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Production of the mycelial phase of *Malassezia in vitro*

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To study the pathogenicity of *Malassezia*, the agent of pityriasis versicolor, it is necessary to obtain the mycelial form *in vitro*. A range of different components and conditions were tested to induce yeast cells of the organism to produce mycelia *in vitro* using different culture media. A mycelial culture medium was developed that consisted of bacteriological peptone, glucose, yeast extract, ox bile, glycerol, glycerol monostearate, Tween 80, squalene, glycine, potassium nitrate, sodium chloride, ferrous sulphate and magnesium sulphate with or without agar. The liquid and solid medium had a pH of 5·6 and the temperature of incubation was 30 °C. Cultures were incubated in air. This medium was able to induce some strains of *Malassezia* to produce up to 40% mycelium *in vitro*. In total, 33 different strains of *Malassezia* obtained from the skin of the healthy individuals and patients with pityriasis versicolor were tested for mycelium production. The strains of *Malassezia* capable of producing mycelium *in vitro* all possessed the serovar A antigen.

Keywords culture medium, *Malassezia*, mycelium, pityriasis versicolor

Introduction

The dimorphism of *Malassezia* species was first suggested by the observation of both yeasts and hyphae in the scales of pityriasis versicolor lesions *in vivo* [1]. However, as there was no method available for growing the mycelial phase *in vitro*, some investigators including Tosti *et al.* [2], believed that the dimorphism only occurred *in vivo*. Contrary to this view, Gordon [1] demonstrated that strains of *Malassezia* with round cells isolated from patients with pityriasis versicolor were able to produce very elongated buds when grown on cornmeal agar with olive oil at 30°C. Salkin & Gordon [3] studied the polymorphism of the organism and found that *Malassezia* strains with round cells occasionally produced short structures resembling germ tubes when cultured on modified Sabouraud glucose agar overlaid with olive oil and incubated at 37 °C. *Malassezia* strains with oval cells, however, only produced slightly elongated buds.

At the same time, other groups were also attempting to induce mycelia in *Malassezia* cultures, using a variety of media. Using yeast morphology agar containing cholesterol or cholesterol esters, Nazzaro-Porro *et al.* [4], were able to induce ~20% of mycelia in four strains of *Malassezia*. The strains, from their own collection or reference isolates, had oval or round cells and were from lesions of pityriasis versicolor (2), 'infected skin' (1) or pig skin (1).

Dorn & Roehnert [5] used an ammonium phosphate buffer supplemented with various salts to attempt to induce mycelia in 25 strains of *Malassezia*. They obtained a maximum of 8·5% mycelia for three of 22 strains with round cells (from patients with pityriasis versicolor), whilst none of the strains with oval cells produced mycelium (three reference strains: one from animal skin and two of unknown origin).

Caprilli & Mercantini [6] repeated the experiments of Nazzaro-Porro *et al.* and obtained similar results using strains isolated from lesions of pityriasis versicolor. Faergemann *et al.* [7] used phosphate buffered saline containing stratum corneum cells and induced 11–17% mycelia in strains with either round or oval yeast cells, originally isolated from pityriasis versicolor or dandruff. When the strains were incubated on stratum corneum in an atmosphere of 7% CO_2, the production of mycelia was increased to 39–48%. In a second system, Faergemann [8] obtained up to 24% mycelia from strains of *Malassezia* with oval cells when they were incubated

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with human stratum corneum in a microaerophilic environment.

Other workers have noted the presence of rare hyphae in cultures of *Malassezia* on Dixon's agar [9], whilst Guillot *et al.* [10] obtained sufficient hyphae from a culture on Lowenstein-Jensen medium supplemented with 1% (v/v) olive oil to carry out ultrastructural studies on the mycelium. Bhattacharyya *et al.* [11] reported that the morphological transition from the yeast to mycelium was easier to obtain in some strains with round cells than in those with oval cells when culture was performed on the medium of Dorn & Roehnert [5]. They obtained 8% mycelia with various strains grown in 10% (v/v) CO_2, but stratum corneum plus 8% (w/v) squalene yielded 60–70% mycelia.

Recent work on the taxonomy of *Malassezia* has defined six lipophilic species, in addition to the non-lipophilic *Malassezia pachydermatis*. Using large subunit ribosomal RNA (rRNA) sequence data and nuclear DNA complementarity, and including strains from various previous classifications, Guillot & Guého [12] defined *M. furfur*, *M. sympodialis*, *M. globosa*, *M. restricta*, *M. obtusa* and *M. slooffiae*. They found that nine strains classified as serovars A (four strains), B (three strains) and C (two strains) [13] fit into the species *M. sympodialis*, *M. globosa* and *M. restricta*, respectively. However, this correlation between the serovars and the new species has not been found for every strain of *Malassezia* examined [14].

