ISHAM INTERNATIONAL SOCIETY FOR HUMAN AND ANIMAL MYCOLOGY

Medical Mycology, 2018, 56, 110–116
doi: 10.1093/mmy/myx014
Advance Access Publication Date: 9 March 2017
Original Article

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Biofilm, adherence, and hydrophobicity as virulence factors in *Malassezia furfur*

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Received 23 November 2016; Revised 24 December 2016; Accepted 6 February 2017

Abstract

Malassezia species are natural inhabitants of the healthy skin. However, under certain conditions, they may cause or exacerbate several skin diseases. The ability of this fungus to colonize or infect is determined by complex interactions between the fungal cell and its virulence factors. This study aims to evaluate "*in vitro*" the hydrophobicity levels, the adherence on a plastic surface and the biofilm formation of 16 clinical isolates of *Malassezia furfur*. Cellular surface hydrophobicity (CSH) levels were determined by two-phase system. The biofilm formation was determined by tetrazolium salt (XTT) reduction assay and by Scanning Electron Microscopy (SEM). Results showed many isolates were hydrophobic, adherent, and producers of biofilm on abiotic surfaces with different capacity. SEM observations confirmed an abundant extracellular matrix after 48 h of biofilm formation. About 63% of strains with high production of biofilm showed medium to high percentage of hydrophobicity and/or adherence. In addition, it has been demonstrated a correlation between hydrophobicity, adherence, and biofilm formation in about 60% of strains examined. These important virulence factors could be responsible of this yeast changing from a commensal to a pathogenic status.

Key words: hydrophobicity, adherence, biofilm, *Malassezia furfur*, *Candida albicans*.

Introduction

Malassezia genus includes a group of lipophilic and mostly lipid-dependent yeasts that are recognized as members of the normal skin mycobiome of both human and other homoeothermic organisms with great significance.[1–4]

The ability of this fungus to colonize or infect has been determined by several molecular methods. The complex interactions between the fungal cell and its virulence factors, and the host tissues and component of the immune system were studied.[5] However, *Malassezia* species are natural inhabitants of the healthy skin; under certain conditions, they may cause or exacerbate several skin diseases.[4,6,7] Furthermore, *Malassezia* species have been associated with systemic infections such as catheter acquired sepsis,

peritonitis, fungemia, and pulmonary infection in patients receiving lipid parenteral nutrition.[8–16] Due to its ability to produce superficial and systemic infections in both immunocompromised and immunocompetent hosts, *Malassezia* is considered an important emerging pathogen.

The adhesion of microbes to host surfaces is a necessity for microbial colonization and infection. It has been recognized that microbial adhesion plays a decisive role in the pathogenesis of microbial infection.[17] Cell surface hydrophobicity (CSH) has a major contribution in microbial adhesion by enhancing hydrophobic interactions between cells and biological or nonbiological surfaces.[18] It has been proved that CSH confers more virulence to the microbes. By enhancing microbial adhesion, CSH enables the pathogen to form biofilm, a community of cells that provides drug resistance and increases virulence.[19–20] CSH also precludes the pathogens from phagocytosis by host immune cells.[21]

Malassezia has the propensity to develop biofilm, which represents a serious health threat and can create a source of persistent infections.[22] Moreover, the increased incidence of systemic infections associated with certain *Malassezia* species emphasizes the need to understand the relationship of these species with the environment and with associated pathologies but also to identify its susceptibility profile.[4,5,9,12–14,23]

The majority of invasive infections caused by *Malassezia* genus reported in literature have been associated with the lipid-dependent *Malassezia furfur* (*M. furfur*). To allow a better knowledge of the pathogenicity of this species, it is important to investigate the expression of virulence factors. In this study, we evaluated the hydrophobicity levels, adherence capacity and biofilm formation of *M. furfur* strains.

