Antagonistic Activity of *Malassezia Spp.* towards Other Clinically Significant Yeast Genera

V. G. Arzumanian, A. Yu. Sergeev, O. V. Shelemekh, I. M. Ojovan, and O. A. Serdiuk

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Antagonistic activity of *Malassezia* yeast towards clinically significant yeast species was studied. Ten *Malassezia* strains exhibited this activity. *M. furfur* strain exhibited maximum activity and the least sensitivity to "foreign" metabolites. *M. globosa* proved to be the most sensitive and the least active. *M. furfur* metabolites exhibited pronounced activity towards 6 *Basidiomycetes* strains. This effect was significantly higher in comparison with antagonistic activity towards 13 *Ascomycetes* species. Studies of a complex of *M. furfur* antagonistic metabolites showed that it has at least two components: thermolabile proteins with molecular weights of 33 and 35 kDa and a thermostable one, proteinase-resistant. In contrast to metabolites of many other yeast species, this substance is more effective against related *Basidiomycetes* microorganisms (*Cryptococcus albicans*), while antagonistic proteins are active mainly towards *Ascomycetes*, such as *Candida albicans*. It was found that mycocin-like activity of *Malassezia* is encoded by chromosomes, but not plasmids.

Key Words: *Malassezia; yeast; antagonism; killer toxins*

The *Malassezia* genus yeast is a part of the bacterial community of human and warm-blooded animal skin [2]. These microorganisms are involved in the pathogenesis of such skin diseases as seborrheic dermatitis, pityriasis versicolor, and even some systemic diseases in immunocompromised carriers.

We found only one report about activity of *Malassezia* (*Pityrosporum ovale*) metabolites towards fungi [11]. An unknown secretion product was active against dermatophytes *Trichophyton rubrum*, *T. schoenleini*, and *Microsporum canis*. This antagonistic activity was thermostable, soluble in chloroform, and not dialyzed.

Two types of killer (anti-yeast) activity related to mycocins and glycolipids were described for yeast. All mycocins released by yeast are proteins or glycoproteins mainly with low molecular weight (10-20 kDa). The corresponding genes are encoded by plasmids or chromosomes [3]. They are usually expressed in acid medium and inactivated at high temperature and by proteinases. Mycocins are active against taxonomically close organisms, mainly within the same species/genus. By contrast, antagonistic glycolipids produced by yeast are characterized by very low molecular weight (about 1 kDa) and wider spectrum of action and are thermo- and proteostabile [4].

Study of skin colonization by various *Malassezia* species in normal subjects showed that the species composition of this yeast changed over 2 years in 3 of 32 subjects. The initial *M. globosa* species was replaced by *M. sympodialis*. We hypothesized that some *Malassezia* species or strains produce factors with antagonistic activity towards other yeast species/genera.

We studied antagonistic activity of *Malassezia spp*. towards clinically significant yeast genera.

MATERIALS AND METHODS

The study was carried out on 10 *Malassezia* strains. Nine of them were isolated from healthy carriers (Nos.

I. I. Mechnikov Institute of Vaccines and Sera, Russian Academy of Medical Sciences, Russia. ***Address for correspondence:*** veraar@mail.ru. V. G. Arzumanian

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1-9), one from a patient with atopic dermatitis (No. 607). These strains were identified [5], karyotyped [10], and analyzed by PCR [9]. The cultures were maintained in modified Dixon's agar (mD) by daily reinoculations. Other yeast strains were obtained from the collection of I. I. Mechnikov Institute.

The production of antagonistic substances by *Malassezia* cultures was studied on solid agar and liquid nutrient media. The yeasts were cultured in solid media (mD) for 7 days at 32°C in petri dishes (4 cm) with 4 ml medium. After culturing, the agar was turned over with a fine steel spatula and fresh yeast test cultures were inoculated onto the other side (50-150 CFU/dish). Dishes with the same medium without preliminary inoculation of *Malassezia* served as the control. After several days the results were recorded and compared with the control.

