BIOLOGIJA. 2007. Vol. 18. No. 1. P. 45–49

Search for biological control agents against *Candida* yeasts and other dermatomycetes

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The objective of this work was to screen the killer action of the bacterial isolates T1x, T2x, T3x, Ux and Ux308 against some dermatomycetes and the genus *Candida* yeast. The bacterial isolates T1x, T2x, T3x, Ux were recovered from spontaneous fruit and berry fermentations and the Ux308 isolate from soil. The raw materials were gathered in Lithuania. The dermatomycetes and *Candida* yeast were isolated from morbid material of patients with diagnosed dermatomycosis and onychomycosis at the Vilnius University Hospital Santariškių Klinikos Diagnostic Centre. The killer ability was tested using routine methods and standard yeast *Saccharomyces cerevisiae* strains. In the course of screening bacterial isolates for killer activity we found that the bacterial isolate T1x exhibited a very strong antifungal action against the dermatomycetes *Trichophyton rubrum*. Extracts of the bacterial isolates T1x, T2x, T3x, Ux and Ux308 showed a killer effect against *Candida* yeast. The growth of dermatomycete *Microsporum canis* was fungistatically affected by bacterial Ux isolate. The results of investigation showed that there is essential to study the biochemical properties of substances which are secreted of the bacterial isolates T1x, T2x, T3x, Ux and Ux308. The fact that the substances from bacterial isolates kill some pathogenic microorganisms allows us to expect their use in medicine. It is possible that the toxins secreted of bacterial isolates could kill also other genera of yeast and micromycetes.

Key words: dermatomycetes, *Candida*, bacteria, killer activity

INTRODUCTION

Fungal infections represent a serious health problem worldwide: 3 to 10% of the human population are attacked by different mycoses such as onychomycoses, dermatomycoses, pilomycoses [1]. In the past few years such fungal infections have spread in Lithuania too. *Candida* yeast and some other micromycetes such as *Trichophyton* Malmsten and *Microsporum* Gruby are the most frequent microorganisms in mycosis patients. They take up an exclusive place among fungal microorganisms and include about 70% of all fungi causing mycoses. Very frequent are infections caused by *Candida* Berkhout, *Rhodotorula* F.C. Harison, *Malassezia* Baillon, *Geotrichum* Link: Fries, *Trichosporon* Behrend, *Cryptococcus* Vuillemin [2].

Various drugs are used for treating mycosis. Antifungal pharmaceuticals are categorized depending on their site of action, on their mechanism of action or their chemical nature. Mostly they are classified by their chemical nature to such groups as grisans (griseofulvin), polyenes-macrolides (amphotericin B, nystatin), pyrimidine derivatives (flucytosine), azoles (imidazoles and triazoles), allylamines (terbinamine), thiocarbamates (tolnaftate, tolciclate), hydroxipiridons (ciclopirox), etc. [3].

Antifungal drugs belong to two groups considering their mechanism of action. Agents of the first group disturb the cell wall and membrane, and the drugs of the second group act on the intracellular processes: cell division, biosynthesis of nucleic acids, proteins and other biologically active substances. Agents of the alylamine, phenylmorpholine and azole groups disturb ergosterol synthesis. Phenylmorpholine combines with proteins Erg24p (Δ_{14}-reductaze) and Erg2p (Δ_8-Δ_7-izomeraze) participating in ergosterol biosynthesis. Antifungal medicines from the azole group inhibit cytochrome P-450 protein Erg11p/Cyp51p. The antifungal drug echinocandin belonging to drugs of a new generation is a chemically modified fungal metabolite. It influences the synthesis of cell wall components, including β-1-3-D-glucan. Antifungal drugs from the sordarin group are known as the inhibitors of protein synthesis [3–5].

The use of chemical antifungal agents in therapy is markedly limited by problems of drug safety, resistance and some side effects, such as allergy.

