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Fungal strains and the development of tolerance against natamycin

Hugo Streekstra [a], Alex E.E. Verkennis [b], Robbert Jacobs [b], Angelina Dekker [a], Jacques Stark [a,1], Jan Dijksterhuis [b,*]

[a] DSM Biotechnology Center, PO Box 1, 2600 MA Delft, The Netherlands
[b] Applied and Industrial Mycology, CBS-KNAW Biodiversity Center, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

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ABSTRACT

Antimicrobial resistance is a relevant theme with respect to both antibacterial and antifungal compounds. In this study we address the possible development of tolerance against the antifungal food preservative natamycin. A selection of 20 fungal species, originating from a medical as well as a food product context, was subjected to increasing concentrations of natamycin for prolonged time, a procedure designated as "training". The range of Minimum Inhibitory Concentrations (M.I.C.) before (1.8–19.2 μM) and after (1.8–19.8 μM) training did not change significantly, but natamycin-exposure caused an increase of M.I.C. in 13 out of 20 tested strains. The average M.I.C. increased from 6.1 to 8.6 μM and 4 strains showed a >2-fold increase of tolerance after training. One strain (of *Aspergillus ochraceus*) also showed increased tolerance to amphotericin B and nystatin. However, two *Fusarium* strains showed similar or even decreased tolerance for these other polyene antifungals.
The work reported here shows that a continuous and prolonged increasing selection pressure induced natamycin tolerance in individual strains. This implies that such a selection pressure should be avoided in the technical application of natamycin to ensure its continued safe use as a food preservative.

1. Introduction

Fungi cause significant losses in the food production chain due to spoilage. A range of anti-fungal compounds is in use to prevent fungal growth. These compounds include weak organic acids, such as sorbate (Plumridge et al., 2004), azole-derivatives, fluorocytes, allylamines, and polyene antifungals (Brul and Coote, 1999). Polyene antifungals, such as amphotericin B, nystatin, filipin, and natamycin, are characterized by the presence of a macrocyclic ring containing a series of conjugated double bonds on one side of the molecule and a number of hydroxyl groups on the opposite side. Additional features may be present, such as the mycosamine and carboxyl groups that determine the amphoteric properties of nystatin and natamycin (Hamilton-Miller, 1973; Bolard, 1986).

The main application of natamycin is to protect the surface of cheese and sausages against fungal development (Stark, 2007). Having been used for decades, it has a reputation of safe use, inhibiting a wide range of fungal species in the micromolar range. Natamycin binds to ergosterol, an important component of the plasma membrane and the growing tips of germinating spores and vegetative hyphae (Van Leeuwen et al., 2008, 2010). The effect is cessation of active fungal growth. Some polyenes, such as amphotericin B and nystatin, form complexes that lead to leakage of the plasma membrane, but natamycin does not permeabilize cells (Te Welscher et al., 2008). Rather, it interferes with ergosterol-dependent cellular processes, such as membrane trafficking and fusion. For instance, it inhibits endocytosis in germinating conidia of *Penicillium discolor* (Van Leeuwen et al., 2009), and the fusion of prevacuolar compartments in *Saccharomyces cerevisiae* (Te Welscher et al., 2010). Natamycin also inhibits transport of amino acids and sugars into cells in a reversible manner (Te Welscher et al., 2012).

In natamycin-containing solutions, spore development is halted at or before isotropic growth. A micro-array study on conidia of *Aspergillus niger* showed that treated cells showed similarities with dormant conidia compared to the controls that had formed germ tubes (Van Leeuwen et al., 2013). Spores of *A. niger* and *P. discolor* are unable to germinate in the presence of natamycin, but are not killed: after 20 h of exposure to ten times the minimal inhibitory concentration, a high percentage of conidia will germinate after removal of the antifungal compound (Van Leeuwen et al., 2010). However, part of the non-pigmented conidia of *Fusarium oxysporum* and *Verticillium fungicola* were killed after such a treatment, showing diversity among fungi in their response.