In order to evaluate the effect of thermal processing, the *Malassezia* colonies were removed (with a slide), agar was washed in sterile distilled water, placed into a sterile flask, and warmed for 20 min in a boiling water bath. Warm agar was then poured into fresh Petri dishes, cooled, and the yeast was inoculated.

In order to evaluate the production of antagonistic substances in liquid medium, culturing was carried out by inoculating *Malassezia spp.* at 25°C into a previously developed synthetic medium containing salts, asparagin, sodium taurocholate, and Twin-40 [1]. After 7 days the cells were separated by centrifugation at 3000 rpm (15 min) and the supernatant was filtered through membrane with 0.22-μ pores (Nucleopore®), lyophilized, and stored at -10°C (LCM). The antagonism of this substance was tested by diluting 200 mg in 200 μl 0.5 M phosphate buffer, pH 4.6 (CCM).

In order to evaluate the effect of *Malassezia* CCM on the growth and viability of yeast cultures, 3 loops (3 mm in diameter) were suspended in 200 μl CCM (200 μl phosphate buffer in the control). The mixture (100 μl) was then put into a tube with 1 ml 2 mM bromocresol purple (BCP) solution in the same buffer and incubated for 1 h at 32°C; the cells were separated by centrifugation and examined under a microscope (×1750). Viable (white) and dead (yellow) cells were counted [7]. A total of 700-1000 cells were analyzed in each experiment.

The CCM was treated with proteinase K as follows: the enzyme was added to 200 μl CCM to a final concentration of 1 mg/ml; the mixture was incubated for 5 h at 50°C; the reaction was stopped by 20-sec incubation of the mixture at 100°C.

Twin-40 was removed by dissolving LCM in distilled water (100 mg/1.25 ml) and subsequent extraction of Twin-40 with n-butanol:chloroform mixture (1:3) from the LCM:mixture in proportion 1:5. The upper, lower, and interphase fractions were collected, dried, and dissolved in 1.25 ml water. Antagonistic activity of LCM fractions was evaluated similarly as for CCM.

Active molecular components were characterized by HPLC analysis of the resultant fractions. Each fraction (3 μl) was dissolved in 100 μl 0.1% trifluoroacetic acid (TFA) and analyzed in a 150×2 mm reverse-phase column of a Jupiter C5 chromatograph (Phenomenex) in the acetonitrile linear gradient (1-60%) for 60 min with 0.1% TFA. The solvents were eluted at a slow rate (0.3 ml/min) at ambient temperature and 210 nM absorption (1 AU equivalent to 500 mV).

Analysis of *Malassezia spp*. plasmids was carried out by pulse-field gel electrophoresis (PFGE) on a ROTAPHOR R22 device (BIOMETRA). The DNA-containing preparation was prepared as described previously [10] with slight modification. Conditions of procedure: 0.9% (weight/volume) agarose gel (Sigma A2929), 180 V (6 V/cm), pulsation interval varying from 120 to 20 sec with a logarithmic angle of 115-95° at 12°C for 24 h. The gels were then stained with ethidium bromide and photographed (Fig. 1).

Lyophilized upper fraction obtained from 200 mg LCM was dissolved in 200 μl distilled water and this solution was added to 200 μl buffer (non-denaturing