The antifungal substances displaying bacteriocidal and fungicidal effects could be found in prokaryotic as well as in eukaryotic cells. A number of yeasts secrete proteins that are lethal to sensitive fungal cells. Fungal cells secreting a killer toxin are resistant to their own toxin but sensitive to other toxins. *S. cerevisiae, Ustilago maydis, Hanseniaspora uvarum, Phaffia rhodozyma, Kluyveromyces lactis* and several *Pichia* species secrete a number of killer toxins [6–8]. Some *S. cerevisiae* yeasts of a killer phenotype have been already applied in wine industry as selected starters in fermentation. The strain *Kluyveromyces phaffii* DBVPG 6076 secreted a wide spectrum of killer toxins against *Hanseniaspora* genus yeasts [9]. *Zygosaccharomyces baillii* secrets zygocin, a protein toxin which shows antifungal effects on pathogenic and phytopatogenic yeasts and some mycromycetes [10]. The antimicrobial activity of plant oils and extracts has been recognized many years ago. It was noted that the essential oil produced from *Thymus revolutus* Celak killed *Candida albicans* and *Candida tropicalis* [11]. It is known that substances extracted from needles and fruits of *Juniperus* L. have a killing effect on the dermatomycetes *Trichophytum rubrum, Microsporum canis*, *Candida* yeasts and *Aspergillus* strains [12]. It is known that some insects, mammals and plants synthesize a number of proteins and peptides that are antifungal. There are hundreds of antifungal peptides and proteins (known in plants) called pathogenesis-related proteins [7, 13]. Little is known about the antifungal factors secreted by bacteria. Therefore, recovering natural materials of biological origin with killer effects on dermatomycetes and other micromycetes is important and promising. Our experiment was focused on the investigation of some substances with the killer (antifungal) potency, secreted by bacterial isolates from spontaneous fermentation and soil.

The objective of this work was to screen the killer action of bacterial isolates T1x, T2x, T3x, Ux and Ux308 against dermatomycetes *Trichophyton rubrum, Microsporum canis* and *Candida* yeasts. Data concerning the adherence to species are not published because of a possible commercial importance of these microorganisms.

Recently we have found six microorganisms secreting killer substances capable to kill some fungous agents of plant diseases [14]. According to these preliminary data, we decided to investigate the toxic properties of bacterial isolates for killer activity and possibility to kill some pathogenic fungi such as *Fusarium, Verticillium albo-artum* and *Venturia ineaqualis*. Thus, it was logical to evaluate the possibility of bacterial isolates to affect human pathogenic microorganisms.

MATERIALS AND METHODS

The experiment was performed with *Trichophyton rubrum* (Castellani) Sabouraud, *Microsporum canis* Bodin dermatomycetes and *Candida* yeasts: *Candida albicans* (Robin) Berkhout, *C. famata* (F. C. Harrison) S. A. Meyer et Yarrows *C. glabrata* (H. W. Anderson) S. A. Meyer et Yarrow, *C. kefyr* (Beijerinck) van Uden et Buckley, *C. lusitaniae* van Uden et do Carmo-Sousa, *C. parapsilosis* (Ashford) Langeron et Talice, *C. tropicalis* (Castellani) Berkhout. The dermomycetes and *Candida* yeasts were isolated from morbid material of patients with the diagnosis of dermatomycosis and onychomycosis at Vilnius University Hospital Santariškių Klinikos Diagnostic Centre. The fungi were screened on Sabouraud Agar and Corn Meal Agar supplemented with antibiotics (Oxoid, England). The identification of yeast strains was performed applying the Candifast and Funichrom methods and diagnostic systems (International Microbio, France) and recommendations [15–17].

The bacterial isolates T1x, T2x, T3x, Ux were obtained from spontaneous fermentation of fruits and different berries [18]. The test isolate Ux308 was prepared from a soil specimen (Kryžkalnis, Lietuva) after repeated cloning at the Laboratory of Biodeterioration Research.

Proteolytic assay. For testing the proteolytic activity of bacterial isolates T1x, T2x, T3x, Ux, Ux308, a broth medium yeast nitrogen base (Difco, USA) supplemented with glucose l g/1, colored skin powder, 3 g/l was used. Bacterial cultures were tested by the stroke method. The plates were incubated for 3 days at 27 ± 1 °C. The clear zone formed around the stroke was evaluated as a positive proteolytic test.

