Medical Mycology 2001, **39**, 395–400 Accepted 13 December 2000

Immuno-crossreactivity of an anti-*Pichia anomala* killer toxin monoclonal antibody with a *Williopsis saturnus* var. *mrakii* killer toxin

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A monoclonal antibody (mAbKT4), produced against the *Pichia anomala* ATCC 96603 killer toxin (*Pa*KT) was used to detect the toxin (*Wm*KT) produced by *Williopsis saturnus* var. *mrakii* MUCL 41968 which inhibits the growth of a *Pa*KT-sensitive *P. anomala* strain MUCL 41969. Immunofluorescence studies revealed that mAbKT4 specifically labels the surface of *P. anomala* and *W. saturnus* var. *mrakii*, suggesting that both taxa secrete a killer toxin bearing a common epitope. Immunoblot analyses of concentrated supernatants from *P. anomala* and *W. saturnus* var. *mrakii* cultures showed that in both taxa mAbKT4 reacts with high molecular weight secreted proteins ranging 85–200 kDa. However, immunoblot experiments showed that the molecular weights of *Pa*KT and *Wm*KT are quite different, indicating that the two toxins are related but not identical molecules.

Keywords antifungal agent, killer toxin, *Pichia anomala*, *Williopsis saturnus* var. *mrakii*

Introduction

Yeast killer toxins are antifungal molecules secreted by a large variety of yeast killer strains [1,2]. The most frequently encountered killer strains belong to the genera *Saccharomyces*, *Candida*, *Kluyveromyces* and *Hansenula*. *Hansenula* species are now classified in the genera *Williopsis* or *Pichia* [3]. *Hansenula* and *Pichia* were first distinguished on the basis of differences in their ability to assimilate nitrate as a sole source of nitrogen. The genus *Williopsis* was mainly characterized by its saturn-shaped ascospores [4]. Further studies demonstrated that *Williopsis* and *Pichia* are polyphyletic.

Based on comparative analysis of 18S ribosomal RNA (rRNA) gene, it is apparent that the genus *Williopsis* should be restricted to the type species *W. saturnus* including the varieties *saturnus*, *mrakii*, *sargentensis*, *suaveolens* and *subsufficiens* [5]. These five varieties have identical 18S rRNA gene sequences and form a taxonomic group distinct from *Pichia* species. Among varieties of *Williopsis saturnus*, structural similarities have been shown between the killer toxins already characterized, especially in *W. saturnus* var. *mrakii* and *W. saturnus* var. *saturnus* [6]. However, no relationships were found between these killer toxins and those secreted by killer strains of *W. beijerinckii* and *W. californica*. In *W. saturnus* var. *mrakii*, killer toxins have been described that show different molecular weights and physiological features. The *W. saturnus* var. *mrakii* killer strain 500 (NCYC, National Collection of Yeast Cultures, Norwich, UK) has been found to produce a killer toxin (K-500) of 1·8–5·0 kDa. K-500 displays an extensive anti-*Candida* activity above 30 °C

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and at pH 4·0 [7]. Another *W. saturnus* var. *mrakii* strain named IFO 0895 (Institute For Fermentation, Osaka, Japan), formerly LKB 169, secretes a proteinaceous toxin called HM-1 of 10·7 kDa which is active between pH 2·0 and pH 11·0 [8]. Finally, a *W. saturnus* var. *saturnus* killer strain (IFO 0117) produces a toxin (called HSK, formerly HYI) of 8·5 kDa, which is active at pH 5·8 [6].

In the genus *Pichia*, which consists of nearly 100 species [9], genomic studies on killer yeasts have been made using restriction fragment length polymorphism and Southern blot hybridization [10]. As described in *Williopsis* species, *Pichia anomala* variants produce strain-specific killer toxins. A halotolerant strain of *P. farinosa* (called KK1) produces a salt-mediated killer toxin (SMK) of 14·2 kDa showing maximum killer activity in the presence of 2 M NaCl [11]. *P. kluyveri* produces a 19 kDa killer toxin with potent antimicrobial activity between pH 2·5 and pH 5·0 [12]. A killer toxin (*Pa*KT) has been studied in the *P. anomala* ATCC 96603 (American Type Culture Collection, Manassas, VA, USA) killer strain (formerly UP25F) using a *Pa*KT-neutralizing monoclonal antibody [13]. *Pa*KT was proven to be active against a wide spectrum of yeast isolates belonging to *Candida albicans*, *P. anomala* and *Saccharomyces cerevisiae*, and was also effective against *Pneumocystis carinii*, an atypical fungal microorganism [14].