The prolonged use of antimicrobial compounds carries the risk of adaptation of micro-organisms to higher concentrations. In the case of natamycin, it is known that some species, and some strains within a species, have a low natural sensitivity, as shown for instance for the genus *Aspergillus* (Lalitha et al., 2008; Xu et al., 2009). This is probably

* Corresponding author at: Applied and Industrial Mycology, CBS-KNAW Fungal Biodiversity, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands.
E-mail address: j.dijksterhuis@cbs.knaw.nl (J. Dijksterhuis).
[1] Present address: Nederlandse Zuivel Organisatie, Benoordenhoutseweg 46, 2596 BC Den Haag, The Netherlands.

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due to a low concentration of, or poor access to, the primary target in the cell membrane - ergosterol (Te Welscher et al., 2008; Van Leeuwen et al., 2008).

A WHO monograph on Food Additives states that obtaining natamycin-resistant isolates is difficult, and that such isolates invariably show reduced metabolic and growth rates in vitro (WHO, 2002). In order to test the development of tolerance of fungi against natamycin we subjected a range of fungi to gradually increasing, sub-lethal concentrations of natamycin for a prolonged time. In cases where an increased tolerance towards natamycin was observed, it was investigated whether this tolerance also extended towards other polyene antifungals, in this case amphotericin B and nystatin.

2. Materials and methods

2.1. Selection of fungal strains

Twenty fungal species were selected for this study (Table 1). Some of these have been studied before with respect to the acquisition of polyene tolerance, such as the yeasts *Saccharomyces cerevisiae* and *Candida albicans* (Te Welscher et al., 2008; Shapiro et al., 2011). *Trichosporon asahii*, *Candida krusei*, *Candida parapsilosis*, *Rhodotorula mucilaginosa* and *Geotrichum candidum* were included as representatives of potentially pathogenic yeasts. The filamentous fungus *Fusarium solani* was included because it is known to cause eye infections and natamycin can be used as treatment. The fungi *Aspergillus terreus* and *Aspergillus fumigatus* are known as opportunistic pathogens, as are *Cladophialophora sp.*, and *Aspergillus (Neosartorya) fennelliae*. We selected two strains of *A. terreus*, one of *A. fumigatus*, one *Cladophialophora potulentorum* strain and a *Neosartorya spinosa* strain for analysis. *Mucor plumbeus* was included as a representative of the Zygomycetes, illustrating a wide range of fungi that are sensitive to natamycin. The remainder of the selected species are associated with problems in the food-chain: *Fusarium oxysporum*, *Colletotrichum musae*, *Trichoderma aggressivum*, *Verticillium fungicola*, and *Penicillium discolor*.

2.2. Growth and determination of the minimum inhibitory concentration

Most fungal strains were obtained from 30% glycerol spore stock solutions (SS, containing 0.5 g Tween 80 and 0.5 g agar/l Demi-water) stored at −80 °C. Four species were taken from lyophilized material containing fungal material mixed with a protective matrix, which was stored at 4 °C. The glass ampullas were broken and fungal cells were resuscitated in malt peptone (MP, containing malt extract and bacto peptone) for 24 h. The *V. fungicola* strain was obtained from the Department of Molecular Microbiology of the University of Utrecht as an agar culture. All fungal material was inoculated on malt extract agar (MEA), and cultivated at 24 °C. After 1 week of growth, the cultures were checked for proper outgrowth, the purity of the culture and morphological characteristics. Cell and spore suspensions were prepared from the MEA-grown cultures by rinsing the agar surface with ACES buffer [10 mM *N*-(2-acetamido)-2-aminoethanesulfonic acid, 0.02% Tween 80, pH 6.8], filtering through sterile glass wool, counting the cells by means of a Bürker-Türk haemocytometer, and diluting to the proper density in ACES buffer. The spores or cells were kept on ice to prevent premature development.