Assay of killer activity. The *S. cerevisiae* strain α'1 (MATα, *leu2-2 [kil-0]*), sensitive to all killers, was used for testing the activity of killer toxin. *S. cerevisiae* killer strains K7 (MAT*a*, *arg9 [kil-K1]*), Rom-K100 (wt, *HM/HM [kil- K2]*), MS300 (MAT į *leu2 ura3- 52 [kil-K28]*) were employed for the control. Killer activity was determined under sterile conditions. The bacterial isolates T1x, T2x, T3x , Ux, Ux308 were spotted on MB agar plates with methylene blue seeded with *S. cerevisiae* or dermatomycetes (as indicator) strains. After the incubation of plates at 20–25 °C for 3–5 days, clear zones of lawn growth inhibition were evaluated. The size of the clear zone was interpreted as killer activity [18].

RESULTS AND DISCUSSION

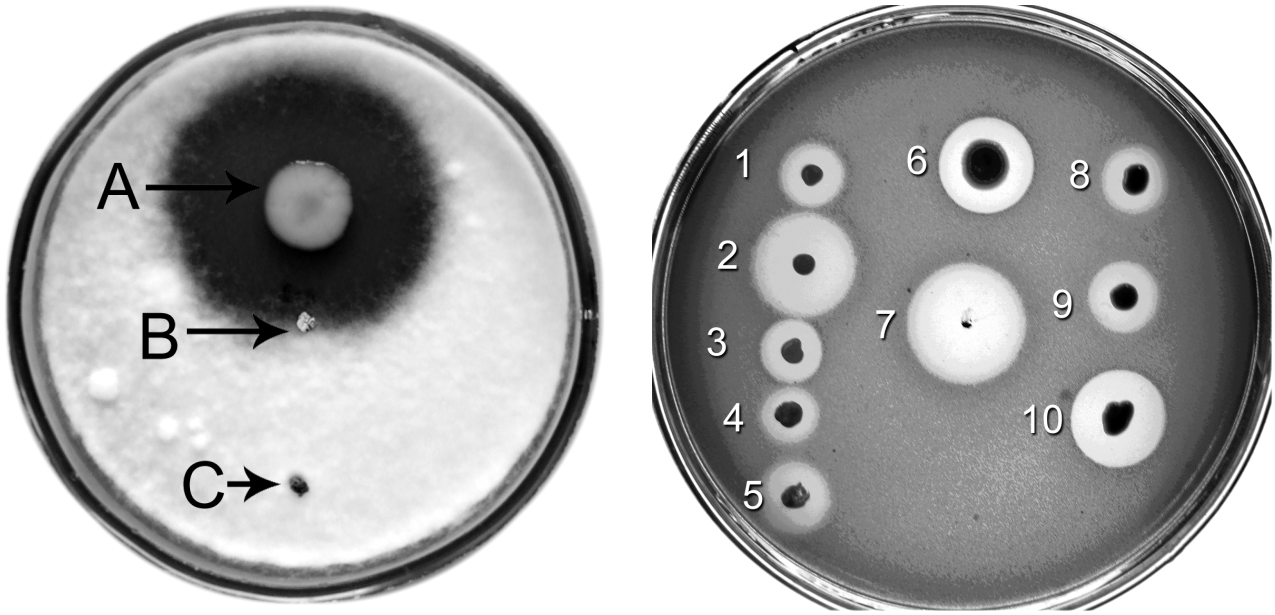
To obtain novel killer substances, we have recently initiated screening the bacterial isolates of spontaneous fermentation for killer activity. We carried out tests with bacterial isolates T1x, T2x, T3x, Ux and Ux308. All these bacterial isolates exhibited activity against *Tr. rubrum* and *M. canis* and *Candida* yeasts. The standard killer strains *S. cerevisiae* K7, Rom K100, MS300 had no antifungal effect (Table).

