Unraveling lipid metabolism in lipid-dependent pathogenic *Malassezia* yeasts

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Unraveling lipid metabolism in lipid-dependent pathogenic *Malassezia* yeasts

Ontrafeling van het lipide metabolisme in lipide-afhankelijke pathogene *Malassezia* gisten

(met een samenvatting in het Nederlands)

Proefschrift

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Porque los sueños se hacen realidad

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Chapter 1

General Introduction

Adriana Marcela Celis

Introduction to the genus *Malassezia*

The genus *Malassezia* belongs to the phylum Basidiomycota and comprises 14 established species as well as 3 species that were first described in 2016 (Table 1) [1–4]. *Malassezia* yeasts are part of the microbiome of healthy human skin but they have also been associated with dermatological conditions like dandruff (D), seborrheic dermatitis (SD), and pityriasis versicolor (PV) [5,6]. Moreover, they have been associated, albeit at low incidence, with systemic infections in patients receiving intravenous lipid therapy such as neonates. As such, they are recognized as opportunistic pathogens [7–10]. *Malassezia* is characterized by lipid-dependency due to the lack of cytosolic fatty acid synthase (FAS). This multifunctional enzyme is required for de novo synthesis of fatty acids (FAs) in a multistep process producing typically palmitic acid that serves as a precursor of (very) long-chain FAs [11,12]. Pathogenicity of *Malassezia* has been related to several factors including the ability to produce enzymes such as esterases, lipases, lipoxygenases and proteases. These enzymes that enable growth of these yeasts on the host skin lead to changes in sebum composition. For instance, release of FAs from triglycerides can result in inflammation, irritation, and scaling in susceptible individuals (Figure 1) [13–15]. Genome sequence analysis of *Malassezia* species revealed possible mechanisms to adapt to the host such as to its immune system [1,2,16,17]. Yet, a relation of these mechanisms to the disease process has in most cases not been shown.

Malassezia spp: Taxonomy and Epidemiology

Since the first description of *Malassezia* by Eichstedt in 1846 many taxonomic revisions have been made based on phenotypic and molecular tools [18]. Currently, 14 well established *Malassezia* species and 3 new species have been described [3,4,19–26]. They belong to the phylum Basidiomycota, subphylum Ustilaginomycotina, and class Malasseziomycetes. *Malassezia* is closely related with the class Ustilaginomycetes that consists almost exclusively of plant pathogens such as *Ustilago maydis* [27,28]. *Malassezia* yeasts are characterized by their lipophilic and lipid-dependent metabolism [13]. It is the most abundant yeast skin commensal, representing 50% – 80% of the total skin fungi. *Malassezia* is most common in areas rich in sebum such as the face and scalp but also on skin poor in sebum such as toe web space and hand palms [29,30]. The establishment of *Malassezia* species as normal members of the skin microbiota begins early after birth [31]. Culturing and non-culture methods showed differences in the distribution of the species [32,33]. Age and gender are associated with changes in *Malassezia* composition of the skin likely due to differences in the activity of sebaceous glands [15,31]. Presence of *Malassezia* increases in males between

Table 1. The genus *Malassezia* consists of 14 established species as well as 3 species described for the first time in 2016

Phylogenetic Cluster [2]	Species	Reference
A	*M. furfur*	Gueho et al. 1996 [20]
	M. obtusa	Gueho et al. 1996 [20]
	M. yamatoensis	Sugita et al. 2004 [23]
	M. japonica	Sugita et al. 2003 [22]
B	*M. globosa*	Gueho et al. 1996 [20]
	M. restricta	Gueho et al. 1996 [20]
	M. sympodialis	Simmons and Gueho 1990 [19]
	M. dermatis	Sugita et al. 2002 [21]
	M. caprae	Cabañes et al. 2007 [25]
	M. equina	Cabañes et al. 2007 [25]
	M. nana	Hirai et al. 2004 [23]
	M. pachydermatis	Gueho et al. 1996 [20]
C	*M. slooffiae*	Gueho et al. 1996 [20]
	M. cuniculi	Cabañes et al. 2011 [26]
Not included	*M. arunalokei sp. Nov*	Prasanna et al. 2016 [4]
Not included	*M. brasiliensis sp. Nov*	Cabañes et al. 2016 [3]
Not included	*M. psittaci sp. Nov*	Cabañes et al. 2016 [3]

15-18 years of age. The increase in abundance on skin of females can occur already at the age of 10-12 years, after which the load of *Malassezia* may decrease [31]. *Malassezia* species are also part of the normal microbiota of animal skin [34,35] and can be isolated from very diverse environments including deep hydrothermal vents and stony corals [36]. These findings have led to the reevaluation of this genus and suggest that more species exist in niches that contain lipid sources [2,35,37].

The skin functions in the innate defense against pathogens due to its low water content, acidic pH, its microbiota, and antimicrobial lipids (i.e. free FAs) [38]. Any changes in these conditions or composition may provide pathogens and even commensals an opportunity to cause disease. The lipid dependency of *Malassezia* on the one hand and the antimicrobial activity of these compounds on the other hand show the successful adaptation of these yeasts to the skin. Its capacity to adapt is also illustrated by its exposure to other residents of the skin such as *Propionibacterium acnes* and *Staphylococcus aureus* [30].

A recent phylogenic analysis distributed *Malassezia* species in clusters A, B, and C (Table 1) [2]. Cluster A consists of the anthropophilic species *M. furfur* that is associated with mild dermatological conditions such as PV and more

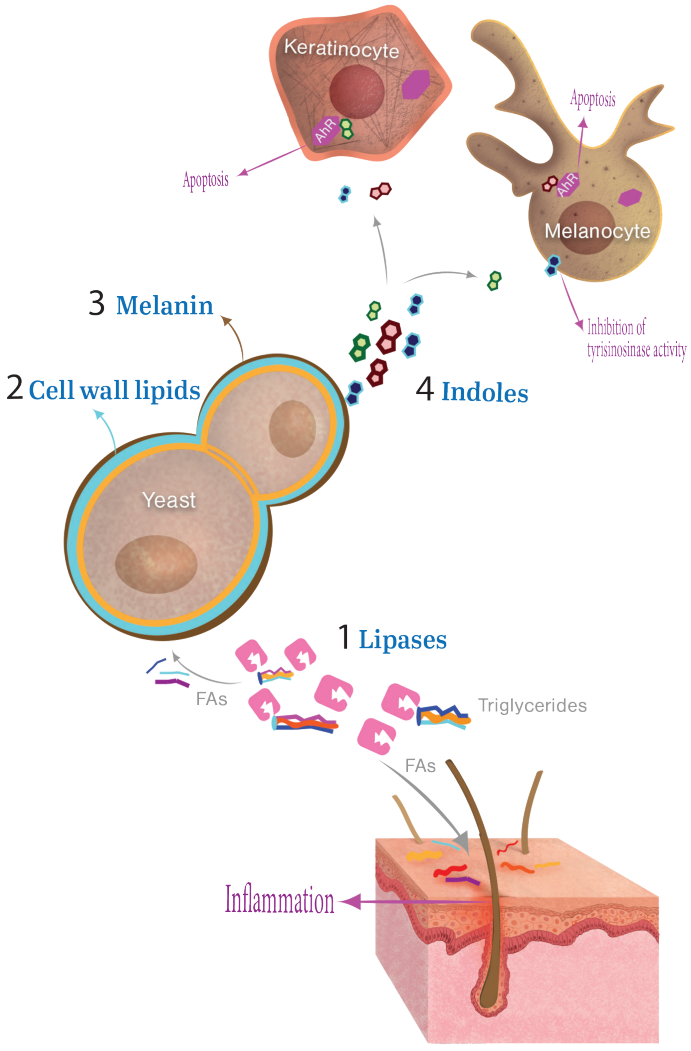


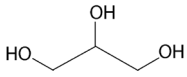
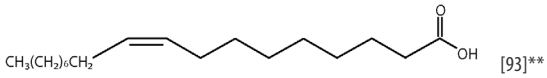
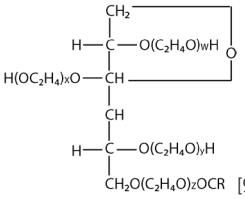
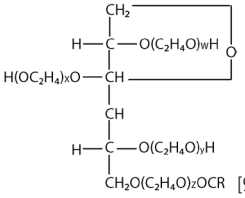
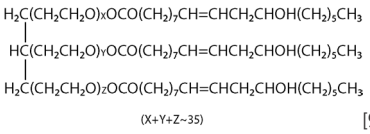
Figure 1. Molecules of *Malassezia* interacting with the host: 1. *Malassezia* obtains fatty acids (FAs) from the host by lipase activity. 2. These FAs are used, in part, to form the lipid layer at the outer part of the cell wall. 3. L-DOPA is required to form melanin in the cell wall and is also suggested to induce a dimorphic switch during infection. 4. Indoles such as malassezin and indirubin are potent ligands of the aryl hydrocarbon receptor (AhR), leading to the apoptosis of melanocytes and the inhibition of tyrosinase, a key enzyme of melanin synthesis in melanocytes. Inflammation is amongst others proposed to be due to action of unsaturated fatty acids on keratinocytes. Adapted from [14,35]

severe conditions such as bloodstream infections [7,18,39]. It also includes other anthropophilic species that are less frequently isolated from healthy humans such as *M. japonica, M. yamatoensis,* and *M. obtusa.* These species have been isolated from the skin of atopic dermatitis (AD) or SD patients [2,5,31]. Cluster B is represented by *M. globosa* and *M. restricta* that are the most abundant species on healthy human skin and *M. sympodialis* and *M. dermatis* that are slightly less common on healthy individuals. These 4 species have also been associated with dermatological diseases such as D/SD, AD, and PV [5,31]. A subcluster of cluster B encompasses zoophilic species such as *M. caprae, M. equina, M. nana,* and *M. pachydermatis.* The latter species is particularly associated with otitis in canines and bloodstream infections in humans [31,34,35]. Cluster C is defined by *M. cuniculi* and *M. slooffiae* that are commonly isolated from animals [26,40]. *M. slooffiae* is also isolated with low frequency from healthy and lesioned human skin.

The demanding nutritional requirements hampered the description and identification of *Malassezia* species. The implementation of complex media such as modified Dixon agar mDA (Table 2) that support *Malassezia* growth was the first step to solve these problems. Notably, *M. pachydermatis* is a less demanding species that can also grow on Sabouraud agar (SA). This medium contains peptone with traces of lipids such as palmitic acid [2,41]. Identification of *Malassezia* species is mainly based on morphological characteristics as well as biochemical tests such as utilization of the non-ionic detergents Tween 20, 40, 60, and 80, or Cremophor EL (Table 2, 3) and catalase, urease, and β-glucosidase activity (Table 3) [20,41].

Molecular tools, however, are superior to identify *Malassezia* species, preventing a 13.8% misidentification by the other methods [31]. Molecular tools have also been used to study *Malassezia* biodiversity and community structure on the human skin, as well as the epidemiology related to this genus [31,42]. The molecular tools include pulsed field gel electrophoresis of chromosomes [43], PCR-based methods (RAPD, PCR-RFLP, AFLP) [43–46], and DNA sequence based methods of D1/D2 domains of the large subunit rDNA and the internal transcribed spacer (ITS) regions and the intergenic spacer (IGS) region [47–50]. For instance, patient material containing a complex microbiome was used for qPCR [51] and Luminex [31] analysis to study the epidemiology of *Malassezia.* Recently, a matrix-assisted laser desorption / ionization–time of flight (MALDI-TOF) database was implemented to reliably identify *Malassezia* species by mass spectrometry [52,53].

Table 2. Composition of the culture media used for isolation and identification of *Malassezia* species [41]

Medium* or test	Composition	Additional description & molecuar structure
Modified Dixon agar mDA*[39]	3.6% malt extract 2.0% desiccated oxbile	Bile composition (water 92 g/dl, bile salts 6 g/dl, bilirubin 0.3 g/dl, cholesterol 0.3 to 0.9 g/dl, FA 0.3 to 1.2 g/dl, lecithin 0.3 g/dl and 200 meq/l inorganic salts [86]. Lecithins: phospholipids, glycolipids or triglyceride. Glycerophospholipids as phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, and phosphatidic acid [91].
	1.2% agar 0.6% peptone	
	0.2% glycerol	 [92]
	0.2 % Oleic acid	 [93]**
	1.0% Tween 40	 [94]**
Utilization of Tween 20, 40, 60, 80, or Cremophor EL**	Tween 20 (Polyoxyethylene (20) sorbitan monolaurate) Tween 40 (Polyoxyethylene sorbitan monopalmitate) Tween 60 (Polyoxyethylene (20) sorbitan monostearate) Tween 80 (Polyoxyethylene (20) sorbitan monooleate)	 [94]**
	Cremophor EL (CrEL or castor oil)	 [95]

**Structural formula: where w + x + y + z = approx. 20 and RCO- is the fatty acid moiety

Dermatological diseases associated with *Malassezia*

Malassezia has been traditionally linked with dermatological diseases because of the isolation of these yeasts from infected skin and the reduction of the load of *Malassezia* and the recovery of the lesions with antifungal treatments [54]. PV is the only skin disease for which a pathogenic role of *Malassezia* is established being mainly related with *M. globosa* [39]. Exogenous factors such as humidity, sweat, and heat are associated with the onset of disease [5,6]. PV is characterized by the presence of hypo- or hyperpigmented macules in the neck, trunk, and arms without inflicting inflammation. The mechanisms of skin color changes are not completely understood. *Malassezia* produces indoles such as malassezin, indirubin, indolo[3,2-b]carbazole [ICZ], formylindolo[3,2-b]carbazole from tryptophan. These indoles are potent ligands of the aryl hydrocarbon receptor (AhR), which is a ligand dependent transcription factor [13,55]. The activation of the AhR signal transduction pathway by the *Malassezia* indoles leads to apoptosis of melanocytes and the inhibition of tyrosinase that is a key enzyme of melanin synthesis (Figure 1) [5,13]. Recently, the AhR receptor has been related with carcinogenesis, immune regulation, and mediation of ultraviolet radiation damage, illustrating the importance to explore its interaction with *Malassezia* [14,35].

Malassezia is considered to play an important role in D/SD, however this is not fully accepted. D/SD are common abnormal skin conditions characterized by flaking and itch. Their incidence ranges between 1-3% in the general population, while patients with acquired immunodeficiency syndrome (AIDS) show an incidence of 30–83% [56]. Costs of treatment of these diseases are considerable but the socioeconomic impact is even higher [57]. *M. globosa* and *M. restricta* are the most frequent species isolated from scalp of individuals with D/SD [58]. *Malassezia* releases lipases, aspartyl proteases, phospholipases C, and acid sphingomyelinases that hydrolyze lipid sources like triglycerides to obtain FAs for growth. The unsaturated FAs resulting from the enzymatic action can give rise to irritation and are assumed to exacerbate the disease in susceptible individuals [57,59]. However, *M. globosa* and *M. restricta* were shown to metabolize the unsaturated oleic acid [60], suggesting that unsaturated FAs are not the etiological factors in D/SD [60]. Notably, *M. globosa* and *M. restricta* lack Δ3,2-enoyl CoA isomerase (EC 5.3.3.8) that is involved in catalyzing an important step in catabolism of unsaturated FAs. Possibly, *Malassezia* contains a Δ3,2-enoyl-CoA isomerase that is structurally distinct from that of other fungi or it uses an alternative biochemical route to degrade unsaturated FAs [60,61]. It should also be noted that *M. globosa* lacks a Δ9-desaturase (EC 1.14.19.2) gene. This suggests it is not capable to synthesize unsaturated FAs, and therefore, has

Table 3. Physiological characteristics of *Malassezia* species

Species	Cell morphology	Utilization of tween				Cremophor EL	β- glucosidase activity	Catalase reaction	Growth in mDA	Growth in SA	Growth	
		20	40	60	80						At 37°C	At 40°C
M. furfur	Globose, ellipsoidal, cylindrical	+	+	+	+	+	+	+	+	-	+	+
M. sympodialis	Ellipsoidal	- w	+	+	+	- (w)	+	+	+	-	+	+
M. globosa	Globose	-b	-	-	-	-	-	+	+	-	-(w)	-
M. restricta	Ellipsoidal, globose	-	- c	- c	-	-	-	-	+	-	v	-
M. obtusa	Ellipsoidal,cylindrical	-	- c	- c	-	-	+	+	+	-	+	-
M. slooffiae	Ellipsoidal,cylindrical	+	+	+	w	-	-	+	+	-	+	+
M. dermatis	Ellipsoidal, globose	+	+	+	+ (w)	-	-	-	+	-	+	-
M. japonica	Globose,ellipsoidal	-b	-b	+	+	w	+	+	+	-	+	-
M. nana	Ellipsoidal	- b	+	+	+	-	+	+	+	-	+	+
M. yamatoensis	Ellipsoidal	+	+	+	+	- (w)	-	+	+	-	+	+
M. equina	Ellipsoidal	w[b]	+	+	+	-	-	+	+	-	w	-
M. caprae	Globose,ellipsoidal	-b(+)	+a	+ b	+b(-)	-	(+),-	+	+	-	-,(w)	-
M. cuniculi	Globose	-	-	-	-	- (w)	-	+	+	-	+	+
M. pachydermatis	Ellipsoidal	+a	+a	+	+	+b	(+), -	(+), w, (-)	+	+	+	+
M. arunalokei sp. nov	Ovoid, globose	-b	-b	-b	v	(-)b	-	-	+	NI	+	-
M. brasiliensis sp. nov	Ovoidal, ellipsoidal	+	+	+	+	+	-	+	+	-	+	+
M. psittaci sp. nov	Globose, ovoidal	+	+	+	+	+	-	-	+	-	-	-

Data are from references [3,4,20] SA: Sabouraud agar, mDA: modified Dixon agar, NI: not included in the description. Growth is indicated with: +, positive; -, negative; v, variable; w, weak; ( ) indicate rare deviations

[a]Growth may be inhibited near the well where the substrate is placed

[b]Growth may occur at some distance from the well where the substrate is placed

[c]Opaque zone may occur

[d]*Malassezia* species have a very narrow optimum growth temperature range (32°C-34°C) and do not survive very long below 28°C on regular culture media [39]

to import it from its environment [61]. Additional studies should be done in order to clarify the true role of the unsaturated FAs in the etiology of DS.

