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Sensitivity of different Zygomycetes to the *Penicillium chrysogenum* antifungal protein (PAF)

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The effects of the *Penicillium chrysogenum* low molecular mass antifungal protein (PAF) on selected fungal species belonging in the Zygomycetes were investigated. A total of 21 fungal isolates from 15 different genera (*Absidia, Actinomucor, Cokeromyces, Gilbertella, Micromucor, Mortierella, Mucor, Mycotypha, Rhizomucor, Rhizopus, Saksenaea, Syncephalastrum, Thamnostylum, Umbelopsis* and *Zygorhynchus*) were tested. The inhibitory potentials of PAF at 50 μg ml^{-1} on the germination of the sporangiospores and at 6.25–50 μg ml^{-1} on the hyphal extension were examined on different culture media. From among the fungi regarded as opportunistic human and/or animal pathogens, PAF exhibited inhibitory effects against *Absidia*, *Mortierella*, *Rhizomucor* and *Rhizopus* species.

Most filamentous fungi actively secrete proteins with various functions into their environment during vegetative growth (PEBERDY 1999). These proteins include molecules which exert biological activity against different groups of microbes. The structures and modes of action of the antimicrobial peptides (amphipathic and hydrophobic α-helices, β-sheet peptides and small proteins, peptides with irregular amino acids composition, peptides with thio-ether rings, peptaibols and macrocyclic cystine knot peptides) are very diverse (EPAND and VOGEL 1999, SELITRENNIKOFF 2001, THEIS and STAHL 2004). A novel antifungal peptide was recently isolated from the culture supernatant of the filamentous fungus *Penicillium chrysogenum* and characterized; it was named the *Penicillium* antifungal protein (PAF; MARX *et al*. 1995, KAISERER *et al*. 2003). PAF is secreted as a small molecular mass, highly basic cysteine-rich protein, which inhibits the germination, hyphal growth and conidiogenesis of several filamentous fungi. In terms of cell morphology, PAF-treated mycelia are generally characterized by swollen, short hyphae with multiple branches, a fragmented cytoplasm and the accumulation of nuclei at broken hyphal tips (KAISERER *et al.* 2003). PAF evokes K^+ efflux from the fungal cells (KAISERER *et al.* 2003) and is internalized by target fungi (OBERPARLEITER *et al.* 2003).

The effects of PAF on fungal species belonging in the Zygomycetes have so far been investigated on only a very limited number of isolates. In an earlier study, isolates of *Mucor circinelloides* and *Mucor genevensis* were found to be insensitive to PAF (KAISERER *et al.* 2003). However, no further data have been published concerning the sensitivities of other Zygomycetes, and especially those known to be pathogenic to humans and animals.

The number of cases of zygomycosis (an opportunistic fungal infection caused by members of the Zygomycetes) has increased dramatically during the past several years. The main risk factors for this disease are poorly controlled diabetes mellitus, haematologic malignancy, solid organ or bone marrow transplantation, prolonged steroid use, metabolic acido-

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sis, desferrioxamine B therapy, and severe and prolonged neutropenia. The major forms of zygomycosis are rhinocerebral, pulmonary, gastrointestinal, primary cutaneous and disseminated infections (RIBES *et al*. 2000). The prognosis of this infection is usually poor and mortality rates can approach 100%, depending on the patient's underlying disease and the type of zygomycosis. Nowadays, zygomycosis is one of the most frequent mycotic diseases caused by non-*Aspergillus* moulds (RIBES *et al*. 2000, NUCCI 2003, PRABHU and PATEL 2004). Unfortunately, these fungi have a substantial intrinsic resistance to most of the widely used antifungal drugs (e.g. azoles). Although there have been some promising results with certain new azole compounds (especially posaconazole; SUN *et al.* 2002), amphotericin B is still the only effective drug for the therapy of zygomycosis in clinical routine. However, the application of amphotericin B is seriously limited by its severe side-effects, especially, in the event of prolonged treatment (GEORGOPAPADAKOU 1998, SUN *et al.* 2002). There is therefore a substantial demand for new types of compounds with antifungal activity against the pathogenic species of Zygomycetes.

