore germination is isotropic growth. During this process, also called swelling, the diameter of the spore increases 2-fold or more. It involves water uptake and a decrease in the microviscosity of the cell (Dijksterhuis *et al.*, 2007). Moreover, molecules are directed to the cell cortex to enable addition of new plasma membrane and cell wall (Momany, 2002). At the same time, isotropic growth is concomitant with metabolic activities such as respiration, DNA, RNA, and protein synthesis (Mirkes, 1974; Osherov and May, 2001). The next phase is a transition to polarized growth. During this phase, the morphogenetic machinery is redirected to the site of polarization. This machinery includes the cytoskeleton, the vesicle trafficking system, landmark proteins, signaling pathways and endocytic partners like RhoGTPase modules, polarisome and Arp2/3 complexes (d'Enfert, 1997; Momany, 2002; Harris and Momany, 2004; Harris, 2006). The final stage of germination comprises the emergence of a germ tube. Sterol-rich domains are formed in the plasma membrane at the tips of germ tubes. Together with sphingolipids they are thought to form liquid-ordered domains known as lipid rafts that are able to associate with

certain types of proteins (e.g. GPI-anchored proteins) (Alvarez *et al.*, 2007). At later stages of development the growth speed of the germ tube increases and the functional organization of the hyphal tip area acquires its full potential as judged by zones of endocytosis and exocytosis and the presence of the Spitzenkörper (Taheri-Talesh *et al.*, 2008; Köhli *et al.*, 2008). By branching and interhyphal fusions (Glass *et al.*, 2004) a fungal mycelium is established.

Genera of the order Eurotiales (e.g. *Penicillium*, *Aspergillus* and *Paecilomyces*) produce numerous single-celled conidia that are abundant in air samples (McCartney and West, 2007). These genera are associated with food spoilage and are able to form a wide panel of mycotoxins (Frisvad *et al.*, 2007). In addition, they can act as opportunistic pathogens (Burrell, 1991). *Aspergillus niger* is occurring as a worldwide food spoiler and can also infect harvested crops (Snowdon, 1990). It is used as a model system due to the availability of a genome sequence and whole genome microarrays (Pel *et al.*, 2008). Here, the transcriptome of conidia of *A. niger* was studied during germination in the presence or absence of natamycin. Natamycin is a polyene antifungal compound. It specifically binds to ergosterol, but does not induce membrane permeability (Te Welscher *et al.*, 2008; Chapter 3). In addition, we observed in germinating conidia of *Penicilium discolor* that natamycin inhibits the endocytic process in a time and dose dependent manner (Chapter 3).

# MATERIALS AND METHODS

***Organism and growth conditions***

The *A. niger* strain N402 (Bos *et al.*, 1988) and its derivative RB#9.5 were used in this study. The latter strain expresses a gene encoding a fusion of sGFP and the histon protein H2B under regulation of the *mpdA* promoter (R. Bleichrodt, unpublished results). For spore isolation, strains were grown for 12 days at 25°C on complete medium (CM) containing per liter: 6.0 g $NaNO_3$, 1.5 g $KH_2PO_4$, 0.5 g KCl, 0.5 g $MgSO_4$, 25 mM glucose, 0.5% casamino acids, 1% yeast extract and 200 µl trace elements (containing per liter: 10 g EDTA, 4.4 g $ZnSO_4 \cdot 7H_2O$, 1.01 g $MnCl_2 \cdot 4H_2O$, 0.32 g $CoCl_2 \cdot 6H_2O$, 0.315 g $CuSO_4 \cdot 5H_2O$, 0.22 g $(NH_4)_6Mo7O_{24} \cdot 4H_2O$, 1.47 g $CaCl_2 \cdot 2H_2O$, and 1.0 g $FeSO_4 \cdot 7H_2O$). 1.5% agar was added to solidify the medium. Conidia were harvested in ice-cold ACES-buffer (10mM ACES, 0.02% Tween-80, pH 6.8). To this end, the colony surface was gently rubbed with a sterile Drigalski spatulum and the conidial suspension was filtered through sterile glass wool. Conidia were washed in ice-cold ACES-buffer, resuspended in CM and kept on ice until further processing on the same day.

***Microscopy***

Samples of liquid cultures were placed on poly-L-lysine (Sigma) coated coverslips to immobilize germinating conidia (Chapter 2). After removal of the medium, the coverslips

with the immobilized conidia were placed upside-down onto an object glass overlaid with a thin (< 0.5 mm) layer of 2% water agar. Any remaining liquid was removed with filter paper. Images were captured with a Zeiss Axioskop 2 plus microscope (Zeiss, Germany) equipped with a HBO 100W mercury lamp and a Photometrics Cool SNAP camera using standard FITC and TRITC filters, respectively.

***HPLC analysis***

Germinating conidia were frozen at -80ºC and homogenized with a Qiagen Tissuelyser® (2 min at 30 strokes $sec^{-1}$; Qiagen, Venlo, The Netherlands) using a stainless steel grinding jar (Qiagen, Venlo, The Netherlands) cooled with liquid nitrogen. After an additional round of grinding with 1 ml milliQ, the samples were thawed and quickly transferred to a 2 ml Eppendorf tube. Samples were centrifuged at 4°C for 30 min at 20.817 x g. The supernatant was stored at -80°C until analysis. Prior to HPLC analysis samples were filtered through an Acrodisc® 0.2 µm PTFE syringe filter (Sigma-Aldrich, Zwijndrecht, The Netherlands). A volume of 10 µl was subjected to HPLC analysis using the Waters 717 plus autosampler equipped with a 515 HPLC pump with control module II (Waters Corporation, Etten-Leur, The Netherlands). The mobile phase consisting of 0.1 mM Ca EDTA in water was maintained at a flow rate of 0.5 ml $min^{-1}$. The Sugar Pak I ($Ca^{2+}$ cation-exchange column) was kept at 65°C with a Waters WAT380040 column heater module (Laborgerätebörse GmbH, Burladingen, Germany). Sugars and polyols were detected with an IR 2414 refractive index detector (Waters Corporation, Etten-Leur, The Netherlands). As standards, trehalose, mannitol, D-(+)-glucose, glycerol, erythritol and arabitol were used (Sigma). Peak integrations and quantitive calculations were performed with the Waters Empower software (Waters Corporation, Etten-Leur, The Netherlands).

***ESR spectroscopy***

Germinating conidia were centrifuged at 8000 rpm for 2 min. The supernatant was discarded and the conidia resuspended in 25 µl perdeuterated TEMPONE - potassium ferricyanide solution (1 mM and 120 mM, respectively). Micro-viscosity was determined and calculated as described in Chapter 4.

***RNA extraction***

For isolation of RNA and phenotypic analysis 3 x $10^9$ conidia were inoculated in 300 ml CM in 500 ml Erlenmeyers. Cultures were shaken at 125 rpm in the absence or presence of 3 or 10 µM natamycin. Natamycin was freshly made as a 10 mM stock in 85% DMSO (Brik, 1981). 15 ml culture medium was sampled at each time point from each of the three cultures and pooled to obtain 4.5 x $10^8$ germinating conidia. The conidial suspensions were centrifuged at 1100 x g at 5°C for 5 min. The medium was carefully discarded, and the pellet immediately

frozen in liquid nitrogen. The pellet was stored at -80°C.

Total RNA was extracted from dormant and germinating conidia that had been pelleted. The frozen samples were placed in a stainless steel grinding jar in liquid nitrogen (Qiagen, Venlo, The Netherlands) and homogenized with the Qiagen Tissuelyser® (2 times 2 min at 30 strokes $sec^{-1}$). Dormant and germinating conidia were homogenized for an additional time with 2 ml RLT buffer (supplied with the Qiagen RNeasy® Maxi kit). The whole sample of timepoints 0 and 2 h (i.e. 3 x 15 ml culture), half of the 4 h and a third of the 6 h and 8 h samples were used to obtain RNA. The material was transferred to a 50 ml Greiner tube and the protocol of the RNeasy® Maxi kit (Qiagen) was performed according to the manufacturer's instructions with some modifications. In short, 15 ml RLT buffer supplemented with 170 µl ß-mercaptoethanol was added, followed by vortexing and thawing of the samples. After centrifugation at 3000 x g for 10 min at 4ºC, the supernatant was transferred to a new 50 ml Greiner tube with 15 ml of 70% ethanol, mixed thoroughly, and applied to an RNeasy Maxi column. After centrifugation for 5 min at 3000 x g, the fluid was discarded and 10 ml of RW1 was added. The column was washed twice with 10 ml RPE buffer and centrifuged for 2 and 10 min at 3000 x g, respectively. 800 µl of RNase-free water was added to the column. After 2 min the column was centrifuged for 3 min followed by an additional elution with 800 µl of RNase-free water. The volume of the eluens was reduced to approximately 100-400 µl with a SpeedVac® (Savant DNA 110). Subsequently, 600 µl of 2x T&C lysis buffer (Epicentre, Landgraaf, The Netherlands) was added and the mixture was incubated on ice. After 5 min, 350 µl of MPC Protein Precipitation Reagent (Epicentre, Landgraaf, The Netherlands) was added, vortexed vigorously, and centrifuged for 10 min at 12.000 x g and 4°C. The supernatant was transferred to a clean microcentrifuge tube and gently mixed with 1000 µl isopropanol. The RNA was precipitated at 12.000 x g at 4°C for 10 min. The isopropanol was carefully removed and the pellet air-dried for 5 min and subsequently resuspended in 100 µl RNase-free water. After addition of 700 µl of RLT buffer (without ß-mercaptoethanol) and 500 µl ethanol (96-100%) the RNeasy® Mini kit (Qiagen) procedure was done according to the RNA Cleanup protocol. The concentration of RNA was measured with the Nanodrop ND-1000 spectrophotometer (NanoDrop Tech., Wilmington, USA). The quality was assayed with an Agilent 2100 Bioanalyzer™, using an RNA Nano LabChip® (Agilent Technology, Palo Alto, CA, USA).

***cDNA labeling, microarray hybridization and data analysis***

cDNA labeling, microarray hybridization, and scanning were performed at ServiceXS (Leiden, The Netherlands) according to Affymetrix protocols. In brief, 2 µg of high quality total RNA was used to generate Biotin-labeled antisense cRNA with the Affymetrix Eukaryotic One-Cycle Target Labeling and Control reagents. Quality of the cRNA was assayed using the Agilent 2100 Bioanalyzer™. Subsequently, labeled cRNA was used for

the hybridization to Affymetrix *A. niger* Genome Genechips. After an automated process of washing and staining, absolute values of expression were calculated from the scanned array using the Affymetrix Command Console v1 software. For data analysis the GeneSpring v10 software package was used. The CEL files with the Affymetrix data were imported into GeneSpring and RNA normalization with a baseline correction of the median of all arrays was applied. Experimental grouping were made with biological triplicates per group. A Principal Component Analysis was performed to calculate the correlation between arrays. An unpaired t-test was performed on the combined triplicates to investigate differential expression. The Functional Catalogue (FunCat; Munich Information Center for Protein Sequence) was used for systematic classification of genes according to their cellular and molecular functions (Ruepp *et al.*, 2004).