AD is a chronic and inflammatory skin disease. It is characterized by severely itchy, red, and dry skin that also may include steps of remission and deterioration [5,58]. The prevalence has increased to 15–30% in children and 2–10% in adults [62]. Pathogenesis is multifactorial and related with a disturbed skin barrier and with genetic and environmental factors such as life style, stress, allergens, and the skin microbiome. *M. sympodialis* is frequently isolated from AD patients. Anti-*Malassezia* IgE antibodies have been detected in these patients but not in healthy individuals. Currently 13 allergens are characterized, 3 from *M. furfur* and 10 from *M. sympodialis* [5,58].

Malassezia folliculitis is an inflammatory papulopustular eruption that occurs on the back or front of the upper trunk [5]. Triglyceride hydrolysis by the yeast leads to an inflammatory reaction in the hair follicles [14]. The diagnosis can be misleading due to similarity with other forms of folliculitis caused by bacteria. The increase of folliculitis in the clinical practice underlines the need for a right diagnosis [5,18,31]. Other dermatological diseases such as psoriasis, onychomycosis and confluent and reticulated papillomatosis have also been associated with *Malassezia*. However, a causal relationship is still purely hypothetical because it is based on colonization of affected areas with the yeast [5,18,31].

Malassezia can be considered an opportunistic yeast and emergent pathogen. Bloodstream infections caused by *M. furfur* and *M. pachydermatis* have been reported since 1980 [8,9,63]. Fungemia is associated with the use of intravenous lipid feeds and affects critically ill low-birth-weight infants and immunocompromised children and adults [64,65]. Colonization and pathogenicity of this yeast are related with adherence properties, possibly mediated by the lipid layer that is found at the outer surface of *Malassezia* cells. This layer has also been reported to allow immune system evasion, suppression of cytokine release, and reduction of phagocytic uptake and killing [13,66].

Physiology and biochemistry

The principal metabolic trait of *Malassezia* that has pressed the adaptation mechanism to the human and animal host is its lipid dependence. Research has therefore especially been focused on lipid metabolism to understand the mechanisms of *Malassezia* to sustain growth and maintain commensalism and pathogenicity [2]. The importance of lipids is also illustrated by the “capsule” formed by these molecules at the outer part of the *Malassezia* cell wall (Figure 1). This capsule is assumed to play a major role in commensalism and pathogenicity.

Carbohydrate metabolism strategies may also be linked to the adaptation of the host. For instance, comparative genomics indicated that a large set of genes involved in carbohydrate metabolism such as glycosyl hydrolases are missing in the *Malassezia* genomes [2].

Lipid metabolism

The 14 *Malassezia* genomes lack the genes encoding the cytosolic fatty acid synthase complex (FAS), which explains why these yeasts cannot synthesize the FA palmitate *de novo* [1,2]. To overcome this, *Malassezia* should obtain FA sources from the culture medium or directly from their host. Human sebum is a complex mixture of triglycerides, FAs, wax esters, sterol esters, cholesterol, cholesterol esters, and squalene [57,59]. These sources are exploited by *Malassezia* by secreting lipases and phospholipases to release FAs. Other secreted hydrolases such as aspartyl proteases, and acid sphingomyelinases support the exploitation of other components of the human skin [2,16,17]. After FA uptake and concomitant activation to coenzyme A derivatives by acyl-CoA synthetases, *Malassezia* metabolizes or modifies FAs. They are used in the synthesis of lipids to build up cell membranes [67], to synthesize triglycerides and / or sterol esters, or they can be degraded via the β-oxidation pathway [13,60] (Figure 2). Intracellular FAs are

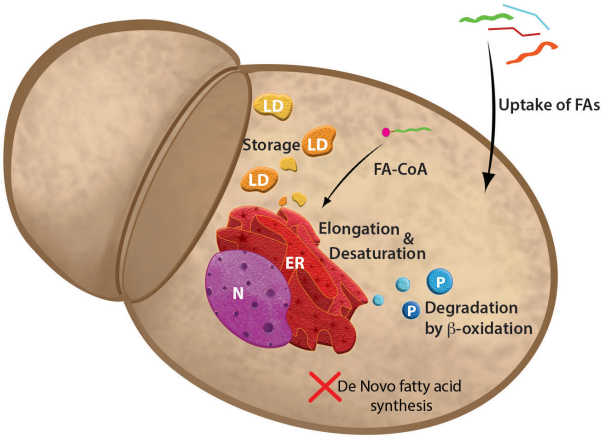


Figure 2. Overview of organelles involved in processing of fatty acids (FAs) taken up from the external milieu by *Malassezia* spp. N: nucleus (Regulation of biosynthetic pathways); ER: endoplasmic reticulum (FA elongation, desaturation and acylation of lipid backbones); P: peroxisome (degradation of FA via β-oxidation) LD: lipid droplets (lipid storage). De novo fatty acid synthesis in the cytosol does not proceed due to the absence of the fatty acid synthase complex

activated to acyl-CoA derivatives by Faa1, Faa2, Faa3, Faa4, and Fat1. These acyl-CoA synthetases can have different localization and substrate specificity [68]. The elongation steps up to C26 occurs in the endoplasmic reticulum (ER) by subsequent addition of 2 carbons from malonyl-CoA by the elongases ELO1, ELO2, and ELO3 [69,70]. Desaturation that involves the introduction of double bonds into acyl chains also occurs in the ER. For instance, palmitic acid and stearic acid are converted by the Δ9-desaturase OLE1 to their corresponding mono-unsaturated FAs palmitoleic acid (C16:1) and oleic acid (C18:1) [68–70]. Degradation of FAs via β–oxidation occurs in the peroxisomes and involves 4 steps. The first step is the oxidation of acyl-CoA to trans-2-enoyl-CoA catalyzed by the acyl-CoA oxidase. Trans-2-enoyl-CoA is converted to 3-ketoacyl-CoA with 3R-hydroxyacyl-CoA as an intermediate. Thiolase cleaves 3-ketoacyl-CoA in the final step of the β–oxidation to acetyl-CoA and a C2-reduced acyl-CoA. Two additional steps are required for β-oxidation of unsaturated FAs. These steps are catalyzed by the enzymes Δ3,2-enoyl -CoA isomerase and 2,4 Dienoyl-CoA reductase [12,70].

Physiological assessments indicated that *Malassezia* could use both saturated and unsaturated FAs [71–73]. Yet, recent in-silico and in-vitro analysis of lipid metabolism in *M. globosa* and *M. restricta* showed that *M. globosa* has an intact β-oxidation pathway but lacks Δ3,2-enoyl-CoA isomerase to degrade unsaturated FAs such as oleic acid. Nevertheless, both strains removed oleic acid when grown at very high density in chemically defined medium with low amounts of oleic acid. This did not support the hypothesis that this FA has a role in D/SD [60]. However, the conclusions may not be correct. The media that were used also contained polysorbates such as Tween 20, 40, 60 and 80, which may have been used as lipid source. In addition, Dixon medium was used during pre-growth, which might result in accumulation of other FAs in yeast cells present in this medium. The presence of additional FAs during (pre-)growth might support growth of *Malassezia* and result in uptake and conversion of oleic acid, an otherwise fungistatic FA if present in high amounts, into a nontoxic form.

Cell wall and dimorphism

The *Malassezia* cell wall is a very thick multilayered structure. It consist of ~70% sugars, ~10% protein, and 15–20% lipids [74]. The sugars consist of chitin/chitosan, β-(1,3)-glucans, β-(1,6)-glucans, galactofuran and mannan structures [75–77]. The cell wall protects the cells against environmental stress like high osmolarity and mediates adherence to the host surface [66,78].

Moreover, it allows evasion of phagocytosis and downregulation of the inflammatory immune response [79–82]. The lipid layer at the outer part of the cell wall shield cell wall components of *Malassezia* that induce an inflammatory response [80,81]. Lipid encapsulated yeast cause a low production of the proinflammatory cytokines, IL-6, TNF-α, IL-8 and IL-1α and a high production of the anti-inflammatory cytokine IL-10 by keratinocytes and peripheral blood mononuclear cells (PBMCs). Removing the lipid capsule of the cell wall results in increased levels of IL-6, IL-8, and IL-1α, while IL-10 levels decrease [83].

The capacity of *Malassezia* to switch from the yeast to a filamentous form may contribute to the establishment of disease similarly as described for the dimorphic fungus *Candida albicans* [29,30]. *M. globosa* hyphae were shown in PV skin lesions and in primary cultures of these samples. Yet, filamentous growth could not be induced in vitro. In contrast, high CO2 conditions and medium supplemented with glycine, cholesterol, and cholesterol esters induces hyphal growth in in vitro cultures of *M. furfur* and *M. sympodialis* [39,82].

Metabolites

Malassezia metabolites can impact pathophysiology and exacerbate skin conditions. Products resulting from lipase activity are such a class of molecules. Genome analysis showed that phospholipases and lipases are the most expanded lipolytic families in *Malassezia* in comparison with other fungi (Table 4). Expression of lipases has been shown in vivo. *LIP1* from *M. globosa* was detected by RT-PCR in samples from human scalp [84]. This finding was corroborated by quantitative real-time PCR of 4 lipase genes in samples from individuals without *Malassezia* lesion, and patients with SD that were either (SD + HIV) or not (SD) infected with human immunodeficiency virus. The 4 genes were expressed in all samples but 3 of them (*Mgl0797, Mgl0798*, and *Mflip1*) were up-regulated in SD and SD+HIV suggesting a role of the encoded lipases in the establishment of disease [85]. Polyketide synthases (PKSs) genes are also present in *Malassezia* species. PKSs are mechanistically and structurally related to FA synthases and have been associated with the biosynthesis of unique lipids or glycolipid conjugates in *Mycobacterium turberculosis* [86,87].

Pigments such as melanin are described as fungal pathogenicity factors [88]. A phenoloxidase was identified in *M. furfur* suggesting that L-DOPA melanin can be produced by this fungus similar to the opportunistic fungal pathogen *Cryptococcus neoformans*. Indeed, production of L-DOPA melanin was recently shown in *Malassezia*. Interestingly, L-DOPA is associated with yeast to hypha transition [89,90]. It remains to be determined if the same source of L-DOPA that

is used for pigment production in skin is also used by *Malassezia* spp and required for pathogenesis. Tryptophan derived indole pigments have been particularly described in *M. furfur*. Malassezin is produced in vitro when *M. furfur* is grown in the presence of the amino acid tryptophan in a selective medium. It is a potent ligand of AhR and implicated in the pathogenesis of the hypopigmented forms of PV because of the capacity to inhibit melanin synthesis [35,74]. Other pigments such as pityriacitrin, pityrialactone, pityriarubins have been proposed to be involved in the inhibition of the respiratory burst in neutrophils or to filter UVA, UVB and UVC [74,82].

Table 4. Number of predicted *Malassezia* genes encoding lipolytic and proteolytic enzymes (Adapted from [2]). MP: *M. pachydermatis*; MSL: *M. slooffiae*; MJ: *M. japonica*; MO: *M. obtusa*; MF: *M. furfur*; ME. *M. equina*; MG: *M. globosa*; MC: *M cuniculi*; MN: *M. nana*; MR: *M. restricta*; MS: *M. sympodialis*; MD: *M. dermatis*; MY: *M. yamatoensis*

Protein families	*Malassezia* species and number of gene copies (predicted secreted proteins)												
	MP	MSL	MJ	MO	MF	ME	MG	MC	MN	MR	MS	MD	MY
PF03583.9 Secretory lipase	13	13	11	10	8-9	8	6-8	5-8	7	4	4-13	4	11
PF01764.20 Lipase (class 3)	6	7	8	10	5-6	12	5-8	8-9	7	6-7	6-12	6	4
PF0057.19 Metalloproteases	6	6	5	6	6	5-6	6	6	6	6	6	6	6
PF00026.18 Aspartyl protease	9	13	13	9	9-8	8	18-22	5-7	8	14	5-7	5	7
Acid sphingomyelinase	4	4	4	4	4	4	4	4	4	4	4	4	4
PF04185.9 Phosphoesterase family (Including phospholipase C enzymes EC:3.1.4.3, and acid phosphatases EC:3.1.3.2)	5	4	4	3	1	4	6	5-4	4	7	4-5	4	0

Scope of this thesis

Lipid dependence is a main factor determining the human and animal skin as an ecological niche for *Malassezia* yeasts. Lipid metabolism may also contribute to disease development. Lipid dependence and metabolism were studied in this Thesis to contribute to the understanding of mechanisms involved in *Malassezia* commensalism and pathogenicity.

Chapter 2A describes the genome sequence of *M. pachydermatis* CBS1879. This species was considered to be the only lipid independent *Malassezia* species due to the capacity to grow on Sabouraud agar medium. Genome analysis showed the absence of the FA synthase genes as was shown before in other *Malassezia* species. This suggested that *M. pachydermatis* could not grow in the absence of a lipid source. In **Chapter 2B** the growth of *M. pachydermatis* and 3 other *Malassezia* species on a variety of FA sources in complex and chemically defined minimal media is compared. Palmitic acid showed to have a fungicidal effect on *M. pachydermatis*, while oleic acid had a fungistatic effect. However, mixtures of saturated and unsaturated FA did sustain growth. These results showed that this species needs a combination of different FAs to support full growth. The results underscore that *M. pachydermatis* is not able to grow in the absence of FAs while palmitic acid, the major FA in Sabouraud, does by itself not support growth of this species.

Lipid-synthesis pathways of *Malassezia furfur,* the atypical *M. furfur* isolate, *M. pachydermatis, M. globosa,* and *M. sympodialis* were reconstructed in silico using genome data and Constraints Based Reconstruction and Analysis **(Chapter 3)**. To this end, the genomes of *M. furfur* CBS 1878 and the atypical *M. furfur* 4DS isolate were sequenced. The predicted lipid metabolism reactions were similar to the other *Malassezia* strains despite the differences in their genome size. Proteomic profiling was utilized to integrate protein profiling with flux distributions. Flux differences were observed in the production of riboflavin in *M. furfur* and in the biosynthesis of glycerolipids in the atypical variant of *M. furfur* and *M. sympodialis*. The results obtained via these metabolic reconstructions also predicted defects in the assimilation of palmitic acid in *M. globosa, M. sympodialis, M. pachydermatis,* and the atypical isolate of *M. furfur*, but not in *M. furfur.* These predictions were validated by physiological characterization, showing the power of metabolic network reconstructions to provide new clues about the metabolic versatility of *Malassezia*.

Chapter 4 describes the connection of lipid dependent metabolism and the presence of lipid droplets (LD) in *Malassezia* in general and *Malassezia furfur*

in particular. Lipidomic analysis showed that these lipid droplets contain triglycerides but no sterol esters (SEs). However genome analysis indicating the presence of genes encoding proteins for esterification of ergosterol, indicating that regulation prevents SE storage. In addition, genomic analysis confirmed the presence of all genes required for the formation of the main precursor for synthesis of triglycerides.

New insights in the pathophysiology of *Malassezia* spp obtained by genome sequencing and metabolic reconstruction require experimental validation. To this end, genetic tools are needed. **Chapter 5** describes a highly efficient *Agrobacterium*-mediated genetic transformation system for *M. furfur* and *M. pachydermatis*. A binary T-DNA vector with the hygromycin B phosphotransferase (*hpt*) selection marker and the green fluorescent protein gene (*gfp*) was introduced in these yeasts by combining the transformation protocols of *Agaricus bisporus* and *Cryptococcus neoformans*. The T-DNA was mitotically stable in approximately 80 % of the transformants after 10 times sub-culturing in the absence of hygromycin selection. Results are summarized and discussed in **Chapter 6**

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Chapter 2

Malassezia pachydermatis: Genome and physiological characterization of lipid assimilation

Chapter 2A

Draft Genome Sequence of the animal and human pathogen *Malassezia pachydermatis* CBS 1879

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Abstract

Malassezia pachydermatis is a basidiomycetous yeast that causes infections in humans and animals. Here, we report the genome sequence of *Malassezia pachydermatis* CBS 1879 that will facilitate the study of mechanisms underlying pathogenicity of the only *Malassezia* species that is claimed to be non-lipid dependent.