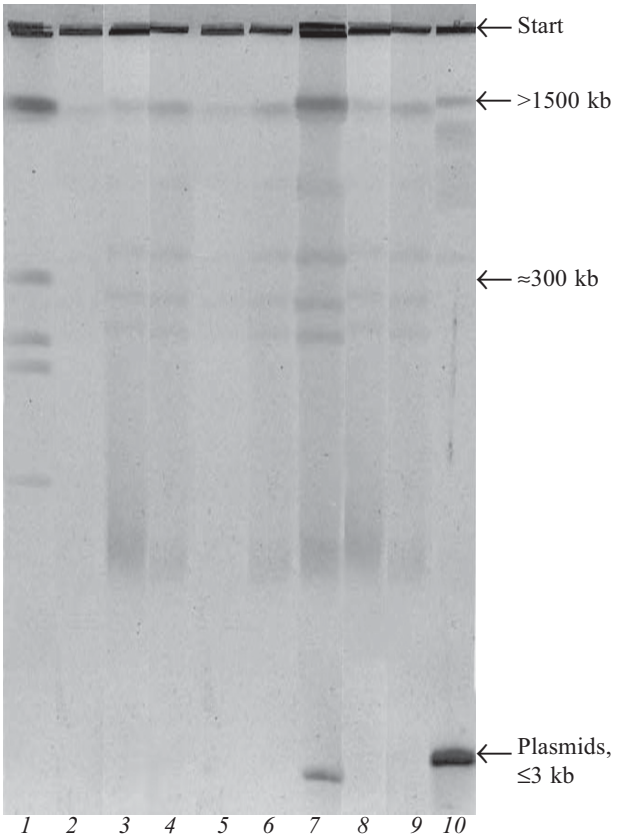

Fig. 1. PFGE analysis of *Malassezia spp*. cultures DNA: *M. furfur* No. 607 (carrier *1*); *M. sympodialis* (carriers *2-8*); *M. globosa* (carriers *9-10*).

conditions). The aliquots of this mixture were then fractionated by electrophoresis in 5-27.5% SDS-PAAG density gradient after Laemmli [8]. Low-molecular-weight reference specimens (Amersham-Pharmacia) served as the markers. Peptides and proteins were stained with silver. The molecular weights of unknown proteins were calculated by calibration curve plotted using the data obtained with the label.

RESULTS

Cross antagonistic activity of 10 *Malassezia* strains isolated from patients *vs.* the same strains is presented in Table 1.

Some of the tested strains were sensitive to metabolites released by other cultures. The highest sensitivity we observed was the absence of growth over 20 days. Medium antagonistic activity presented as reduction of CFU compared to the control. The following characteristics of the strains were used: stability (percentage of active strains preventing any growth of the test strain) and antagonistic activity (percentage of test strains demonstrating the absence of any growth under the effect of an active strain).

According to these criteria, *M. furfur* No. 607 was the most stable and active strain, while *M. globosa* No. 9 was the most sensitive (least stable) and least active.

Strain No. 607 exhibited the highest growth rate of all the studied cultures: the exponential phase in mD liquid medium lasted for just 17-24 h (36-48 h for all *M. sympodialis* strains and 72-96 h for *M. globosa* strain No. 9). Warming of the agar after *M. furfur* No. 607 growth led to reduction of antagonistic activity from 90 to 10%. Plasmid analysis detected plasmids in antagonistically most inert *M. sympodialis* No. 6 and *M. globosa* No. 9 strains (Fig. 1). A stringent inverse correlation between antagonistic activity and presence of plasmids was noted (correlation coefficient p=-0.829).

The most active strain, *M. furfur* No. 607, was used for evaluation of the effects on other yeast genera (Table 2). A total of 19 *Asco*- and *basidiomycetes* were tested. *M. furfur* No. 607 suppressed the yeast growth, which presented by delayed colony formation or formation of just microcolonies less than 0.1 mm in diameter (*vs.* 1-2 mm in the control) or complete absence of growth. Warming of the agar after culturing of *M. furfur* No. 607 reduced the antagonistic effect of its metabolites. The number of colonies and their size were greater in culturing on warmed agar than on turned-over agar. *Basidiomycetes* were more sensitive to *M. furfur* No. 607 metabolites than *Ascomycetes*.

The dynamics of the effects of *Malassezia* metabolite on other yeast species was evaluated by incubation of their cells with CCM from *M. furfur* No. 607 and subsequent staining with BCP (Fig. 2).