Recently, a yeast killer strain (Mycothèque de l'Université Catholique de Louvain 41968 [MUCL, Louvain, Belgium]) formerly designated K36 [15–17], first considered to be a *P. anomala* strain, has been shown to be a *W. saturnus* var. *mrakii* strain as demonstrated by its formation of saturn-shaped rather than hat-shaped ascospores in a sporulation medium. Since the toxin produced by this killer strain is also active against *C. albicans*, *P. anomala*, *S. cerevisiae* and *P. carinii*, we investigated the killer activity of *W. saturnus* var. *mrakii* MUCL 41968 toxin (*Wm*KT) using the *Pa*KT-neutralizing monoclonal antibody (mAbKT4). The results presented in this study indicate that the killer toxins produced by *W. saturnus* var. *mrakii* MUCL and *P. anomala* ATCC 96603 are related but not identical.

Materials and methods

Yeast identification and characterization

Strains were characterized according to the Belgium Coordinated Collection of Microorganisms (BCCM/MUCL, Louvain; Belgium) automated identification system employing both physiological criteria and morphological features [18]. For morphological characterization, the strains were grown on YPDA (yeast 2%, peptone 1%, glucose 0·5%, agar 3%). Germ tube formation was obtained with Dalmau's technique [19] by seeding the strains on PDA (potato 20%, glucose 2%, agar 2%) medium. To induce sporulation, strains were seeded on McClary's acetate agar medium [20]. Results were computed using the ALLEV identification software [18].

Killer toxin production

As a preculture, *P. anomala* ATCC 96603 and *W. saturnus* var. *mrakii* MUCL 41968, were cultured at 26 °C for 48 h at 70 rpm in 100 ml of Sabouraud broth (tryptone 5%, meat peptone 5%, glucose 20%) and buffered at pH 4·6 with 0·1 M citric acid and 0·2 M sodium phosphate. For production of killer toxin, a 250 ml flask containing 90 ml of Sabouraud broth was seeded with 10 ml of this preculture and incubated at 26 °C for 72 h at 70 rpm.

Killer toxin activity assay

The *P. anomala* ATCC 96603 or *W. saturnus* var. *mrakii* MUCL 41968 killer cells were harvested by centrifugation at 4500 *g* for 10 min. The supernatants containing the secreted *Pa*KT or *Wm*KT were filtered through a 0·45 µm filter (Millipore S.A, Saint-Quentin-en-Yvelines, France) and concentrated 50-fold by using an ultrafiltration cell under N_2 pressure with a 10 kDa cut-off YM-10 membrane (Millipore S.A.). Aliquots (50 µl) of each concentrated supernatant were mixed into 200 µl liquid Sabouraud broth containing 5×10^4 cells of a killer toxin sensitive *P. anomala* strain, MUCL 41969, seeded into wells of 96-well microtiter plates (Costar, Brumath, France). The plates were incubated at 26 °C for 18–20 h. Following incubation, the optical density at 630 nm was measured using an automatic plate recorder (Bio-tek Instruments, Winooski, VT, USA). Killer toxin activities were measured by observation of a reduction in growth of the sensitive strain compared to a toxin-free microculture control.

Immunofluorescence assay

A monoclonal *P. anomala* killer toxin-neutralizing antibody (mAbKT4) was used in immunofluorescence assays (IFA) to localize the secretion sites of *Pa*KT and *Wm*KT on the surface of the yeast killer producing cells [13]. The *P. anomala* ATCC 96603 and *W. saturnus* var. *mrakii* MUCL 41968 yeast cell suspensions were centrifuged (10 000 *g* for 4 min) and pellets were resuspended in sterile distilled water. The resulting suspensions were adjusted to a final concentration of approximately 5×10^3 cells μl^{-1}. Twenty microlitres of each standardized

