Adaptation of *Aspergillus niger* to several antifungal agents

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Adaptation of *Aspergillus niger* to short-term stress induced by three antifungal agents [amphotericin B (AMPH), miconazole (MCZ), and ketoconazole (KCZ)] was observed and evaluated quantitatively using individual hyphae. Spores were inoculated onto a poly-L-lysine-coated glass plate making up the base of a culture vessel. Potato dextrose broth (PDB) was added and the vessel incubated for 24 h at 28 °C. The growth rate of an arbitrarily selected test hypha was measured automatically. Exposure to AMPH (0.075 µg ml⁻¹) stopped the growth of the hypha. After washing with PDB, the same concentration of AMPH was applied again. The growth of the test hypha was not inhibited. This phenomenon was defined as adaptation to the short-term stress of AMPH. Similarly, adaptation was observed with MCZ (0.01 µg ml⁻¹) and KCZ (0.5 µg ml⁻¹). The time required for the test hypha to re-start growth after washing with PDB depended upon the concentration of MCZ or KCZ, but not upon the concentration of AMPH.

Keywords: adaptation, *Aspergillus niger*, antifungal agents

INTRODUCTION

Living cells tolerate a particular stress after repeated exposure to it, and are then said to be adapted. In the case of fungi, studies have demonstrated adaptations to chemical stresses such as peroxide (Collison & Dawes, 1992), ketoconazole (Lenhart & Merkunova, 1989; Ryley *et al.*, 1984) and potassium sorbate (Schroeder & Bullerman, 1985). However, these studies were carried out by the evaluation of increasing tolerance upon repeated exposure to sub-inhibitory levels of chemicals with a series of fungal transfers, and these stresses were regarded as long-term ones. Because it is difficult to analyse the changes taking place in mycelia (Lenhart, 1973), adaptation studies using filamentous fungi at the mycelial level have rarely been performed.

A system for the evaluation of dynamic growth of a single hypha has been developed (Yamada *et al.*, 1992) and its

applicability to the assessment of the antifungal activity of volatile compounds and antifungal agents has been demonstrated (Oh *et al.*, 1992, 1993a, b). The growth rate of a single hypha was found to be a useful indicator of activity for filamentous fungi. The response of the same hypha to repeated exposure to stress could be evaluated successively, and was applied to the study of adaptation to short-term salt stress (Park *et al.*, 1993). In the present work, the adaptation of *Aspergillus niger* to the short-term stress imposed by several antifungal agents was observed and evaluated quantitatively at the single-hypha level.

METHODS

Organism and antifungal agents. *Aspergillus niger* IFO6661 was maintained at 4 °C on potato dextrose agar slants (PDA, Difco). The fungus was precultured on PDA slants at 28 °C for 7 d prior to use. Amphotericin B (AMPH) and miconazole (MCZ) were purchased from Sigma and ketoconazole (KCZ) from Biomol Research Laboratories. Stock solutions were prepared at 20 mg ml⁻¹ in dimethyl sulphoxide (DMSO) and stored at -20 °C. These stock solutions were used within 7 d. Test solutions of AMPH, MCZ and KCZ were prepared by

Abbreviations: AMPH, amphotericin B; MCZ, miconazole; KCZ, ketoconazole.

Fig. 1. System for measuring dynamic hyphal growth: 1, glass plate coated with poly-L-lysine; 2, test mycelium; 3, potato dextrose broth medium (about 1 ml); 4, medium inlet; 5, medium outlet; 6, light source; 7, culture vessel; 8, objective lens of microscope; 9, charge coupled device (CCD) camera; 10, image digitizer; 11, microcomputer; 12, computer display; 13, video tape recorder; 14, TV monitor; t_i , time of measurement; l_i , length of hypha at time t_i .