The isolate T1x displayed a very strong killer activity against the important dermatomycetes *Tr. rubrum* under assay conditions (Fig. 1, I). The T1x isolate formed clear inhibition growth zones on the lawn of

Table. **Effects of bacterial isolates and standard killer strains *Saccharomyces cerevisiae* on dermatomycetes and *Candida* yeast**

Strains	Bacterial isolates					Controls		
	T1x	T2x	T3x	Ux	Ux308	K7	Rom-K100	MS300
Trichophyton rubrum	++	-	-	-	-	-	-	-
Microsporum canis	-	-	-	+	-	-	-	-
Candida albicans	+	+	+	++	+	-	-	-
Candida famata	-	-	-	+	+	-	-	-
Candida glabrata	+	+	+	+	+	-	-	-
Candida kefyr	+	+	+	+	+	-	-	-
Candida lusitaniae	+	+	+	-	-	-	-	-
Candida parapsilosis	+	+	+	+	+	-	-	-
Candida tropicalis	+	+	+	-	+	-	-	-

The size of clear zone (diameter, mm): ++ (>10 mm); + (<10 mm).



I. **II.**

Fig. 1. Killer activity of bacterial isolates against (I) *Trichophyton rubrum* dermatomycetes ((A – T1x; B – Ux; C – Ux308)) and (II) sensitive *Saccharomyces cerevisiae* α'1 strains (controls: 1 – Rom K 100, 2 – M 437, 3–4 – K7, 5 – MS 300, 6 – T1x, 7 – Ux

Tr. rubrum with a diameter more than 20 mm. The standard killer strains S. c*erevisiae* Rom K100, K7, MS300 had no antifungal effect (Fig. 1, II).

It is worth noting that the killer effect of the bacterial isolate Ux against *M. canis* was short-lived. Clear lysis zones were visible only on the first incubation day. The secreted substances of standard killer *S. cerevisiae* strains, just like the bacterial isolates T1x, T2x, T3x and Ux308, had no inhibiting effect on the growth of *M. canis.* The antifungal activity of the bacterial isolates T1x, T2x, T3x, Ux308 against *C. kefyr* and *C. parapsilosis* was confirmed by formation of various size cleared lysis zones. The bacterial Ux isolate killed *C. albicans* forming inhibition zones with a diameter of up to 10 mm. The bacterial isolates T1x, T2x, T3x, Ux308 exhibited a moderate action against *C. albicans* (Fig. 2). The standard killer *S. cerevisiae* strains did not exhibit any killer activity.

The moderate effect was established for bacterial isolates T1x, T2X, Ux and Ux308 when they were cultivated on yeasts *C. kefyr* and *C. parapsilosis* and formed lysis zones of about 5–10 mm. The Ux and Ux308 isolates were active against *C. famata, C. glabrata* yeasts. Clear zones on the lawn of these yeasts with a diameter up to 4–8 mm were seen. The secreted substances of standard killer *S. cerevisiae* strains, like the bacterial isolates T1x, T2x, T3x, had no inhibiting effect on the growth of *C. famata* and *C. glabrata* yeasts. The T1x formed clear zones with a diameter of about 7–8 mm on the gazone of *C. lusitaniae*, while the substances of the bacterial isolates Ux, Ux308 and standard killer *S. cerevisiae* strains did not exhibit any killer activity in

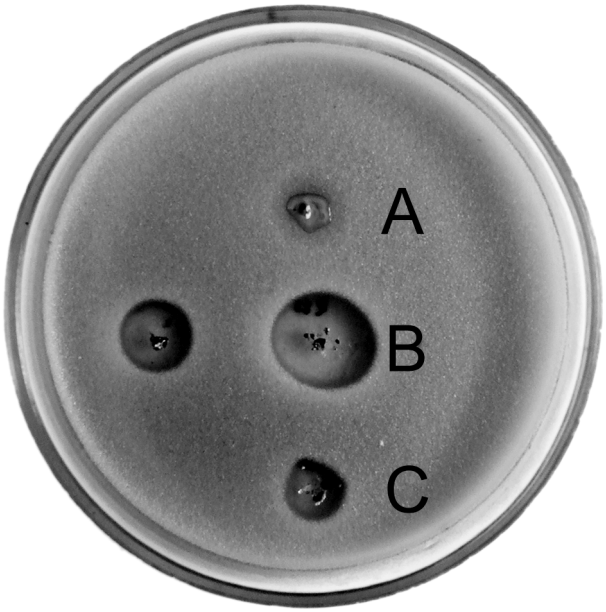


Fig. 2. Killer effects of bacterial isolates on *Candida albicans* yeast: A – T3x, B – Ux, C – Ux308

case of *C. lusitaniae*. The bacterial isolates T1x, T2x, T3x and Ux308 killed *C. tropicalis* (lysis zones 5–10 mm), whereas Ux and *S. cerevisiae* did not produce any killer effect in such cases (Table).