Introduction

M. pachydermatis is claimed to be the only non-lipid-dependent species of *Malassezia*. All other 13 species [1] of this genus are obligate lipophilic and require fatty acids for growth. This is due to the lack of a fungal type fatty acid synthase [2]. *M. pachydermatis* is able to assimilate fatty acids (FAs) from the growth medium and can thus be considered a facultative lipophilic species [3]. The molecular mechanisms underpinning this behavior are not yet clear.

M. pachydermatis is a member of the microbiota of animals. It is an opportunistic pathogen of dogs causing dermatitis and otitis externa. *M. pachydermatis* has also been implicated in human blood stream infections [4,5]. The genomes of the obligate lipophilic species *M. globosa*, *M. restricta* and *M. sympodiales* have been reported [2,6]. The genome of *M. pachydermatis* will help us to reveal why this species is facultative lipophilic.

Materials and Methods

M. pachydermatis genomic DNA was extracted as previously described [7]. The DNA was sequenced with the Illumina HiSeq 2000 platform at ServiceXS (Leiden, the Netherlands). Two runs with 120 bp paired-end reads on 250 bp fragments were performed following standard Illumina protocols with a 280-fold genome coverage. Reads were quality controlled with FastQC [8] and trimmed using Flexbar [9]. *De novo* assembly was performed using CLC Assembly Cell (CLC bio, Denmark). The resulting contigs were scaffolded using SSPACE_Basic [10] and gaps were filled with GapFiller [11]. The genome was annotated using Maker2 [12] and we made use of a set of 109.264 previously reported *Ustilaginomycotina* proteins, 1413 ESTs from *Malassezia spp*, and CEGMA [13]. The homology-based predictor GeneMark and the ab-initio predictors SNAP [14] and Augustus [15] were used to predict genes. In order to train Augustus and SNAP we ran MAKER 2 consecutive times; the initial annotation output from MAKER was converted into a model for SNAP and a training set for Augustus, which was used in the subsequent run. Functional annotation of the predicted genes was performed by Blast2GO [16], which involved Blast and InterProScan annotation [17,18]. The whole-genome shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession number LGAV00000000. The version described in this paper is LGAV01000000.

Results and Discussion

The final assembly of the genome of *M. pachydermatis* consisted of 148 contigs that were linked by pair-end reads into 91 scaffolds, 28 of which were longer than 1 kb. The maximum contig and scaffold length were 1,466,538 and 1,489,072

bp, respectively, and the N50 was 0.64 Mbp and 1.3 Mbp, respectively. The genome size was 8.15 Mbp with a GC content of 55.17%. A total of 4202 protein encoding genes were predicted with an average size of 1581 bp. The coding regions corresponded to 81% of the genome. CEGMA showed that 97.18% of the eukaryotic core genome was present in the genome [13].

Lipid degrading enzymes play an important role in the host invasion process of *M. pachydermatis* [19,20]. A total of 50 such enzyme encoding genes were identified in the genome, including 35 lipases and 15 esterases (Table 1). Most interestingly, a typical fungal fatty acid synthase gene was not detected in the genome. Its absence dismisses the hypothesis that presence of such a gene explains the lipid independent nature of *M. pachydermatis*. Instead a polyketide synthase, homologous to fatty acid synthases [21], was detected that showed 75% identity with its bidirectional homologue of *M. sympodialis*. How *M. pachydermatis* is able to grow in the absence of FAs is subject for future research.

Table1. *Malassezia pachydermatis* genes encoding lipolytic enzymes

Gene	Go enrichment	Annotation	Function
Malapachy0298	GO:0005975	Carbohydrate esterase family 4 protein	Esterase
Malapachy0339	GO:0006629	Triglyceride lipase-cholesterol esterase	Esterase
Malapachy0369	GO:0016787	Phospholipase carboxylesterase family protein	Esterase
Malapachy0396	GO:0006629	Plc-like phosphodiesterase	Esterase
Malapachy0599	GO:0051186	Acyl- thioesterase ii	Esterase
Malapachy0928	GO:0006629	Plc-like phosphodiesterase	Esterase
Malapachy1321	GO:0016787	Carboxylesterase family protein	Esterase
Malapachy1810	GO:0008150	Carbohydrate esterase family 1 protein	Esterase
Malapachy2653	GO:0005975	Plc-like phosphodiesterase	Esterase
Malapachy2674	GO:0008150	Related to palmitoyl-protein thioesterase 1	Esterase
Malapachy3104	GO:0008150	Esterase lipase	Esterase
Malapachy3669	GO:0055086	Cyclic-amp phosphodiesterase	Esterase
Malapachy3749	GO:0003674	Thioesterase family	Esterase
Malapachy4055	GO:0005737	Dhh phosphoesterase	Esterase
Malapachy4219	GO:0008150	Protein phosphatase methylesterase	Esterase
Malapachy1632	GO:0051186	Probable peroxisomal acyl-coenzyme a thioester hydrolase 1	Lipase
Malapachy2697	GO:0003674	Progesterone binding protein	Lipase
Malapachy0238	GO:0006629	Hypothetical secretory lipase (family lip)	Lipase

Gene	Go enrichment	Annotation	Function
Malapachy0295	GO:0006629	Hypothetical secretory lipase (family lip)	Lipase
Malapachy0315	GO:0006629	Hypothetical secretory lipase (family lip)	Lipase
Malapachy0481	GO:0006629	Hypothetical secretory lipase (family lip)	Lipase
Malapachy0498	GO:0006629	Hypothetical secretory lipase (family lip)	Lipase
Malapachy1294	GO:0006629	Hypothetical secretory (family lip)	lipase
Malapachy1312	GO:0006629	Hypothetical secretory lipase (family lip)	Lipase
Malapachy1317	GO:0006629	Hypothetical secretory lipase (family 3)	Lipase
Malapachy1955	GO:0006629	Hypothetical secretory lipase (family lip)	Lipase
Malapachy2050	GO:0006629	Hypothetical secretory lipase (family lip)	Lipase
Malapachy2052	GO:0006629	Hypothetical secretory lipase (family lip)	Lipase
Malapachy2356	GO:0006629	Hypothetical secretory lipase (family lip)	Lipase
Malapachy2723	GO:0006629	Hypothetical secretory lipase (family 3)	Lipase
Malapachy2741	GO:0016787	gdsl family lipase	Lipase
Malapachy2743	GO:0016787	gdsl family lipase	Lipase
Malapachy2948	GO:0006629	Hypothetical secretory lipase (family 3)	Lipase
Malapachy2949	GO:0006629	Hypothetical secretory lipase (family 3)	Lipase
Malapachy3407	GO:0006629	Hypothetical secretory lipase (family lip)	Lipase
Malapachy3408	GO:0006629	Hypothetical secretory lipase (family lip)	Lipase
Malapachy3413	GO:0006629	Triacylglycerol lipase	Lipase
Malapachy3425	GO:0006629	Hypothetical secretory lipase (family 3)	Lipase
Malapachy4203	GO:0006629	Hypothetical secretory lipase (family lip)	Lipase
Malapachy0399	GO:0006629	Lysophospholipase plb1	Phospholipase
Malapachy0514	GO:0008150	Hypothetical phospholipase c	Phospholipase
Malapachy0515	GO:0008150	Hypothetical phospholipase c	Phospholipase
Malapachy0761	GO:0008150	Probable spo14-phospholipase d	Phospholipase
Malapachy1091	GO:0003674	Phospholipase a-2-activating protein	Phospholipase
Malapachy1627	GO:0005737	Related to lysophospholipase	Phospholipase
Malapachy2561	GO:0006629	Cytosolic phospholipase	Phospholipase
Malapachy3821	GO:0008150	Phospholipase c p1 nuclease	Phospholipase
Malapachy3976	GO:0008150	Hypothetical phospholipase c	Phospholipase
Malapachy4264	GO:0008150	Hypothetical phospholipase c	Phospholipase
Malapachy4270	GO:0016787	Hypothetical phospholipase c	Phospholipase

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Chapter 2B

Physiological characterization of lipid assimilation in *M. pachydermatis*

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Abstract

Malassezia pachydermatis causes skin infections in animals and bloodstream infections in humans. In contrast to other *Malassezia* species, this yeast was regarded as lipid independent. However, genome sequencing indicated the absence of genes encoding the α and β subunits of the fungal type fatty acid synthase complex, indicating that this species is lipid dependent. In this study, a physiological characterization of lipid metabolism of *M. pachydermatis*, *M. furfur*, atypical *M. furfur,* and *M. sympodialis* was performed. *M. pachydermatis* grew on Sabouraud medium without lipid supplementation but was unable to grow on defined medium in the presence of free fatty acids such as palmitic acid. In fact, the latter showed to be fungicidal for all strains except *M. furfur*. In addition, fungistatic activity of oleic acid was observed for all strains, while growth in combination with palmitic acid alleviated, at least partly, its toxic effect in the case of *M. pachydermatis*, *M. furfur*, and atypical *M. furfur*. *M. sympodialis* was shown to be the most demanding species since supplementation of minimal medium with palmitic and / or oleic acid did not restore growth.

Introduction

M. pachydermatis is a normal member of the microbiota of animal skin. In addition, it is found in the external ear canal in dogs and is therefore considered a zoophilic species. This yeast is associated with otitis externa and dermatitis in dogs, and can cause blood stream infections in human, especially in neonates [1–3]. Weidman described *M. pachydermatis* in 1925 as a species that does not depend on lipids for its growth [4]. Multi-locus phylogenetic analysis showed that *M. pachydermatis* is highly diverse [5,6]. Genotypic subtypes have been clustered in 2 different clades. One clade comprises mainly isolates from dogs, while the other clade includes isolates from other domestic animals including cats. The observation that genotypes are associated with specific hosts suggests that *M. pachydermatis* has adapted to its ecological niche [7].

The genome sequence of *M. pachydermatis* did not reveal a fungal type fatty acid synthase [8,9], which was peculiar considering the fact that this yeast, unlike other *Malassezia* species, can grow on Sabouraud medium without additional lipids. Detailed analysis, however, revealed that Sabouraud contains 0.6% lipid, with 6 μg of palmitic acid (PA) per gram of peptone and lower amounts of other fatty acids. The presence of these lipid traces could support growth of *M. pachydermatis* [9].

M. pachydermatis can grow as small or large colonies with ellipsoidal or short cylindrical cells [4,10]. The small colony phenotype of *M. pachydermatis* isolates from dogs did not grow after 2 passages on Sabouraud medium, while the large colony type still showed growth [11]. Oleic acid could not be detected in cells of small colonies and linoleic acid content was lower when compared to large colonies. Further analysis revealed that oleic and linoleic acid inhibited the mean generation time of growth of the large colony phenotype on Sabouraud medium, inducing changes in the lipid composition including production of new fatty acids [11]. Lipid profiles of *M. pachydermatis* isolates from dogs with otitis externa were also different when compared to isolates from dogs without symptoms. After culturing on Sabouraud medium with 0.5% Tween 60 and 2% olive oil isolates from dogs without symptoms contained more oleic acid and mono-unsaturated lipids when compared to isolates from dogs that did show symptoms. In addition, a higher content of ergosterol esters and triglycerides was reported. These findings suggest adaptations of the isolates that cause disease [12].

In this report lipid-dependency was studied of 4 *Malassezia* species, including *M. pachydermatis*. It is shown that combinations of palmitic acid and oleic acid support growth of *M. pachydermatis* but that they individually lead to fungicidal and fungistatic effects, respectively.

Materials and Methods

Chemicals

Chemicals were purchased from Sigma (St Louis, MO, USA) unless otherwise indicated.

Strains and growth conditions

The reference *Malassezia* strains *M. pachydermatis* CBS 1879, originally isolated from a dog with otitis externa, *M. furfur* CBS 1878 and *M. sympodialis* CBS 7222 were purchased from the Fungal Biodiversity Center (Westerdijk Institute, Utrecht, The Netherlands). These strains and the isolate of *M. furfur* with atypical assimilation of Tween 80 (4DS) (from now on referred to as atypical *M. furfur*) [13] were recovered at 33 °C using modified Dixon agar (mDixon agar; 36 g L^{-1} mycosel agar [BD, Franklin Lakes, NJ, USA], 20 g L^{-1} Ox bile, 36 g L^{-1} malt extract [Oxoid, Basingstoke, UK], 2 mL L^{-1} glycerol, 2 mL L^{-1} oleic acid, and 10 mL L^{-1} Tween 40) [4]. Physiological characterization was done on cells grown on minimal medium (MM; containing per liter: 10 mL K-buffer pH 7.0 [200 g L^{-1} K2HP04 and 145 g L^{-1} KH2PO4], 20 mL 30 g L^{-1} MgSO4.7H20, 15 g L^{-1} NaCl, 1 mL 1% CaCl2.2H2O [w/v], 10 mL 20% glucose [w/v], 10 mL 0.01% FeSO4 [w/v], 5 mL spore elements [100 mg L^{-1} ZnSO4.7H2O, 100 mg L^{-1} CuSO4.5H2O, 100 mg L^{-1} H3BO3, 100 mg L^{-1} MnSO4.H2O, l00 mg L^{-1} Na2MoO4.2H2O], and 2.5 mL 20% NH4NO3 [w/v]) [14], containing 1.5% bacteriological agar (Oxoid).

Physiological characterization of lipid assimilation

Strains were grown on mDixon agar at 33 ºC for 7 days, after which 2 loops of cells from colonies were suspended in 3.0 mL sterile water. To determine growth on Tween and fatty acids the cell suspension was added to 18 mL melted Sabouraud or MM agar (with a temperature of 50 ºC), and poured in a 9-cm Petri dish [4]. After solidifying, 10 holes were made with a 2-mm diameter punch. Holes were filled with 15 µl MM (control) or MM containing 4.2 mM Tween 20 (≥ 40% purity), Tween 40 (≥ 90% purity), Tween 60 (≥ 40-60% purity), Tween 80 (≥ 58% purity), palmitic acid (≥ 98% purity) (Merck, Darmstadt, Germany), oleic acid (≥ 78% purity) (Carlo Erba, Val de Reuil, France), mixtures of palmitic and oleic acid (4.2 mM of each), glycerol (≥ 99% of purity), or cremophor EL. The emulsion of palmitic acid was supplemented with non-metabolizable 1% Brij-58 [14]. Plates were incubated for 7-10 days at 33 ºC in a moist environment [4]. For growth curves, the 3 ml cell suspension in sterile water was added to 27 mL MM containing 4.2 mM palmitic acid (Merck) supplemented with 1% Brij-58 and / or

oleic acid (Carlo Erba). After 3 days of growth at 33 ºC at 180 rpm, 0.3 mL culture was used to inoculate 29.7 mL of fresh MM containing either 4.2 mM palmitic acid supplemented with 1% Brij-58, oleic acid, or mixtures of 4.2 mM palmitic (with 1% Brij-58) and 4,2 mM oleic acid. Growth was monitored for 7 days by determining CFU by plating on mDixon plates.

Results and Discussion

The absence of genes encoding the fatty acid synthase complex indicates that *M. pachydermatis* is lipid dependent despite the fact that it was generally assumed to be lipid independent (Chapter 2A). *M. pachydermatis* grows on Sabouraud agar in contrast to all other *Malassezia* species. This characteristic was related to the presence of trace amounts of lipids like palmitic acid [9]. In this study, a lipid-assimilation assay of *M. pachydermatis*, *M. furfur*, atypical *M. furfur* and *M.sympodialis* was performed by growing on Sabouraud and MM plates in the absence or presence of lipidic components. Growth of *M. pachydermatis* on Sabouraud was not strongly, if at all, stimulated by the addition of 4.2 mM Tween 20, Tween 40, Tween 60, Tween 80, palmitic acid, oleic acid, glycerol, cremophor EL, or mixtures of 4.2 mM palmitic and 4.2 mM oleic acid. In fact, a halo was observed around the hole with palmitic acid (Figure 1A). This halo was also observed in the case MM agar was used, indicating that growth is inhibited by palmitic acid (Figure 1B). The residual growth of *M. pachydermatis* on MM plates is most likely due to the fact that these cells were directly derived from mDixon plates with lipids from this medium still associated to or stored in the yeast cells. Similar background growth was observed with the other 3 *Malassezia* species in the case of MM plates. In agreement, *M. pachydermatis, M. sympodialis,* and atypical *M. furfur* could not grow in liquid MM containing palmitic acid (C16:0) (Figure 1C,F,L). In fact, prolonged incubation of these strains resulted in a strong decrease of viability based on CFU counting. Apparently, palmitic acid is fungicidal for these strains under these conditions but not for *M. furfur* that remained viable (Figure 1I) or could even slightly grow on palmitic acid in the lipid assimilation assay (Figure 1G,H). This results are in agreement with previous reports [9].

The differences in the utilization of palmitic acid could be related with adaptation mechanisms to the host. While *M. furfur* especially colonizes human skin that contains a relatively high amount of palmitic acid in its sebum, *M. pachydermatis* is isolated from animals with sebum that mainly contains margaric (C17:0), stearic (C18:0), oleic (C18:1), and linoleic (C18:2) acids [11,15]. *M. pachydermatis* did not grow in MM containing oleic acid (Figure 1B,C) but no

decrease in viability was observed. Similar results were obtained for atypical *M. furfur* (Figure 1H,I,J,L). Apparently, oleic acid is fungistatic for these strains under these conditions. Notably, a combination of palmitic and oleic acid did support growth, albeit lowly, and survival of *M. pachydermatis* in liquid MM. Yet, some decrease of viability was observed upon prolonged incubation. Apparently, the fungicidal effect of palmitic acid was reduced but not abolished under these conditions (Figure 1C). In accordance, the lipid-assimilation assay still showed a halo with this mixture of fatty acids indicating inhibition of growth of *M. pachydermatis*. In contrast, the mixture of palmitic and oleic acid resulted in increased growth of atypical *M. furfur* in liquid MM (Figure 1L) and the lipid-assimilation assay (Figure 1J,K: Table 1) as compared to growth on either fatty acid alone.