Methods

Microorganisms and growth conditions

The neotype strain *M. furfur* CBS 7019 and 16 clinical *M. furfur* isolates from pityriasis versicolor, seborrheic dermatitis and atopic dermatitis lesions, were included in this study (Table 1). All isolates were deposited in the culture collection (IMR-M) of the Departamento de Micologia, Instituto de Medicina Regional, Universidad Nacional del Nordeste, Argentina. All isolates were previously identified by polymerase chain reaction–restriction fragment length polymorphisms (PCR-RFLP). Amplification was performed by using generic primers, forward 5′-TAA CAA GGA TTC CCC TAG TA-3′ and reverse 5′-ATT ACG CCA GCA TCC TAA G-3′. The primers successfully amplified the target part of 26S rDNA from all tested *Malassezia* strains, providing a single PCR product of the expected size (approximately 580 bp). Amplified DNA products were subjected to restriction fragment length polymorphism (RFLP) using *Cfo* I (Promega) in a first identification step. [24]

Table 1. Clinical isolates of *M. furfur*.

Strains	Lesion
IMR-M 010	Pytiriasis versicolor
IMR-M 129	Pytiriasis versicolor
IMR-M 227	Pytiriasis versicolor
IMR-M 299	Pytiriasis versicolor
IMR-M 360	Seborrheic dermatitis
IMR-M 361	Seborrheic dermatitis
IMR-M 375	Seborrheic dermatitis
IMR-M 445	Pytiriasis versicolor
IMR-M 446	Pytiriasis versicolor
IMR-M 459	Pytiriasis versicolor
IMR-M 529	Pytiriasis versicolor
IMR-M 649	Pytiriasis versicolor
IMR-M 655	Pytiriasis versicolor
IMR-M 676	Pytiriasis versicolor
IMR-M 678	Pytiriasis versicolor
IMR-M 824	Seborrheic dermatitis

IMR-M: Instituto de Medicina Regional, *Malassezia*.

Hydrophobicity assay

CSH levels were determined by two-phase system.[25] The yeasts *Malassezia* cells were grown in Leeming-Notman modified broth at 32°C for 72 h. Subsequently, cells were washed twice with sterile saline buffer (with 0.5% Tween 20) and resuspended in 0.05 M sodium phosphate buffer (pH 7.2) at a final concentration of 2×10^6 cells/ml. Cell suspension (2 ml), adjusted to an OD_{600} of 0.7, was transferred to a glass tube containing 50 or 500 µl octane (Sigma Aldrich) and mixed for 1 min by gentle vortexing. After separation of the phases, the aqueous phase was carefully transferred to a cuvette and the OD_{600} was measured. The percent of hydrophobicity was calculated by subtraction of measurements in aqueous phase respect to control. The value considered for each strain was the average of three independent biological replicates, with five measurements per individual cell culture.

Adherence assay

In order to examine the adherence capacity to plastic surface, the yeasts were grown for 72 h at 32°C in Leeming-Notman modified broth, washed twice with sterile PBS (10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4 with 0.5% Tween 20) and then resuspended at 37°C in RPMI 1640 modified for *Malassezia* plus 10% FBS at 7.5×10^2 cells/ml. After incubation for 3 h at 37°C in six-well polystyrene plates

(Corning Incorporated, Corning, NY) followed by extensive washing, 1 ml of Leeming-Notman Agar medium modified was poured into each well and let solidify. After incubation for 72 h at 37°C, colonies were counted, and the results were expressed as a percentage of the inoculum size. The inoculum size for each cell suspension was confirmed by plating aliquots of the culture directly in Leeming-Notman Agar medium modified plates.

Biofilm formation

Biofilm formation was tested in presterilized polystyrene flat-bottom 96-well microtiter plates (Corning, NY). A *Malassezia* cell suspension (1×10^7 cells/ml), grown as described above, was incubated for 24 and 48 h at 37°C.