yeast cells suspension were put into the wells of an IFA slide and left to dry and fix for 45 min at 50 °C. Twenty-five microlitres of mAbKT4 diluted 1:200 in phosphate-buffer saline (PBS) were added to each well and incubated for 35 min at 37 °C in a humid chamber. The slides were then washed three times with PBS (10 min each) and 25 µl of fluorescein isothiocyanate (FITC)-goat anti-mouse immunoglobulin (Ig)G (Sigma-Aldrich, Saint Quentin Fallavier, France) diluted 1:50 were allowed to react for 1·5 h at 37 °C in a humid chamber. The slides were then washed three times in PBS (10 min each) and mounted with a coverslide with mounting medium containing glycerol, mowiol and Dabco (Sigma-Aldrich). The slides were then observed using epifluorescence microscopy (Axiophot 2, Zeiss S.A., Le Pecq, France). As negative controls, the IFA were carried out using PBS in place of mAbKT4 and cells were stained using Evans Blue 1% (Argene Biosoft, Varilhes, France) diluted 1:10000 in PBS.

SDS-PAGE and immunoblotting

The 50-fold concentrated supernatants prepared as described above were subjected to sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a 4–15% linear acrylamide gel [21]. After SDS-PAGE, proteins were transferred onto nitrocellulose membranes (Bio-Rad S.A., Ivry-sur-Seine, France) as described by Towbin *et al* [22]. Killer toxin proteins were then detected with mAbKT4. Immune complexes were revealed with alkaline phosphatase-linked goat anti-mouse IgG (Roche Diagnostics S.A., Meylan, France).

Results

Yeasts identification and characterization

At the morphological level, both *P. anomala* 96603 and *W. saturnus* var. *mrakii* MUCL 41968 appeared on YPD plates as cream colored colonies with a mat surface, soft texture, convex shape, furrowed surface and entire margin. The vegetative cells were spherical to ovoid for the two strains but occasionally ellipsoid cells could be observed for *P. anomala* 96603. Both strains developed a pseudomycelium on a Dalmau plate. Nevertheless, *P. anomala* 96603 and *W. saturnus* var. *mrakii* MUCL 41968 grown on McClary's acetate agar were distinguished by their ascospores, which were hat-shaped and saturniform, respectively. In the two strains, two to four ascospores measuring 2–3 × 1·5–2 µm formed per ascus and had smooth, thin walls. They were liberated soon after formation. Despite similarities in vitamin requirements, the isolates showed some different carbon fermentation and assimilation features (Table 1).

The morphological and physiological features of the two isolates were compared with entries in the BCCM database. The computer-assisted system developed for the identification of yeast at MUCL showed that *P. anomala* ATCC 96603 displayed a 100% similarity with the reference strain *P. anomala* MUCL 28639. For *W. saturnus* var. *mrakii* MUCL 41968, a 97·8% similarity was observed with reference strain *W. saturnus* var. *mrakii* MUCL 31950.

Killer toxin assay

When *P. anomala* MUCL 41969 was incubated with either of the killer toxins, a cytocidal effect was observed, suggesting that *Pa*KT and *Wm*KT strongly inhibited the growth of the susceptible strain by reducing the number of viable cells present. These observations confirmed our previous results, which showed that these killer toxins were active against *P. anomala* MUCL 41969 yeast cells and could be completely neutralized using mAbKT4 [13–15]. The killer activity of both toxins was more effective when cultures were in the exponential phase of growth, suggesting an effect on cellular reproduction.

Killer toxin immunodetection

As shown in Figure 1, the binding of mAbKT4 at the surface of the yeast killer cells indicated an accumulation of the toxin in the cell wall during the secretion process. This trend was clear even though the labelling density varied from cell to cell. In our study, IFA did not show any differences in mAbKT4 fixation at the surface of *P. anomala* or *W. saturnus* var. *mrakii* killer strains, suggesting the presence of a common epitope shared by *Wm*KT and *Pa*KT as well as a comparable expression.

Immunoblotting experiments performed on concentrated culture supernatants of *P. anomala* and *W. saturnus* var. *mrakii* killer strains showed that mAbKT4 reacts with high-molecular weight proteins present in both samples (Fig. 2). In accordance with a previous study [13], the mAbKT4 reacted with a *P. anomala* secreted protein displaying an apparent molecular weight of 115 kDa. However, in the *W. saturnus* var. *mrakii* supernatant, mAbKT4 reacted with two proteins exhibiting apparent molecular weights of 85 and 200 kDa.