In order to determine the pathogenic mechanisms of mycelial phase *Malassezia*, the causative agent of pityriasis versicolor, it is imperative to be able to grow the hyphae *in vitro*. The aim of this study was to obtain an appropriate method for the consistent production of hyphae by *Malassezia* spp.

Materials and methods

Strains

In total, 33 *Malassezia* strains were used in this study: nine strains (three strains of each of serovars A, B and C), isolated from the skin of healthy individuals with no history of skin diseases and receiving no antimicrobial therapy at the time of sampling; four strains (one of serovar A, two of serovar B and one of serovar C), all from normal human skin, obtained from Dr H. R. Ashbee's collection (Division of Microbiology, Leeds University, Leeds, UK); eight strains (seven of serovar A and one of serovar B) isolated from pityriasis versicolor lesions (Mycology Reference Centre, Department of Microbiology, Leeds General infirmary, Leeds, UK); and 12 strains from the Mycology Laboratory, University of Glasgow, Glasgow, UK. The 12 Glasgow strains included five from normal human skin (three serovar A, one serovar B and one non-serotypable), six from pityriasis versicolor (four serovar A and two serovar B) and one serovar A strain from a psoriatic lesion. The details of the strains are shown in Table 1.

Culture media and components

Bacto agar and Yeast morphology agar were obtained from Difco (Detroit, MI, USA). Leeming and Notman agar culture medium was prepared as previously described [15]. Cholesterol, cholesteryl stearate, oleic acid and triolein were obtained from Fluka (Gillingham, UK). Ergosterol, Nile blue sulphate, squalene, sodium taurocholate, sodium chloride, glycine, histidine, methionine, tryptophan and polyoxyethylene sorbitans (Tween 20, Tween 40, Tween 60 and Tween 80) were obtained from Sigma (Poole, UK). Glycerol monostearate, di-ammonium hydrogen orthophosphate, ammonium dihydrogen orthophosphate, potassium nitrate, magnesium sulphate-7-hydrate, ferrous sulphate-7-hydrate, monobasic potassium phosphate and calcium chloride were obtained from BDH (Liverpool, UK).

Preparation of culture media

Numerous formulations were tested as suitable media for mycelium production. Each culture medium was prepared by the addition of the appropriate components to distilled water. The pH of the medium was adjusted to 4·5, 5·6 or 6·2, depending on the type of culture medium, and the components were fully dissolved by gentle heating. The culture medium was then sterilized by autoclaving. Appropriate filter-sterilized amounts of the heat-sensitive components such as cycloheximide, chloramphenicol, ultra-high temperature (UHT) treated milk, ergosterol or amino acids were then added to the culture medium. The solid or liquid culture media (without agar) were prepared by dispensing the medium into petri dishes (9 cm) or aliquoting into 250 ml flasks respectively. The strains of *Malassezia* to be tested were then inoculated into both solid and liquid culture medium and incubated at the temperature (29, 30 or 34 °C) and appropriate atmospheric condition (aerobic, anaerobic or 5%, 7%, 10% and 15% [v/v] in air CO_2) to be tested. The liquid culture media were incubated in an orbital shaker incubator at 160 rpm. All the cultures were checked after 3 days and then every 2 days for a total of 3 weeks for mycelium production by light microscopy.

Estimation of mycelium production by Malassezia

Malassezia was routinely stained using fuchsin and methylene blue solutions (1% w/v). Nile blue sulphate