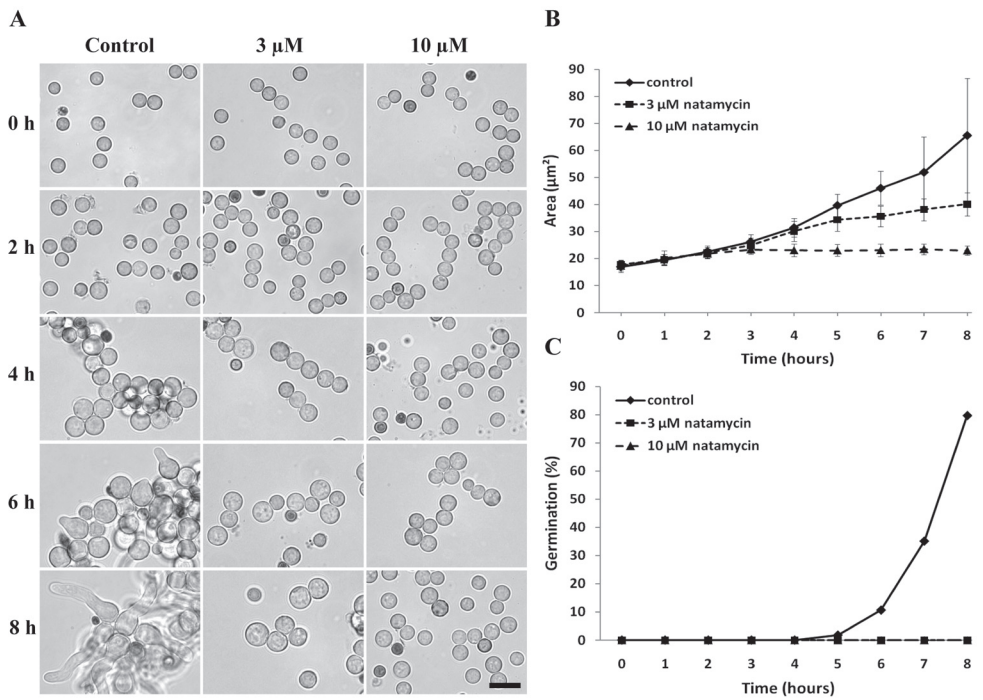



**Figure 1.** Effect of natamycin on germination of *A. niger* conidia. Morphology **(A)**, increase in surface area **(B)** and percentage of conidia forming germ tubes **(C)** in the absence or presence of 3 µM and 10 µM natamycin. Bar represents 10 µm.

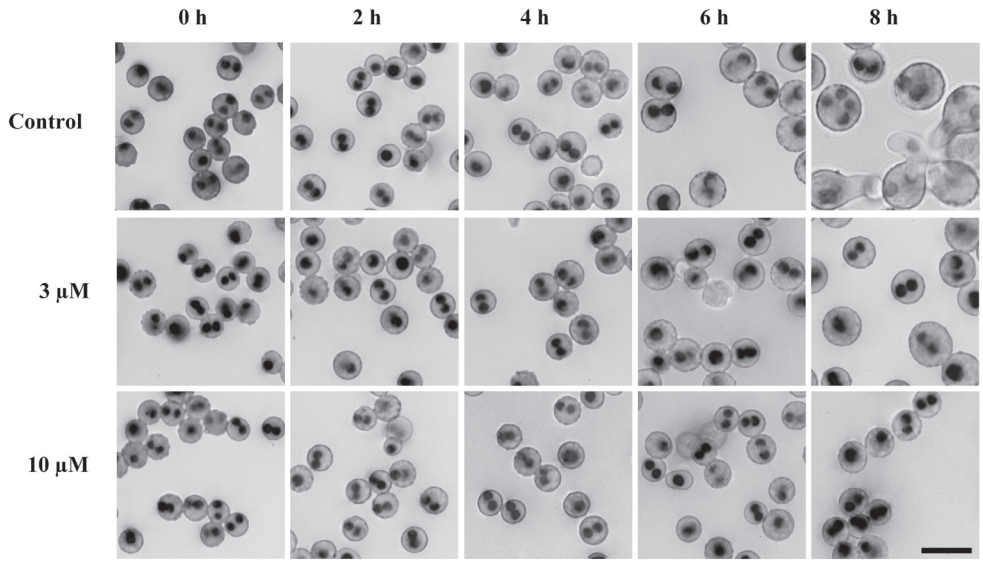



**Figure 2.** Nuclear division in germinating conidia of the *A. niger* strain RB#9.5 in the absence or presence of 3 µM and 10 µM natamycin. Bar represents 10 µm.

# RESULTS

***Morphological responses to natamycin during conidial germination***

Conidial germination of *A. niger* has a maximal rate between 30 – 34°C, with more than 90% germination after 6 h (Abdel-Rahim and Arbab, 1985). In this study *A. niger* was grown at 25°C to increase separation of the different stages of germination in time (Fig. 1A). During the first 2 h after addition of medium, the size of the spores increased from 17 to 22.5 µm² (Fig. 1B). The surface area of conidia increased further to 46 µm² between 2 and 6 h. After 6 h, 10% of the germinating conidia had signs of germ tube emergence. By 8 h, over 80% had formed germ tubes (Fig. 1C). In the presence of 10 µM natamycin, the surface area of conidia remained 23 µm² and germ tubes were not formed throughout culturing (Fig. 1C). At 3 µM natamycin, isotropic swelling did occur, but to a lower extent than the untreated cells. However, no polarization and germ tube formation was observed during the 8 h incubation time (Fig. 1C). These results show that natamycin can inhibit isotropic growth (at higher concentrations) and polarization and germ tube formation (at low concentrations).

An *A. niger* reporter strain (RB#9.5) expressing a fusion of the H2B histon protein and the sGFP protein under control of the *mpdA* promoter was used to monitor nuclear division (Fig. 2). Dormant conidia were predominately binucleate (85%), whereas a small

fraction was uninucleate. Nuclear division was shown to occur after 7 h of germination, prior to the formation of germ tubes. After 8 h, 42% and 34% of the germinating conidia contained 3 or 4 nuclei, respectively. 10% and 14% of the conidia still had 1 or 2 nuclei. Conidia did not show mitosis after 8 h of incubation when incubated with 3 or 10 µM natamycin.

***Intracellular viscosity and compatible solutes during germination***

Dormancy of fungal spores has been correlated to the microviscosity of the interior of the cell. Microviscosity was analysed by electron spin resonance (ESR) using the spin probe perdeuterated TEMPONE (PDT) to determine whether natamycin keeps the spores in a dormant state when added to the culture medium. The spectra included a narrow and a broad component. The narrow component represents the cytoplasmic signal and was detectable after subtraction of the broad component originating from cell wall located PDT (Dijksterhuis *et al.*, 2007). The narrow spectrum showed a central line ($h_0$), flanked by a low-field line



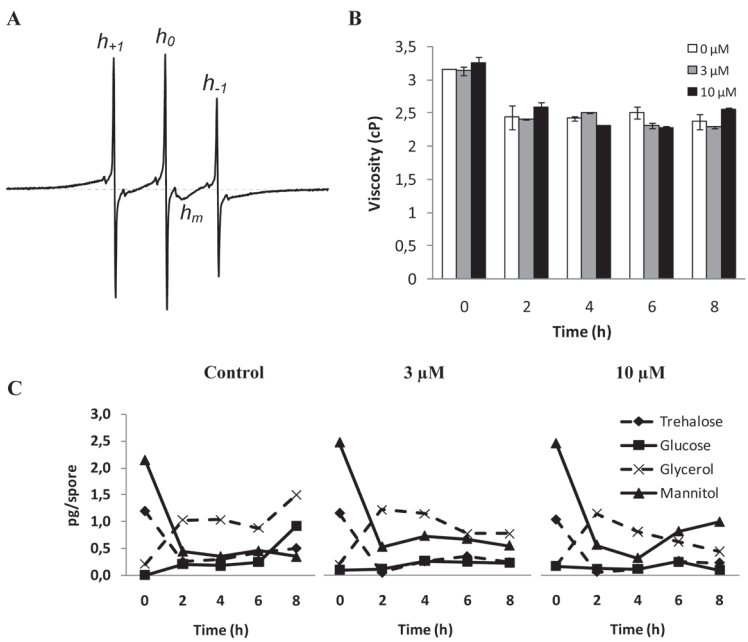



**Figure 3.** Effect of natamycin on the cellular viscosity and compatible solutes. Electron Spin Resonance (ESR) on conidia of *A. niger* germinating in the absence or presence of 3 µM and 10 µM natamycin. **(A)** Conidia contain melanin, which is paramagnetic and therefore has its own ESR signal ($h_m$). $h_0$ and $h_{-1}$ represent the low-field and high-field lines, respectively, which are used to calculate microviscosity. Microviscosity (means ± S.D.) **(B)** and profiles of glucose and the compatible solutes trehalose, glycerol and mannitol **(C)** during germination in the presence or absence of 3 µM and 10 µM natamycin.

($h_{-1}$), high-field line ($h_{+1}$) and an m-line ($h_m$). The latter represents melanin which has its own paramagnetic properties (Fig. 3A; Dijksterhuis *et al.*, 2007). Central and high-field components were used to calculate rotational correlation time and the viscosity (η) based on the Stocks-Einstein equation (see Material and Methods). The effective viscosity of dormant conidia was 3.16 cP for the control and 3.14 and 3.26 cP for the 3 µM and 10 µM natamycin treated conidia, respectively. After 2 h of germination, viscosity decreased with 20-30%, irrespective of the presence of natamycin (Fig. 3B). As germination proceeded, no significant change in viscosity was observed. These results indicate that conidia do not remain dormant in the presence of natamycin.

The intracellular viscosity of dormant conidia correlates with the levels of trehalose and mannitol. In germinating conidia trehalose is rapidly degraded within the first 2 h, after which the concentration increased to 42% of the initial level. Similar results were obtained when spores were incubated with natamycin (Fig. 3C). Mannitol showed a similar rapid degradation within the first 2 h but its concentration remained low in case of untreated conidia. Mannitol levels of conidia treated with 3 µM natamycin restored to 20% of the initial level. In contrast, the concentration of mannitol restored to 40% of the initial level in the presence

**A**

| Time | µg/$10^8$ conidia | 260/280 | 260/230 | 260/270 |
|---|---|---|---|---|
| **0 h** | 7.9 ± 1.4 | 2.1 | 2.3 | 1.24 |
| **2 h** | 19.7 ± 12.0 | 2.2 | 2.5 | 1.26 |
| **4 h** | 31.1 ± 12.0 | 2.2 | 2.5 | 1.25 |
| **6 h** | 58.6 ± 2.4 | 2.1 | 2.4 | 1.23 |
| **8 h** | 52.9 ± 7.8 | 2.1 | 2.4 | 1.22 |