After biofilm formation, the medium was aspirated, and nonadherent cells were removed by thoroughly washing the biofilm three times with sterile PBS. A semiquantitative measurement of biofilm formation was made by using an XTT [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide]-reduction assay.[26] XTT (Sigma) was dissolved in PBS at 0.5 g/l. The solution was sterilized through a 0.22-μm pore size filter. Prior to each assay, the XTT solution was thawed and supplemented with menadione (10 mM stock dissolved in acetone to a final concentration 1 μM; Sigma). An aliquot of 1 ml of the XTT-menadione solution was added per well, and the plates were incubated for 2 h at 37°C. A sample (500 μl) was then transferred from each well into a fresh 12-well plate (to eliminate interference of cells with colorimetric readings) and the colorimetric change resulting from XTT reduction was measured at 490 nm.

Scanning electron microscopy (SEM)

For SEM observations, *M. furfur* cells were grown on Leeming-Notman Agar medium (see above) for 72 h at 32°C. After being washed twice in calcium and magnesium-free phosphate-buffered saline (PBS), a 2.5×10^6 cells concentration was transferred on polyurethane material and incubated for 24 h and 48 h. Subsequently, cells were fixed for 20 min at room temperature with 2.5% (v/v) glutaraldehyde in 0.01 M cacodylate buffer (pH 7.4) containing 2% (w/v) sucrose. Then, they were post-fixed with 1% (w/v) OsO4 for 1 h, dehydrated using an ethanol gradient and critical point dried in CO2. Samples were examined under a Joel 5800 LV (Tokyo, Japan) scanning electron microscope at Servicio de Microscopia Electrónica (Universidad Nacional del Nordeste, Argentina).

Table 2. Hydrophobicity and adherence in *M. furfur* clinical isolates.

Strains	% Hydrophobicity	% Adherence
IMR-M 010	81.9 ± 5.5	76.7 ± 5.7
IMR-M 129	24.8 ± 1.1	45.0 ± 7.0
IMR-M 227	49.1 ± 5.2	58.4 ± 2.3
IMR-M 299	1 ± 0.1	49.0 ± 6.5
IMR-M 360	39.4 ± 4.8	40.8 ± 0.3
IMR-M 361	47.9 ± 2.2	40.7 ± 6.4
IMR-M 375	50.1 ± 5.6	51.6 ± 6.2
IMR-M 445	33.1 ± 2.5	78.7 ± 2.3
IMR-M 446	70.2 ± 20.7	57.9 ± 19.9
IMR-M 459	46.8 ± 8.9	54.9 ± 8.7
IMR-M 529	53.5 ± 0.1	48.6 ± 9.8
IMR-M 649	76.6 ± 27.4	39.8 ± 0.28
IMR-M 655	83.3 ± 14.6	46.4 ± 3.4
IMR-M 676	58.6 ± 11.3	38.8 ± 3.2
IMR-M 678	60.3 ± 8.5	30.7 ± 3.3
IMR-M 824	57.6 ± 3.2	29.2 ± 1.1
CBS 7019	41.6 ± 3.8	64.5 ± 4.5

Percentage of plastic adherent cells and cell surface hydrophobicity values measured with a two phase system. Mean values of % hydrophobicity and adherence ± SD of three independent experiments.

Statistical analysis

All experiments were performed in triplicate and the values presented are the mean with standard deviation. Student's *t*-test was used for comparison between 24 and 48 h biofilm formation. P value $< .05$ was considered statistically significant (*) while $P < .001$ as highly significant (**) for comparisons.

Results

Hydrophobicity and adherence

Results of CSH obtained by a two-phase system for the CBS 7019 *M. furfur* and 16 clinical isolates (IMR-M) of *M. furfur* are shown in Table 2. Hydrophobicity was variable among the isolates tested and it ranged from 1 ± 0.1% to 83.25 ± 14.6%. In particular, four isolates (IMR-M 010, 446, 649, and 655) showed high hydrophobicity with values of 83.3 ± 14.6, 81.9 ± 5.5, 76.6 ± 27.4, and 70.2 ± 20.7% respectively. Ten isolates (IMR-M 227, 360, 361, 375, 459, 529, 676, 678, 824, and CBS 7019) presented medium hydrophobicity with values among 39.4 ± 4.8 to 60.3 ± 8.5%. Only three isolates (IMR-M 129, 299, and 445) showed low hydrophobicity values. Although the hydrophobicity is an important factor to adherence, the adherence on plastic surfaces was evaluated as well. Adherences values presented in Table 2 also display variability with a range between 29.2 ± 1.1 to78.7 ± 2.3%, and all strains were adherent. Six clinical