Materials and methods

Strains and media: In this study the effects of PAF on 21 fungal isolates from 16 different genera were investigated: *Absidia corymbifera* (SZMC 2010), *Actinomucor elegans* (NRRL 1706), *Cokeromyces recurvatus* (CBS 168.59), *Gilbertella persicaria* (M)101638(+), *Micromucor ramanniana* (NRRL 5844), *Mortierella elongata* (NRRL 5513), *Mortierella nanthalensis* (NRRL 5842), *Mortierella wolfii* (NRLL 28640), *Mucor hiemalis f. luteus* (NRRL 3632), *Mucor racemosus* (WRL CN(M)304), *Mycotypha africana* (NRRL 2978), *Rhizomucor miehei* (NRRL 5282), *Rhizomucor pusillus* (WRL/CN(M) 231), *Rhizopus microsporus* var. *oligosporus* (NRRL 514), *Rhizopus oryzae* (CBS 112.07), *Rhizopus stolonifer* (TJM 24B2), *Saksenaea vasiformis* (FSU 870), *Syncephalastrum racemosum* (SZMC 2011), *Thamnostylum piriforme* (MUFS 025), *Umbelopsis isabellina* (NRRL 1757) and *Zygorhynchus macrocarpus* (NRRL 2663). *Aspergillus niger* (IMI 381727) and *Aspergillus terreus* (NRRL 30313) were used as controls for a PAF-sensitive and a PAF-insensitive strain, respectively. Fungal strains were maintained on malt extract medium (MA; 0.5% malt extract, 0.5% yeast extract, 0.5% glucose, 1.0% KH_2PO_4) slants (solidified with 1.5% agar) at 4 °C. Tests for hyphal growth and spore germination inhibition were performed in MA, complete medium (CM; 0.2% peptone, 0.1% yeast extract, 0.1% NZ-amine A, 2% glucose, 0.05% KCl, 0.04% $MgSO_4 \cdot H_2O$, 0.15% KH_2PO_4, pH 6.5), yeast extract-glucose medium (YEG; 0.1% yeast extract, 0.5% KH_2PO_4, 1% glucose) and minimal medium (MM; 1% glucose, 0.5% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4$). Solid culture media always contained the same additives and were solidified with 1.5% agar.

Purification of PAF: PAF was purified via a modification of the method described by MARX *et al.* (1995). Briefly, *P. chrysogenum* NCAIM 00237 was grown in a sucrose (20 g l^{-1}) – $NaNO_3$ (3 g l^{-1}) minimal medium for 72 h at 25 °C with shaking (4.2 Hz) (MARX *et al.* 1995). After harvesting of the mycelia by centrifugation and separation of the low molecular weight protein fraction in Amicon Stirred Cells (V = 50 ml, Biomix PBTK ultrafiltration discs, size exclusion limit M_r = 30000; MILLIPORE, Billerica, MA, USA), PAF was purified by ion-exchange chromatography on a CM Sephadex Fast Flow column (2 × 18 cm, equilibrated with 50 mM sodium phosphate buffer, pH = 6.6, flow rate 1 ml min^{-1}, t = 4 °C; AMERSHAM-Pharmacia, Uppsala, Sweden). PAF was eluted with a NaCl gradient (0.05 – 1.0 M) prepared in the equilibrating buffer. The quality of the PAF preparation was always checked by SDS-PAGE on pre-cast Novex 16% Tris/Glycine gels (Invitrogen Life Technologies, Carlsbad, CA, USA). Protein bands were visualized with Coomassie Brilliant Blue R staining.

Antifungal activity assays: The effect of PAF on the germination efficiency of sporangiospores was examined in different culture media. Sporangiospores (10^4 ml^{-1}) were incubated with PAF (50 µg ml^{-1}) in a total volume of 0.2 ml of various liquid culture media (MA, CM, YEG or MM) for 18 h at 25 °C (except for *R. miehei* and *R. pusillus*, which were incubated at 37 °C), pelleted for 15 min at 10000 g, washed with 1.5 ml aliquots of culture media (KAISERER *et al.* 2003), and resuspended in a concentration of 10^3 sporangiospores ml^{-1} culture medium. Treated spores were plated onto appropriate solid

culture media in three replicates in volumes of 25 µl, 50 µl and 100 µl. Untreated sporangiospores were plated as controls. Colonies were counted after 24 h and 48 h of incubation at the temperatures indicated above.

An agar diffusion technique was used to estimate the degree of inhibition of hyphal extension by PAF. Solid culture media were overlaid with 5 ml of medium (CM, YEG, MA or MM, containing 1.5% agar) kept liquid at 44 °C after autoclaving and inoculated with 10^5 sporangiospores. 100 µl aliquots of a serial PAF dilution (6.25, 12.5, 25 and 50 µg ml^{-1}) were filled into wells which had been drilled into the solidified overlayer. PAF-free culture media were used as negative controls. The diameters of the inhibition zones were documented after incubation for 24 h and 48 h at 25 or 37 °C, as indicated above.