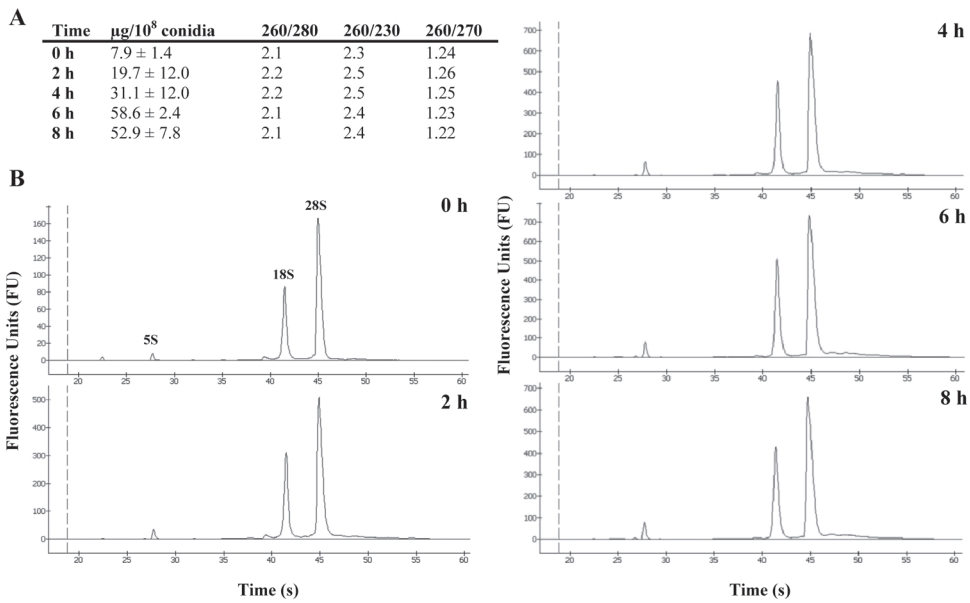



**Figure 4.** Quality control of RNA extracted from dormant and germinating conidia. **(A)** Spectrophotometric data showing concentration and quality relevant ratios. **(B)** Agilent 2100 Bioanalyzer™ electropherograms.

of 10 µM natamycin. Levels of glycerol had increased 7-fold after 8 h in untreated conidia. In contrast, levels decreased 1.6- and 2.4-fold in the presence of 3 µM and 10 µM natamycin, respectively (Fig. 3C). Arabitol and erythritol, two other fungal compatible solutes were not detected during germination. These data show that natamycin affects the level of compatible solutes in spores.

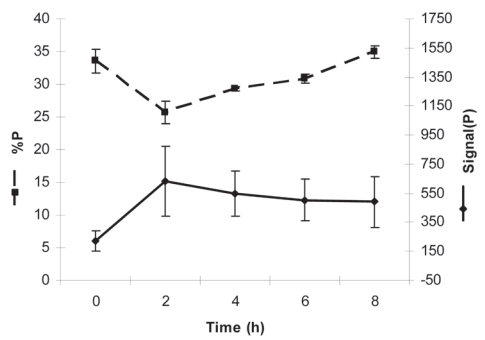
***Transcriptional profiling during germination***

Most methods for RNA isolation from fungal tissue require phenol or phenol based reagents like TRIzol® (Invitrogen, Breda, The Netherlands). However, using this method we were unable to extract RNA from dormant conidia and from conidia in early stages of germination. Therefore, a RNA extraction method for conidia of *A. niger* was developed (see Materials and Methods) resulting in high quality intact RNA (Fig. 4). This method was used to isolate RNA from dormant conidia (0 h), and during conidial activation (2 h), isotropic growth (4 h), polarized growth (6 h) and after germ tubes had been formed (8 h). RNA samples from three independent biological replicates were used for hybridization of Affymetrix microarray chips representing 14,509 open reading frames of *A. niger*. Each experiment had conidia pooled from three independent cultures. Dormant conidia contained transcripts from 33.5% of the genes (Fig. 5A). However, the average intensity of the signal was rather low (220 AU). As soon as conidia were activated, the percentage of genes with transcripts decreased to 25.7%, while the average signal increased with 34.7% compared to dormant conidia. Between 2 and



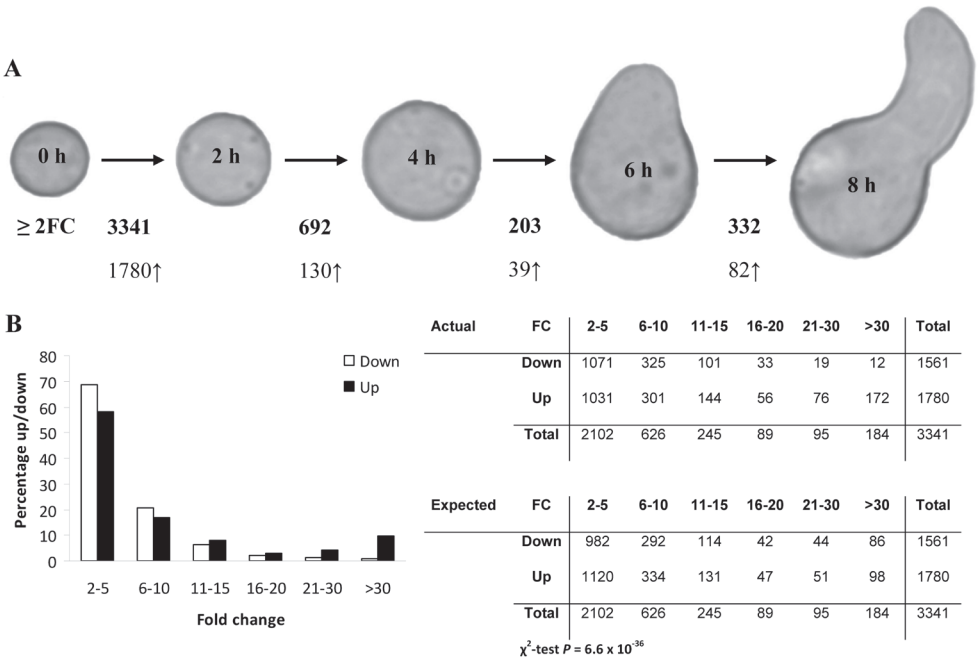
**Figure 5.** RNA complexity, average hybridization signal **(A)** and correlation of expression **(B)** during germination of conidia of *A. niger*. RNA complexity is expressed as % of probes with a present call. Correlation was determined by principal component analysis.

**A**




**B**

| | 0 h | 2 h | 4 h | 6 h | 8 h |
|---|---|---|---|---|---|
| **0 h** | 1.00 | 0.34 | 0.36 | 0.36 | 0.38 |
| **2 h** | | 1.00 | 0.96 | 0.93 | 0.86 |
| **4 h** | | | 1.00 | 0.99 | 0.94 |
| **6 h** | | | | 1.00 | 0.96 |
| **8 h** | | | | | 1.00 |




| Actual | FC | 2-5 | 6-10 | 11-15 | 16-20 | 21-30 | >30 | Total |
|---|---|---|---|---|---|---|---|---|
| | Down | 1071 | 325 | 101 | 33 | 19 | 12 | 1561 |
| | Up | 1031 | 301 | 144 | 56 | 76 | 172 | 1780 |
| | Total | 2102 | 626 | 245 | 89 | 95 | 184 | 3341 |

| Expected | FC | 2-5 | 6-10 | 11-15 | 16-20 | 21-30 | >30 | Total |
|---|---|---|---|---|---|---|---|---|
| | Down | 982 | 292 | 114 | 42 | 44 | 86 | 1561 |
| | Up | 1120 | 334 | 131 | 47 | 51 | 98 | 1780 |
| | Total | 2102 | 626 | 245 | 89 | 95 | 184 | 3341 |

$\chi^2$-test $P = 6.6 \times 10^{-36}$

**Figure 6.** Overview of differential expression during germination. **(A)** The course of germination representing the number of genes with ≥ 2-fold change (bold), and up/down regulation per interval. **(B)** The distribution of up and down regulation per fold change interval for the developmental process between 0 and 2 h. Results are confirmed by $\chi^2$-test analysis.

8 h of growth, the percentage of expressed genes gradually increased to 35.0%. The average signal remained unchanged compared to the 2 h sample. Principal Component Analysis (PCA) showed that the RNA profile of dormant conidia was most different from the other stages (Fig. 5B). The correlation coefficients increased from 0.38 for 0 h vs 8 h to 0.86 for 2 h vs 8 h, to 0.94 for 4 h vs 8 h, and to 0.96 for 6 h vs 8 h. Taken together, these data show that the largest change in the transcriptome is occurring during the first 2 h of germination.

***Comparison of gene expression during conidial germination***

A surprisingly high number of 3341 genes showed more than 2-fold difference in expression between 0 and 2 h, 1780 genes were up-regulated and 1561 genes were down-regulated (Fig. 6A). The relative number of up-regulated genes was even higher in the group of genes that had a 11- to 30-fold change (276 up vs 153 down), and this became even more prominent at a fold change > 30 (172 up vs 12 down) (Fig. 6B). This observation is in agreement with the fact that the average number of transcripts per gene in dormant conidia is low compared to later stages (Fig. 5A). During conidial germination, the number of differentially expressed genes

| Table 1. FunCat category no. (description) | Interval 0 h vs 2 h | 2 h vs 4 h | 4 h vs 6 h | 6 h vs 8 h |
|---|---|---|---|---|
| 01 (Metabolism) | 11.9 | 12.9 | 17.8 | 18.1 |
| 02 (Energy) | 2.7 | 3.5 | 2.5 | 3.2 |
| 03 (Cell cycle & DNA processing) | 10.4 | 15.6 | 13.5 | 7.8 |
| 04 (Transcription) | 5.4 | 3.1 | 1.8 | 4.5 |
| 05 (Protein synthesis) | 5.2 | 1.8 | 1.2 | 1.1 |
| 06 (Protein fate) | 5.8 | 6.8 | 4.9 | 5.4 |
| 08 (Cellular transport & transport mechanisms) | 4.9 | 4.3 | 2.5 | 3.2 |
| 10 (Cellular communication/signal transduction mechanisms) | 8.2 | 9.3 | 9.8 | 7.6 |
| 11 (Cell rescue, defense & virulence) | 3.8 | 4.8 | 3.4 | 3.9 |
| 13 (Regulation of/interaction with cellular environment) | 1.4 | 1.5 | 1.2 | 1.3 |
| 14 (Cell fate) | 1.5 | 1.8 | 2.8 | 1.7 |
| 40 (Subcellular localisation) | 13.1 | 13.5 | 13.5 | 13.2 |
| 99 (Unclassified proteins) | 21.2 | 16.3 | 22.1 | 25.7 |

0-10 11-20 21-30 31-40 41-50 51-60 61-70 71-80 81-90 91-100

Values given in bold represents percentage of differentially expressed genes (≥ 2 FC) present in a functional category per interval.
Grayscale code represents the percentage of up-regulated genes.



gradually decreased to 692 and 203 genes when the 2 and 4 h stages and the 4 and 6 h stages were compared, respectively. In contrast, during outgrowth of the germ tube (between 6 and 8 h) the number of differentially expressed genes increased to 332 (Fig. 6A). Remarkably, for each time interval between 2 and 8 h, differentially expressed genes were primarily down-regulated (81.2%, 80.8% and 75.3%, respectively).

The functional gene categories (FunCat) metabolism, protein fate, cellular transport, cell rescue and regulation of interaction with cellular environment were all up-regulated during the first 2 h of germination (Table 1). This was also observed for the category unclassified proteins, representing approximately 20% of the genes. Genes belonging to the category cell cycle and DNA processing showed marked up-regulation between 4 and 6 h of germination, whereas the category metabolism was down-regulated. The categories transcription and protein synthesis both showed a clear decrease in expression after the first 2 h but an increase in expression after 6 h. Notable is also the up-regulation in the category cellular communication after 6 h of germination.