Discussion

The monoclonal antibody mAbKT4 has been shown to specifically neutralize the activity of a killer toxin

Table 1 *W. saturnus* var. *mrakii* MUCL 41968 and *P. anomala* ATCC 96603 growth factor and assimilation/fermentation tests as seen in microplates

	W. mrakii MUCL41968	*P. anomala* MUCL41969		*W. mrakii* MUCL41968	*P. anomala* MUCL41969		*W. mrakii* MUCL41968	*P. anomala* MUCL41969
Fermentation								
D-glucose	+	+	α,α-trehalose	−	−	Raffinose	−	+
D-galactose	−	+	Melibiose	−	−	Inulin	−	−
Maltose	−	+	Lactose	−	−	Soluble starch	−	−
Methyl α-D-glucosidase	−	−	Cellobiose	−	+	D-xylose	−	−
sucrose	−	+	Melezitose	−	−			
Carbon assimilation								
D-glucose	+	+	Arbutin	+	+	Myo-inositol	−	−
D-galactose	−	+	Melibiose	−	−	D-glucono-1,5-lactone	+	+
L-sorbose	−	−	Lactose	−	−	2-keto-gluconate	−	−
D-glucosamine	−	−	Raffinose	−	−	5-keto-gluconate	−	−
D-ribose	−	−	Melezitose	−	+	D-gluconate	+	+
D-xylose	+	−	Inulin	−	−	D-glucuronate	−	−
L-arabinose	−	−	Soluble starch	?	?	D-galacturonate	−	w
D-arabinose	−	−	Glycerol	+	+	DL-lactate	+	+
L-rhamnose	w	−	Erythritol	−	+	Succinate	+	+
Sucrose	w	+	Ribitol	−	w	Citrate	w	+
Maltose	−	+	Xylitol	−	−	Methanol	w	−
α,α-trehalose	−	w	L-arabinitol	−	−	Ethanol	+	+
Methyl α-D-glucoside	−	+	D-glucitol	−	+	Propane 1,2 diol	+	+
Cellobiose	+	−	D-mannitol	−	+	Butane 2,3 diol	w	+
Salicin	+	+	Galactitol	−	−			
Nitrogen assimilation								
Nitrate	+	+	L-lysine	+	+	Creatinine	−	−
Nitrite	+	+	Cadaverine	+	+	Glucosamine	−	−
Ethylamine	+	+	Creatine	−	−	Imidazole	−	−
Vitamin assimilation								
w/o Vitamins	+	w	w/o Thiamin	+	+	w/o Niacin	+	+
w/o Myo-inositol	+	+	w/o Biotin and thiamin	+	+	w/o P-aminobenzoic acid	+	+
w/o Ca pantothenate	+	+	w/o Pyridoxin	+	+			
w/o Biotin	+	+	w/o Pyridoxin and thiamin	+	+			

−, negative; +, positive; ?, unknown; w, weak; w/o, without.

produced by *P. anomala* ATCC 96603 [13]. In this study, the previously described killer strain *P. anomala* K36 [15–17], here identified as *W. saturnus* var. *mrakii* strain MUCL 41968, was shown to produce a similar toxin recognized in immunofluorescence studies by mAbKT4. Immunoblotting experiments showed that the antigenic determinants produced by *P. anomala* ATCC 96603 and *W. saturnus* var. *mrakii* MUCL 41968 had higher molecular weights than killer toxins secreted by other *Pichia* or *Williopsis* isolates. Nevertheless, the toxins produced by the two yeast isolates exhibited different molecular weights. Although these results show that *Pa*KT is not produced by *W. saturnus* var. *mrakii* MUCL 41968, its toxin was also recognized and neutralized by mAbKT4 as *Pa*KT is. Furthermore, its antibiotic properties were also close to those of *Pa*KT as it is also active against *C. albicans*, *P. anomala*, *S. cerevisiae* and *P. carinii* [23].

Further molecular characterization of *Pa*KT and *Wm*KT should confirm if they are structurally related, as has been reported for killer toxins produced by various strains of the *W. saturnus* complex. For instance, peptidic homology of 87% was reported between killer toxins HM-1 and HYI produced by *W. saturnus* var.