Results and discussion

Twenty-one fungal strains belonging in the Zygomycetes were tested for sporangiospore germination after PAF treatment (Table 1) and for hyphal growth in the presence of PAF (Table 2). Both tests were highly effective for monitoring of the inhibitory potential of PAF against Zygomycetes (Figs. 1 and 2).

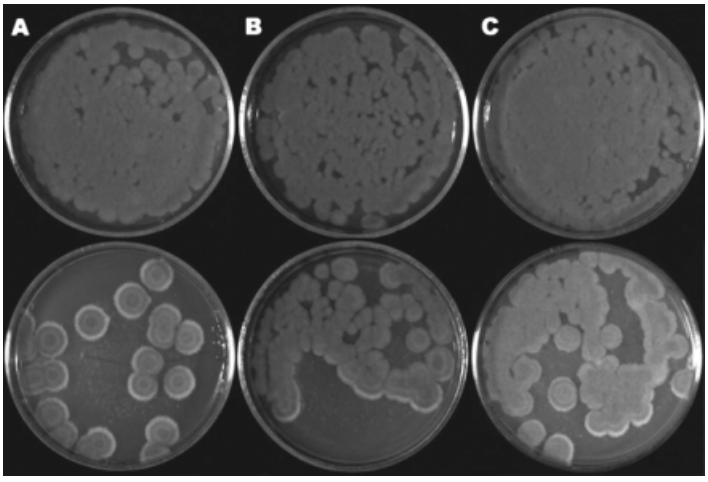

Fig. 1
Effect of PAF on the rate of germination of *M. africana* sporangiospores. Plates inoculated with the same amount of untreated (upper row) or PAF-treated (50 µg ml^{-1} PAF, 25 °C, 18 h, lower row) sporangiospores are shown. From the sporangiospore suspensions (10^3 spores ml^{-1}), 25 µl (**A**), 50 µl (**B**) and 100 µl (**C**) aliquots were plated onto solid MA medium

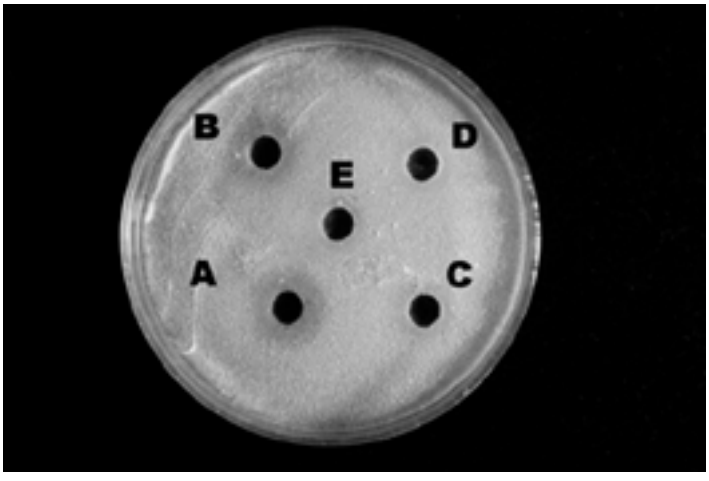

Fig. 2
Inhibition of *M. elongata* hyphal growth by PAF, tested on a CM culture medium plate after incubation at 25 °C for 48 h. The wells in the CM agar containted 100 µl PAF, diluted in CM at concentrations of 50 µg ml^{-1} (**A**), 25 µg ml^{-1} (**B**), 12.5 µg ml^{-1} (**C**) and 6.25 µg ml^{-1} (**D**). 100 µl CM without PAF was used as negative control (**E**)

Table 1
Inhibition of sporangiospore germination by PAF (50 µg ml^{-1}) treatment

Species	CM	YEG	MA	MM
Absidia corymbifera	+++	++	+	++
Actinomucor elegans	+	–	–	+
Cokeromyces recurvatus	–	–	–	–
Gilbertella persicaria	–	+	–	+
Micromucor ramanniana	+++	+++	+++	+++
Mortierella elongata	++	++	+	+++
Mortierella nanthalensis	+++	+++	+++	+++
Mortierella wolfii	++	++	++	+++
Mucor hiemalis f. luteus	+	–	–	+
Mucor racemosus	+	–	+	–
Mycotypha africana	+++	+++	++	+++
Rhizomucor miehei	+	–	++	++
Rhizomucor pusillus	+++	++	+	++
Rhizopus microsporus var. *oligosporus*	+++	–	++	+++
Rhizopus oryzae	+++	+	–	++
Rhizopus stolonifer	+	–	–	+
Saksenaea vasiformis	–	+	–	–
Syncephalastrum racemosum	+	–	–	+
Thamnostylum piriforme	+	-	+	++
Umbelopsis isabellina	+++	+++	+++	+++
Zygorhynchus macrocarpus	++	++	+	+++
Aspergillus niger	+++	+++	+++	+++
Aspergillus terreus	–	–	–	–