***Specific transcriptional changes associated to conidial germination***

*Sterol biosynthesis:* Out of 21 genes with a putative function in ergosterol biosynthesis 10 were up-regulated between 0 and 2 h after inoculation. Among these genes are a hypothetical protein (An11g11120; strong similarity to *Schizosaccharomyces pombe* SPBC646.08c), a C-5 sterol desaturase (An16g02930; strong similarity to *Saccharomyces cerevisiae erg3*), an ergosterol biosynthesis protein (An02g07570; strong similarity to *S. cerevisiae kes1*) and a sterol O-acyltransferase (An18g04660; strong similarity to *S. cerevisiae sat1*). With an excess of sterol, the later enzyme catalyses fatty acid esterification of sterols to steryl esters, which are stored in lipid droplets. Genes that were down-regulated are a C-8 sterol isomerase (An01g03350; strong similarity to *Neurospora crassa erg1*), a sterol transmethylase (An14g01590; strong similarity to *Candida albicans erg6*), a C-14 sterol reductase

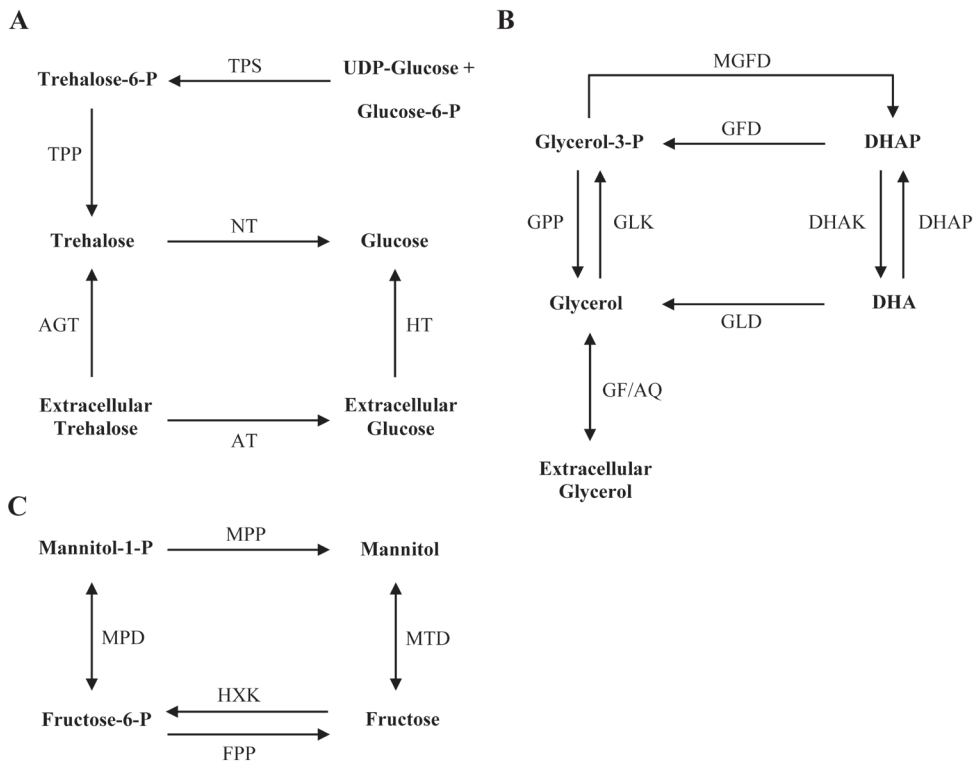



**Figure 7.** Overview of compatible solute metabolism (Ruijter *et al.*, 2003; Pel *et al.*, 2007). **(A)** Trehalose pathway: TPS, trehalose 6-phosphate synthase; TPP, trehalose 6-phosphate phosphatase; NT, neutral trehalase; AT, acid trehalase; AGT, trehalose transporter; HT, glucose transporter. **(B)** Glycerol pathway: MGFD, mitochondrial glycerol 3-phosphate dehydrogenase; GFD, glycerol 3-phosphate dehydrogenase; GPP, glycerol 3-phosphate phosphatase; DHAP, dihydroxyacetone phosphate phosphatase; GLD, glycerol dehydrogenase; DHAK, dihydroxyacetone kinase; GLK, glycerol kinase; GF / AQ, glycerol facilitator/aquaporin. **(C)** Mannitol pathway: MPP, mannitol 1-phosphate phosphatase; MPD, mannitol 1-phosphate dehydrogenase; HXK, hexokinase; FPP, fructose 6-phosphate phosphatase; MTD, mannitol dehydrogenase.

(An01g07000; strong similarity to *S. cerevisiae erg24*) and a C-3 sterol dehydrogenase (An18g05470; similarity to *S. cerevisiae erg26*). With each following interval (i.e. 2 h vs 4 h, 4 h vs 6 h and 6 h vs 8 h, respectively) the number of differentially expressed genes decreased to 6, 5, and 3 genes. These genes were all down-regulated. Taken together, these results show that the primary changes in expression of sterol related genes are within the first 2 h of germination.

*Endocytosis related gene expression:* Endocytosis takes place in *Penicillium discolor* within 4.5 h of conidial germination (Chapter 3). Proteins like SNARE and VPS that mediate fusion

of cellular transport vesicles and vacuoles are involved in this process. During the first 2 h of germination of conidia of *A. niger*, 12 out of 13 differentially expressed genes related to these proteins were up-regulated. These included An02g05390 (strong similarity to *S. cerevisiae* t-SNARE *sec9*), An04g05980 (strong similarity to *S. cerevisiae* v-SNARE *vti1*), An02g05380 (strong similarity to *S. cerevisiae* vacuolar protein sorting-associated protein *vps33*), An08g00740 (strong similarity to *S. cerevisiae* vacuolar protein sorting-associated protein *vps20*) and An04g04950 (strong similarity to *S. cerevisiae* vacuolar protein sorting-associated protein *vps13*). As germination proceeded, only 0-2 genes were differently expressed.



*Genes related to compatible solutes:* In filamentous fungi trehalose biosynthesis occurs by the action of trehalose-6-phosphate synthase (TPS) where UDP-glucose is linked to glucose-6-phosphate resulting in trehalose-6-phosphate (Fig. 7A) (d'Enfert *et al.*, 1999; Avonce *et al.*, 2006). In the next step, the phosphate is removed by trehalose-6-phosphate phosphatase (TPP) resulting in the formation of trehalose. Degradation of trehalose is performed extracellularly by acid trehalase or intracellularly by neutral trehalase. Interestingly, between 0 and 2 h, two genes encoding proteins involved in trehalose synthesis, TPS (An14g02180; trehalose-6-phosphate synthase, *tpsB*) and a subunit of TPS (An08g10510; trehalose-6-phosphate synthase subunit 1, *tpsA*) were up-regulated 16-fold and 18-fold, respectively. The enzyme neutral trehalase (An01g09290; strong similarity to *Aspergillus nidulans treB*), which is responsible for degrading trehalose into glucose, was 81-fold up-regulated between 0 and 2 h. Between 2 and 4 h and between 6 and 8 h, neutral trehalase was down-regulated 2.8-fold and 2.1-fold, respectively. This could explain the observed increase of the trehalose level between 2 and 8 h of germination.


**Figure 8.** Hierarchical cluster analysis of the transcriptomes of conidia incubated for 0 to 8 h in minimal medium in the absence or presence of 3 µM or 10 µM natamycin.

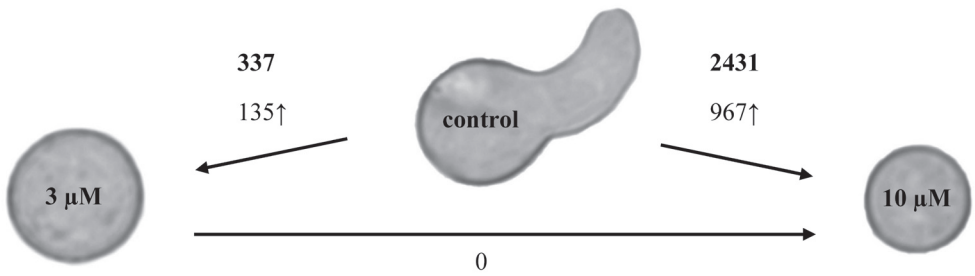


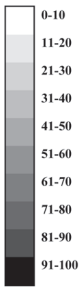

**Figure 9.** Overview of ≥ 2-fold change differential expression of conidia germinated for 8 h in the absence and presence of 3 μM or 10 μM natamycin.

Mannitol is degraded into fructose, which enters the glycolysis were it is converted into glyceraldehyde-3-phosphate via fructose-1,6-diphosphate (Fig. 7C). These intermediates can enter the glycerol metabolism (Fig. 7B). Three differentially expressed genes predicted to function in mannitol degradation were up-regulated from 0 h to 2 h: An02g07610 with strong similarity to a mannitol transporter, An03g02430 with strong similarity to mannitol dehydrogenase and An02g05830 with strong similarity to mannitol-1-phosphate dehydrogenase (MPD). Glycerol biosynthesis occurs through two different pathways each starting with dihydroxyacetone phosphate (DHAP) (Pel *et al.*, 2007). In the first pathway, DAHP is converted into glycerol-3-phosphate by the action of glycerol-3-phosphate dehydrogenase (GPD) but this reaction can be reversed by mitochondrial glycerol 3-phosphate dehydrogenase (MGFD). The phosphate of glycerol-3-phosphate is then removed by glycerol-3-phosphate phosphatase (GPP) resulting in glycerol. This reaction can be reversed by glycerol kinase (GLK). In the second pathway, DAHP is converted into dihydroxyacetone (DHA) by dihydroxyacetone phosphate phosphatase. Subsequently, glycerol is synthesized by the catalytic activity of glycerol dehydrogenase (GLD). 7 genes functioning in glycerol metabolism were differentially expressed between 0 and 2 h. Of these 6 were up-regulated including GLD (An01g06970), DHAK (An14g06500), GFD (An15g07390) and MGFD (An08g00210). No up-regulation was observed between 2 and 8 h of germination, but genes like GLD (An01g06970), MGFD (An08g00210), GLK (An04g04890) and a glycerol facilitator/aquaporin (An13g02350) were down-regulated.

***Transcriptional responses to natamycin***

RNA from conidia that had been germinated for 2 h or 8 h in the presence or absence of natamycin was hybridized to whole genome microarrays. Hierarchical Cluster Analysis (HCA) showed that natamycin hardly affected gene expression in conidia that had been germinated for 2 h (Fig. 8). In contrast, conidia that had germinated for 8 h in the presence of

| Table 2. FunCat category no. (description) | 8 h vs 8 h [10 µM] |
|---|---|
| 01 (Metabolism) | 16.9 |
| 01.02 (Nitrogen & sulfur utilization | 1,4 |
| 01.03.16.01 (RNA degradation) | 0,3 |
| 01.04.01 (Phosphate utilization) | 0,2 |
| 01.05.01 (C-compound & carbohydrate utilization) | 9,6 |
| 01.06.01 (Lipid, fatty acid & isoprenoid biosynthesis) | 3.3 |
| 01.20.01 ( Metabolism of primary metabolic sugars derivatives) | 0,3 |
| 02 (Energy) | 2.6 |
| 02.13.01 (Anaerobic respiration) | 0,04 |
| 02.13.03 (Aerobic respiration) | 0,9 |
| 03 (Cell cycle and DNA processing) | 8.9 |
| 03.01.03 (DNA synthesis and replication) | 0,6 |
| 03.03.01 (Mitotic cell cycle & cell cycle control) | 2,0 |
| 04 (Transcription) | 4.7 |
| 04.05.01 ( messenger RNA synthesis) | 5,2 |
| 05 (Protein synthesis) | 3.3 |
| 5.04 (Translation) | 1,2 |
| 5.07 (Translation control) | 0,5 |
| 06 (Protein fate) | 5.2 |
| 08 (Cellular transport and transport mechanisms) | 5.3 |
| 08.07 (Vesicular transport) | 1,3 |
| 10 (Cellular communication / signal transduction mechanisms) | 7.8 |
| 11 (Cell rescue, defense & virulence) | 4.6 |
| 13 (Regulation of / interaction with cellular environment) | 1.4 |
| 14 (Cell fate) | 1.4 |
| 40 (Subcellular localisation) | 12.2 |
| 99 (Unclassified proteins) | 20.8 |




Values given in bold represents percentage of differentially expressed genes (≥ 2 FC) present in a functional category.
Grayscale code represents the percentage of up-regulated genes.