Cryptococcus albidus No. 220 and *R. mucilaginosa* No. 132 cultures rapidly lost their viability: 16.5 and 32.3% cells, respectively, remained viable after the first hour of incubation. Other cultures were less sensitive to the treatment: *M. globosa* No. 9 lost just 67% of its viability after 24 h, *T. ovoides* lost 84%, *C. albicans* No. 1135 and *Geotrichum sp.* N M lost 20

TABLE 1. Antagonistic Cross-Activity of *Malassezia* Evaluated in Experiments on Solid Agar Medium

Test strains	Control dishes	Active strains											S, %
		607	607*	1	2	3	4	5	6	7	8	9	
607	■	▒	▒	▒	▒	▒	▒	■	■	▒	▒	▒	100
1	■		▒									■	10
2	■		▒			▒		▒	▒	▒		■	50
3	■		▒						▒			■	20
4	■		▒									■	10
5	■		▒			▒	▒	▒	▒	▒		▒	60
6	■		▒									▒	10
7	■		▒						▒			■	20
8	■		▒		▒	▒	▒	▒	▒	▒		■	70
9	■											▒	10
AA, %		90	10	90	80	60	70	60	40	60	90	0	

Note. ■ compact growth; □ no growth over 20 days; ▒ slow growth: microcolonies or few colonies *vs.* control. 607*: boiled agar. AA: antagonistic activity, S: stability.

and 14% of their viability, respectively. These results also evidence a higher sensitivity of *Basidiomycetes* in comparison with *Ascomycetes* to *M. furfur* No. 607 metabolites. Moreover, these cultures exhibited a stringent correlation between the number of colonies on overturned agar and percentage of living cells after 24-h treatment with CCM (p=0.953).

The impact of CCM dose for antagonistic activity of *M. furfur* No. 607 was evaluated on the most sensitive *Cr. albidus* strain No. 975 (Fig. 3).

Resistance of *M. furfur* No. 607 CCM to high temperature and protease K was evaluated on cultured *M. globosa* strain No. 9 (Table 3). The cells were incubated with a standard dose of CCM for 24 h. Buffer, Twin-40 and bovine bile solutions in buffer (concentration as in culture medium), and 10-fold concentrated Twin-40 and bovine bile in buffer served as controls 1, 2, and 3. About $^{2}/_{3}$ cells died after CCM treatment, while CCM treatment with protease K reduced its activity by half. The effect of high temperature was about the same.

The presence of proteins in water-soluble fraction of *M. furfur* No. 607 CCM was detected by HPLC and SDS-PAAG. After transition of Twin-40 into the lower (water-insoluble) phase during extraction the upper and intermediate phases were analyzed. According to HPLC, 3 peptide-like compounds were present in the upper phase, emerging after 16, 24, and 28 minutes, respectively, for peaks 1, 2, and 3. The same substances in low levels were detected in the interphase. Control sample from fresh medium did not contain these components. Electrophoresis of *M. furfur* No. 607 CCM soluble components in SDS-PAAG also showed at least two protein bands (about 33 and 35 kDa). No metabolites of this kind were detected in *M. sympodialis* No. 6 supernatant.

Activities of upper and intermediate fractions were evaluated separately on 3 strains most sensitive to *M. furfur* No. 607 metabolites: *M. globosa* No. 9, *Cr. albidus* No. 975, and *R. mucilaginosa* No. 132. Antagonistic activity of the upper phase towards *M. globosa*

TABLE 2. Antagonism of *M. furfur* No. 607 towards *Asco-* and *Basidiomycetes* Yeast in Experiments in Solid Media

Yeast, species/collection No.	Appearance of colonies, days			Result on day 7, CFU (% of control)	
	control dishes	warmed agar	turned-over agar	warmed agar	turned-over agar
Cryptococcus albidus No. 975	2	7	20**	m***25.9	0
Cryptococcus albidus No. 220	2	3	7	s****64.5	s 2,2
Rhodotorula mucilaginosa No. 132	3	2	7	s 100	m 12,5
Rhodotorula aurantiaca No. 786	3	2	7	s 100	m 12,5
Trichosporon cutaneum No. 566	1	2	7	s 60.9	m 69,6
Trichosporon ovoides No. 18	1	3	20	s 30	0
Geotrichum sp. No. 1206	1	2	20	s 100	0
Geotrichum sp. No. M	2	2	2	s 75	m 100
Candida albicans No. 628	1	2	4	100	s 100
Candida albicans No. 689	1	2	4	100	s 93.2
Candida albicans N 735	1	2	4	65	s 100
Candida albicans No. 741	1	2	4	80	s 76.5
Candida albicans No. 815	1	2	4	89.4	m 97
Candida albicans No. 820	1	2	4	100	s 100
Candida albicans No. 860	1	2	4	100	s 100
Candida albicans No. 927	1	2	4	100	s 100
Candida albicans No. 932	1	2	4	96.8	m 100
Candida albicans No. 934	1	2	4	83.2	m 83.2
Candida albicans No. 1135	1	2	4	100	83.9