Natamycin (DSM Food Specialties, Delft, The Netherlands) with a purity of 91% was used for the preparation of a stock solution in 85% DMSO (dimethylsulfoxide, Sigma, St Louis, USA). A quantity of 7.3 mg natamycin was dissolved in 2 ml solution, to achieve a concentration of 5 mM (see also Brik, 1981). The natamycin was completely dissolved after 2 to 3 h, and the stock solution was kept in tin foil for UV protection. This stock solution was diluted in double strength malt extract broth (MEB) which was subsequently diluted to normal strength. Then, a serial dilution in MEB was made in microtiter plates to give a final volume of 200 μl. The natamycin concentration ranged from 0.6 to 6.6 μM in steps of 0.6 μM for all strains, with the exception of the least sensitive strains 10 and 11, for which the concentration range was 5.4 to 11.4 μM (also in 0.6 μM intervals). Each well was inoculated with 10 μl of spore solution containing 10^4 conidia or cells, each strain in three-fold.

The microtiter plates were incubated in the dark at a temperature of 24 °C. Growth was checked by visual inspection after 3 and 5 days, and after 5 days also with a stereomicroscope (Nikon ZOOM AZ100) at 100× magnification. Each strain was inoculated in minimally three rows. The lowest concentration in which no growth was observed was designated as the Minimum Inhibitory Concentration (M.I.C.), and the range of concentrations in which fungal growth was affected was assessed.

In order to investigate whether the M.I.C. value on MEB is also indicative for growth on agar medium, agar was supplemented with

Table 1
Fungal strains used in this study.

	Fungal species	Strain code[a]	Source of isolation	Stock
1	*Saccharomyces cerevisiae*	82I1	Fruits of the forest	SS, −80 °C
2	*Candida parapsilosis*	13B8	Tortilla	SS, −80 °C
3	*Candida albicans* var. *albicans*	CBS 5144	Rectum, Denmark	Lyophilized culture
4	*Candida krusei*	CBS 573; 105F6	–	SS, −80 °C
5	*Rhodotorula mucilaginosa* var. *mucilaginosa*	CBS 326	Type of *R. rubra*; atmosphere	Lyophilized culture
6	*Trichosporon asahii*	133H8	Tea leaves	SS, −80 °C
7	*Geotrichum candidum*	CBS 590.96	Feces	Lyophilized culture
8	*Fusarium solani*	149H7; CBS 115659	–	SS, −80 °C
9	*Aspergillus terreus*	8G3	Almond	SS, −80 °C
10	*Aspergillus terreus*	17A1	Fungizone-containing medium (azole)	SS, −80 °C
11	*Aspergillus fumigatus*	60H1	Indoor air, Eindhoven	SS, −80 °C
12	*Cladophialophora potulentorum*	133A1	Sports drink	SS, −80 °C
13	*Neosartorya spinosa*	3E7	*Ex*-cardboard	SS, −80 °C
14	*Penicillium discolor*	CBS 183.88	Cheese	SS, −80 °C
15	*Mucor plumbeus*	161E3	Roquefort cheese	SS, −80 °C
16	*Aspergillus ochraceus*	48E4	[b]	SS, −80 °C
17	*Verticillium fungicola*	MES12712	*Agaricus*	Agar culture
18	*Colletotrichum musae*	CBS 116870	Banana, Florida, USA[c]	Lyophilized culture
19	*Fusarium oxysporum* f. sp. *tulipae*	CBS 116593	Tulip bulb	SS, −80 °C
20	*Trichoderma aggressivum*	122 C5/CBS 123782	*Agaricus*	SS, −80 °C

[a] Codes are taken from strains of the CBS-KNAW Fungal Biodiversity Centre (CBS number) or from the working collection of the department of Applied and Industrial Mycology of the same institute.
[b] As identified with DNA sequences from ITS, β-tubulin and calmodulin.
[c] Epitype.