3 or 10 µM natamycin clearly showed a difference in gene expression compared to any stage grown in the absence of the antifungal.

FunCat analysis showed that genes involved in translation and translational control including elongation factors and ribosomal proteins were up-regulated when conidia had been incubated in 3 or 10 µM natamycin for 8 h (Fig. 9 and Table 2). Interestingly, transcription of genes related to DNA synthesis and the cell cycle were also increased. Yet, nuclear division was not observed in conidia treated with natamycin. Genes related to aerobic respiration also showed increased expression. The number of up-regulated genes in this class mainly represented cytochrome oxidase related proteins. These genes included An08g01550 (strong similarity to *Saccharomyces cerevisiae* subunit of cyctochrome c oxidase *cox19*), An02g09930 (strong similarity to *S. cerevisiae* subunit VI of cytochrome c oxidase *cox6*) and An102g13360 (strong similarity to *S. cerevisiae* assembly factor of cytochrome c oxidase *sco1*). The cycA gene encoding cytochrome c showed an 11-fold increased expression.

*Plasma membrane related expression:* After 8 h of germination 12 out of 14 genes related to sterol biosynthesis were up-regulated when control conidia were compared with conidia that had been germinating in the presence of 10 µM natamycin. These genes included erg1 (An01g03350; strong similarity to *Neurospora crassa* C-8 sterol isomerase), *erg5* (An01g02810; strong similarity to *S. cerevisiae* P450 sterol Δ-22 desaturase), *erg3* (An16g02930; strong similarity to *S. cerevisiae* C-5 sterol desaturase) and *erg25* (An03g06410; strong similarity to *S. cerevisiae* methyl sterol oxidase) which were up-regulated 12.3-, 16.9-, 41.2- and 44.5-fold, respectively. In contrast, in the presence of 3 µM natamycin only a single gene (An04g04210; strong similarity to Δ24-sterol-C-methyltransferase) was highly up-regulated (9.4-fold).

Sterols can reduce membrane fluidity at high temperatures and are able to prevent membrane gelling at low temperatures (Hains, 2001). Membrane fluidity can also be mediated by changes in unsaturated fatty acid levels, a function provided in part by fatty acid desaturases (Laoteng *et al.*, 1999). Six of these genes were up-regulated including *odeA* (28.2-fold). In addition, genes representing a fatty acid synthase alpha subunit (An01g00060; strong similarity to *Schizosaccharomyces pombe fas2p*) and a acetyl-CoA carboxylase (An12g04020; strong similarity to *Schizosaccharomyces pombe* SPAC56E4,04c) were upregulated 7.2- and 5.3-fold, respectively. Sphingolipids are important constituents of the plasma membrane. For instance, together with ergosterol they form lipid rafts which are important in anchoring membrane proteins, and they mediate cell-cell interactions and act as enzyme co-factors (Hannun and Bell, 1989). In the presence of 10 µM natamycin, *sur2* (An01g10030; homologue of the *S. cerevisiae* sphinganine C4-hydroxylase Sur2p) was up-regulated 5-fold, whereas expression of an ortholog of a sphingolipid Δ4 desaturase (An04g01320) was increased 2-fold.

*Endocytosis related gene expression influenced by natamycin:* The endocytic process in germinating conidia of *P. discolor* was inhibited by natamycin in a time- and dose-dependent manner (Chapter 3). After 8 h of germination, 9 genes related to SNARE and VPS proteins were down-regulated when control conidia were compared with conidia that had been exposed to 10 µM natamycin. These genes included An12g07570 (strong similarity to *S. cerevisiae* synaptobrevin *snc2*), An02g05380 (strong similarity to *S. cerevisiae* vacuolar protein sorting-associated protein *vps33*), An04g04950 (strong similarity to *S. cerevisiae* vacuolar protein sorting-associated protein *vps13*) and An11g04400 (strong similarity to *S. cerevisiae* vacuolar protein sorting-associated protein *vps27*). No differential expression was observed for genes related to SNARE and VPS proteins when conidia were incubated for 8 h in 3 µM natamycin.

*Compatible solutes:* After the initial increase of glycerol levels between 0 and 2 h in conidia that germinated in the presence of 3 or 10 µM natamycin, we observed a decrease in glycerol

content between 2 and 8 h. After germinating in the presence of 10 μM natamycin for 8 h, expression of GLD (An01g06970) was decreased 2.1-fold. The glycerol kinase (GLK; An04g04890) that converts glycerol into glycerol-3-phosphate was decreased 14.8-fold. In contrast, two putative glycerol facilitator/aquaporin genes, An02g13250 and An13g02350 were up-regulated 2.6- and 8.5-fold, respectively. Their encoded proteins shuttle glycerol through membranes. After germinating in the presence of 3 μM natamycin for 8 h, the glycerol facilitator/aquaporin An13g02350 was up-regulated 8.9-fold and was the only differentially expressed gene related to glycerol biosynthesis. Although mannitol levels increased during conidial germination in the presence of 10 μM natamycin, a striking 3.5-fold down-regulation of MPD (An02g05830) was observed. This gene encodes mannitol-1-phosphate dehydrogenase involved in mannitol synthesis (Fig. 7B).



# DISCUSSION

In this study the transcriptome of *A niger* was analyzed during germination of its conidia. For this, an RNA extraction protocol was developed. The fact that extraction was performed from a fixed number of cells, enables us to quantify the amount of RNA per conidium and even per volume of the cell. Dormant conidia (0 h) contain approx. 1.4 fg $\mu m^{-3}$ RNA, which increases to 2.4, 4.6, and 7.5 fg $\mu m^{-3}$ between 2-6 h of germination and decreases to 4.2 fg $\mu m^{-3}$ after 8 h of incubation in medium. 60000 mRNA transcripts are present in a cell of *S. cerevisiae* (Zenklusen *et al.*, 2008). This would be equal to a density of 4.9 fg $\mu m^{-3}$ of RNA assuming a cell volume of 50 fL and a relative abundance of mRNA of 20% of the total RNA population (Johnston *et al.*, 1979). This indicates that the RNA content of conidia resembles that of yeast cells. Dormant conidia contain a relatively low number of transcripts per gene as deduced by the average hybridization signal. However it contains a relatively high complexity compared to later stages of germination. Transcripts of 34% of the genes were detected in dormant conidia, which is in good agreement with the finding that 42% and 27% of the genes had transcripts in dormant conidia of *Fusarium graminearum* (Seong *et al.*, 2008) and *Aspergillus fumigatus* (Lamarre *et al.*, 2008). It should be noted that these numbers are lower compared to the percentage of active genes in the vegetative mycelium of *A. niger,* which was found to be 53% (Levin *et al.*, 2007). It is thought that the mRNAs in dormant conidia function as a pool of pre-packed mRNAs primed for translation (Osherov *et al.*, 2002; Lamarre *et al.*, 2008). This would enable the conidium to respond quickly and specific after the onset of germination.

The compatible solutes trehalose and mannitol were rapidly degraded between 0 and 2 h of incubation in complete medium, while levels of glycerol and glucose increased. This indicates an increase in central carbon metabolism during early stages of germination.

Concomitantly, the microviscosity of the germinating spore had dropped. Changes in microviscosity and compatible solutes levels were accompanied by changes in the transcriptome. A number of 8 out of the 13 gene categories had the highest upward change during early germination. These functional categories included metabolism and energy, protein fate, cellular transport / transport mechanisms, communication/signal transduction, interaction with the environment, and cell fate. Notably, 12 out of 13 genes encoding proteins mediating vesicular transport and vesicle fusion (i.e. SNARES and VPS) were up-regulated at least 2-fold.

Conidia are in a phase of extensive swelling between 2 and 6 h of incubation in medium. During this period no changes in levels of compatible solutes were observed. The transcriptome of conidia germinated for 2, 4 or 6 h showed a high correlation with an overall down-regulation in every functional category except for genes related to cell cycle and DNA processing. The latter is explained by the fact that mitosis takes place after 6-7 h of germination.

Between 6 and 8 h levels of trehalose and glucose increased, which could be explained by the 2-fold down-regulation of neutral trehalase, the trehalose degrading enzyme. After mitosis has occurred, mRNA levels increased of genes related to transcription as well as protein synthesis and cellular communication/signal transduction mechanisms. Taken together, the largest transcriptional changes occur within the first 2 h of germination when spores are activated. This is followed by a *status quo* in the transcriptome, during which the spore develops (isotropic growth, polarization establishment) and prepares itself for mitosis. This points to a relatively loose connection between the transcriptional program and the morphological changes during these stages. Xu *et al.* (2004) observed a similar pattern with spores of the protozoan *Dictyostelium discoidium,* where germination was accompanied by two large transcriptional transitions, one after induction and another during germ tube emergence.

Natamycin inhibits ergosterol functioning. Interestingly, this antifungal compound did not affect the transcriptome of conidia between 0 and 2 h and also had no effect on early germination (0-2 h) and breaking of dormancy as indicated by levels of compatible solutes and microviscosity. This indicates that conidia are not able to detect, or react, to the presence of natamycin at this stage. After 8 h incubation in 3 μM natamycin, conidia were unable to initiate polarized growth, whereas 10 μM natamycin even blocked isotropic swelling. Levels of mannitol had also raised when conidia had been exposed to 10 μM natamycin. Interestingly, genes related to cytochrome c showed a higher expression too. Both mannitol and cytochrome c are known to quench reactive oxygen species (Smirnoff and Cumbles, 1984; Giannattasio *et al.*, 2008). This suggests that natamycin somehow evokes formation of these forms of oxygen.

Despite the morphological consequences, cells still actively expressed genes of

the categories energy, protein synthesis and cell cycle when exposed to 10 μM natamycin. Moreover, these spores show a higher expression of genes involved in sterol synthesis and fatty acid desaturases (Laoteng *et al.*, 1999) that may be needed to maintain membrane fluidity (Hains, 2001). Together with sterols, sphingolipids participate in plasma membrane segregation forming microdomains or lipid rafts (Pike, 2004). In fungi, these sterol rich domains are asymmetrically distributed and can be found in membranes at sites of cytokinesis and polarized growth (Wachtler, 2003; Martin and Konopka, 2004; Chapter 2). The increased expression of genes involved in ergosterol and sphingolipid biosynthesis could indicate an attempt in re-establishment of microdomain function. Myriocin that inhibits sphingolipid biosynthesis resulted in increased ergosterol staining in conidia of *P. discolor* (Chapter 2). This also suggests that depletion of either sphingolipids or ergosterol results in the increased formation of the other raft component.