Note. *Control: mD; **20: no growth over 20 days and longer; ***m: microcolonies; ****s: colonies smaller than in the control.

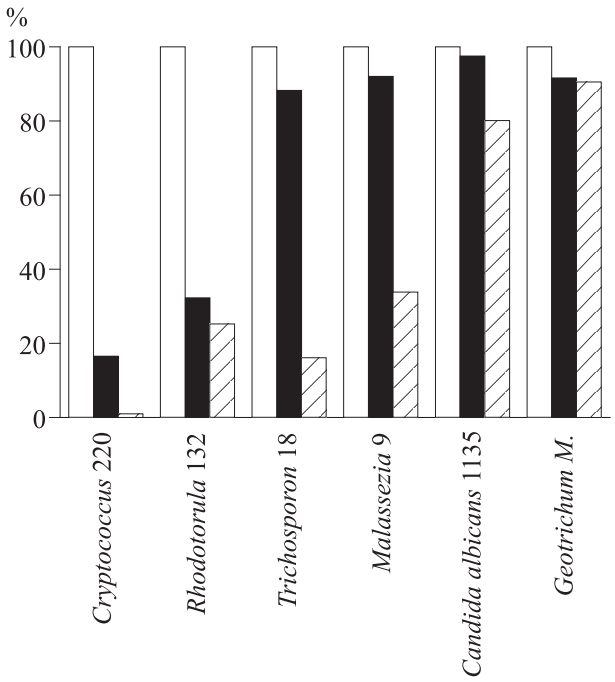

Fig. 2. Effects of *M. furfur* No. 607 CCM on yeast strains, evaluated by bromocresyl purple staining. Ordinate: percentage of viable cells compared to the control. Duration of cell exposure with CCM: light bars: 0 h; dark bars: 1 h; cross-hatched bars: 24 h.

No. 9 was 91.1±2.2% viable cells, *vs.* just 14.4±1.0% and 9.3±0.1% towards the two other strains, respectively. On the other hand, the interphase significantly suppressed viability of *M. globosa* (13.4±1.8% living cells) and less so of *R. mucilaginosa* (63.1±0.1%) and of *Cr. albidus* No. 975 (23.0±1.4%).

We studied several aspects of *Malassezia spp.* yeast antagonism to various yeast genera. The results indicate that *Malassezia spp.* really produce protective substances characteristic of the majority of the genera studied previously [3]. Antagonistic activity was detected in 10 studied *Malassezia* strains. Activities and sensitivities of different strains were different, depending on the species and growth rate: *M. furfur* No. 607 culture, growing most rapidly, was the most active antagonist (towards 90% tested strains) and the most resistant (100%) to metabolites of other *Malassezia* species. Contrary to this, *M. globosa* strain No. 9 exhibited the least growth rate, zero activity, and the highest sensitivity to other strains. Hence, presumably, the growth rate of *Malassezia spp.* correlates with the production/diffusion of antagonistic metabolites into agar.

Since the antagonistic effect of *Malassezia* metabolites decreased significantly, but did not disappear completely under the effect of high temperature, presumably, these metabolites have two constituents: thermolabile and thermostable. It is most likely that the *Malassezia* active metabolites are presented by peptide compounds: mycocins and/or glycolipids. Experiments on solid and nutrient media demonstrated a more pronounced antagonistic effect of *M. furfur* No. 607 metabolites against 6 *Basidiomycetes* than against 13 *Ascomycetes*. Exposure of metabolites to high temperature reduced antagonistic activity of all tested strains, particularly towards *C. albicans*. This seems to be justified, because mycocins produced by *Basidiomycetes* are mainly effective towards the yeast of the same taxonomic group, but not towards *Ascomycetes* [3].