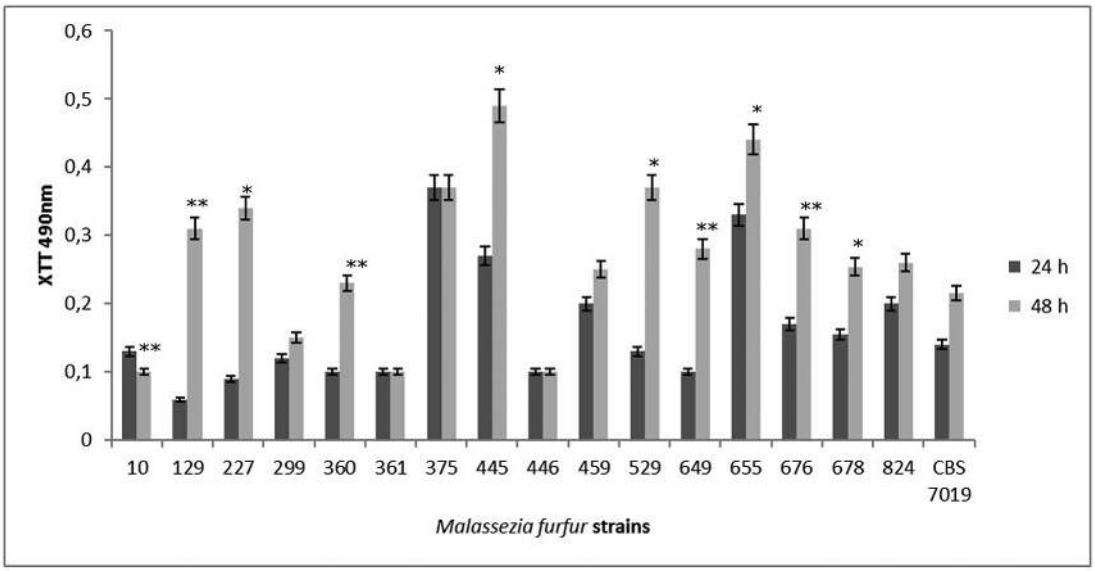

Figure 1. Histograms of biofilm formation of clinical isolates (IMR) and the reference strain CBS 7019 of *M. furfur* after 24 and 48 h measured by XTT.

isolates (IMR-M 010, 227, 375, 446, 445, 459), including the laboratory strains CBS 7019, showed high values of adherence ranged between 51.6 ± 6.2% and 78.7 ± 2.3%. The other isolates presented values of adherences ranging between 29.2 ± 1.1% and 49.0 ± 6.5%.

It is interesting to note that three of four isolates from seborrheic dermatitis (IMR-M 360, 361, and 375) showed medium values of hydrophobicity and adherence (see Table 2).

Biofilm formation

Figure 1 shows the biofilm formation after 24 and 48 h by XTT assay of all the strains tested. Every isolate of *M. furfur* was able to form biofilm after 48 h. Biofilm formation showed high variability among the different isolates studied. Indeed, 13 isolates presented a linear increase of metabolic activity over time; three (IMR-M 361, 375 and 446) showed the same metabolic activity; and one strain (IMR-M 010) a low reduction in development.

In particular, eight clinical isolates (IMR-M 129, 227, 375, 445, 529, 649, 655, and 676) were high producers of biofilm with values ≥ 0.30 OD at 490 nm. Five isolates (IMR-M 360, 459, 678, 824, and CBS 7019) were medium (0.15–0.25) producers. Only four (IMR-M 010, 299, 361, 446) were low (<0.15) producers.