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Chapter 6

# Summary and General Discussion

Fungi cause enormous food losses worldwide due to crop infection and food spoilage. Contamination by fungi often starts with dispersal vehicles that include air- and waterborne spores (conidia). A crucial step in fungal contamination is the process of germination, which ultimately results in mycelial growth throughout the food product. To prevent fungal spoilage, organic acids like acetate, citrate, malate, propionate and sorbate are used. However, acquired resistance has been reported for the major food preservatives sorbate and benzoate in various fungi, including species of *Penicillium* and *Zygosaccharomyces* (Kinderlerer and Hatton, 1990; Golden and Beuchat, 1992). Natamycin (pimaricin) has been used for decades to suppress fungal development on surfaces of cheeses and sausages and to treat fungal infections. This antifungal belongs to the polyene antibiotics, which are characterized by the possession of a macrocyclic ring containing a series of conjugated double bonds at one side and functional hydroxyl groups on the other side of the molecule. Surprisingly, despite the long use of natamycin, its mode of action is an enigma. In this Thesis the mode of action of this antifungal was studied, particularly during germination of conidia. For this, conidia of *Penicillium discolor* and *Aspergillus niger* were used as a model system. The fungal genera *Aspergillus* and *Penicillium* are major food spoiling fungi that produce a variety of mycotoxins (Frisvad *et al.*, 2007). Their conidia are the most abundant fungal spores in the air.

***Sterol localization in germinating conidia***

Early observations have shown that conidia are less sensitive to polyene antibiotics than growing fungal hyphae. Russell *et al.* (1975; 1977) showed that freshly harvested conidia of *Aspergillus fumigatus* were insensitive to the polyene amphotericin B but sensitivity gradually increased during the course of germination (Russell *et al*., 1975). This correlated with sterol levels (Russell *et al*., 1977). This prompted me to study sterol localization during germination. The fluorescent sterol marker filipin was used for this purpose and its reliability as an ergosterol marker was evaluated by means of different techniques including the use of yeast mutant strains (**Chapter 2**). A novel protocol including a short incubation in the dye (see also Takeda *et al.*, 2004) was combined with lowering of the temperature and quick acquisition of micrographs. Ergosterol could not be detected by filipin staining in dormant and swollen conidia. However, after 6 h of incubation in medium, a fluorescent cap was observed at the presumptive site of germ tube emergence. The fluorescence intensity of the cap increased as the formation of the germ tube proceeded. This indicates an important role of ergosterol in polarized growth (Fig. 1). Sterols associate with sphingolipids into a liquid ordered phase ($L_o$) surrounded by largely unsaturated phospholipids in a liquid-disordered ($L_d$) state. This phase segregation results in the formation of micro-domains or lipid rafts (Maxfield, 2002; Pike, 2004; Simons and Ikonen, 1997). It has been suggested that the ergosterol-enriched cap functions as a lipid raft and captures landmark proteins that organize the hyphal tip and as such guide polarization (Martin and Konopka, 2004; Li *et al.*, 2006;

Alvarez *et al.*, 2007; Takeshita *et al.*, 2008). However, it has also been suggested that filipin staining indicates the presence of free ergosterol and as such is not related to raft formation (Jin *et al.*, 2008). According to the latter view, these membranes are more prone to membrane fusion, which is important for exocytosis (see also Dijksterhuis *et al.*, unpublished results). Myriocin that inhibits sphingolipid biosynthesis resulted in increased filipin staining and ergosterol content of conidia of *P. discolor* (**Chapter 2**). This suggests that depletion of either sphingolipids or ergosterol (see **Chapter 5**) results in the increased formation of the other raft component.

Airborne conidia of *A. niger* and *P. discolor* were still viable after prolonged natamycin treatment, while up to 80% of the waterborne conidia of *F. oxysporum* and *V. fungicola* were killed as judged by viable counts and fluorescent staining with the viability dye TOTO-1 (**Chapter 4**). The waterborne conidia showed immediate or very early staining (< 2 h) with filipin, indicating that ergosterol was already present at early germination. In addition, the waterborne conidia showed a markedly lower microviscosity than freshly harvested airborne conidia. The lower microviscosity and the early presence of ergosterol indicate that freshly harvested waterborne conidia are in a more "germinated" state than airborne conidia. This is also reflected in a faster formation of germ tubes in the case of *F. oxysporum* and *V. fungicola*.



***Natamycin as a potential inhibitor of sterol function***

The use of large unilamellar vesicles (LUV) and mutant strains of *Saccharomyces cerevisae* showed that natamycin binds specifically to ergosterol (Te Welscher *et al.*, 2008). Upon binding, natamycin does not permeabilize the plasma membrane as was shown by permeability assays on sterol containing liposomes, spherules, and cells from *Acholeplasma laidlawii* and *S. cerevisiae* (Weissmann and Sessa, 1967; De Kruijff *et al.*, 1974; Teerlink *et al.*, 1980; Te Welscher *et al.*, 2008). This was confirmed in germinating conidia of *P. discolor* by influx assays of the endocytosis marker FM4-64 and FC-ions (**Chapter 3**). In contrast, other polyenes such as filipin and nystatin do compromise the integrity of the membrane by binding to ergosterol. From these data it is concluded that natamycin interferes with ergosterol functioning.

Ergosterol seems to have multiple functions in fungal cells, including endocytosis, vacuole fusion, polarity, and morphogenesis (Kato and Wickner, 2001; Heese-Peck *et al*., 2002; Mysyakina and Funtikova, 2007; Takeshita *et al*., 2008). In conidia of *P. discolor* endocytosis was observed after 3 - 4.5 h of germination, which coincided with an increase of cellular ergosterol (**Chapter 3**). Inside the germinating spores putative endosomes could be observed within minutes after loading with the fluorescent dye FM4-64 as observed earlier in cells of *S. cerevisiae*, *Neurospora crassa*, and *Ustilago maydis* (Vida and Emr 1995; Wedlich-Söldner *et al*., 1995; Fisher-Parton *et al*., 2000; Fuchs *et al*., 2006). Conidia of the

rice pathogen *Magnaporthe grisea* showed already endocytosis within 3 min after hydration (Atkinson *et al.*, 2002). This indicates that these airborne spores, like the waterborne spores, are in a more germinated state than dormant conidia of *A. niger* and *P. discolor*.

The role of sterols in endocytosis was demonstrated in *S. cerevisiae erg* mutants that display different sterols in the cell. These Δ*erg* cells did take up FM4-64 but the dye was not transferred via endosomal compartments to the vacuole (Heese-Peck *et al*., 2002). In **Chapter 3** it was shown that formation of putative endosomes was disturbed after a pre-treatment with micromoles of natamycin in a dose- and time dependent manner. Using the *S. cerevisiae erg* mutants it was shown that saturation of the side chain of the ergosterol molecule has strong effects on endocytosis. Interestingly, the saturated B-ring of the molecule was found important for receptor internalization and for binding of ergosterol to natamycin (Te Welscher *et al.*, 2008). As a consequence, the Minimal Inhibitory Concentration (MIC) of natamycin for the *S. cerevisiae erg* mutants was much higher when sterols had no desaturations in the B-ring (Te Welscher *et al.*, 2008). Taken together, these data indicate that natamycin is a very specific novel inhibitor of endocytosis, which does not cause extensive cell damage as filipin and nystatin (Marella *et al.*, 2002). It should be noted that natamycin did not only have an effect on early endocytosis but that it also affected later stages of this process. This was indicated by the fact that vacuoles were highly fragmented in most yeast *erg* mutants (Te Welscher, unpublished results) and in conidia of *P. discolor* upon natamycin treatment (data not shown). Addition of sterol can stimulate the fusion of wild type vacuoles *in vitro* (Heese-Peck *et al.*, 2002; Kato and Wickner, 2001). Sec18p-driven release of sec17p from the vacuolar membrane is important for vacuole fusion. Addition of nystatin, filipin and amphotericin B block the *in vitro* vacuole fusion and prevented the release of Sec17p (Kato and Wickner, 2001).

***The effect of natamycin on germination of conidia***

Micro-array analysis showed that 34% of the genes have transcripts in dormant conidia of *A. niger* (**Chapter 5**). This mRNA complexity is in line with that in dormant conidia of *Fusarium graminearum* (Seong *et al.*, 2008) and *Aspergillus fumigatus* (Lamarre *et al.*, 2008). The pool of mRNAs may enable the conidium to respond quickly after breaking dormancy (Osherov *et al.*, 2002; Lamarre *et al.*, 2008). It should be noted, however, that the average number of transcripts per gene was relatively low in the dormant conidia (**Chapter 5**). It takes 8 h for a conidium to form a germ tube upon exposure to medium. The largest transcriptional changes occur during the first 2 h of incubation. Eight out of 13 functional categories of genes including metabolism and energy, protein fate, cellular transport/transport mechanisms, communication/signal transduction, interaction with the environment, and cell fate were up-regulated in this period. However, the complexity of the mRNA decreased since only 26% of the genes gave a present call in the micro-array analysis. Interestingly, changes in

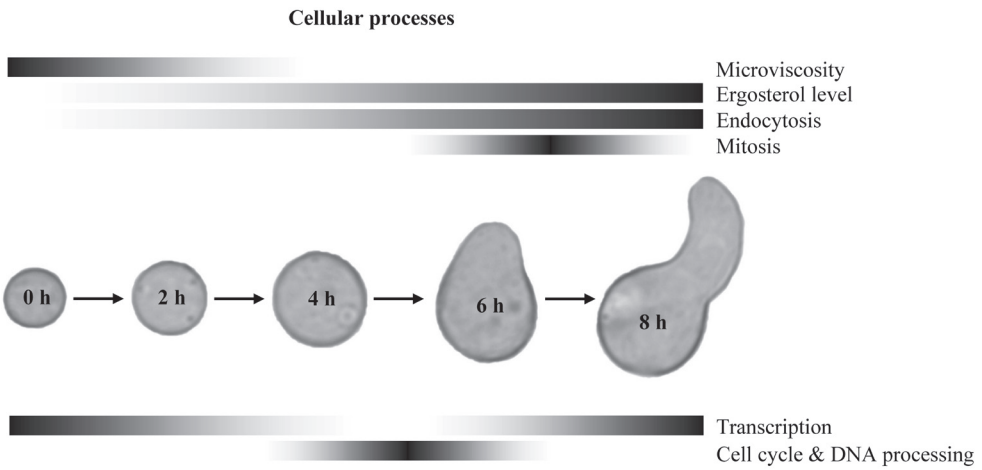




**Figure 1.** Overview of the temporal cellular processes during conidial germination of *Penicillium discolor* and *Aspergillus niger*.

the mRNA pool were not accompanied by morphological changes. However, central carbon metabolism increased as indicated by the degradation of the compatible solutes trehalose and mannitol and the increase of glycerol and glucose levels. Concomitantly, the microviscosity of the cytoplasm dropped (Fig. 1). Levels of compatible solutes and microviscosity did not change between 2 and 6 h (**Chapter 5**). During this time interval the extent of differential expression had decreased strongly and every functional category was down-regulated except for genes related to cell cycle and DNA processing. This can be explained by the fact that the conidia that grew isotropically (swelling) prepared themselves for mitosis that took place after 6-7 h of germination. After 8 h, more than 80% of the conidia showed germ tubes. This was accompanied by an increase of mRNA levels related to transcription, protein synthesis and cellular communication/signal transduction. The fact that the amount of differential expression as well as up-regulation of transcripts is low between 2-8 h suggests that there is a relatively loose correlation between the transcriptional program and the timing of morphological development during germination (Fig. 1).