It was found that the effect *M. furfur* No. 607 metabolites time- and dose-dependent. Of the *Basidiomycetes*, the most sensitive were *Rhodotorula mucilaginosa* No. 132 and *Cr. albidus* No. 975, even in comparison with the sensitive *M. globosa* strain No. 9.

Thermal processing of *M. furfur* No. 607 antagonistic substances, as well as protease K treatment, led to a significant reduction of antagonistic activity, which attests to protein nature of these substances. Separation of this complex with a mixture of organic solvents resulted in 3 fractions: upper (water-soluble), intermediate, and lower (water-insoluble). The upper phase presumably contained proteins, the intermediate one glycolipids. Two proteins with molecular weights of about 33 and 35 kDa were detected in the water-soluble fraction by HPLC and SDS-PAAG electrophoresis. However, a less active *M. sympodialis* No. 7 culture did not produce such proteins. The upper phase was more active towards *Cr. albidus* No. 975 and *Rh. mucilaginosa* No. 132, while the interphase was active against *M. globosa* No. 9. Hence, presumably, the glycolipid component of antagonistic activity is aimed at closely related *Basidiomycetes*, while the protein component is directed towards genetically distant objects. This characteristic of *Malassezia* distinguishes it from many other fungi [6].

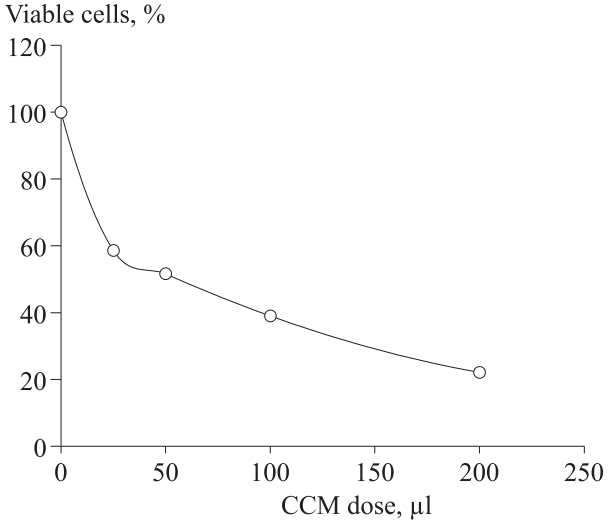

Fig. 3. Relationship between survival of *Cr. albidus* No. 975 cells and the dose of *M. furfur* No. 607 metabolite (1-hour treatment). Ordinate: percentage of viable *Cr. albidus* No. 975 cells.

TABLE 3. Proteo- and Thermostability of *M. furfur* No. 607 Metabolite, Evaluated in Culture of *M. globosa* No. 9

Treatment variant	% of viable cells
Buffer (control 1)	62.9±5.2
Twin-40+bovine bile (control 2)	65.0±8.5
Twin-40+bovine bile (×10; control 3)	59.1±7.5
CCM	20.4±6.0
CCM+protease K	38.5±7.1
Autoclaved CCM*	31.5±4.9

Note. *CCM was autoclaved for 30 min under 0.5 atm pressure (about 120°C).

According to DNA analysis, only 2 of 10 strains have plasmids: *M. globosa* No. 9 and *M. sympodialis* No. 6, the presence of plasmids inversely correlating with antagonistic activity. Hence, mycocin-like activity of *Malassezia*, in contrast to the majority of previously studied yeast genera, seems to be encoded not by plasmids, but by chromosomes.

The function of *Malassezia* antagonistic metabolites is most likely dubious: protection of the resident population of microorganisms, on the one hand, and maintenance of symbiont relationships with the carrier, on the other.

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