It is not clear which mechanisms underlie the fungistatic and fungicidal activities. It is tempting to speculate that individual fatty acids create an imbalance in the fatty acid compositions of lipids and affect the integrity of the membranes and / or membrane-protein interactions, thereby compromising essential membrane functions. Indeed, palmitic acid affected cell integrity since microscopic observations revealed strong reduction of intact cells (data not shown). The fact that the fungistatic fatty acid oleic acid is antagonistic to the fungicidal activity of palmitic acid suggests that oleic acid blocks a process that

Table 1. Lipid assimilation assay for *Malassezia* species in Sabouraud (A) and MM (B) agar. T: Tween; PA: Palmitic acid; OA: Oleic acid; G: Glycerol; Cr: Cremophor EL at a concentration of 4.2 mM. -, no growth; +/-, weak growth ; +, good growth ; ++, very good growth; *: Inhibition halo; n.d., not discernible

A

Strains	T20	T40	T60	T80	PA	OA	PA+OA	G	Cr
M. pachydermatis	n.d.	n.d	n.d.	n.d.	-*	n.d.	n.d.*	n.d.	n.d.
M. sympodialis	-	+/-	+/-	+/-	-	-	+/-	-	-
M. furfur	+/-	++	++	++	+/-	-	+/-	-	+/-
Atypical *M furfur*	-	-	+/-	++	-	-	++	-	+/-

B

Strains	T20	T40	T60	T80	PA	OA	PA+OA	G	Cr
M. pachydermatis	+	++	-*	+	-*	-	-*	-	+/-
M. sympodialis	-	-	-	-	-	-	-	-	-
M. furfur	-	++	++	++	+/-	-	++	-	+/-
Atypical *M furfur*	-	+/-	+/-	++	-	-	++	-	+/-

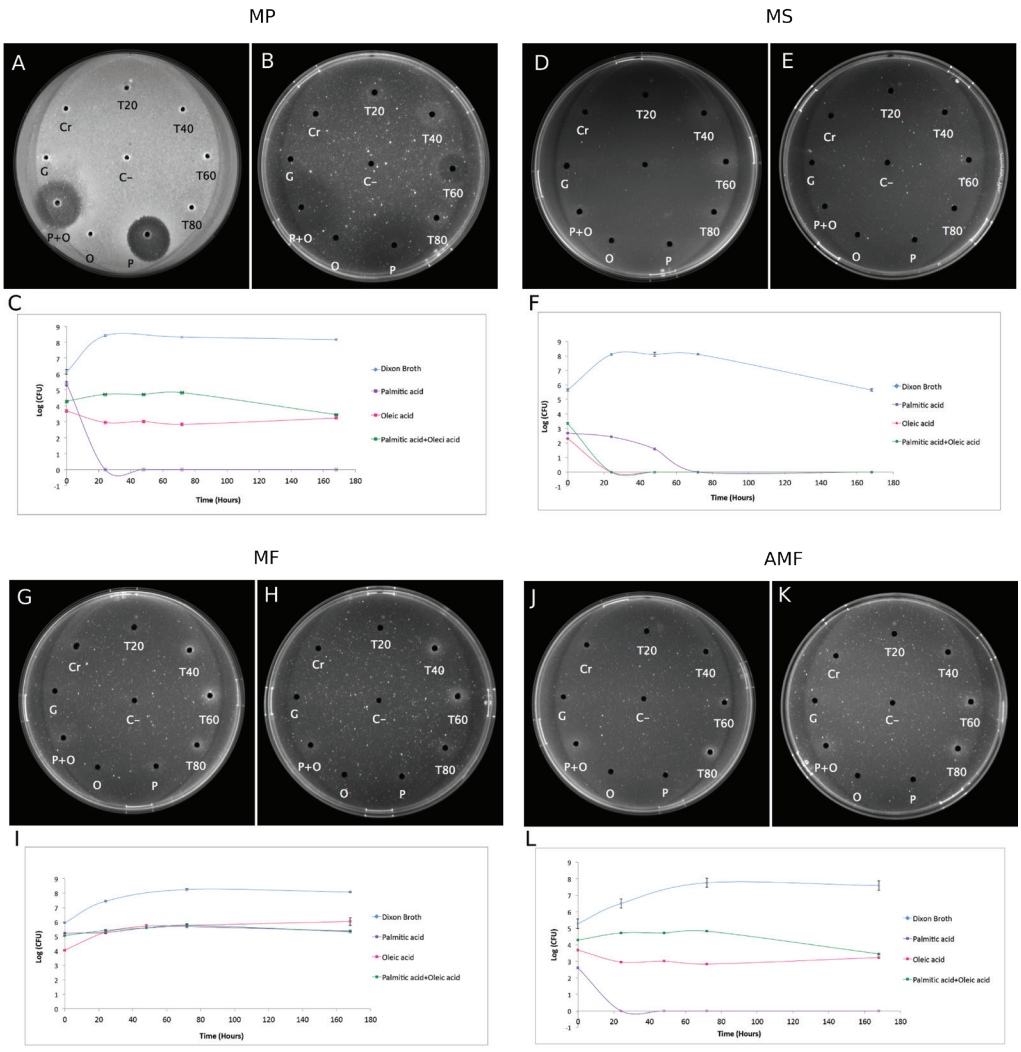

Figure 1. Lipid assimilation assay for *Malassezia* species in Sabouraud (A, D, G, J) and MM agar (B, E, H, K) and growth curves in MM (C, F, I, L). T20: Tween 20; T40: Tween 40; T60: Tween 60; T80: Tween 80; P: Palmitic acid C:16; O: Oleic acid C:18; P+O: Palmitic acid+Oleic acid; G: Glycerol; Cr: Cremophor EL. MP, *M. pachydermatis*, MS, *M. sympodialis*, MF, *M. furfur*, AMF, atypical *M. furfur*

leads to cell death and lysis. More research is required to elucidate the mechanisms how fatty acids interfere or stimulate fungal growth, which is especially relevant considering the variation in lipid sources in the natural environment. Variations in these compositions could favor growth of specific *Malassezia* species.

Cremophor EL showed slight stimulation of growth of *M. pachydermatis*, atypical *M. furfur,* and *M. furfur* (Figure 1B,G,H,I,J and Table 1). The lack of a mycostatic effect of Cremophor EL, as compared to oleic acid, might be due to the fact that oleic fatty acids in Cremophor EL are only slowly released from its backbone by lipases secreted by these fungal species. We also observed growth of *M. pachydermatis* in presence of Tween 40, a donor of palmitic acid (Figure 1A, B) and to a lesser extend of atypical *M. furfur* (Figure 1K). Possibly, palmitic acid is slowly released from Tween 40 thereby alleviating the fungicidal effect. Alternatively or in addition, growth on Tween 40 might be due to the presence of small amounts of other fatty acids since the purity of Tween 40 is around 90%. It is not clear why growth of *M. pachydermatis* in presence of Tween 60 is inhibited on MM (Figure 1B) but not on Sabouraud plates. This might be due to the presence of other fatty acids in the peptone containing Sabouraud [9] (Figure 1A).

The growth profile of *M. sympodialis* deviated from the other 3 strains showing it has different lipid requirements. *M. sympodialis* could not grow on any of the lipid compounds in MM (Figure 1E, F and Table 1), whereas slight growth was observed on Sabouraud with either Tween 40, 60, 80, or a mixture of palmitic acid and oleic acid. Notably, this strain was regarded to be less demanding as compared to the other species based on positive lipid-assimilation tests with Tweens [4]. The difference between these and our results might be due to the amount of supplemented lipidic components which were pure reagents and adjusted to 4.2 mM, respectively.

Secreted lipolityc enzymes play an important role in growth in the in vitro and natural situation since fatty acids need to be released from complex lipidic structures such as triglycerides, lipids, Tweens and Cremophor. Wu et al. (2015) hypothesized that expansion of lipases and phospholipases gene families are related with *Malassezia* niche-specificity. *M. pachydermatis* have 5 and 13 genes encoding phosphoesterases and secretory lipases respectively, while *M. furfur* has only 1 and 8 of these genes, respectively [9]. Although *M. furfur* has less genes encoding lipolytic enzymes it could growth efficiently with different fatty acid supplementation. This indicates that differences in lipid metabolism are not (only) related with these gene numbers or their encoding enzyme activities. Therefore, other mechanisms related to lipid synthesis pathways should be explored, as well as uptake and metabolic conversion of fatty acids by the different *Malassezia* species.

Conclusions

Understanding lipid dependency of *Malassezia* will help to understand how these yeasts establish themselves as part of the skin microbiota, which adaptation mechanisms are involved, and how, and whether, lipid metabolism impacts the shift to pathogenicity. The complex nutritional requirements of *Malassezia* have delayed the full comprehension of its lipid metabolism. This was further hampered by the presence of fatty acids in Sabouraud medium and the need for pure lipids [2]. Moreover, presence of residual lipids associated to cells or stored in cells previously grown in a complex medium such as Dixon broth, is probably a complicating factor. Monitoring growth or viability in liquid MM after inoculation from a MM-based inoculum is possibly a better method to reduce the presence of residual lipids in medium and cells. In addition, it allows metabolic analysis of labeled fatty acid species in combination with lipidomics.

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Chapter 3

Metabolic reconstruction and characterization of the lipid metabolism of *Malassezia spp.*

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Abstract

Malassezia species are lipophilic and lipid-dependent yeasts belonging to the human and animal microbiota. Typically, they are isolated from regions rich in sebaceous glands. They have been associated with dermatological diseases such as seborrheic dermatitis, tinea versicolor, atopic dermatitis, and folliculitis. The genomes of *Malassezia globosa, Malassezia sympodialis,* and *Malassezia pachydermatis* lack the genes related to fatty acid synthesis. Here, the lipid-synthesis pathways of these species, as well as *Malassezia furfur,* and an atypical *M. furfur* variant were reconstructed using genome data and Constraints Based Reconstruction and Analysis. To this end, the genomes of *M. furfur* CBS 1878 and the atypical *M. furfur* 4DS were sequenced and annotated. The resulting enzyme commission numbers and predicted reactions were similar to the other *Malassezia* strains despite the differences in their genome size. Proteomic profiling was utilized to integrate protein profiling with flux distributions. Flux differences were observed in the production of steroids in *M. furfur* and in the metabolism of butanoate in *M. pachydermatis.* The predictions obtained via these metabolic reconstructions also suggested defects in the assimilation of palmitic acid in *M. globosa, M. sympodialis, M. pachydermatis,* and the atypical variant of *M. furfur*, but not in *M. furfur.* These predictions were validated via physiological characterization, showing the predictive power of metabolic network reconstructions to provide new clues about the metabolic versatility of *Malassezia*.

Introduction

Malassezia species are lipophilic and lipid-dependent yeasts that are frequently encountered in the human and animal microbiota. Usually, they are isolated from regions rich in sebaceous glands. They have been associated with dermatological diseases such as seborrheic dermatitis, tinea versicolor, atopic dermatitis, and folliculitis [1]. The increasing number of *Malassezia* isolations from systemic infections shows that members of this genus are emerging opportunistic agents [2,3].

Lipid dependency of *Malassezia* species has been confirmed through the genome sequences of 14 species within the genus [4–7]. The genomes lack the cytosolic fatty acid synthase (FAS) gene, thus explaining why they cannot synthesize the fatty acid palmitate *de novo*. On the other hand, lipase, phospholipase, and sphingomyelinase genes that are involved in the release of fatty acids from the host are present, enabling lipid synthesis in *Malassezia* species [6]. Genes homologous to FAS genes have also been identified, but they have been predicted to have different functions [5,6].

The *Malassezia* genus shows different lipid-assimilation phenotypes as well as differences in the number of lipids that are required in the growth medium. *Malassezia furfur* usually assimilates different kinds of Tween including Tween 20, 40, 60, and 80. However, atypical strains have been identified, including strains that can only assimilate Tween 80 [8], and strains that are reported to be lipid-independent [9], although in the latter case, growth was only tested in Sabouraud glucose agar medium, which still contains lipids [7]. *Malassezia pachydermatis* was also believed to be lipid-independent [10]. However, recent findings show that this species is actually a more versatile lipid-dependent yeast also lacking a FAS gene [5,7]. The *Malassezia* assimilation assay is widely used to determine the lipid requirement of strains [1]. For instance, growth on Tween is indicative of a role being played by external lipase(s) that release the fatty acid tail from the non-ionic detergent. Analysis of the genomes of *Malassezia* spp. revealed a collection of genes encoding lipases, phospholipases, and sphingomyelinases [4–7] that are most likely involved in the release of fatty acids from a variety of lipid compounds, for example, such as those produced by the sebaceous glands in the skin of the host. Uptake of these fatty acids and their subsequent use in various lipid-biosynthesis routes is required to sustain growth of *Malassezia* species.

Genomic data and Constraints Based Reconstruction and Analysis (COBRA)-based models [11] can be used to reconstruct the cellular metabolism of an organism in a mathematical model. Such networks can be used to predict a cell's behavior under different conditions or disorders [12]. Flux balance analysis (FBA) models are constraint-based approaches suitable for studying the range of

possible phenotypes of a metabolic system [11]. These approaches have been used to study pathogens such as *Mycobacterium tuberculosis*, *Plasmodium falciparum,* and *Candida glabrata* [13–16].

The aim of this work was to investigate the genomic and metabolic differences between reference strains of 4 *Malassezia* species and an atypical *M. furfur* strain. To this end, we used metabolic modeling, integrating genomics and proteomics data. The results show flux differences in the production of steroid in *M. furfur* and the metabolism of butanoate in *M. pachydermatis*. In addition, defects in the assimilation of palmitic acid in *M. globosa, M. sympodialis, M. pachydermatis,* and the atypical variant of *M. furfur* (4DS), were suggested, but not for *M. furfur* CBS 1878. These predictions were validated via culturing on defined media.

Materials and Methods

Chemicals

All chemicals were obtained from Sigma (St Louis, MO, USA) unless otherwise indicated.

Strains and growth condition

The reference *Malassezia* strains *M. globosa* CBS 7986, *M. sympodialis* CBS 7222, *M. pachydermatis* CBS 1879, and *M. furfur* CBS 1878 were purchased from the Fungal Biodiversity Center (Westerdijk institute, Utrecht, The Netherlands), and used in this study. In addition, a previously reported isolate of *M. furfur* with atypical assimilation of Tween 80 (4DS) (from now on referred to as atypical *M. furfur*) [8] was used. Strains were recovered in modified Dixon agar (mDixon): 36 g L^{-1} mycosel agar (BD, USA), 20 g L^{-1} Oxbile (Sigma Aldrich, USA), 36 g L^{-1} malt extract (Oxoid, UK), 2 mL L^{-1} glycerol (Sigma Aldrich, USA), 2 mL L^{-1} oleic acid (Sigma Aldrich, USA), and 10 mL L^{-1} Tween 40 (Sigma Aldrich, USA) for 4 to 5 days at 33 °C [1]. The genomes of *M. globosa* [6], *M. pachydermatis* [5], and *M. sympodialis* [4] have been sequenced and are available in GenBank under the accession numbers GCA_000181695.1, GCA_001278385.1, and GCA_000349305.2.

DNA extraction, sequencing and genome assembly

Genomic DNA was extracted as described [8] and sequenced at the Beijing Genomics Institute (Shenzhen) using the Illumina HiSeq 2000 platform. Two runs of 100-bp paired-end reads and 200-bp insert-size libraries were undertaken following standard Illumina protocols. The quality of the reads was analyzed using FastQC software [17] and trimmed and filtered based on quality using Flexbar

[18]. *De novo* assembly was performed using the CLC Assembly Cell software [19] using default parameters. The resulting contigs were scaffolded using SSPACE_Basic script [20], discarding scaffolds < 1,000 bp. Gaps in the scaffolds were filled with the GapFiller script [21]. Assembly statistics such as N50, N75, L50, L75, and GC content, were computed with the QUAST software [22]. The average genome coverage was calculate by mapping the reads to the assembly with Bowtie [23] using default parameters and calculating the coverage per base using BEDTools [24]. This whole-genome shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession numbers MATP00000000 and LMYL00000000. The version described herein is LMYL01000000.

Assembly comparisons

Assemblies of the *M. furfur* strains were aligned with each other and with the genomes of *M. globosa* [6], *M. sympodialis* [4], and *M. pachydermatis* [5] using Nucmer (a maximum gap between 2 adjacent matches in a cluster of 90 bp and a minimum length of a maximal exact match of 20 bp) and Promer (a maximum gap between 2 adjacent matches in a cluster of 30 amino acids and a minimum length of a maximal exact match of 6 amino acids) that evaluates the 6-frame translation of the nucleotide sequence. Mummer [25] alignments were plotted and the genome coverage per nucleotide was calculated with the BEDTools suite Coverage tool [24]. The percentage of matches in each pairwise comparison was computed using custom python scripts.