Table 1 Strains used in the study of mycelium production by *Malassezia*

Strain	Source	Where isolated	Serovar	Species name if known
21.HK.CH.L*	Healthy individual	Normal skin	A	
24.CC.F.L*	Healthy individual	Normal skin	A	
28.GM.CH.L*	Healthy individual	Normal skin	A	
7.LM.F.S	Healthy individual	Normal skin	B	
31.ML.CH.S	Healthy individual	Normal skin	B	
34.CJ.F.L	Healthy individual	Normal skin	B	
19.BD.F.L	Healthy individual	Normal skin	C	
38.KA.F.S	Healthy individual	Normal skin	C	
55.AH.F.S	Healthy individual	Normal skin	C	
13 (RA)	H. R. Ashbee's collection	Normal skin	A	*M. sympodialis*
16.8 (RA)	H. R. Ashbee's collection	Normal skin	B	*M. globosa*
21.3 (RA) (=CBS† 7990)	H. R. Ashbee's collection	Normal skin	B	*M. globosa*
42.2 (RA) (=CBS 7877)	H. R. Ashbee's collection	Normal skin	C	*M. restricta* type‡
CBS 6001§	CBS	Pityriasis versicolor	A	*M. furfur*
GM 51$	T. Bhattacharyya's collection	Pityriasis versicolor	B	*M. globosa*
GM 101$ (=CBS 7971)	T. Bhattacharyya's collection	Normal skin	A	*M. slooffiae*
GM 110$ (=CBS 7875)	T. Bhattacharyya's collection	Normal skin	B	*M. slooffiae*
GM 215$ (=CBS 7876)	T. Bhattacharyya's collection	Normal skin	A	*M. obtusa* type
GM 216§$	T. Bhattacharyya's collection	Pityriasis versicolor	A	*M. obtusa*
GM 301$	T. Bhattacharyya's collection	Normal skin	A	*M. sympodialis*
GM 340$	T. Bhattacharyya's collection	Pityriasis versicolor	B	*M. sympodialis*
GM 451$	T. Bhattacharyya's collection	Normal skin	NS	*M. pachydermatis*
Hook§	T. Bhattacharyya's collection	Psoriasis lesion	A	*M. furfur*
L 251	T. Bhattacharyya's collection	Pityriasis versicolor	A	
01333.1§	T. Bhattacharyya's collection	Pityriasis versicolor	A	
1.PV.MYC	Clinical isolate	Pityriasis versicolor	A	
2.PV.WY§	Clinical isolate	Pityriasis versicolor	A	
3.PV.MWY	Clinical isolate	Pityriasis versicolor	A	
4.PV.SA	Clinical isolate	Pityriasis versicolor	B	
5.PV.LY	Clinical isolate	Pityriasis versicolor	A	
6.PV.MI	Clinical isolate	Pityriasis versicolor	A	
7.PV.MT	Clinical isolate	Pityriasis versicolor	A	
8.PV.MYC§	Clinical isolate	Pityriasis versicolor	A	

*, Produced elongated buds, but not mycelium on new culture medium;
†, CBS, Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands;
‡, type, strain ex type of species;
$, originally isolated by Dr G. Midgley;
§, produced mycelium on new culture medium.
NS, Non-serotypable.

was used to stain *Malassezia* cells to differentiate them from lipid globules. The Nile blue sulphate was prepared by dissolving 100 mg of powder in 10 ml distilled water followed by the addition of 20 ml ethanol. The rate of mycelial production was determined by counting cells in three different samples (100 cells per sample) using an improved Neubaur haemocytometer (Weber, Liverpool, UK) and light microscopy (magnification ×400).

Results

Nazzarro-Porro method

The first attempt to produce mycelium was carried out using the medium described by Nazzaro-Porro *et al*. [4]. At the same time, the effects of different concentrations of the components of the Nazzaro-Porro culture medium were tested by adding cholesterol, cholesteryl stearate, glycerol monostearate and oleic acid to Leeming and Notman's culture medium [15].

The growth of yeast cells of the *Malassezia* strains on the Nazzaro-Porro culture medium was slow and after day 10 following inoculation only a few colonies had formed. The lipid components were spread on the surface of the medium. Because of the high concentration of lipids in both the solid and liquid culture media, light microscopic observation of the yeast cells was difficult and most of the yeast cells were trapped by lipid globules. Hence, direct observation of microscope slides

was carried out after Nile blue sulphate staining for 10 min in a humid atmosphere. None of the strains produced mycelium on this medium.

Yeast cells of some of the serovar A strains isolated from pityriasis versicolor, were capable of producing a few elongated buds (at a rate of 2–3 elongated yeast cells per 100 yeast cells) on Leeming and Notman's culture medium [15] supplemented 1 mg cholesterol ml^{-1}, 0·75 mg cholesteryl stearate ml^{-1}, 1 mg glycerol monostearate ml^{-1} and 4% (v/v) oleic acid at pH 4·5 or 6·2. Incubation was at 30 or 34 °C in air or 5% (v/v) CO_2 in air for 5 days in solid culture medium and 36 h in liquid culture medium.