It is interesting to note that about 63% of isolates with high production of biofilm presented from medium to high percentage of hydrophobicity (range 49.1 ± 5.2% to 83.3 ± 14.6%) and/or adherence (38.8 ± 3.2% to 78.7 ± 2.3%); the exception was IMR-M 129 with low hydrophobicity values and medium values of adherence.

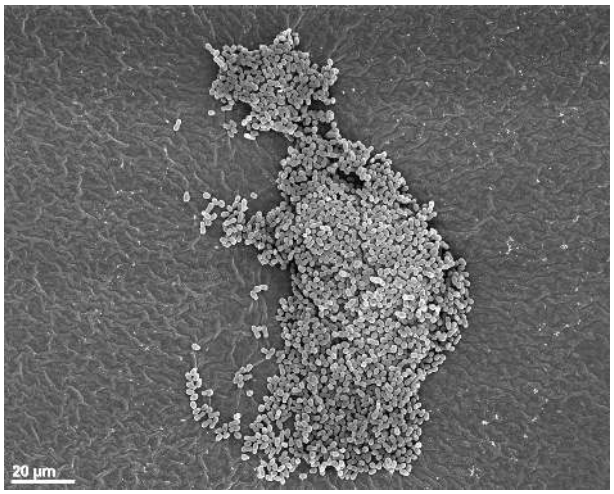

Figure 2. Scanning electron microscopy. *M. furfur* (IMR-M 459) cells on polyurethane catheter after 24 h (×600).

Two strains (IMR-M 010 and 446) with high hydrophobicity and adherence were low producers of biofilm. All isolates with medium production of biofilm (IMR-M 360, 459, 678, 824, and CBS 7019) showed medium values of hydrophobicity and adherence.

SEM observations confirmed the results obtained above. Figure 2 shows *Malassezia* cells on polyurethane catheter after 24 h. Figure 3 shows the evident biofilm formation after 48 h. In particular, after 24 h of incubation no extracellular matrix was formed (Fig. 2), only an aggregation of adherent cells was observed. However, after 48 h, the biofilms were characterized by abundant extracellular matrix covering the yeast cells (Fig. 3), organized in multi- or monolayer structures.

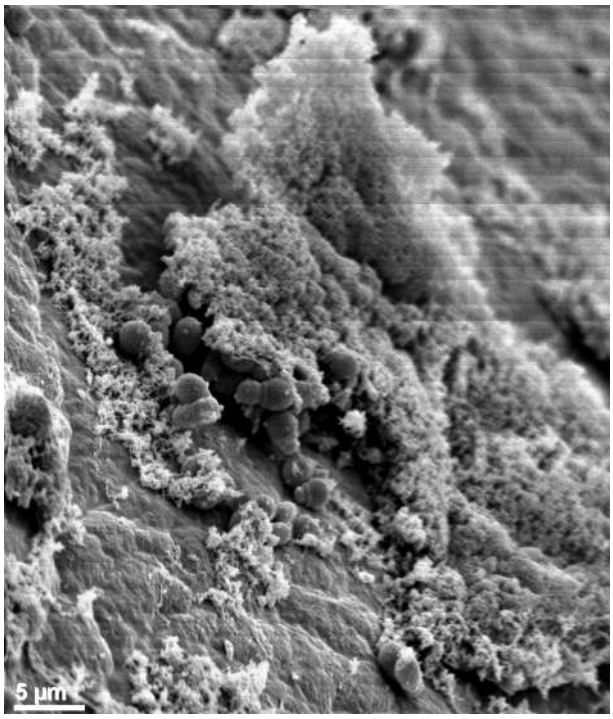

Figure 3. Scanning electron microscopy. *M. furfur* (IMR-M 459). Biofilm with extracellular matrix formed after 48 h (×1800).

Discussion

Currently, the knowledge about the biological proprieties and pathogenicity of the lipophilic *Malassezia* yeasts is scarce. This study shows for the first time the cellular hydrophobic surface, the adherence and the biofilm formation of various isolates of *M. furfur*, the most frequently specie associated with systemic infections within the genus *Malassezia*.