Annotation

The assemblies of the *M. furfur* strains were annotated using the MAKER 2 pipeline [26]. To this end, a set of 109,264 previously reported Ustilaginomycotina proteins was used. In addition, genes predicted by CEGMA [27] and GeneMark [28], 1,413 ESTs from *Malassezia* spp., and results from the *ab initio* gene predictors SNAP [29] and Augustus [30] were used as genetic evidence for the annotation. MAKER was run 2 consecutive times. The first run included proteins, ESTs, and predicted genes to identify genes within the scaffolds. The first output obtained from MAKER was converted into a model for SNAP and a training set for Augustus. Subsequently, the *ab initio* results were provided as an input model for the second MAKER run. The statistics of the resulting annotation were calculated with genome tools [31].

Functional annotation of the predicted genes was performed using Blast2GO [32], which included BlastX [33] and an InterProScan annotation [34]. To determine the number of duplicated proteins in the *M. furfur* genomes, CD-Hit [35] was run with an identity threshold of 90%. Non-coding repeated sequences

within the genomes were analyzed using RepeatMasker (http://repeatmasker.org) by running them against the RepBase library.

To assess whether genes involved in the formation of free fatty acid precursors are absent in the *M. furfur* genomes, as is the case for *M. globosa*, *M. sympodialis,* and *M. pachydermatis*, 2,382 fungal and bacterial genes encoding FAS and 954 PKS genes were compared with the predicted genes and proteins of the genomes using Blastp and Blastn [33] (parameters by default). Further validation of missing InterProScan domain was manually done by comparing the missing domain sequence from the *Malassezia* species presenting the domain with the predicted protein of the other species.

Phylogenetic analysis

FastTree 2.1.9 [36] was used to reconstruct phylogeny of the available *Malassezia* species [4–7], To this end, genes in each genome assembly were predicted using Augustus 3.0.2 [30] with the parameters that were previously optimized for *M. furfur* CBS 1878. *Ustilago maydis* was used as an outgroup [37]. Highly conserved genes were identified using BUSCO 2.0 [38] using the gene set "fungi odb9." These sequences of each species were concatenated, aligned using MAFFT version 7.309 [39], and the well-aligned regions were extracted using Gblocks 0.91b [40]. This resulted in 62,988 amino acid positions that were used as input for FastTree 2.1.9.

Metabolic network reconstruction

The predicted proteins were compared to the KEGG database [41] using the KAAS server [42] and Blast2GO [32] to obtain corresponding EC numbers. These numbers were used to retrieve the associated reactions from KEGG and to map the corresponding metabolic pathways. The directionality of each reaction was determined using the literature, the MetaCyc database [43], and the Gibbs free energy obtained with the group contribution method [44]. The reaction nomenclature was converted to the Metanetx identifiers in order to have a more cohesive nomenclature in the network [45]. Furthermore, the metabolic core was determined as the reactions shared among the 5 strains.

Compartmentalization and curation

The predicted enzymes were analyzed with the subCELlular LOcalization predictor (CELLO) [46], a peptide localization predictor that uses support vector machines based on n-peptide compositions. The significant compartment was selected and added to the reactions of the enzyme. Transport reactions were added to the network according to the genome annotation and the literature review.

The metabolites with production and consumption problems were identified and missing data were imputed using an iterative approach with the GapFind and GapFill algorithms [21] implemented in the General Algebraic Modeling System (GAMS) [47] using the minimal media defined for iMM904 *Saccharomyces cerevisiae* [48] supplemented with oleic acid and glucose as the sole carbon source. In addition to a manual curation based on the literature and visualization in Cytoscape [49], an in-house exchange-reaction database and the complete metabolic reactions' repository for Metanetx [45] were used to detect unconnected metabolites, and to calculate the topological statistics of the network.

Flux balance analysis

A stoichiometric matrix (*S*) was obtained from the metabolic network using in-house Perl scripts to obtain a system of linear equations, $S*v = 0$, where v is the flux vector. System constraints included the lower and upper bounds of reaction fluxes. The system allowed 0.000 to 1,000 mmol gDW^{-1} h^{-1} for irreversible reactions and −1,000 to 1,000 mmol gDW^{-1} h^{-1} for reversible reactions. The system was solved to identify the theoretical limits for different fluxes in the metabolic system using GAMS (General Algebraic Modeling System, GAMS Development Corp., Washington, DC) software. The linear Programming presented here was developed with solver CPLEX 12.6.0.0 with an optimization of tolerance of 10^{-6}. A modified biomass production reaction (S1Table) of iMM904 *Saccharomyces* cerevisiae as an objective function [48] were fixed as parameters for the optimization process. Furthermore the same media used in the GapFill process, the minimal media defined for iMM904 *Saccharomyces cerevisiae* [48] supplemented with oleic acid and glucose (uptake rate of 10 mmol $gDW^{-1}h^{-1}$) in anaerobic conditions (oxygen uptake rate of 2 mmol $gDW^{-1}h^{-1}$) was used for the simulation. This optimization problem aimed to solve the maximization of the flux through the reaction of biomass used as objective functions. The resulting flux distributions were filtered with a cutoff of ± 0.05 mmol gDW^{-1} h^{-1} to allow a better visualization of the reactions that can carry fluxes and plotted as a heatmap in R [50].

Proteomic profiling

Protein extraction was carried out as described [51]. Strains were grown at 180 rpm and 33 °C on DB (20 g L^{-1} Oxbile), 36 g L^{-1} malt extract (Oxoid), 6 g L^{-1} Peptone (Oxoid), 2 mL L^{-1} glycerol, 2 mL L^{-1} oleic acid, 10 mL L^{-1} Tween 40, and 500 mg L^{-1} chloramphenicol. Aliquots of 5 mL were taken in the early exponential and early stationary phase and centrifuged at 26,000 *g* for 10 min. The resulting pellet was washed 3 times with PBS, after which it was resuspended in extraction

buffer (1:10 cell to extraction buffer v/v ratio; 125 mM ammonium bicarbonate, 20 mM ε-aminocaproic acid, 5 mM ethylenediaminetetra-acetic acid, and 1 mM phenylmethylsulfonyl fluoride [Thermo Scientific, St Peters, MO, USA]). Cells were disrupted by vortexing for 10 min with 4-mm silica beads and centrifuged at 26,000 *g* and 4 °C for 10 min. Proteins in the supernatant were precipitated with 20% TCA [52]. The amount of protein was quantified using a Nanodrop (Thermo Scientific) and visualized by SDS-PAGE electrophoresis. The protein extraction was carried out 5 times per sample.

The extracted proteins were sent to the proteomic center of the University of California at Davis (http://proteomics.ucdavis.edu/). Protein profiling for each sample was carried out using the mass spectrometer Michrom HPLC Paradigm type, the Q-mass spectrometer ionization Proxeon Exactive nano-spray, and the Easy-LC II HPLC.

The identification and annotation of the proteins was performed using Scaffold (Proteome Software Inc., Portland, OR) and proteins were compared against the Uniprot Ustilaginomycotina proteins and the *Malassezia* spp. predicted proteins. The mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE [53] partner repository with the dataset identifier PXD004523. The resulting proteins were clustered with CD-Hit to compare the proteins among the samples. These clusters were analyzed statistically with R using the normalized total spectra count [50]. To validate the network, proteins related to the reactions in the genomic model were selected (Excluding those metabolic and transport reactions added with GapFill that did not have an associated enzyme) and compared to the abundance of the proteins in each extract using at least duplicates with 95% identity.

Physiological characterization of lipid assimilation

To determine the growth on different Tween varieties and with fatty acids, the strains were first grown on mDixon at 33 ºC for 7 days. The fungal cells were suspended in 3 ml of water with 0.1% Tween 80 used to inoculate 27 mL of MM (containing per liter: 10 mL K-buffer pH 7.0 [200 g L^{-1} K2HP04, 145 g L^{-1} KH2P04, 20 mL M-N [30 g L^{-1} MgS04.7H20, 15 g L^{-1} NaCl, 1 mL 1% CaCl2.2H20 [w/v], 10 mL 20% glucose [w/v], 10 mL 0.01% FeS04 [w/v], 5 mL spore elements [100 mg L^{-1} ZnS04.7H20, 100 mg L^{-1} CuS04.5H20, 100 mg L^{-1} H3B03, 100 mg L^{-1} MnS04.H20, l00 mg L^{-1} Na2Mo04.2H20, and 2.5 mL 20% NH4N03 [w/v] [78]) containing 4 mM Tween (20, 40, 60 or 80), or 4 mM oleic acid, or palmitic acid (Merck, Darmstadt, Germany) supplemented with 1% Brij-58, an emulsifier that is not metabolized. It did not support the growth of the FAS mutant of the yeast *S. cerevisiae* [54] that was grown for 3 days at 33 ºC. Subsequently, 0.3

mL was used to inoculate 29.7 mL of fresh MM medium containing either 4 mM Tween 20, 40, 60, 80, oleic acid and / or palmitic acid in 1% Brij 58, with mDixon broth as the positive control. Growth was followed during 8 days in the medium containing Tween 40, palmitic acid, oleic acid, or mixtures of palmitic and oleic acid by determining the colony-forming units by plating on mDixon plates with subsequent incubation at 33 °C. In the medium containing Tweens and oleic acid, the optical density (OD) at 600 nm was measured and plating aliquots of the liquid cultures on mDixon plates at 33 °C was used to determine the viability of the cells after 8 days of growth. The fatty acids used for culturing were analyzed for composition via a gas chromatography–flame ionization detector (GC-FID); separation was reached using an RTX-Wax column (30 m × 0.25 mm × 0.5 um) of RESTEK®. FAMEs were identified by comparing their retention times with those identified with a Supelco® 37 Component FAME Mix standard. Quantification was intended as a relative concentration. Palmitic acid (Merck) contained 98% palmitic acid and 2% elaidic acid, an unsaturated acid. The oleic acid (Carlo Erba) contained 78% oleic acid and, in addition, we detected polyunsaturated fatty acids (10% of linoleic acid), unsaturated fatty acids (3% palmitoleic acid and 2% elaidic acid), and saturated fatty acids (6% of palmitic acid and 1% heptadecanoic acid).

Results

Genome assembly and pairwise comparisons

The draft genomes of *M. furfur* CBS 1878 and the atypical *M. furfur* 4DS were assembled from a shotgun Illumina HiSeq 2000-Paired data set using the CLC-assembler [19]. The assemblies yielded 2,084 scaffolds (N50 = 23 kb) and 3,577 scaffolds (N50 = 42 kb), respectively, corresponding to nuclear genomes of 14.19 Mbp and 10.38 Mbp. The summary of the genome assembly statistics calculated by QUAST [22] is shown in Table 1.

A pairwise Nucmer comparison at the nucleotide level showed that *M. furfur* and the atypical *M. furfur* had more repetitions and / or duplications in their genomes than *M. globosa, M. pachydermatis,* and *M. sympodialis* did (Figure S1 and Table 2), with 30.8% and 7% of the genomes of *M. furfur* and of the atypical *M. furfur* strains, respectively, representing multiple matches or repetitions. In contrast, less than 1% of the genomes of *M. globosa, M. pachydermatis,* and *M. sympodialis* corresponded to multiple matches.

The Nucmer alignment showed significant sequence divergence between the 4 species. The biggest difference was observed for *M. globosa,* which only showed 0.8% identity using exact matches of at least 20 bp when compared to *M. furfur*

or the atypical *M. furfur* genomes. This was < 1.2% when the *M. globosa* genome was compared to *M. pachydermatis* and *M. sympodialis*. As expected, the most similar strains were *M. furfur* and the atypical *M. furfur* strains with a similarity > 80% (Table 2). A higher degree of similarity was observed when evaluating the 6 translation frames using Promer (Figure S2 and Table 3). The biggest differences were found when *M. furfur* and *M. sympodialis* were compared, with 70.7% zero matches. The second highest difference was between *M. globosa* and *M. furfur,* with approximately 48% of zero matches. As expected, the highest similarity was observed in the case of the *M. furfur* strains with only 3.4% of zero matches.

To establish the taxonomic position of the 2 reported *M. furfur* sequences in this study with the already published *Malassezia* genomes, we built a phylogenetic tree using highly conserved genes (Figure 1). This phylogeny showed that atypical *M. furfur* clustered with *M. furfur* CBS 7982 and our sequence of *M. furfur* CBS 1878 clustered with the previously reported sequence of this strain.

Table 1. Assembly statistics calculated by QUAST [18] and BEDtools [19]. The genome assembly was performed using the CLC-assembler [17] and scaffolding was carried out using Sspace [20] and Gapfiller [21]

Assembly	*Malassezia furfur*	Atypical *Malassezia furfur*
Number of contigs (≥ 0 bp)	6968	17882
Number of contigs (≥1000 bp)	1249	877
Total length (≥ 0 bp)	15780944	14951138
Total length (≥ 1000 bp)	13644665	8614553
# Scaffolds	2084	3577
Largest Scaffold	110895	562614
Total length	14194927	10380899
GC (%)	63.95	63.01
N50	23366	42453
N75	10269	2449
L50	171	50
L75	400	295
Number of Ns in the assembly (Per 100 kbp)	41.75	127.69
Average coverage	605x	861x

Table 2. Percentage of the genome with zero (0), one (1), or multiple (> 1) matches in the Nucmer genome alignment [21] with a maximum gap between two adjacent matches in a cluster of 90 bp and a minimum length of a maximal exact match of 20 bp

Nucmer	*Atypical M. furfur*			*M. furfur*			*M. globosa*			*M. pachydermatis*			*M. sympodialis*		
	0	1	> 1	0	1	> 1	0	1	> 1	0	1	> 1	0	1	> 1
Atypical M. furfur	0.1 %	92.9 %	7.0 %	3.9 %	43.2 %	52.8 %	98.1 %	1.9 %	0.0 %	96.6 %	3.3 %	0.0 %	94.6 %	5.4 %	0.0 %
M. furfur	12.6 %	75.1 %	12.3 %	0.0 %	69.1 %	30.8 %	96.4 %	3.6 %	0.1 %	95.1 %	4.9 %	0.0 %	93.2 %	6.7 %	0.0 %
M. globosa	99.2 %	0.8 %	0.0 %	99.2 %	0.6 %	0.2 %	0.0 %	99.7 %	0.3 %	98.8 %	1.2 %	0.0 %	98.7 %	1.3 %	0.0 %
M. pachydermatis	97.5 %	2.5 %	0.0 %	97.5 %	1.6 %	1.0 %	98.7 %	1.3 %	0.0 %	0.0 %	99.8 %	0.2 %	96.6 %	3.4 %	0.0 %
M. sympodialis	95.6 %	4.4 %	0.0 %	94.8 %	3.1 %	2.1 %	98.4 %	1.5 %	0.0 %	96.4 %	3.6 %	0.0 %	0.2 %	98.9 %	0.9 %

Table 3. Percentage of the genome with zero (0), or one or more (1) matches in the Promer genome alignment [22] with a maximum gap between two adjacent matches in a cluster of 30 amino acids and a minimum length of a maximal exact match of six amino acids

PROMER	Atypical *M. furfur*		*M. furfur*		*M. globosa*		*M. pachydermatis*		*M. sympodialis*	
	0	1	0	1	0	1	0	1	0	1
Atypical *M. furfur*	0.10 %	99.90 %	3.40 %	96.60 %	49.60 %	50.40 %	44.50 %	55.50 %	44.50 %	55.50 %
M. furfur	11.80 %	88.20 %	0.00 %	100.00 %	76.70 %	23.30 %	73.10 %	26.90 %	70.70 %	29.30 %
M. globosa	49.50 %	50.50 %	48.60 %	51.40 %	0.00 %	100.00 %	38.50 %	61.50 %	39.60 %	60.40 %
M. pachydermatis	40.30 %	59.70 %	39.20 %	60.80 %	33.60 %	66.40 %	0.00 %	100.00 %	28.40 %	71.60 %
M. sympodialis	37.50 %	62.50 %	35.70 %	64.30 %	31.80 %	68.20 %	26.00 %	74.00 %	0.20 %	99.80 %

Assembly annotation

A total of 10,203 and 12,131 protein-encoding genes were predicted for *M. furfur* and the atypical *M. furfur* strain using multiple lines of evidence (*ab initio* predictors, expressed sequence tags [ESTs] from *M. globosa*, and protein alignments). Functional annotation showed that both *M. furfur* strains contained twice the number of proteins when compared to the other *Malassezia* species. Yet, the number of Enzyme Commission (EC) numbers and reactions were similar (Figure 2). The average genome length, exon length, and the number of exons per gene for the 5 genomes analyzed are presented in Figure 2.

The genomes of *M. furfur* and the atypical *M. furfur* contained 7,570 and 10,434 protein clusters, respectively, as predicted with CD-Hit [35]. The total number of non-coding repetitions found using RepeatMasker was approximately 1.61% and 1.32% for *M. furfur* and the atypical *M. furfur* strain, respectively. Most repetitions were found to be low-complexity repeats and long terminal repeat (LTR) elements.