Fig. 2. Parameters related to the recovery of hyphal growth: T_{EXPO} , time between introduction of an antifungal agent and washing with medium containing no antifungal agents; τ_{OFF} , lag time from termination of exposure until growth rate begins to change in response to washing; t_{OFF} , lag time from termination of exposure until growth rate reaches another steady level; $t_{\text{OFF}} - \tau_{\text{OFF}}$, lag time from the re-start of hyphal growth until growth rate reaches another steady level. The bold line represents the response of hyphal growth.

diluting the stock solution with potato dextrose broth (PDB, Difco) before use. Final concentrations of DMSO in the test drug preparations were less than 0.25% (v/v).

System for measuring dynamic hyphal growth in liquid medium. The system originally developed by the authors (Yamada *et al.*, 1992) was improved to measure vegetative hyphal growth in liquid medium. The experimental setup is shown in Fig. 1. The system consists of a microscope (model CK2, Olympus), a charge coupled device camera (CCD camera, model HCB-1450, Flovel), an image digitizer (model FDM4-

256, Photron), a microcomputer (model PC-9801FX, NEC), a video tape recorder (model PV-S98, NEC), and two monitors (model PC-TV353, NEC). The culture vessel used in this study was designed for tracing vegetative hyphae in liquid medium. The bottom of the culture vessel was made of a thin glass plate (thickness 0.12–0.17 mm). The inner surface of this plate was coated with poly-L-lysine to which spores and hyphae adhered throughout the experiment. This enabled continuous observation of growing hyphae without focus adjustment during the test period. Coating was done by pouring 0.01% (w/v) solutions of filter-sterilized poly-L-lysine (poly-L-lysine hydrobromide M_r 70000–150000, Sigma) onto the glass plates and incubating for 2 h at 25 °C, then removing the solution and allowing the plates to dry horizontally in a sterile-air-flow cabinet under UV irradiation. The culture vessel containing test mycelium was placed on the microscope stage, and the analogue image obtained through the microscope was converted to a digital image by the image digitizer, then processed into a binary image. From the binary image, the edge points of all hyphae were detected by analysing the light intensity distribution. An appropriate hypha was then selected for continuous observation. Microscope images were obtained every 30 s. The position of the hypha was determined and its growth rate calculated.

Assay of adaptation to short-term antifungal stress. *A. niger* spores from a 7-d-old culture grown on a PDA slant at 28 °C were harvested in sterile saline containing 0.05% (v/v) Tween 80. After vigorous agitation, the suspension was filtered through glass wool. Spores were washed three times with sterile distilled water and resuspended in sterile distilled water at a concentration of 2×10^8 spores ml^{-1} . One microlitre of this suspension was placed onto the poly-L-lysine-coated glass plate of the culture vessel and left for 30 min. The suspension was removed and the glass plate rinsed with 10 ml sterile distilled water. Adhesion of about 10^3 spores onto the glass plate was confirmed by microscopy. Adhered spores were not dislodged during the introduction of medium or sterile distilled water. One millilitre of PDB was added and the culture vessel incubated for 24 h at 28 °C. In this time each spore formed a colony with a diameter of about 5 mm. The culture vessel was mounted on the microscope stage, and the medium was replaced by 1 ml fresh PDB and allowed to stand for 30 min at 28 °C. A hypha that was growing horizontally on the glass plate was selected and its growth rate was measured for 30 min. After confirming that the growth rate was stable, the medium was replaced by PDB containing an antifungal agent. The hypha was exposed to the agent for 30 min (AMPH) or 60 min (MCZ and KCZ), and then washed with PDB containing no antifungal agent. Adaptation of a hypha to an antifungal agent was confirmed by repeated treatment with the same agent. In all experiments, the antifungal activity of each agent was checked on an untreated hypha immediately after adaptation was confirmed.

Parameters related to the recovery of hyphal growth. A typical response curve is illustrated in Fig. 2. The growth rate of a single hypha was stable during the pre-exposure period. Following introduction of an antifungal agent, the growth rate decreased and reached another steady level; i.e. growth cessation in this case. After washing with medium containing no antifungal agent, the hypha re-started growth after a certain lag time. The growth rate then increased, and finally became stable. The following parameters can be defined to describe this hyphal growth response (Oh *et al.*, 1992).

1. T_{EXPO} represents the time between the introduction of an antifungal agent and washing with medium containing no antifungal agent.