Determination of the effect of squalene and triolein on mycelium production by Malassezia

The effects of different concentrations of squalene and triolein on mycelium production were tested by adding these compounds to the culture media of Leeming & Notman [15] and Nazzaro-Porro *et al.* [4]. Squalene 1% (v/v) added to both culture media had a minor effect, inducing some *Malassezia* strains isolated from pityriasis versicolor patients to produce a few elongated buds.

Determination of the effect of ergosterol and sodium taurocholate on mycelium production by Malassezia

The effects of different concentrations of ergosterol and sodium taurocholate (STC) on mycelium production were tested separately and in combination by adding these materials to the culture media of Leeming & Notman [15] and Nazzaro-Porro *et al.* [4]. Only one *Malassezia* strain, isolated from a pityriasis versicolor patient, was capable of producing a few elongated buds in Leeming and Notman medium supplemented with squalene 1% (v/v) and ergosterol (12 μg ml^{-1}).

Dorn and Roehnert method

The medium of Dorn & Roehnert [5] was reported to be able to induce *Malassezia* yeast cells to produce mycelia *in vitro*. Therefore, this culture medium was prepared. The strains isolated from healthy individuals produced no mycelium on this medium. The rate of mycelium production by clinical isolates in liquid medium was higher than on solid culture medium. The mycelial phase colonies of the *Malassezia* strains on solid medium were white to creamy in colour, round, 3–6 mm in diameter, flattened and shiny. The percentage of mycelium production was between 5% and 10% depending on the strain. The strains designated Hook and GM216 produced hyphae from an average of 10% of yeast cells, but only 5% of yeast cells of strain CBS6001 (Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands) produced them. Mycelia were short and some of them were curved and branched. The lengths of hyphae were between 15 and 25 μm. The yeast cells tended to aggregate with each other to form clumps, and the hyphae were usually intermingled through the yeast network. The results obtained using this culture medium identified factors that might have an important role in yeast to mycelium transformation. They were culture medium pH, magnesium, iron, Tween 80, amino acids and incubation temperature.

Mycelial culture medium

Finally, after numerous attempts using the components of Leeming and Notman culture medium [15] supplemented with components from the Dorn and Roehnert culture medium [5], individually and in combination at different concentrations, a modified mycelial *Malassezia* culture medium was prepared containing the components listed in Table 2. Glycine, cycloheximide, chloramphenicol and UHT milk were sterilized by filtration and then added to the mycelial culture medium following sterilization by autoclaving.

The mycelial culture medium was able to induce mycelial formation in some *Malassezia* strains from lesions but not in the isolates from healthy skin. Three to five days after the initial inoculation onto solid medium, the growth of both yeast and mycelial phases was observed. The colonies were 2–4 mm in diameter, round, flattened, wrinkled, powdery, and yellow to dull white (Fig. 1). The percentage of hyphal production was 20–30%. After subculture one or two more times on

Table 2 Components of mycelial culture medium

Component	Amount
Bacteriological peptone	10 g l^{-1}
Glucose	5 g l^{-1}
Yeast extract	0·1 g l^{-1}
Ox bile	4 g l^{-1}
Glycerol	1 ml l^{-1}
Glycerol monostearate	0·5 g l^{-1}
Tween 80	1 ml l^{-1}
Squalene	1 ml l^{-1}
Potassium nitrate	1 g l^{-1}
Sodium chloride	1·3 g l^{-1}
Ferrous sulphate	0·01 g l^{-1}
Magnesium sulphate	0·15 g l^{-1}
Glycine	3·75 g l^{-1}
Agar No.1 (for solid medium)	12 g l^{-1}
Distilled water to	1 l
Cycloheximide	5 ml l^{-1}
Chloramphenicol	4 ml l^{-1}
UHT milk	10 ml l^{-1}
pH	5·6
Incubation condition	30 °C, aerobic