Adherence to an inert surface is an important step in biofilm formation, and it involves different factors, such as hydrophobicity. Fungal CSH may play an important role in pathogenesis, and it is mediated by mannoproteins associated to cell wall. The relative hydrophobicity can be useful in predicting the propensity of different strains to form biofilm, as in *Candida parapsilosis* and *Candida glabrata*, where CSH measurements represent a simple and reliable method to predict biofilm formation.[27] In fact, some authors reported high values of CSH in *Candida albicans* as virulence factors that contribute to adhesion on biological or nonbiological surfaces.[28] As there are limited references about these virulence factors of *M. furfur* or other *Malassezia* lipodependant yeasts, discussion of our results was made considering other yeasts such as *Candida* spp.[29]

Whereas the hydrophobicity is an important factor for adherence on the host tissue, we evaluated also the adherence on plastic surfaces. Our results clearly show the high variability of hydrophobicity in *Malassezia* cells, in accordance with values reported by Akaza et al.[30] The strain IMR-M 299, whose hydrophobicity was not possible to measure (Table 2), proved to be an exception. It is known that hydrophobic *Candida albicans* cells are more adherent than hydrophilic cells on different host tissues.[31] Indeed, in this study, about 40% of the strains tested showed high values of adherence. In particular, we have demonstrated that about 60% of the isolates displayed similar range values in both hydrophobicity and adherence.

A wide variety of fungi has demonstrated their ability to colonize surfaces and form biofilm. Most studies on fungal biofilms were focused on *Candida albicans* and other *Candida* species.[28,32–33] More recently, several authors have reported studies about other yeasts, such as *Cryptococcus neoformans*, *Cryptococcus gattii* and *Malassezia pachydermatis*.[34,35] In our study all isolates were able to form biofilm as shown in Figure 1. These results are in accordance with the studies on *Malassezia pachydermatis* obtained by Figueredo et al.,[36] but it is important to consider that *Malassezia pachydermatis* is not a lipodependent yeast.

Bumroongthai et al.,[35] for *Candida parapsilosis* and *Malassezia pachydermatis* co-cultures, obtained biofilm values corresponding to our results for *M. furfur*, but it is significant to note that values obtained with *M. furfur* were higher than results obtained only with *Malassezia pachydermatis* using different staining methodology (Alamar Blue).

SEM observations showed that extracellular matrix was absent in the first 24 h of biofilm formation (Fig. 2) but an abundant extracellular matrix was observed after 48 h (Fig. 3). The same results were reported by Figueredo et al.[36] for *Malassezia pachydermatis*. It is important to take into account the extracellular matrix generation observed in this study, because it might be responsible for the emergence of antifungal resistance[22] in addition to the low antifungal susceptibility to common clinical drugs showed by this species.[23,37]

Colonization is further enhanced by the pathogen's virulence factors, including the adherence properties that favor colonization and proliferation, followed by biofilm formation in central vascular catheters.[39] *Malassezia* fungemia is usually associated with lipid-solution administration through venous catheters, and *M. furfur* is the *Malassezia* species that most frequently emerges as catheter colonizer. Therefore, it is important to highlight the ability of this specie to form biofilm, as observed in this study. In addition, the lipodependent character with strict nutritional requirements of *M. furfur* makes it a fungus difficult to detect in routine laboratory cultures.[12–13,38–40]

In conclusion, our results suggest that all clinical isolates of *M. furfur* were hydrophobic and adherent on an abiotic surface with varying degrees of success. These characteristics are involved also in the high ability to form biofilm

observed in this study. Indeed, we have demonstrated a correlation between hydrophobicity, adherence and biofilm formation in about 60% of the strains examined, as reported by others authors for *Candida albicans*.[27,28,32] These important virulence factors could be responsible to change from commensal to pathogen status of this yeast.

The revision of the genus *Malassezia* has opened up new questions about the pathogenicity of *Malassezia* species.

Acknowledgment

The authors are grateful to Cristina Buffone to editorial assistance.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this manuscript.

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