2. τ_{OFF} represents the lag time from the termination of exposure until the growth rate begins to change in response to washing.
3. t_{OFF} represents the lag time from the termination of exposure until the growth rate reaches another steady level.
4. $t_{\text{OFF}} - \tau_{\text{OFF}}$ represents the lag time from the re-start of hyphal growth until the growth rate reaches another steady level.

RESULTS

Hyphal growth of *A. niger* and effect of DMSO

The growth rate of each hypha of *A. niger* became stable after 24 h incubation at 28 °C. In each experiment, the growth rate was recorded for 30 min before the medium was changed. The average growth rate during the pre-exposure period was $3.64 \pm 0.82 \mu\text{m min}^{-1}$ (mean $\pm$ SD, $n = 129$).

Since DMSO was used as a solvent, its effect on hyphal growth rate was checked before examining the action of the antifungal agents. As shown in Fig. 3, DMSO had no appreciable effect at concentrations below 0.25 %. The growth rate was retarded at concentrations above 0.5 %, and finally inhibited at 10 %. In subsequent experiments therefore, the final concentration of DMSO in the culture vessel was kept below 0.25 %.

Effect of antifungal agents on *A. niger* hyphal growth

To determine the concentration range at which each antifungal agent affected *A. niger* hyphal growth, PDB containing various concentrations of antifungal agents was introduced after the pre-exposure period. As shown in Fig. 4(a), hyphal growth was affected immediately by AMPH at concentrations above $0.075 \mu\text{g ml}^{-1}$. At AMPH concentrations of 0.075 and $0.1 \mu\text{g ml}^{-1}$, hyphal growth ceased, but returned to normal about 20 min later. In subsequent experiments, $0.075 \mu\text{g ml}^{-1}$ was chosen as an

Fig. 3. Effect of DMSO on *A. niger* hyphal growth. At the time indicated by the arrow, PDB was replaced by PDB containing DMSO at concentrations of (%) 0.25 (—), 0.5 (---), 5.0 (····), 10.0 (— · —). Experiments were done in triplicate for each concentration. Each line shows a representative result obtained with the respective concentration.

Fig. 4. Effect of (a) AMPH, (b) MCZ and (c) KCZ on *A. niger* hyphal growth. At the time indicated by the arrow, PDB was replaced by PDB containing various concentrations of each antifungal agent. Concentrations ($\mu\text{g ml}^{-1}$) were: AMPH, 0.05 (—), 0.075 (— · —), 0.1 (---), 0.125 (····); MCZ, 0.001 (— · —), 0.01 (—), 0.1 (---), 1.0 (····); KCZ, 0.1 (— · —), 0.5 (—), 1.0 (---), 5.0 (····). Experiments were done in triplicate for each concentration. Each line shows a representative result obtained with the respective concentration.

effective concentration. Introduction of MCZ at concentrations above $0.01 \mu\text{g ml}^{-1}$ caused hyphal growth to cease within about 30 min (Fig. 4b), and it did not re-start within 60 min. KCZ at concentrations above $0.5 \mu\text{g ml}^{-1}$ caused hyphal growth to cease within about 50 min (Fig. 4c). In subsequent experiments, concentrations of 0.01 and $0.5 \mu\text{g ml}^{-1}$ were chosen for MCZ and KCZ, respectively.

Adaptation of *A. niger* hyphae to antifungal agents

The inhibition of hyphal growth caused by the first treatment with $0.075 \mu\text{g ml}^{-1}$ AMPH did not occur following the second treatment with the same concentration

Fig. 5. Adaptation of *A. niger* hyphal growth to (a) AMPH, (b) MCZ and (c) KCZ. At the arrows marked 'AMPH', 'MCZ' and 'KCZ', PDB was replaced by PDB containing 0.075 μg AMPH ml⁻¹, 0.01 μg MCZ ml⁻¹ and 0.5 μg KCZ ml⁻¹, respectively. At the arrows marked 'Washing', PDB containing an antifungal agent was replaced by PDB. Experiments were done in triplicate. The data are representative results obtained with the respective antifungal agent.

of the agent (Fig. 5a). Similarly, hyphal growth was inhibited by the first treatment with 0.01 μg MCZ ml⁻¹ and 0.5 μg KCZ ml⁻¹ but recovered after washing and was not appreciably affected by the second treatment with the same agents at the same concentrations (Fig. 5b, c).