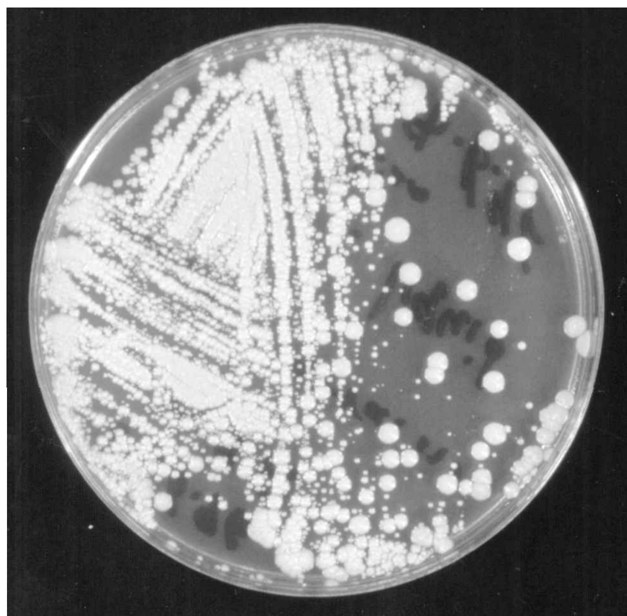

Fig. 1 The colonial morphology of *Malassezia* serovar A (GM 216) grown on the mycelial culture medium (magnification ×3).

mycelial culture medium, the percentage increased to around 40%. Branching hyphae and hyphal septa were observed in some of the mycelial states. The lengths of hyphae were 15–50 μm, average 32·5 μm (Fig. 2). The yeast cells tended to clump and most of the hyphae were seen around the clumps. The colour of the liquid medium 1–2 days after inoculation changed from creamy yellow to dark yellow. *Malassezia* isolates generally grew very quickly in liquid medium in comparison to their growth on solid medium. The effect of different Tween formulations (Tween 20, 40, 60 and 80) was compared by separately adding each of them to the mycelial culture medium. Only Tween 80 increased mycelium production. Incubation in air or in various CO_2 concentrations did not significantly alter the production of mycelium.

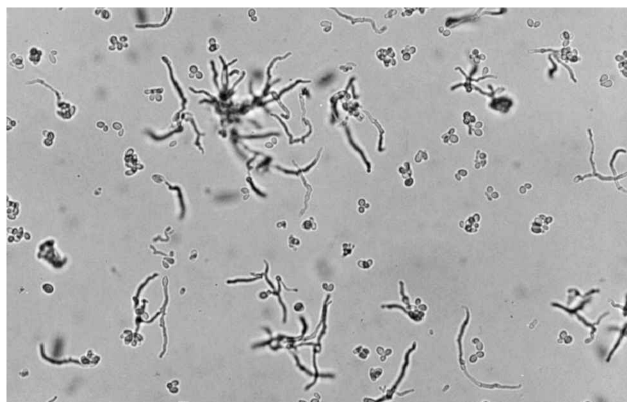

Fig. 2 Branched, septate hyphae produced by *Malassezia* serovar A (GM 216) grown in mycelial culture medium (magnification ×400).

The ability of Malassezia *strains to produce mycelium*

A total of 33 *Malassezia* strains were tested for their ability to produce mycelium on the newly developed medium. Only a few were able to produce elongated buds and even fewer were able to produce mycelium *in vitro*. They were *M. furfur* strain CBS 6001, *M. obtusa* strain GM216, and unidentified strains 01333.1, 2.PV.WY and 8.PV.MYC, all from pityriasis versicolor lesions, and the Hook strain, an *M. furfur* isolate from a psoriatic lesion. When the strains were serotyped [13] they were all serovar A, although none of the identified strains were *M. sympodialis* as would be expected from the data of Guillot & Guého [12]. They were able to produce a proportion of up to 30% mycelial forms, and this could be increased to 40% by subculture on the mycelial culture medium.

Discussion

Leeming and Notman *Malassezia* culture medium [15] has been shown to support the growth of a wider number of strains than other culture media. Therefore, this medium was used as the basal medium for investigation of the effects of different nutrients on mycelium production. Nazzaro-Porro *et al*. [4] suggested that cholesterol, cholesteryl stearate, glycerol monostearate and oleic acid might be important in inducing yeasts to form mycelium. They are the principal constituents of epidermal lipids and may alter the rigidity of the aliphatic polymethylenic chains of phospholipids, allowing modifications to the shape of the cell membrane and, therefore, inducing hyphal formation. In this study, although these components were used in different basal media, none of them were able to induce *Malassezia* to produce hyphae. Ergosterol is important in fungal membranes and in maintaining the integrity of fungal structures [16,17], and it was therefore tested in the culture medium, but showed no effect on mycelium production.

Cunliffe [18] reported that squalene was used by *Malassezia* species as a source of energy. In this study, squalene, a combination of squalene and ergosterol and a combination of squalene and STC were studied. Squalene induced short filaments. Ran *et al*. [19] suggested that the addition of STC to culture medium would induce mycelium formation. In the present study the results with STC (which may act as a lipase activator) were conflicting and mycelial production was inhibited when 1 or 2% STC was added to the basal culture medium.