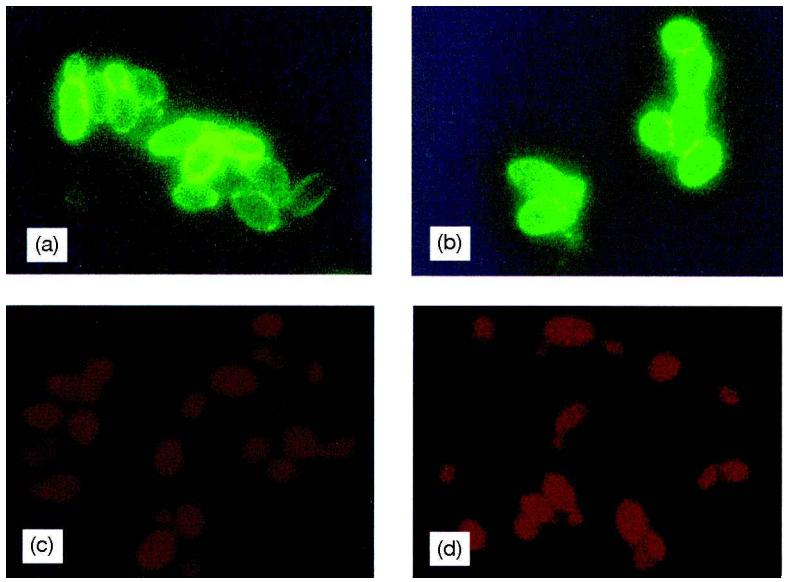

Fig. 1 Immunofluorescence assays (IFA). (a) *P. anomala* (ATCC 96603) and (b) *W. saturnus var. mrakii* (MUCL 41968) labeled with the anti-*P. anomala* killer toxin-neutralizing monoclonal antibody mAbKT4. Killer cells were characterized by a punctuate labeling pattern with concentrated label in defined areas. Similar patterns were observed on the outer surfaces of killer *P. anomala* and *W. saturnus* var. *mrakii* cells. As negative controls, the IFA were carried out on (c) *P. anomala* (ATCC 96603) and (d) *W. saturnus* var. *mrakii* (MUCL 41968) using phosphate-buffered saline (PBS) in place of mAbKT4. Cells were stained with Evans Blue 1% diluted 1:10000 in PBS. Magnification ×1500.

mrakii and *W. saturnus* var. *saturnus* [24]. In addition, Southern blot analyses using gene fragments of *HMK* and *HSK* showed cross-hybridization with genomic DNA fragments of *W. saturnus* var. *mrakii*, *W. saturnus* var. *saturnus* and *W. saturnus* var. *subsufficiens*. These results suggested that these killer strains have sequences homologous to *HMK* and *HSK*, even if their toxins are not identical to each other [6].

The results of this study show that *Pa*KT and *Wm*KT share an epitope that may be involved in their antifungal properties as demonstrated by the killer activity of anti-idiotypic monoclonal antibodies directed against mAbKT4 [25,26]. KT-anti-idiotypic antibodies were produced by immunization against the variable domain (idiotype) of mAbKT4, but they were also isolated from vaginal fluid of patients with recurrent vaginal candidosis

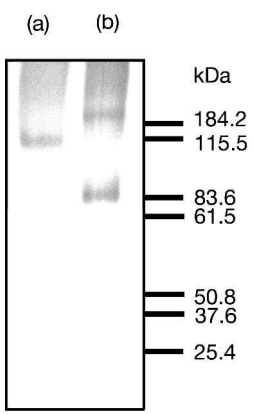

Fig. 2 Western blot analysis using mAbKT4 to probe 50× concentrated culture supernatant of *P. anomala* ATCC 96603 (a) and *W. saturnus* var. *mrakii* MUCL 41968 (b). Molecular weights expressed in kDa are shown in the right margin.

[27]. Moreover, these different anti-idiotypic molecules mimic the mAbKT4-sensitive anti-microbial activity of *Pa*KT. The fact that *Wm*KT is also recognized and neutralized by this antibody suggested that the common epitope is involved in the toxic activity. Further analyses of *Wm*KT and *Pa*KT molecules should reveal the relationships between their structures and lead us to understand their evolutionary and functional relations.

Acknowledgements

We would like to thank Dr O. Legeay from the Lille Pasteur Institute for fruitful discussion and criticism. C. Guyard is supported by a Ph.D. grant from the Région Nord Pas-de-Calais (France) and the Institut Pasteur de Lille (France). BCCM/MUCL (Belgian Coordinated Collections of Microorganisms/Mycothèque de L'UCL) is supported by the contract C2/10/007 of the prime Minister's services, Scientific, Technical and Cultural Affairs (SSTC).

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