+++: highly sensitive (75–100% inhibition of germination); ++ sensitive: (50–75% inhibition of germination); +: slightly sensitive (25–50% inhibition of germination); –: insensitive (0–25% inhibition of germination). Inhibition efficiency was calculated in comparison with untreated controls

In a majority of the cases of zygomycoses caused by members of the order Mucorales, *Rhizopus*, *Mucor*, *Absidia* and *Rhizomucor* species were identified as the causative agents of these opportunistic infections. *Apophysomyces*, *Cunninghamella*, *Cokeromyces*, *Saksenaea* and *Syncephalastrum* spp. have also been confirmed as potential opportunistic human pathogens, while *Mortierella* spp. (e.g. *M. wolfii*) are regarded as true animal pathogens (RIBES *et al.* 2000). The antifungal activity of PAF was tested on the whole range of human and/or animal pathogenic Zygomycetes, with the exceptions of *Apophysomyces* and *Cunninghamella*.

In accordance with earlier studies (KAISERER *et al.* 2003, MARX 2004), all the *Mucor* strains tested (*M. hiemalis f. luteus* and *M. racemosus*) were insensitive to PAF in both sporangiospore and hyphal extension tests (Tables 1 and 2). Similarly, *A. elegans*, *C. recurvatus*, *G. persicaria*, *R. stolonifer*, *S. vasiformis* and *S. racemosum* were also practically insensitive to PAF, independently of the culture conditions and inhibition tests applied (Tables 1 and 2). On the other hand, *A. corymbifera*, *M. ramanniana*, *Mortierella* sp., *M. africana*, *Rhizomucor* sp., *R. microsporus* var. *oligosporus*, *R. oryzae*, *T. piriforme* and *U. isabellina* were sensitive to PAF.

The sensitivity of some of the Zygomycetes to PAF depended on the culture medium and the inhibition test selected, e.g. in the cases of *M. africana*, *R. pusillus* and *U. isabellina*, the inhibitory effect on hyphal growth was usually weaker than that on sporangiospore germination (Tables 1 and 2). The highest degrees of inhibition were recorded on the MM culture medium. The background of the dependence of the antifungal effect of PAF on the composition of the culture media has not yet been clarified; it is possibly explained, for instance by

Table 2
Inhibition of fungal growth in the presence of PAF

Species	CM	YEG	MA	MM
Absidia corymbifera	++	++	+++	++
Actinomucor elegans	+	–	–	–
Cokeromyces recurvatus	–	–	–	–
Gilbertella persicaria	–	–	–	+
Micromucor ramanniana	++	++	++	+++
Mortierella elongata	+++	+++	++	+++
Mortierella nanthalensis	++	++	+	+++
Mortierella wolfii	+++	+++	+++	+++
Mucor hiemalis f. luteus	–	–	–	+
Mucor racemosus	–	–	–	–
Mycotypha africana	++	+	++	+++
Rhizomucor miehei	+	+	–	++
Rhizomucor pusillus	+	+	–	++
Rhizopus microsporus var. *oligosporus*	+++	+++	++	+++
Rhizopus oryzae	+	++	–	++
Rhizopus stolonifer	–	–	–	+
Saksenaea vasiformis	–	+	–	+
Syncephalastrum racemosum	–	–	–	+
Thamnostylum piriforme	++	+	+	+++
Umbelopsis isabellina	–	+	+	++
Zygorhynchus macrocarpus	+++	+++	++	+++
Aspergillus niger	+++	+++	+++	+++
Aspergillus terreus	–	–	–	–

+++: highly sensitive (5–7 mm inhibition zone), ++: sensitive (3–4 mm inhibition zone), +: slightly sensitive (1–2 mm inhibition zone), –: insensitive (no inhibition). Inhibition zones were determined at the highest PAF concentration

the presence of certain constituents in the media which may interfere with the activity of PAF (e.g. salts; KAISERER *et al.* 2003, MARX 2004). Further studies are planned to clarify this question.

Among the Zygomycetes regarded as opportunistic human and/or animal pathogens, PAF was effective against *Absidia*, *Mortierella*, *Rhizomucor* and *Rhizopus*. These results support the assumption that PAF and similar low molecular mass basic antifungal proteins produced by filamentous fungi (MARX 2004) should be considered promising candidates in future antifungal drug research.

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