To search for genes related to lipid biosynthesis, a total of 2,382 FASs (i.e. 179 fungal and 2,203 bacterial) were compared with the predicted proteins of the *M. furfur* strains using blastp [33]. No FAS genes were identified in the 2 genomes, but several polyketide synthase (PKS) genes (3 in *M. furfur* and 1 in atypical *M. furfur*) were found (Figure 3). The PKSs from the 5 species shared most of the domains, with the exception of *M. globosa,* which lacked the S-adenosyl-L-methionine-dependent methyltransferase domain, and *M. furfur,* which lacked the ketoreductase domain, acyl carrier protein-like domain, NAD binding domain, and the thioester reductase-like domain in its 3 predicted PKSs. Out of 954 fungal homologues, the PKS of the basidiomycete *Paxillus involutus* was the most similar to that of the atypical *M. furfur,* with an identity of 31%. The most similar PKSs to those of *Malassezia* were found in basidiomycetes. The closest

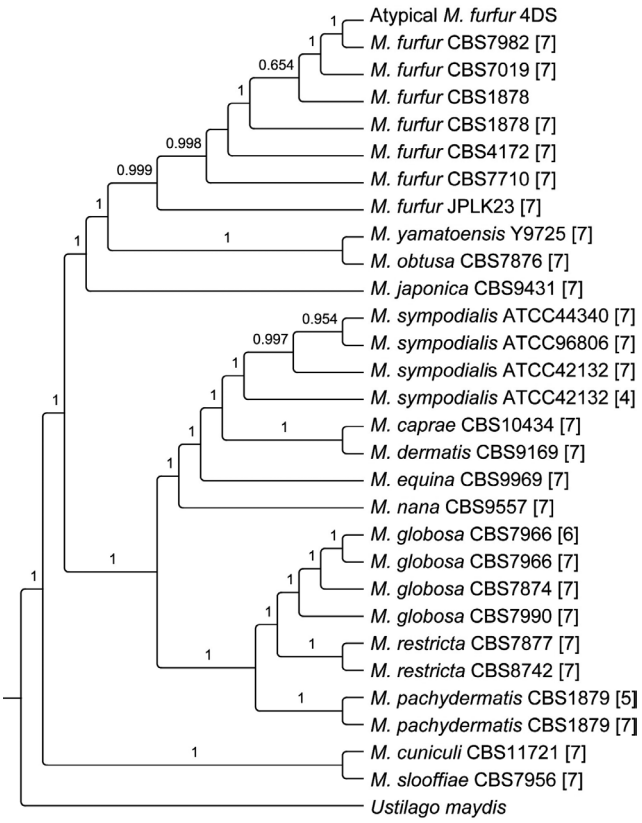
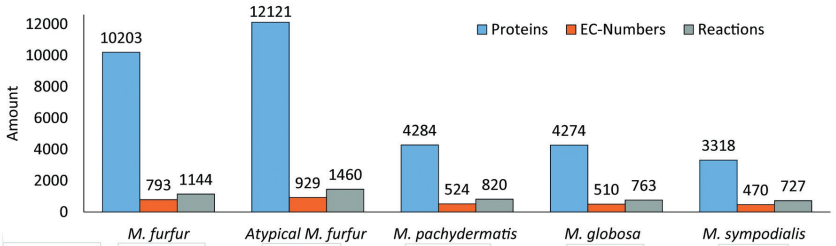


Figure 1. Phylogenetic analysis of *Malassezia* reconstructed from highly conserved genes identified with BUSCO 2.0 [36]



	M. furfur	Atypical *M. furfur*	*M. pachydermatis*	*M. globosa*	*M. sympodialis*
Genome size	14.19	10.38	8.16	8.93	7.67
Average gene length	1214.30	827.58	1575.78	1486.92	1593
Average exon length	602.02	483.99	674.66	974.94	772
Average exon per gene	1.93	1.64	2.25	1.49	1.9

Figure 2. Annotation data for the *Malessezia* genomes. The predicted proteins of the *M. furfur* strains were obtained from MAKER [23]. For all strains, ECs were obtained from KAAS (KEGG Automatic Annotation Server) [42] and Blast2GO [32], the reactions were retrieved from the KEGG database, and the annotation statistics were calculated with genome tools [31]

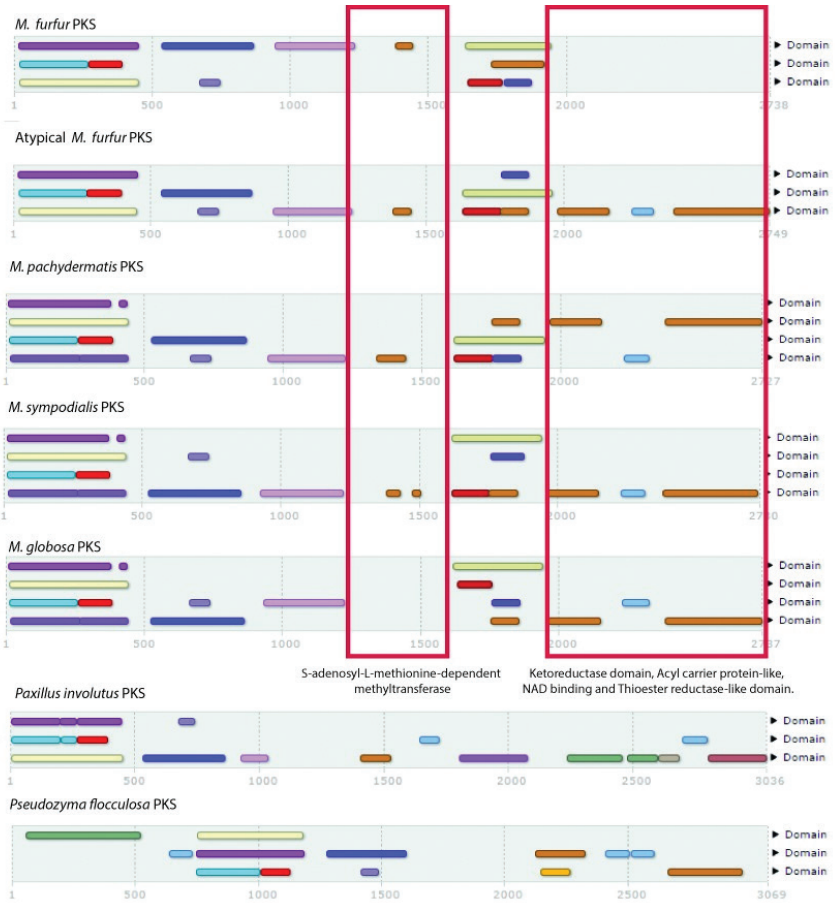


Figure 3. Domains obtained with Interproscan [30] of the 5 polyketide synthases (PKSs) of *Malassezia* species compared to *Paxillus involutus* and *Pseudozyma flocculosa*

homologue of the PKS of *M. furfur* was from *Hydnomerulius pinastri,* with an identity of 29.8%, while an *Auricularia delicata* homologue was most similar to the PKS of *M. pachydermatis* and *M. sympodialis,* showing 29.9% and 31% similarity, respectively. In the case of *M. globosa,* the most similar PKS was that of *Coniophora puteana,* with an identity of 26.8%.

The distribution of the genes in the genomes of the 5 *Malassezia* strains was similar when analyzing 13 categories of metabolic genes (Figure 4). The metabolic core (Figure S3) represented 628 reactions that were shared between the strains. These were mainly distributed in carbohydrate, amino acid, and lipid metabolism. When examining the lipid metabolism in more detail, arachidonic acid biosynthesis was only present in the atypical *M. furfur* strain. Differences in the fungal steroid biosynthesis were also found. Around 10 reactions involved in

this pathway were present in the *M. furfur* strains, which was higher than those in the other species with less than 6 reactions. Similarly, 36 reactions associated with fatty acid degradation were detected in the atypical *M. furfur* strain, a higher number than that found in the other species studied (23–31 reactions) (Figure 4).

Network construction and curation

The database search and the group contribution method used to calculate the Gibbs free energy predicted that 15–26% of the reactions of the 5 *Malassezia* strains were irreversible. Compartmentalization analyses showed a similar distribution of reactions among the species. However, differences were observed in the number of reactions when the *M. furfur* strains were compared to the other species. This corresponded to cytoplasmic and mitochondrial reactions (Figure 5). After the gap-filling approach to reduce the network pathologies, around 250 reactions were added to *M. globosa* and *M. sympodialis,* 300 to *M. pachydermatis,* 600 to *M. furfur,* and 660 to the atypical *M. furfur* strain. The final networks ended up with 2,162 metabolites in *M. globosa*, 2,303 in *M. sympodialis*, 1,838 in *M. pachydermatis*, 3,103 in *M. furfur*, and 3,642 in the atypical *M. furfur* strain.

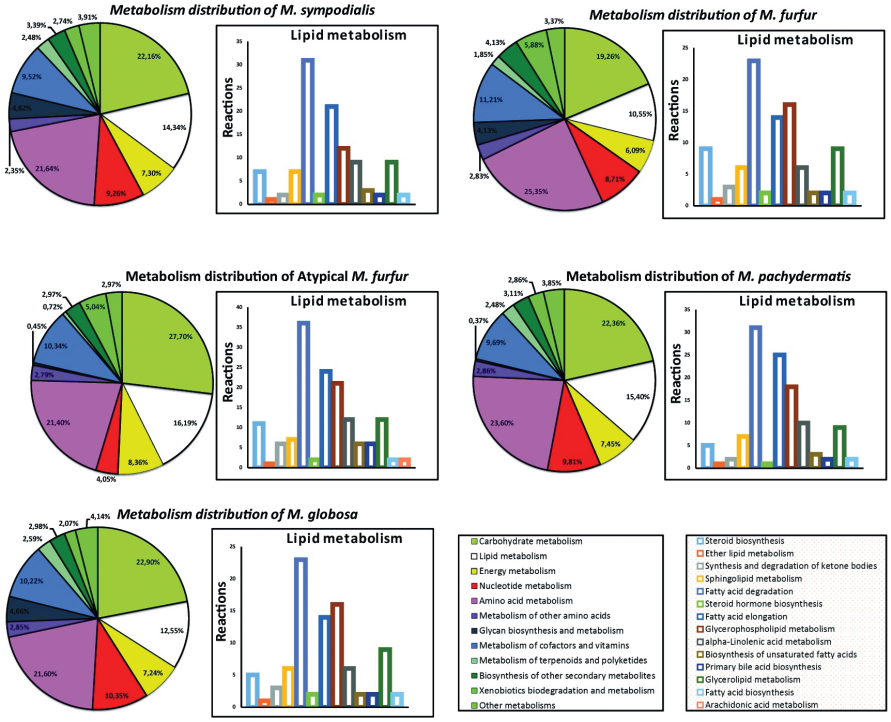


Figure 4. Metabolism distribution predicted from *Malessezia* species and the expanded distribution of lipid metabolism

The final metabolic networks (File S1) were visualized in Cytoscape using the compartment as a discrete mapping category to color the nodes. A topological analysis was performed (Figure 6). The clustering coefficients were around 0.14 and 0.18 and the diameter of the networks ranged between 10 and 15 nodes.

Flux balance analysis

FBA was carried out and the resulting predicted biomass fluxes that were used as objective functions are shown in Table 4. The highest biomass production was found in the atypical *M. furfur* strain at 2.84 mmol gDW^{-1} h^{-1} and the lowest in *M. sympodialis* at 0.089 mmol gDW^{-1} h^{-1}. The number of reactions that the flux carried varied among the species, ranging from 36% in the atypical *M. furfur* strain to 42% in the *M. sympodialis* strain.

The flux distribution of each network is represented in Figure 7 for visualization as a heatmap, the resulting clusters were found based on the flux carried by each reaction, with *M. globosa* and *M. sympodialis* having the most similar flux distribution. The main differences between *M. furfur* and the other strains were reactions involved in valine, leucine, and isoleucine degradation, and pyrimidine and purine metabolism. Differences in the lipid-metabolism reactions were also found. *M. furfur* displayed high fluxes in reactions related to fatty acid degradation and elongation, which involved the conversion of hydroxyacyl-CoA to trans-2-enoyl-CoA. These were higher in comparison to those observed in the atypical *M. furfur s*train. Several reactions associated with fungal steroid biosynthesis were found in the atypical *M. furfur* and *M. furfur* isolates. Despite the atypical *M. furfur* isolate having a larger number of these reactions, they had higher fluxes in the *M. furfur* strain. In the atypical *M. furfur* strain, higher fluxes were found in reactions involved in the degradation of maltose, fructose, and starch. *M. globosa* and *M. sympodialis* showed similar metabolic behavior. However, some differences in *M. sympodialis* corresponding to changes in the pyruvate and gluconeogenesis pathways, as well as the pathways involved in the degradation of long-chain fatty

Table 4. Predicted biomass flux with the flux balance analysis (FBA)

Strain	Biomass (mmol gDW^{-1} h^{-1})
M. furfur	1.280
Atypical *M. furfur*	2.840
M. pachydermatis	0.742
M. globosa	1.087
M. sympodialis	0.090

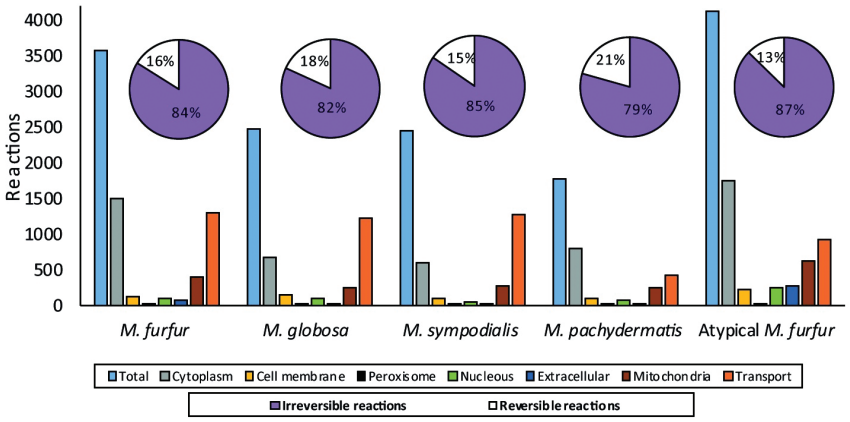


Figure 5. Distribution of *Malessezia* reactions after compartmentalization with the subCELlular LOcalization predictor (CELLO) [46] and the percentage of reversible and irreversible reactions determined from the Gibbs free energy of the reactions calculated with the group contribution approach and from the literature

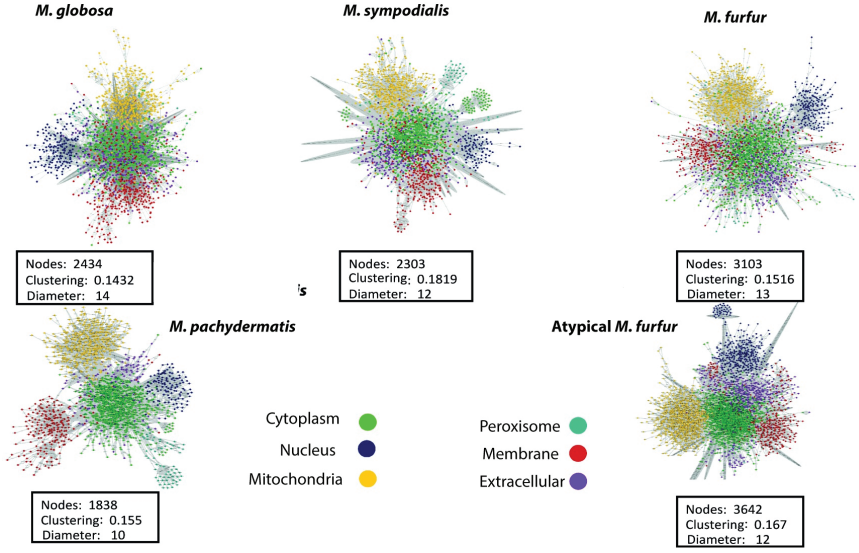
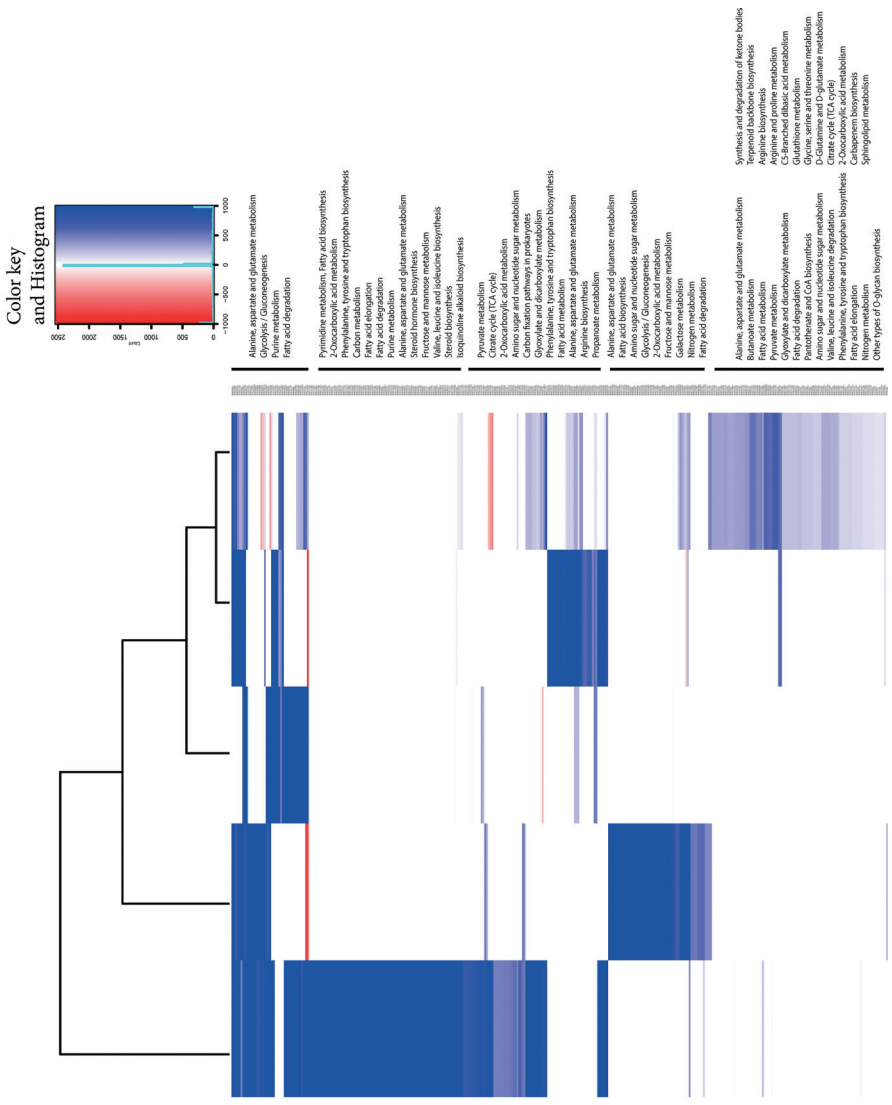