Different Tween formulations can be used as a source of carbon and lipid by *Malassezia* [3]. Of the Tween compounds tested in this study, only Tween 80, which consists of 70% oleic acid, stimulated mycelial production. Oleic acid alone had no such effect. Plotkin *et al.* [20] reported that the enzymatic activity and growth of *Malassezia* was initially induced and then inhibited by increasing the concentration of Tween 80. Therefore, the concentration of this substance in the culture medium may be important. Dorn & Roehnert [5] used 40 and 50 ml l^{-1} of Tween-80 in solid and liquid media, respectively. In the present study, only 1 ml l^{-1} of Tween-80 was necessary to induce mycelial production in the medium.

The role of glycine in the induction of hyphae was not clear. Glycine is taken up by microorganisms by an active transport system. In the present study, it was shown to be more effective than methionine, histidine or tryptophan for the induction of mycelium.

The effect of Mg^{2+} on the formation of germ tubes by *Candida albicans* was described by Walker *et al.* [21] who stated that 'magnesium plays a central role in regulation of dimorphism in *C. albicans* through a combination of its effect on enzyme activation and cytoskeletal organization'. An inductive effect on lipase activity by *M. furfur* was described by Plotkin *et al.* [20]. In the present study, a lack of $MgSO_4$ in the medium prevented mycelium production. A similar effect was found when $FeSO_4$ was deleted from the medium. Therefore, it can be concluded that at least one form of these components is essential for the production of mycelium in *Malassezia*.

Faergemann [8] suggested that CO_2 concentration during incubation was an important factor for the growth of mycelium, but this was not supported by our study. It made no difference whether cultures were incubated in 5 or 15% CO_2 or under aerobic conditions. Results did not differ at different pH, and therefore pH 5·6 was chosen since it is the physiological pH of human skin.

Three major studies have been carried out on induction of mycelium in *Malassezia in vitro*. In the study of Dorn & Roehnert [5], all three isolates producing filaments were from pityriasis versicolor lesions and had round yeast cells. Nazzaro-Porro *et al.* [4] induced filaments in one of two round-celled strains isolated from pityriasis versicolor lesions. They also found two strains with oval cells (CBS 4162 from the ear of a pig and CBS 5334 from infected skin), produced short mycelium in defined culture medium. Faergemann *et al.* [7] and Faergemann [8] used six strains, obtained from the American Type Culture Collection (Manassas, VA, USA), all of which had been isolated from lesions of pityriasis versicolor or dandruff. Thus, all strains of *Malassezia* previously induced to form mycelia *in vitro* were isolated initially from diseased skin.

In the present study, only five of 33 strains were able to produce mycelium and all were obtained from lesional skin. It may be that some *Malassezia* strains are able to produce hyphae *in vitro* and some are not. Therefore, for studying mycelium production by *Malassezia* species *in vitro*, the strain tested may be very important.

The strains that produced mycelium were all serovar A, but none were *M. sympodialis*, as would be expected from the work of Guillot & Guého [12]. Serovar A may also encompass *M. furfur* and *M. obtusa* strains [14] as seen in this present study. Indeed, to date, no strains of *M. sympodialis* have been induced to form mycelium *in vitro*.

Two recent publications have suggested that *M. globosa* may be the causative agent of pityriasis versicolor. Crespo Erchiga *et al.* [22] found that *M. globosa* was the most common species from pityriasis versicolor when scales from patients were cultured on Dixon's agar. However, neither the sampling method nor the culture media used were optimal, and the findings that cultures from normal skin were negative in 85% of cases from the forehead and 53% from the shoulders casts doubt on the sensitivity of their methods. A second group also concluded that *M. globosa* was the 'pathogenic species of pityriasis versicolor' [23]. Examination of their data shows that *M. globosa* was isolated from 55% of pityriasis versicolor lesions on the trunk and 51% of normal skin on the trunk, and therefore, there were no significant differences in prevalence of the *M. globosa* between healthy and lesional skin. Thus, their data does not support their contention that 'overgrowth by *M. globosa* ... causes the lesions of pityriasis versicolor'.

In conclusion, the data from this study suggest that only limited numbers of strains of *Malassezia* are able to produce hyphae *in vitro* and that those that do possess the serovar A antigen. This may be an extremely important observation since it has implications for the aetiology of pityriasis versicolor. It merits further investigation with a wider range of strains from normal and lesional skin, in regard to their species and their capacity to form hyphae *in vitro* and *in vivo*.

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