The presence of natamycin (3 μM or 10 μM) did not affect the transcriptome, morphology, composition of compatible solutes and microviscosity during the first 2 h of incubation in medium (**Chapter 5**). This is in line with the fact that ergosterol could not be detected by filipin staining in the spores of *P. discolor* and *A. niger* at this stage of development (**Chapter 2**). In contrast, filipin staining did detect ergosterol in spores of *P. discolor* that had been incubated in medium for 6 h and that were in the transition to

polarized growth. From this it was expected that natamycin should have an effect on later stages of germination. Indeed, after 8 h incubation in 3 μM natamycin, conidia were unable to initiate polarized growth, whereas 10 μM natamycin even blocked isotropic swelling. These phenotypic effects were accompanied by major changes in gene expression. For instance, natamycin treated spores showed a higher expression of genes involved in sterol synthesis and fatty acid desaturases. The proteins encoded by these genes may be needed to maintain membrane fluidity (Hains, 2001). Moreover, the increased expression of genes involved in ergosterol and sphingolipid biosynthesis could be an attempt to reestablish microdomain function. It is also interesting to note that levels of mannitol inside natamycin treated conidia were higher than in the untreated control. Mannitol is known to quench reactive oxygen species (Smirnoff and Cumbles, 1984; Giannattasio *et al.*, 2008), which raises the possibility that natamycin evokes formation of these forms of oxygen. This hypothesis is strengthened by the fact that genes related to cytochrome c are up-regulated when conidia are treated with natamycin. Cytochrome c too is known to quench reactive oxygen species.

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Chapter 7

# Samenvatting
# Nawoord
# Curriculum vitae
# List of publications

# SAMENVATTING

Schimmels veroorzaken wereldwijd ernorme verliezen van voedsel door middel van na-oogstziekten en voedselbederf. Contaminatie door schimmels begint meestal door middel van sporen (conidia) die via lucht of water worden verspreid. Een cruciale factor in contaminatie door schimmels is het ontkiemingproces dat resulteert in de vorming van een mycelium door het voedselproduct. Om voedselbederf te voorkomen worden organische zuren als acetaat, citraat, malaat, propionaat, en sorbaat gebruikt. Desondanks is resistentie beschreven tegen de meest gebruikte conserveermiddelen sorbaat en benzoaat in verscheidene schimmels zoals *Penicillium* en *Zygosaccharamocyes* soorten. Natamycine (pimaricine) wordt al decennia gebruikt om schimmelgroei te onderdrukken op oppervlakten van kaas en worst. Dit antischimmelmiddel behoort tot de polyeen antibiotica die gekarakteriseerd worden door de aanwezigheid van een macrocyclische ring met geconjugeerde dubbele bindingen aan de ene zijde en functionele hydroxyl groepen aan de andere zijde van het molecuul. Ondanks dat natamycine al langdurig in gebruik is, is het werkingsmechanisme een mysterie. In dit proefschrift is getracht het werkingsmechanisme van natamycine te doorgronden, met name gedurende de ontkieming van conidia. Hiervoor zijn conidia van *Penicillium discolor* en *Aspergillus niger* gebruikt als modelsysteem. Schimmelsoorten binnen de genera *Penicillium* en *Aspergillus* zijn beruchte bederfschimmels van voedselproducten die een verscheidenheid aan mycotoxinen kunnen uitscheiden. Conidia van deze schimmels behoren tot de meest voorkomende schimmelsporen in de lucht.

***Ergosterol in de ontkiemende schimmelsporen***

Eerder onderzoek heeft aangetoond dat conidia minder gevoelig zijn voor polyeen antibiotica dan groeiende schimmeldraden. Versgeoogste conidia van *Aspergillus fumigatus* zijn ongevoelig voor de polyene amphotericine B maar de gevoeligheid nam geleidelijk toe tijdens ontkieming. Ook het gehalte aan sterolen in de cel nam toe tijdens ontkieming. Omdat polyene antibiotica binden aan sterolen is de lokalisatie van sterol bestudeerd tijdens de ontkieming van conidia. Het fluorescente, sterolbindende filipine is voor dit doel gebruikt. In onze eerste studie is de betrouwbaarheid van filipine als sterol merker bepaald door middel van een scala aan technieken waarbij zelfs mutante giststammen zijn gebruikt (**Hoofdstuk 2**). Hier is een verbeterd protocol voor filipine kleuring ontwikkeld, dat bestaat uit een verkorte incubatie met filipine, het gebruik van een lage temperatuur en snel microscopiëren. Ergosterol, een belangrijk sterol in schimmels, kan niet worden aangetoond in het plasmamembraan van rustende (pasgeoogste) conidia en gedurende de eerste uren van ontkieming. Echter, na 6 uur ontkieming was een fluorescente aankleuring zichtbaar op de locatie waar de kiembuis verschijnt. De intensiteit van de fluorescentie van deze ‘kap’ nam toen naarmate

de kiembuis uitgroeide. Dit geeft een indicatie dat ergosterol een belangrijke rol speelt in deze gepolariseerde groei. Sterolen zijn in staat om samen met zogenaamde sphingolipiden te associëren in een bepaalde staat van orde ($L_o$) die worden omgeven door (onverzadigde) phopsholipiden die in een staat van wanorde verkeren ($L_d$). Dit resulteert mogelijk in de formatie van micro-domeinen ('eilanden') die ook wel 'lipid rafts' worden genoemd. Het zou kunnen zijn dat de ergosterol verrijkte structuren in schimmelsporen fungeren als een 'lipid raft' die zogenoemde oriëntatie eiwitten kunnen invangen die de vorming van de kiembuis organiseren. Andere onderzoekers opponeren dat filipine-kleuring de aanwezigheid van vrij ergosterol aantoont, en daardoor niet gerelateerd is aan de formatie van de micro-domeinen. In overeenstemming met deze visie zijn deze membranen meer geneigd tot membraanfusie, wat belangrijk is voor exocytose (de fusie van membraanblaasjes met de plasmamembraan en een belangrijk process voor schimmelgroei). Myriocine heeft een remmende werking op de vorming van sphingolipiden wat resulteerde in een hogere intensiteit van filipine fluorescentie en ergosterol gehalte voor conidia van *P. discolor* (**Hoofdstuk 2**). Dit suggereert dat een gemis aan sphingolipiden of ergosterol (**Hoofdstuk 5**) resulteert in een verhoogde synthese van het andere micro-domein component.



Conidia die verspreid worden via de lucht zoals die van *A. niger* en *P. discolor* blijven levensvatbaar na een langdurige behandeling met natamycine, terwijl tot 80% van de door middel van water verspreide conidia van de schimmels *Fusarium oxysporum* en *Verticillium fungicola* afsterven zoals aangetoond met een bepaling van de levensvatbaarheid door middel van kolonie-telling alsmede fluorescentie aankleuring van dode cellen (met de kleurstof TOTO-1) (**Hoofdstuk 4**). De in water verspreide conidia laten een onmiddelijke of zeer vroege kleuring (< 2 uur) zien met filipine. Dit geeft een indicatie dat ergosterol al aanwezig is tijdens het begin van de ontkieming. Tevens laten waterverspreide conidia een opvallend lagere microviscositeit (stroperigheid in de cel) zien dan luchtverspreide conidia. De lagere microviscositeit en de vroege aanwezigheid van ergosterol suggereren dat de in water verspreide conidia al direct na het oogsten in een meer 'ontkiemde' staat verkeren dan lucht verspreide conidia. Dit wordt ook bevestigd door de snellere ontkieming in het geval van *F. oxysporum* en *V. fungicola*.

***Natamycine als een potentiële remmer van sterol functies***

Experimenten waarin gebruik wordt gemaakt van zogenaamde 'large unilamellar vesicles' (LUV, grote kunstmatige membraanblaasjes), alsmede mutante stammen van de bakkersgist *Saccharomyces cerevisiae* laten zien dat natamycine specifiek aan ergosterol bindt. Na binding is natamycine niet in staat het plasmamembraan permeabel (lek) te maken zoals permeabiliteit experimenten met sterol bevattende liposomen (membraanblaasjes), 'spherules', en cellen van *Acholeplasma laidlawii* en *S. cerevisiae* hebben aangetoond. Dit is bevestigd door de instroom van FC-ionen (ferrycianide) en FM4-64, een zogenaamde endocytose marker, in

ontkiemende conidia van *P. discolor* te meten (**Hoofdstuk 3**). In tegenstelling tot andere polyeen antibiotica namelijk filipine en nystatine, is natamycine niet in staat om de integriteit van het plasmamembraan nadelig te beïnvloeden na binding met sterol. Aan de hand van deze data is geconcludeerd dat natamycine werkt doordat het de functie van ergosterol belemmert zonder de membraan lek te maken.

Ergosterol lijkt meerdere functies te vervullen in de schimmelcel waaronder endocytose, vacuolefusie, polariteit en morphogenese. In conidia van *P. discolor* was endocytose (afsnoering van membraanblaasjes van de membraan naar binnen toe) aantoonbaar na 3 – 4.5 uur ontkieming. Dit valt samen met een verhoging van cellulair ergosterol (**Hoofdstuk 3**). In ontkiemende conidia waren endosomen (de plaats waar de membraanblaasjes verzamelen) waar te nemen, binnen enkele minuten nadat de cellen gekleurd waren met FM4-64. Dit is tevens in eerdere studies met de schimmels *S. cerevisiae*, *Neurospora crassa* en *Ustilago maydis* aangetoond. In conidia van de rijst pathogeen *Magnaporthe grisea* is endocytose waarneembaar 3 minuten na bevochtiging. Dit kan betekenen dat deze sporen, net als de water verspreide conidia, in een verdere staat van ontkieming verkeren dan lucht verspreide conidia.

De rol van sterolen gedurende endocytose is aangetoond in de *S. cerevisiae erg* mutanten, die een gewijzigde sterolsamenstelling vertonen. Deze *Δerg* cellen zijn niet in staat om FM4-64 op te nemen (‘endocyteren’) en te transporteren via endosomen naar de vacuole (het eindstation van endocytose). In **hoofdstuk 3** is uiteengezet dat de vorming van endosomen verstoord raakt na een voorbehandeling met natamycine in een dosis- en tijd afhankelijk proces. Door gebruik te maken van de *erg* mutanten is gedemonstreerd dat de verzadiging van de zijketen van het ergosterolmolecuul sterke effecten heeft op de endocytose. Belangwekkend, de verzadigde B-ring van ergosterol blijkt een rol te spelen in receptor gestuurde internalisatie en voor de binding tussen ergosterol en natamycine. Verder blijkt dat de minimale inhiberende concentratie (MIC) van natamycine op *S. cerevisiae erg* mutanten verhoogt naarmate sterolen minder onverzadigingen in hun B-ring hebben. Samengevat, deze data suggereren dat natamycine een specifieke remmer is van endocytose in schimmels zonder de cel ernstig te beschadigen zoals filipine en nystatine dat wel doen. Naast het remmende effect van natamycine op vroege endocytose blijken ook latere processen beïnvloed te worden. Dit wordt gesuggereerd door het feit dat vacuoles een gefragmenteerd voorkomen hebben in de meeste *S. cerevisiae erg* mutanten (ongepubliceerde resultaten) en in conidia van *P. discolor* na behandeling met natamycine (niet vertoonde resultaten). Tevens kan de toevoeging van sterol de fusie van vacuolen *in vitro* stimuleren. Het vrijkomen van het eiwit sec17p uit het vacuole membraan wordt aangedreven door een ander eiwit sec18p en is van belang voor de fusie van vacuolen. Nystatine, filipine en amphotericine B blokkeren na toevoegen de *in vitro* vacuole fusie en voorkomen het vrijkomen van sec17p.