Figure 6. Compartmentalized metabolic network of *M. globosa* (A), *M. sympodialis* (B), *M. pachydermatis* (C), *M. furfur* (D), and the atypical *M. furfur* (E) visualized in Cytoscape [49]


Color key
and Histogram
2500
2000
1500
1000
500
0
−1000
−500
0
500
1000
Count
Alanine, aspartate and glutamate metabolism
Glycolysis / Gluconeogenesis
Purine metabolism
Fatty acid degradation
Pyrimidine metabolism; Fatty acid biosynthesis
2-Oxocarboxylic acid metabolism
Phenylalanine, tyrosine and tryptophan biosynthesis
Carbon metabolism
Fatty acid elongation
Purine metabolism
Fatty acid degradation
Alanine, aspartate and glutamate metabolism
Starch and sucrose metabolism
Fructose and mannose metabolism
Valine, leucine and isoleucine biosynthesis
Steroid biosynthesis
Isoquinoline alkaloid biosynthesis
Pyruvate metabolism
Citrate cycle (TCA cycle)
2-Oxocarboxylic acid metabolism
Amino sugar and nucleotide sugar metabolism
Carbon fixation in photosynthetic organisms
Glyoxylate and dicarboxylate metabolism
Phenylalanine, tyrosine and tryptophan biosynthesis
Fatty acid metabolism
Alanine, aspartate and glutamate metabolism
Arginine biosynthesis
Pentose phosphate pathway
Alanine, aspartate and glutamate metabolism
Fatty acid biosynthesis
Amino sugar and nucleotide sugar metabolism
Glycolysis / Gluconeogenesis
2-Oxocarboxylic acid metabolism
Fructose and mannose metabolism
Carbon metabolism
Nitrogen metabolism
Fatty acid degradation
Alanine, aspartate and glutamate metabolism
Butanoate metabolism
Fatty acid metabolism
Pyruvate metabolism
Glyoxylate and dicarboxylate metabolism
Fatty acid degradation
Pantothenate and CoA biosynthesis
Propanoate metabolism
Valine, leucine and isoleucine degradation
Phenylalanine, tyrosine and tryptophan biosynthesis
Fatty acid elongation
Nitrogen metabolism
Other types of O-glycan biosynthesis
Synthesis and degradation of ketone bodies
Terpenoid backbone biosynthesis
Arginine biosynthesis
Arginine and proline metabolism
C5-Branched dibasic acid metabolism
Glutathione metabolism
Glycine, serine and threonine metabolism
D-Glutamine and D-glutamate metabolism
Citrate cycle (TCA cycle)
2-Oxocarboxylic acid metabolism
Carbapenem biosynthesis
Sphingolipid metabolism

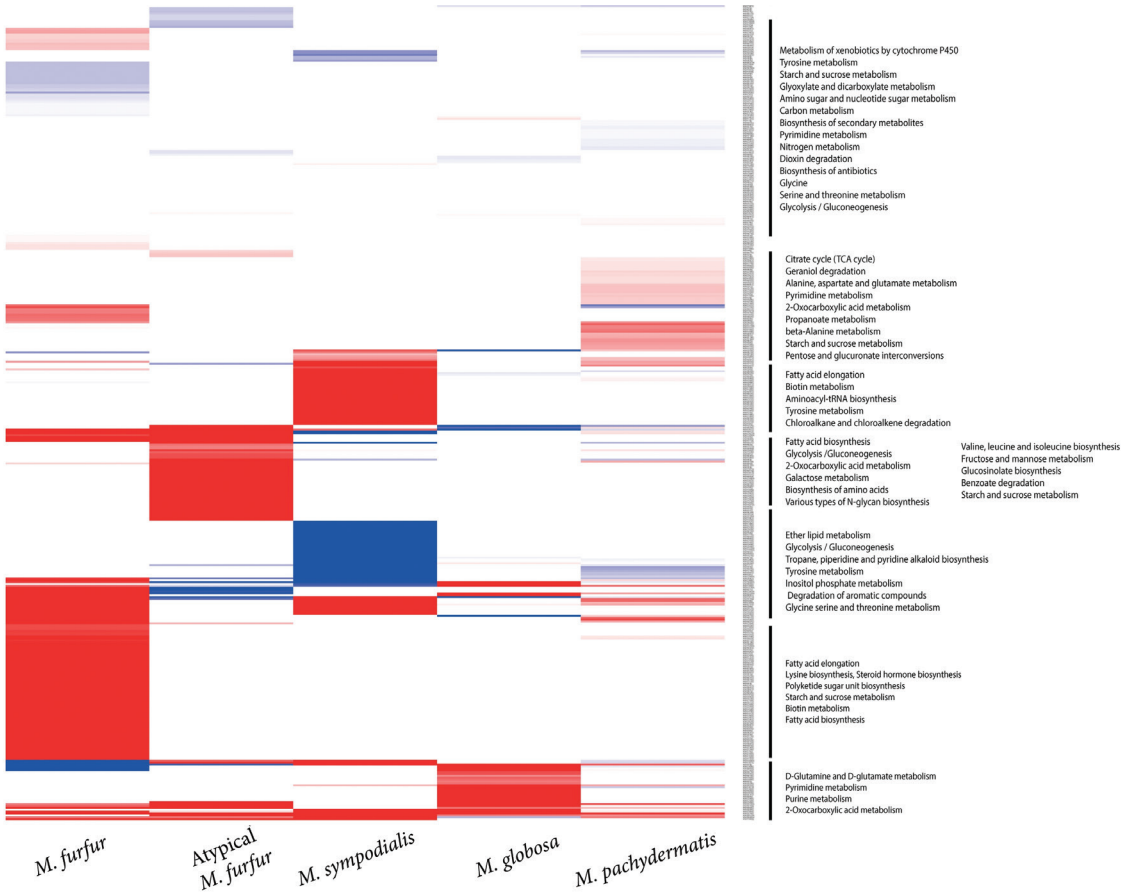


Figure 7. Flux distribution of *Malessezia* species resulting from the flux balance analysis of each metabolic network reaction filtered for visualization with a cutoff of ± 0.05 mmol gDW^{-1} h^{-1}

acids into acyl-CoA, and in the conversion of hydroxyacyl-CoA to trans-2-enoyl-CoA were found. Finally, in *M. pachydermatis,* there were differences in the core metabolism of pyruvate and butanoate, and in the biosynthesis of phenylalanine, tyrosine, and tryptophan.

As lipid assimilation differs among *Malassezia* species, and lipid metabolism may be related to the pathogenesis of these yeasts, differences in lipid assimilation among the species was further evaluated in the model. In reactions involved in fatty acid biosynthesis such as MNXR2003 involved in the conversion of hexadecanoic acid to Palmitoyl-CoA (ATP + Hexadecanoic acid + CoA <=> AMP + Palmitoyl-CoA + Diphosphate) higher fluxes were found in *M. furfur*, even though all the strains' genomes contain the genes that encode the enzymes that catalyzes these reactions.

Proteomic validation

An average number of 8,864 ± 2,400 *M. furfur* peptides was found during the exponential phase (T1), corresponding to an average of 1,865 ± 154 proteins. The lowest number of peptides (828 ± 456) was obtained with *M. globosa* in the stationary phase (T2), corresponding to an average of 116 ± 60 proteins. Consensus proteins were defined as proteins present in at least duplicates with a quantitative identity of ≥ 95%. The number of these proteins differed among the strains. *M. furfur* in the stationary phase had the highest number (1,539 consensus proteins), while *M. globosa* presented the lowest number in the exponential phase (201 consensus proteins). The number of reactions was higher in atypical *M. furfur* in the exponential phase, with 841 reactions, as compared to the other species (Table 5). The principal component analysis (PCA) showed that the proteome profiles behaved differently among the species, with the samples from atypical *M. furfur* and *M. globosa* having the most similar profiles. In addition, the clustering and PCA analyses also showed that the replicates behaved similarly within each species in both the stationary and exponential phases (Figure 8).

Protein validation of the models was conducted and expressed as the percentage of proteins predicted by the model that were detected by proteomic profiling (expressed proteins) as well as the percentage of expressed enzymes that were found to be catalyzing an reaction carrying flux in the model (Figure 9). The most successful model was that of *M. pachydermatis* based on the validation and taking into account the percentage of the total reactions that can be experimentally validated (83 % in *M. furfur*, 80% in *M. globosa*, 73% in *M. sympodialis*, 79 % in *M. pachydermatis* and 78% in Atypical *M. furfur*). In this case, ~ 40% and 30% of the predicted proteins were expressed in the exponential and stationary phase, respectively. The less accurate models were those of *M.*

Table 5. Proteomic profiling results of five strains of *Malassezia* during the exponential (T1) and stationary (T2) phase. A total of four replicates were examined for each strain

Sample	Peptides					Proteins					Consensus proteins	Enzymes	Reactions
	1*	2	3	4	Mean ± SD	1	2	3	4	Mean ± SD			
M. furfur (T1)	12017	8990	6236	8214	8864 ± 2400	2094	1761	1784	1821	1865 ± 154	1284	430	770
M. furfur (T2)	9690	9075	9857	5354	8494 ± 2120	2098	1929	2015	1521	1890 ± 255	1539	517	830
Atypical *M. furfur* (T1)	1893	3188	6240	7099	4605 ± 2466	864	730	1146	1103	960 ± 197	985	301	841
Atypical *M. furfur* (T2)	5055	3971	2349	5286	4165 ± 1339	930	757	582	949	804 ± 171	805	250	789
M. pachydermatis (T1)	7185	9093	7707	8509	8123 ± 845	1130	1314	1165	1265	1218 ± 85	1118	362	716
M. pachydermatis (T2)	858	5213	6156	5245	4368 ± 2380	333	844	1130	997	826 ± 348	763	256	568
M. sympodialis (T1)	2465	3359	4440	5625	3972 ±1366	952	1107	1163	1292	1128 ± 140	1023	337	578
M. sympodialis (T2)	2108	2475	4864	2182	2907 ±1314	816	918	1234	816	946 ± 197	774	268	535
M. globosa (T1)	3737	2414	2200	2586	2734 ± 686	252	247	241	224	241 ± 12	201	101	248
M. globosa (T2)	264	953	736	1360	828 ± 456	71	140	63	190	116 ± 60	216	100	244

*Replicate

globosa and of the atypical *M. furfur* with ~ 10% of the predicted proteins being found. Similarly, when evaluating the number of predicted enzymes that were expressed, *M. pachydermatis* showed the best prediction model with ~ 90% of its expressed enzymes predicted by the flux distribution in both the exponential and stationary phases. *M. globosa,* on the contrary, only showed ~ 50% of its expressed enzymes, as predicted by the model under both growth phases.

Physiological characterization and requirement for lipid compounds for growth

We performed a physiological characterization of *M. globosa*, *M. sympodialis*, *M. pachydermatis*, *M. furfur* CBS 1878 and atypical *M. furfur*. We used minimal medium (MM) instead of rich media such as Sabouraud or potato dextrose agar, since these media contain small amounts of lipids that can sustain growth of *M. pachydermatis* [7]. Culturing was performed in liquid MM media in the presence of oleic acid, palmitic acid, or Tween. Strains were first grown in mDixon containing a variety of lipid sources and Tween 80. To prevent subsequent growth in MM due to the presence of residual lipids either from mDixon or associated with cells, we performed a two-phase growth in MM. First, cells were diluted into MM containing oleic acid, palmitic acid, or 1 of the Tween sources. After 3 days, these cells were diluted again in fresh MM with oleic acid, palmitic acid, or Tween. The results are described in Table 6, and in Figures S4 and S5. *M. furfur* indeed can assimilate palmitic acid or oleic acid as well as all Tween variants (Table 6, Figures S4 and S5), including Tween 40, which represents the C16:0 fatty acid donor in this respect. The atypical *M. furfur* strain however was only able to assimilate Tween 80, Tween 20, and oleic acid. We observed that *M. pachydermatis, M. globosa,* and *M. sympodialis* were able to grow in the first step in MM, however, these strains were not able to grow in the second step in this defined medium supplemented with oleic acid, palmitic acid, or any of the Tween sources (Table 6, Figures S4 and S5). However, growth of these latter strains was sustained in the mDixon medium. Interestingly, whereas *M. furfur* was able to maintain growth in MM with palmitic acid, growth of the atypical *M. furfur* strain*, M. pachydermatis, M. globosa,* and *M. sympodialis* declined rapidly in the second growth step as determined by the colony-forming units (Figure S5). We determined whether *M. pachydermatis* and atypical *M. furfur* could sustain growth in MM with a mixture of palmitic acid and oleic acid (Figure S6). Both strains were able to maintain growth or survive in this mixture of saturated and unsaturated fatty acids.

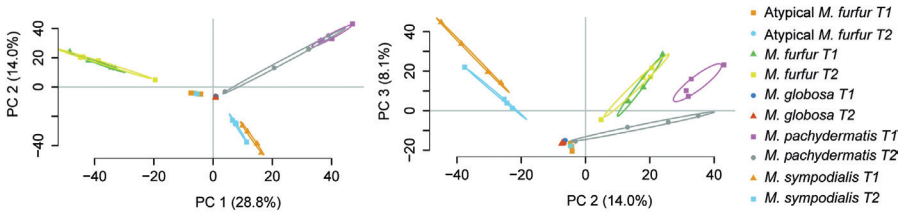


Figure 8. Principal component analysis (PCA) of the expressed protein cluster for 5 *Malessezia* strains at T1 (exponential phase) and T2 (stationary phase) with 4 replicates

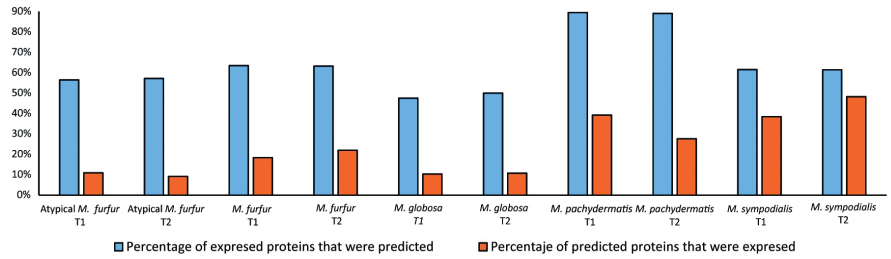


Figure 9. Protein validation as the percentage of *Malessezia* enzymes predicted by the genomic model

Table 6. Physiological characterization of *Malassezia* spp. by culturing in liquid minimal medium (MM) containing either Tw20 (Tween 20), Tw40 (Tween 40), Tw60 (Tween 60), Tw80 (Tween 80), OA (oleic acid), PA (palmitic acid), or DB (Dixon Broth) during the first (A) and second growth step (B). Growth was determined by measuring the absorbance of the culture at 600 nm after 168 h

A.

Strain	Tw20	Tw40	Tw60	Tw80	OA	PA	DB
M. furfur	++	++	+/-	++	+/-	++	++
Atypical *M. furfur*	++	+	+	++	+/-	-	++
M. pachydermatis	+/-	+	++	+	+/-	+	++
M. sympodialis	+/-	+/-	+/-	+/-	+/-	+	++
M. globosa	+/-	+/-	+/-	+/-	+/-	+	++

B.