The results of our study showed that the killer ability of the bacterial isolate T1x was very stabile. It was necessary to control constantly the antifungal properties of isolates T2x and T3x. Based on this observation, we can predict that the determinants of killer possibilities could be located in a plasmid in the case of T2x and T3x isolates. The killer indications of the Ux and Ux308 clones were stable.

In order to clarify whether the killer ability is related to proteolytic activities, we have performed a proteolytic analysis of experimental isolates. The proteolytic activity was shown only by the Ux isolate. This fact allows to suppose that the killer abilities of the isolate Ux could be related to its proteolytic activity.

To evaluate the molecular mass of the material of interest we tried to concentrate it. After centrifugation, samples of the supernatant were concentrated with an Amicon 10 membrane. The supernatant of isolate Ux was concentrated and showed that the molecular mass of the killer substance could be about or more than 10 kDa. The supernatants T1x, T2x, T3x, Ux308 passed through this filter. We predict that the killer substances of the test isolates are of a different molecular mass (more or less than 10 kDa). Antifungal substances of all the test isolates are of different origin, too. A detailed biochemical characterization of bacterial isolates is in progress. Thus, the substances with killer abilities secreted by bacterial isolates could be very interesting in medicine as a prospective agent in treating mycoses caused by human pathogens. We believe that they have a potent antifungal activity against a wide variety of human pathogens. On the other hand, the genes coding such substances could be cloned and used for constructing genetically modified microorganisms.

In summary, our experimental results have shown that the bacterial isolates T1x, T2x, T3x, Ux and Ux308 are capable of killing some fungal agents of human diseases. The data obtained when estimating the antifungal action against nonpathogenic yeasts, plants and human pathogenic fungi allow to suggest that the bacterial isolates produce killer substances within a wide range. Efforts to find the description of analogous materials in the literature were unsuccessful.

ACKNOWLEDGEMENT

This work was supported by the Lithuanian State Science and Studies Foundation.

Received 15 December 2006
Accepted 30 January 2007

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BIOLOGINIŲ KOVOS PRIEMONIŲ PAIEŠKA PRIEŠ DERMATOMICETUS IR *CANDIDA* GENTIES MIELES

S a n t r a u k a

Šio darbo tikslas – nustatyti Tlx, T2x, T3x, Ux ir Ux308 bakterijų izoliatų poveikį dermatomicetams ir *Candida* genties mielėms. Tlx, T2x, T3x, Ux bakterijų izoliatai buvo išskirti iš spontaninių raugų, Ux308 – iš dirvožemio. Dermatomicetai ir *Candida* genties mielės gautos iš dermatomikozėmis ir onichomikozėmis sergančių Vilniaus universiteto ligoninės Santariškių klinikų Dermatovenerologijos centro ambulatorinių ir stacionarinių ligonių patologinės medžiagos. Nustatyta, kad T3x bakterijų izoliatas pasižymi stipriu antagonistiniu poveikiu prieš *Trichophyton rubrum* dermatomicetą. *Candida* genties mieles fungicidiškai veikė Tlx, T2x, T3x, Ux ir Ux308 bakterijų izoliatų išskiriamos medžiagos. *Microsporum canis* dermatomiceto augimą fungistatiškai veikė Ux bakterijos izoliato išskiriamos medžiagos. Tyrimų rezultatai rodo, kad reikalingi biocheminiai Tlx, T2x, T3x, Ux ir Ux308 bakterijų izoliatų išskiriamų toksinų tyrimai. Gauti duomenys gali būti panaudoti medicinoje kuriant genetiškai modifikuotus organizmus.