Influence of exposure period and concentration of antifungal agents on the recovery of hyphal growth

In order to investigate adaptation further, we checked the effect of T_{EXPO} and concentration of the antifungal agents on τ_{OFF} and $t_{\text{OFF}} - \tau_{\text{OFF}}$. As shown in Fig. 6(a), neither τ_{OFF} nor $t_{\text{OFF}} - \tau_{\text{OFF}}$ was influenced by a change in T_{EXPO} of MCZ or KCZ. In contrast, τ_{OFF} became longer as the

Fig. 6. Comparison of parameters related to the recovery of hyphal growth that was previously halted by treatment with MCZ and KCZ. (a) Influence of T_{EXPO} on τ_{OFF} and $t_{\text{OFF}} - \tau_{\text{OFF}}$. ●, 0.01 μg MCZ ml⁻¹; ○, 0.5 μg KCZ ml⁻¹. (b) Influence of concentration on τ_{OFF} and $t_{\text{OFF}} - \tau_{\text{OFF}}$; T_{EXPO} was 60 min. The data are expressed as mean ± SD for three separate experiments. Statistical significance of differences was determined by the Student *t* test. **P* < 0.01 versus minimum concentration tested (MCZ, 0.01 μg ml⁻¹; KCZ, 0.05 μg ml⁻¹).

concentration of MCZ or KCZ increased, as depicted in Fig. 6(b). $t_{\text{OFF}} - \tau_{\text{OFF}}$, however, was not influenced by changes in concentration of MCZ or KCZ.

As shown in Fig. 5(a), hyphal growth at first ceased and then re-started in PDB containing 0.075 μg AMPH ml⁻¹. Therefore experiments on changing T_{EXPO} could not be carried out. On the other hand, as the concentration of AMPH increased, the response of individual hyphae showed large variations: some did not resume growth in spite of washing and in other cases bursting of the tips was observed. Therefore, with AMPH, no reproducible results were obtained concerning the relationship between the above parameters.

Fig. 7. Morphological changes in *A. niger* hyphae after exposure to (a) AMPH, (b) MCZ or (c) KCZ. Arrows indicate the positions of the hyphal tips when each antifungal agent was introduced. Bars represent 150 µm.

Morphological changes

Morphological changes in hyphae caused by antifungal agents are a useful indicator of the activity of the agent. In the present system, a tracing marker was adjusted at the position of the test hypha on a TV screen to trace the movement of the hyphal tip. However, the morphology of the hypha was not analysed quantitatively. If a large change occurred in hyphal morphology during the course of the experiment, it was necessary to check if the tracing marker should be moved. As shown in Fig. 7, AMPH caused no appreciable change in hyphal morphology. In contrast, MCZ and KCZ caused branching at the tips and the sides of hyphae. In the case of these two agents therefore, we moved the tracing marker to the most rapidly growing branch (a single hypha) and continued to measure its growth rate.

DISCUSSION

As shown in Fig. 5, *A. niger* became less sensitive to AMPH, MCZ and KCZ after repeated exposure to the respective agents. This fact was established by continually tracing the growth of the same hypha during repeated changes of the medium. We have defined this phenomenon as adaptation to the short-term stress caused by exposure to the antifungal agents.

The antifungal agents used in this study were a polyene (AMPH) and two imidazoles (MCZ and KCZ). These two types of agent are different in their mechanism of action (Bolard, 1986; Hiratani & Yamaguchi, 1985; Van den Bossche *et al.*, 1978; Yamaguchi & Hiratani, 1984). Since adaptation was observed with every agent, it cannot be ascribed to one specific mechanism.