***Het effect van natamycine op de ontkieming van conidia***

Micro-array experimenten laten zien dat transcripten van 34% van de genen aanwezig zijn in inactieve conidia van *Aspergillus niger*. Deze mRNA complexiteit is in samenspraak met wat is waargenomen in inactieve conidia van *Fusarium graminearum* en *Aspergilus fumigatus*. De aanwezige mRNA voorziening stelt de spore in staat om snel te reageren na activatie. Het gemiddelde aantal transcripten per gen is echter wel relatief laag in deze sporen (**Hoofdstuk 5**). Het duurt 8 uur voor een spore om een kiembuis te vormen na blootstelling aan medium. De grootste transcriptionele veranderingen vinden plaats gedurende de eerste 2 uur van ontkieming. Acht van de 13 functionele categorieën van genen, waaronder stofwisseling en energie, eiwitbestemming, cellulaire transport/transport mechanismen, communicatie/ signaal transductie, interactie met de omgeving en cel bestemming nemen toe. Gedurende deze periode verlaagt de complexiteit van het mRNA vanwege het feit dat 26% van de genen aanwezig is volgens micro-array analyse. Verrassend genoeg, gaan de veranderingen in het transcriptoom (het totaal aan transcripten) niet vergezeld met morphologische veranderingen. De toename van de transcripten van centraal koolstof metabolisme valt samen met de afbraak van compatible bestanddelen zoals trehalose, mannitol en de verhoging van glycerol en glucose waarden. Daarmee gaat een verlaging van de microviscositeit van het cytoplasma gepaard. De waarden van compatible bestanddelen veranderen niet tussen 2 en 6 uur (**Hoofdstuk 5**). Gedurende dit tijdsinterval is de omvang van de differentiële expressie verlaagt en elk functionele categorie negatief gereguleerd, behalve genen gerelateerd aan cel cyclus en DNA ontwikkeling. Dit is te verklaren door het feit dat conidia die isotroop
groeien ('zwellen') zichzelf voorbereiden voor de mitose die na 6 – 7 uur ontkieming plaats vindt. Na 8 uur, vertoont meer dan 80% van de conidia kiembuizen. Dit gaat gepaard met een verhoging van het mRNA ('transcript') gerelateerd aan transcriptie, eiwit synthese en cellulaire communicatie/signal transductie. Het feit dat de omvang van differentiële expressie en de mate van positief gereguleerde genen laag is tussen 2 – 8 uur suggereert dat er lage correlatie is tussen het transcriptionele programma en de temporele morphologische ontwikkeling tijdens de ontkieming (Fig. 1).

De aanwezigheid van natamycine (3 μM of 10 μM) heeft geen effect op het transcriptoom, de morphologie, compositie van compatibele bestanddelen en microviscositeit gedurende de eerste 2 uur incubatie in medium (**Hoofdstuk 5**). Dit komt overeen met het feit dat ondanks een filipine kleuring ergosterol niet detecteerbaar is in de plasmamembraan van conidia van *P. discolor* en *A. niger* in dit stadium van ontkieming (**Hoofdstuk 2**). Dit staat in contrast met de detectie van ergosterol in conidia van *P. discolor* na 6 uur incubatie in medium en gedurende het stadium van polarisatie. Aan de hand van deze gegevens is te verwachten dat natamycine een effect zou kunnen hebben gedurende deze stadia van ontwikkeling. Inderdaad, na 8 uur incuberen in 3 μM natamycine resulteerde dit in een deficiëntie om gepolariseerde groei te initiëren, terwijl 10 μM natamycine daarnaast nog

eens in geblokkeerde zwelling resulteerde. Deze phenotypische effecten werden vergezeld met grote veranderingen in genexpressie. Bijvoorbeeld, natamycine behandelde sporen vertoonden een hogere expressie van genen gerelateerd aan de synthese van sterol, als mede die een rol spelen in de verzadiging van vetzuren. De eiwitproducten van deze genen kunnen mogelijk een rol spelen in het behouden van de vloeibaarheid van het membraan. Tevens, de verhoogde expressie van genen die een rol spelen in ergosterol en sphingolipid biosynthese, kan een poging van de cel zijn om de micro-domein functie te herstellen. Verder is het interessant dat het mannitol gehalte in natamycine behandelde sporen hoger is in vergelijking met onbehandelde sporen. Mannitol is in staat om reactieve zuurstof componenten (ROS) te neutraliseren wat mogelijk een rol suggereert voor natamycine in dit proces. Deze hypothese wordt versterkt door het feit genen gerelateerd aan cytochrome c positief gereguleerd zijn wanneer conidia behandeld zijn met natamycine. Cytochrome c staat ook bekend om de mogelijkheid om reactieve zuurstof afgeleiden te neutraliseren.

# NAWOORD

Eindelijk, de resultaten van vier jaar onderzoek hebben de weg naar het papier gevonden in de vorm van dit proefschrift. ‘Eindelijk’ is een beetje ongelukkig gekozen want ik heb totaal niet het gevoel gehad dat ik uitkeek naar het einde van dit project. Sterker nog, in gedachten dezelfde weg nog eens nawandelend, brengt mij tot de conclusie dat ik enorm heb genoten tijdens de afgelopen vier jaar. Hier hebben collega’s een enorm aandeel in gehad.

Ten eerste mijn promoter Han. Ik heb grote bewondering voor je kennis en de vaardigheid waarmee je alles overziet en aanstuurt. Je hebt me vooral aan het eind, met het schrijven van de manuscripten, een tandje hoger doen schakelen. Dit resulteerde vaak in lappen tekst die enige tijd vergde om te corrigeren. Zie nog steeds het beeld voor me hoe je je hoofd ter hemel wendde toen ik weer een manuscript bij je had ingeleverd. Gelukkig heb je humor en ben dan ook erg dankbaar voor de tijd die je in het nakijken hebt gestoken.



Veruit de meeste dank ben ik verschuldigd aan mijn co-promoter Jan. Ik weet nog goed wanneer ik je voor het eerst zag. Ik liep stage bij Arman en tijdens een seminar waarbij je aanwezig was viel je me toch wel op. Niet eens door de duimgaten in de mouwen van je trui toentertijd, maar door het enthousiasme en passie waarmee je vertelde (Wist je dat glas een vloeistof is). Verhalen van anderen gaven te kennen dat je een aangenaam persoon was om mee samen te werken. Het was dan ook fantastisch toen ik anderhalf jaar later bij je kon komen werken als OIO. De vier jaar vlogen voorbij en heb heel veel geleerd en gelachen. Vooral de brainstorm sessies die gepaard gingen met opgewonden heen en weer lopen en vergeten koffiekopjes en verdwenen pennen. Maar ook de congressen die we hebben bezocht en de mensen die we hebben ontmoet zal ik niet snel vergeten (Wanneer zou het boek van Jimmy Mack uitkomen?). Kortom, ik kijk al uit naar de volgende vier jaar.

Natuurlijk zitten er bij DTO meer collega’s. Rob, bedankt voor de interesse die je als groepsleider hebt getoond en de mogelijkheid dat ik op jouw afdeling heb mogen werken. Ondanks dat de afdeling op zijn kop stond met de reorganisatie heb je het toch laten uitgroeien tot een gezellige en hechte groep. Jos, vooral in het begin heb ik veel aan je kennis en ervaring gehad. Je aanwezigheid als reispartner tijdens congressen was altijd gezellig. Vooral in Cairns hebben we gelachen als we gingen ontbijten bij de wel erg mannenminnende en humoristische kantine medewerker (Yes, I’m really a fun gi). Martin, ik heb je ervaren als een erg hulpvaardige collega met een groot verantwoordelijkheidsgevoel. Je wist mij dan ook altijd tijdens ‘ochtendsessies’ op de hoogte te houden met wat er nog moest gebeuren (Pfff). Wie Martin zegt, zegt Tineke (Biertje?). De aanwezigheid van jou en ijs op het lab heeft mij menigmaal een koude rilling over mijn rug doen gaan. Verder was het altijd gezellig als je even kwam buurten als ik in de bibliotheek zat te werken. Natuurlijk mogen we je

herintroductie van koffiepauzes niet vergeten. Dit heeft uiteindelijk geresulteerd in een bont gezelschap van studenten, analisten en AIO's aan de tafel. Met name Maikel, Henk en Rolf.

Groep DTO is erg dynamisch en vele personen heb ik zien komen en gaan. Toch wil ik er een paar noemen. Carmo, 'kleine snoeperd', sua presença no grupo sempre foi acompanhada de muita diversão e gargalhada. Você nunca entrou no CBS com expressão triste, e penso que eu posso falar por todos do DTO, que isso foi uma grande contribuição para a atmosfera no grupo. Você sempre foi uma companhia agradável durante as conferências, especialmente a IUMS Food Mycology no Key West. Obrigado por tudo. Eu realmente espero que você possa contornar a difícil situação na qual você encontra-se, enquanto você está escrevendo sua própria tese. Eu desejo a você muita sorte e força para concluir seu projeto e eu estou ansioso para sua defesa de du tesis em Portugal. Janos en Tao, ondanks dat jullie op een rustige manier het lab bevolkten heeft dat toch een leuke bijdragen geleverd aan de sfeer in de groep. Natuurlijk mijn stagaires Jeroen en Edwin, naast jullie hulp en inzet was het altijd gezellig en leuk om jullie te zien groeien in het onderzoek. Bedankt daarvoor.

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# CURRICULUM VITAE

Richard van Leeuwen was born on November 7th 1979 in Ede, a town in the middle of the Netherlands. In 1999, after receiving his VWO-diploma from the Neder-Veluwe College in Ede, he started with the study 'Biology' at Utrecht University. During this study the interest for micro-organisms was evoked. This resulted in an undergraduate thesis (The role of pH regulation on protein production in *Aspergillus niger*) under supervision of dr. ir. R. P. de Vries and a scientific internship (Fluorescent reporters to visualize transcription and protein secretion in *Aspergillus niger* and *Schizophyllum commune*) under supervision of dr. A. Vinck at the department of Microbiology. The second internship (FIV vaccine based on live attenuated feline infectious peritonitis virus) was performed under supervision of dr. B. J. Haijema at the department of Veterinary Virology. In December 2004 he graduated and obtained his MSc degree. In January 2005 he started as an 'Onderzoeker-In-Opleiding' (OIO) at the CBS/Fungal Biodiversity Centre, an Institute of the Royal Academy of Arts and Sciences (KNAW) in Utrecht, under the supervision of dr. J. Dijksterhuis. The research during this period on the mode of action of the polyene antibiotic natamycin is described in this thesis. At present he holds a position as postdoctoral researcher to study heatresistance in fungal spores at the CBS/Fungal Biodiversity Centre in collaboration with Utrecht University.

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