As shown in Fig. 6, in the case of MCZ and KCZ, τ_{OFF} was found to be concentration-dependent but T_{EXPO} -independent. These results suggest that, though we do not know exactly what happened within the hyphae, the recovery process is started by the signal of antifungal introduction.

In order to establish if acquired adaptation could be inherited, subcultures were taken from mycelium which had adapted to the three antifungal agents. Spores were picked from these subcultures and the hyphae growing from them were assayed for their susceptibility to each agent. These hyphae were as sensitive as un-adapted hyphae (data not shown). This suggests that the adaptation observed in this study is not heritable.

This is the first study on the adaptation of filamentous fungi to antifungal agents performed at the single-hypha level. The dynamic response of a particular hypha to various agents can be continually monitored and analysed as demonstrated with AMPH, MCZ and KCZ. The results might also have clinical implications in that variations in antifungal concentration in patient serum could lead to phenotypic adaptation. Using the method described here, adaptation to other agents could be studied.

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REFERENCES

- Bolard, J. (1986). How do the polyene macrolide antibiotics affect the cellular membrane properties? *Biochim Biophys Acta* **864**, 257–304.
- Collison, L. P. & Dawes, I. W. (1992). Inducibility of the response of yeast cells to peroxide stress. *J Gen Microbiol* **138**, 329–335.
- Hiratani, T. & Yamaguchi, T. (1985). Mode of antifungal action of oxiconazole nitrate toward *Candida albicans*. *Chemotherapy* **33**, 215–226.
- Lenhart, K. (1973). Population aspects in genetics of dermatophytes. *Acta Univ Palacki Olomuc Fac Med* **65**, 59–74.
- Lenhart, K. & Merkunova, A. (1989). Adaptation of *Microsporum gypseum* to ketoconazole. *Acta Univ Palacki Olomuc Fac Med* **123**, 35–47.

- Oh, K., Matsuoka, H., Sumita, O., Takatori, K. & Kurata, H. (1992). Evaluation of antifungal activity of antimycotics by automatic analyzing system. *Mycopathologia* **118**, 71–81.
- Oh, K., Matsuoka, H., Sumita, O., Takatori, K. & Kurata, H. (1993a). Automatic evaluation of antifungal volatile compounds on the basis of the dynamic growth process of a single hypha. *Appl Microbiol Biotechnol* **38**, 790–794.
- Oh, K., Matsuoka, H., Nemoto, Y., Sumita, O., Takatori, K. & Kurata, H. (1993b). Determination of anti-*Aspergillus* activity of antifungal agents based on the dynamic growth rate of a single hypha. *Appl Microbiol Biotechnol* **39**, 363–367.
- Park, J.-C., Nemoto, Y., Homma, T., Jing, W., Chen, Y., Matsuoka, H., Ohno, H., Takatori, K. & Kurata, H. (1993). Adaptation of *Aspergillus niger* to short-term salt stress. *Appl Microbiol Biotechnol* **40**, 394–398.
- Riley, J. F., Wilson, R. G. & Barrett-Bee, K. J. (1984). Azole resistance in *Candida albicans*. *Sabouraudia* **22**, 53–63.
- Schroeder, L. L. & Bullerman, L. B. (1985). Potential for development of tolerance by *Penicillium digitatum* and *Penicillium italicum* after repeated exposure to potassium sorbate. *Appl Environ Microbiol* **50**, 919–923.
- Van den Bossche, H., Willemsens, G., Cools, W., Lauwers, W. F. J. & Le Jeune, L. (1978). Biochemical effects of miconazole on fungi. II. Inhibition of ergosterol biosynthesis in *Candida albicans*. *Chem Biol Interact* **21**, 59–78.
- Yamada, S., Cao, J., Sumita, O., Kurasawa, K., Kurata, H., Oh, K. & Matsuoka, H. (1992). Automatic antifungal activity analyzing system on the basis of dynamic growth process of a single hypha. *Mycopathologia* **118**, 65–69.
- Yamaguchi, H. & Hiratani, T. (1984). Studies on the mode of antifungal action of bifonazole. *Chemotherapy* **32**, 829–841.

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