Strain	Tw20	Tw40	Tw60	Tw80	OA	PA	DB
M. furfur	++	++	++	++	+	++	++
Atypical *M. furfur*	+	-	-	++	+	-	++
M. pachydermatis	-	-	-	-	-	-	++
M. sympodialis	-	-	-	-	-	-	++
M. globosa	-	-	-	-	-	-	++

-, no growth; +/-, weakly; +, good; ++, very good

Discussion

The lipid-dependent and lipophilic life style of *Malassezia* spp. seems to involve selection pressure to adapt to skin environments rich in lipid sources in human and animal hosts. Many factors can disturb the normal status of these yeast species and lead to disease. Approaches to understand how this disturbance occurs provide clues to manage the negative impacts on the host. Omics studies are necessary to fully comprehend these mechanisms [55]. In this study, the genomes of the previously reported *M. furfur* CBS 1878 [7] and an isolate of *M. furfur* with atypical assimilation of Tween 80 were sequenced. Furthermore, the metabolic networks of 5 *Malassezia* strains were reconstructed via genome annotation, reaction directionality, compartmentalization, and manual curation. This allowed to elucidate differences in the genomes, in the metabolism of fungal steroids, and other pathways among the 4 *Malassezia* species that were studied. Moreover, we carried out FBA on the metabolic reconstructions to measure the potential of each strain to produce biomass. We observed differences in the flux distribution among the species, with a variation in the fluxes of reactions related to lipid metabolism. Furthermore, proteomic profiling was used to validate the results. The validation showed that most of the proteins expressed in the proteomic profiling were predicted. However, not all the model's predictions were corroborated by this approach. Future high-throughput proteomic, metabolomic and modeling approaches are needed to validate the models.

The genome assemblies of *M. furfur* and the atypical *M. furfur* resulted in a genome size of 14.19 Mbp and 10.38 Mbp, respectively, approximately double the size of the genomes of *M. globosa* [6], *M. sympodialis* [4], and *M. pachydermatis* [5]). Differences in the genome size of *M. furfur* CBS 1878 (14.19 Mbp) in comparison to that of the same strain reported by Wu et al. in 2015 (13.5 Mbp) could be due to the assembly and scaffolding methods used in each study. The total length of the resulting contigs longer than 1000 bp in our assembly is around 13.6 Mbp (Table 1); a similar size to the previously reported size of 13.5 Mbp. Phylogenetic analysis conducted in this study established that our *M. furfur* strains are part of the *Malassezia* cluster A that has been previously described. This cluster consists of several strains of this species that were suggested to be a species complex [7]. Particularly, atypical *M. furfur* (10.38 Mbp) clustered with *M. furfur* 7982 (7.7 Mbp), thus showing them to be distantly related to other strains, and suggesting that they are implicated in divergence events [7]. More analysis is required to define the relation of the atypical strain and how it may be implicated in those events.

Additionally, the assembled genomes showed a greater number of scaffolds, indicating a possible fragmentation of the genome [56]. The fragmentation was

also evidenced by the reduction in the average gene size. The fragmentation present in the genome sequence assembly may cause a bias when comparing it to the other genomes and may further generate false-positive protein annotations [57,58]. However, the CD-Hit and EC results provided support for our protein dataset. The CD-Hit analysis showed that the number of protein clusters was close to the number of proteins that, although higher when compared to those of the other species, did not collapse after clustering. Also, the finding of new EC numbers in the genomes, as well as EC numbers previously reported in other *Malassezia* genomes, provided support for our annotation. Additionally, the metabolic reconstruction approach allowed us to reduce the fragmentation bias by determining the possible reactions of each protein according to the domains of each gene. The genome assembly could further be improved by resequencing these 2 genomes with a technique that generates longer reads such as PacBio [59].

Different mechanisms might explain the observed genome size differences. The options might include the presence of non-coding repetitions, possible protein duplications in the genomes, and / or the presence of new proteins. The first explanation could refer to mobile genetic elements such as transposons and retrotransposons as well as low-complex repetitions, as has been reported in other haploid fungi [60]. However, the analyses showed that less than 2% of the genomes presented these kinds of repetitions. To assess whether protein duplications or an increase in the number of unique proteins may explain the increase in the genome size of both *M. furfur* strains studied, we annotated the assemblies. The number of predicted proteins was higher in the genomes of *M. furfur* and the atypical *M. furfur* than in the genomes of *M. globosa, M. sympodialis*, and *M. pachydermatis*.

Differences in the number of predicted proteins could not solely be explained by protein duplications, as mentioned above. The number of protein clusters detected by CD-Hit correlates with the number of total proteins. Among the number of predicted proteins found in *M. furfur* and in the atypical *M. furfur* strain (10,203 and 12,121, respectively) 7,570 and 10,434 protein clusters, respectively, were detected. Thus, although there are indeed protein duplications, most of the predicted proteins represent unique proteins in the genomes of *M. furfur* and the atypical *M. furfur* strains. This is further supported in the case of enzymes by the fact that the unique EC numbers and reactions in both *M. furfur* strains were higher than those in the other species studied.

This diversification in the number of predicted proteins may provide evidence for the metabolic versatility found in *M. furfur*. The increase in unique proteins and the genetic diversification present in the *M. furfur* strains may be due to mating of the yeast, since bipolar mating systems have been previously proposed in *Malassezia spp.* [7], and sexual reproduction has been proposed to promote

genetic variation in other pathogenic fungi [61,62]. These special characteristics can be an advantage in terms of being able to easily adapt to different body sites, even under adverse conditions, such as blood in the case of a fungaemia, which this species has been associated with as well [63]. However, the analysis of metagenomic datasets from different sites on healthy human skin showed that *M. furfur* is less frequently detected than *M. globosa*, *M. restricta,* and *M. sympodialis* [7]. This pattern is suggestive of the metabolic profile of this species leading to a strong and intimate relation with the host due to its complex lipid requirements.

As expected, the FAS was not found in the genomes of either *M. furfur* or the atypical *M. furfur* strains. The PKSs were found to be conserved among the *Malassezia* species, with the exception of *M. globosa,* which lacked 1 domain [6], and the *M. furfur* isolate, which lacked several domains in its 3 predicted PKSs. *Mycobacterium tuberculosis* PKSs have been associated with the biosynthesis of unique lipids or glycolipid conjugates [64], and in *Streptomyces griseus,* with the synthesis of phenolic lipids [65]. Further studies are needed to determine the importance of these unique PKSs in *Malassezia* and their role in the lipid dependency of this genus. They are clearly different from other fungi with an identity of less than 32% with the phylogenetically related basidiomycete *P. involutus* (even with this was the most similar enzyme).

Even though there were differences in the number of predicted reactions among the species, the proportion in each pathway was similar. Carbohydrate and amino acid metabolism, which are part of the core metabolism of an organism, were the most abundant, as is the case for other fungi such as *Aspergillus oryzae* [66] and *Mortierella alpine* [67]. With respect to fungal steroid biosynthesis, we found that the atypical *M. furfur* strain had the highest number of reactions, followed by *M. furfur*. These reactions were almost negligible in the other 3 species studied. Differences in the fungal steroid biosynthesis between *M. furfur* and the other 3 *Malassezia* species studied may be explained by *i)* the production of steroid-like fungal hormones in *M. furfur* for sexual reproduction, as happens in ascomycetes [68]; *ii)* fungal steroids perhaps being a constitutive component of *M. furfur,* as is the case of the fruiting body of the basidiomycete *Sarcodon joedes* [69]; and most likely, *iii)* steroids in *M. furfur* perhaps acting as secondary metabolites, as previously reported in the basidiomycete *Polyporus ellisii* [70]. Finally, the differences in arachidonic acid lipid metabolism found in the atypical *M. furfur* strain may be related to the presence of precursors of eicosanoids that may act as an alternative lipid compound in this strain [71]. In addition, arachidonic acid may act as a mediator of skin inflammation—a previously reported role of *M. furfur* [72].

The topological features of the networks were used to assess their robustness. The node degree distribution (Figures S7 and S8) fits a power law regression and we showed that the 5 networks had free-scale topologies and non-random behavior [73]. The higher clustering and diameter of the *M. sympodialis* and the *M. furfur* CBS 1878 networks imply a more complex network. Yet, the clustering and diameter of these 2 networks were lower than for other yeast metabolic networks such as those of *S. cerevisiae* [48]. This may be due to the reduction in the number of connections among metabolites in each compartment when these are compartmentalized and subdivided as a representation of organelles.

The biomass fluxes found in our study were similar to those reported in other yeasts such as *S. cerevisiae* (0.7388 mmol gDW^{-1} h^{-1}) [74], but because of the lack of biomass data for *Malassezia* species further experimental validation of the growth rate in each species is necessary. The FBA allowed us to observe that even though the atypical *M. furfur* had a higher number of reactions for the steroid biosynthesis pathways, these had higher fluxes in the given condition in *M. furfur* strain CBS 1878. In addition, we found that *M. furfur* strain CBS 1878 displayed high fluxes in reactions related to the degradation and elongation of fatty acids such as the conversion of hydroxyacyl-CoA to trans-2-enoyl-CoA [75] than the atypical *M. furfur* strain did, and this may explain the differences in lipid assimilation observed between these 2 strains. Strikingly, the FBA analysis suggested an important difference in the use of palmitate. *M. furfur* had a high flux in the CoA activation of palmitate, whereas the other species lacked this activity.

FASs are required in most organisms to synthesize fatty acids such as the end product palmitate (C16:0). Notably, *Malassezia* spp. lack a FAS complex and are not able to produce palmitate [5,7]. This phenotype can be complemented with external lipids and Tween, which can act as donors for fatty acids, and after uptake, are directly used and / or elongated into long-chain- or very long-chain fatty acids, desaturated, or degraded via β-oxidation in the peroxisome. We observed that *M. furfur* is able to grow in MM supplemented with Tween 40 or with palmitate, indicating that palmitate is indeed used, and this is in accordance with the FBA predictions. The physiological analysis of the atypical *M. furfur* strain indicates that it cannot use palmitate since the second growth step in liquid medium supplemented with Tween 40 or with palmitate was not sustained. Similar results were obtained for *M. pachydermatis, M. globosa,* and *M. sympodialis* (Figures S4 and S5), which is in agreement with the FBA predictions. These findings could suggest that the transport and activation of palmitic acid in palmitic acid CoA is happening in *M. furfur* however could be hampered in other strains. More studies such Flux variability analysis (FVA) are required to

clarify this. However, previous *in silico* analysis of the fatty acid metabolism of *M. globosa* using integrated microbial genomes (IMGs) confirmed the presence of the complete β-oxidation pathway for the degradation of saturated fatty acids [76]. These differences could be related to differences in the fluxes in reactions related to this pathway in *M. furfur.* The observation that these latter 4 strains did grow in the first step in MM indicates that residual lipids from mDixon and / or associated with cells are sufficient to support the first step. These lipids are depleted in the second growth step in MM, which allows for a real analysis of lipid dependency. The observation that the atypical *M. furfur* cannot grow in MM with Tween 20, 40, and 60, the donors of C12:0, C16:0, and C18:0 fatty acids, respectively, suggests that these saturated fatty acids can not be further elongated and / or desaturated. All strains except *M. furfur* and the atypical variant were not able to grow in MM supplemented with a single Tween or with fatty acid species, whereas they did grow in mDixon. This observation might be explained if we assume that the strains require a mixture of saturated and unsaturated fatty acids, as was similarly observed with *fasl*, *olel* (FAS and fatty acid desaturase minus) mutants of *S. cerevisiae* [77]. Further analysis showed that the atypical *M. furfur* and *M. pachydermatis* indeed was capable to grow or survive in MM with a mixture of palmitic acid and oleic acid.

Studies have suggested that *M. globosa* and *M. restricta* are not capable of synthesizing unsaturated fatty acids due to the lack of a Δ9-desaturase (EC 1.14.19.2) gene [1,6], which is involved in the desaturation of palmitic acid and stearic acid to palmitoleic acid and oleic acid, respectively. However, Gioti et al. [4] found this desaturase gene in the genome of *M. sympodialis*. We also found the same gene in *M. furfur*, atypical *M. furfur,* and *M. pachydermatis*, suggesting the ability of these species to produce unsaturated fatty acids such as oleic acid, thus providing these species with an advantage in terms of their metabolic versatility to rapidly adapt to regions in which the availability of such fatty acid sources is limited. This ability is missing in *M. globosa* and *M. restricta,* which do require these unsaturated fatty acids as additional sources from their host to support their growth. These particular differences highlight the differences in the pathogenic role of these species in the development of certain dermatological diseases in which different species have epidemiological relevance, as is the case for *M. globosa* and dandruff/seborrheic dermatitis (SD) [1]. In this regard, more research is required to determine how these different *Malassezia* spp. use external fatty acids.

The lipid-metabolism reactions among the other species were also divergent. Higher activity in the reactions involved in the degradation of long-chain fatty acids was present in *M. sympodialis* but not in *M. globosa* or *M. pachydermatis.*

These differences may be associated with the phenotypic differences of each species, reflected by the differential lipid assimilation or the differential use of Tweens. We should, however, explore more deeply the physiology and regulation of β-oxidation in this yeast [1,78].

The higher number of reactions related to the butanoate metabolism found in *M. pachydermatis* may be related to the lipid-assimilation versatility of this species. This metabolism is closely related to the synthesis of type II and type III polyketides (which may be precursors of unique lipids) as well as to the fatty acid metabolism [79,80]. Thus, the higher activity of reactions involved in the metabolism of butanoate in *M. pachydermatis* may be reflected in the production of lipids that may not be produced by the other *Malassezia* species and that may be corroborated by lipid profiling of the yeasts.

The proteomic profiling allowed us to validate, on average, 30% of the predicted proteins of the model, implying that the other enzymes that are supposed to be expressed according to the FBA are not present or that their concentrations are substantially low. An average of 70% of the proteins expressed in all the samples were in fact predicted. Given that this technique is still not widely used to validate these kinds of models, the cutoff points and expected percentages are not well known. Furthermore, issues such as the identification of large-scale protein datasets, small protein concentration detection, and the extraction of low soluble proteins such as membrane proteins may affect the profiling [81,82].

Together, the metabolic reconstruction and modeling showed versatility within the genus of *Malassezia*. Flux differences were suggested in the production of steroids in *M. furfur* and in butanoate metabolism in *M. pachydermatis*. The assimilation defects of palmitic acid were suggested in *M. globosa, M. sympodialis, M. pachydermatis,* and in the atypical variant of *M. furfur*. The capability of *M. furfur* to assimilate palmitic acid was confirmed via culturing on defined media.

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Supplementary Information

File S1. The Supplementary Material for this article can be found online at: http://journal.frontiersin.ord/article/10.3389/fmicb.2017.01772/full#supplementary-material

Chapter 4

Identification and characterization of lipid droplets in *Malassezia furfur*

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Abstract

Malassezia furfur is a lipid-dependent yeast that is part of the human skin microbiota. Mechanisms underlying growth on skin including its lipid metabolism are largely unknown. Lipid droplets (LDs) have previously been shown to be present in *Malassezia*. In other organisms, these droplets store mainly neutral lipids such as triglycerides (TGs) and steryl esters (SEs) and are surrounded by a monolayer of phospholipids. Lipidomic analysis revealed the presence of phospholipids as well as TGs in LDs of *M. furfur* but SEs were not detected. TG species 52:4, 54:3, 54:4, and 54:5 were enriched in LDs after growth in the presence of Tween 80 or oleic acid (both donors of C18:1), while TG species 50:1 and 52:2 were enriched after growth in the presence of Tween 40 (donor of C16:0). Together, our results suggest that LDs of *M. furfur* relieve lipotoxicity caused by oleic acid. This would compensate for the inability of this yeast to degrade unsaturated fatty acids via β-oxidation, which is likely explained by the absence of a gene encoding Δ3,2-enoyl-CoA isomerase.

Introduction

Lipid droplets (LDs), also known as lipid particles, lipid bodies, oil bodies, or oleosomes, are highly dynamic organelles that can be produced in nearly all cells when exposed for example to fatty acids [1]. The presence of LDs in *Malassezia* (*Pityrosporum orbiculare)* have been reported [2], but their role has not been studied. LDs are surrounded by a protein containing phospholipid monolayer [3,4] and contain neutral lipids like triglycerides (TGs) and / or steryl esters (SE) depending amongst others on cell type and culture conditions. LDs contribute to a variety of processes including lipid storage, cell signaling, temporary protein storage, cell lipid homeostasis, prevention of lipotoxicity, biosynthesis and secretion of inflammatory mediators such as prostaglandins and leukotriene's, interferon responses, and antigen cross presentation [4–9]. Interest in LDs range from biofuel production by oleaginous yeasts to pathological conditions of humans with obesity and metabolic disorders.

The 14 lipophilic and lipid dependent *Malassezia* species can be part of the human or animal skin microbiota [10,11]. For instance, *Malassezia furfur* is an antropophilic species that have been implied a cause mild dermatological infections such as pityriasis versicolor up to severe bloodstream infections [12,13]. Lipid requirement necessitates *Malassezia* to exploit lipid sources from the host, which explains the high number of genes encoding secreted lipases and phospholipases [14]. LD formation may also be instrumental to cope with the dependence on external lipid sources. LDs could function as lipid reservoirs when lipids become limiting in the environment or could function as a detoxification mechanism when lipids are abundantly present. Here, the role of LDs in *Malassezia* was studied as well as the impact of environmental conditions on abundance and composition of these organelles. Results suggest that LDs of *M. furfur* relieve oleic acid toxicity.

Materials and Methods

Chemicals

Chemicals were purchased from Sigma (St Louis, MO, USA) unless otherwise indicated.

Strains and culture conditions

The reference *M. furfur* strain CBS1878 (Westerdijk institute, Utrecht, The Netherlands) was grown at 33 °C using modified Dixon agar (mDixon agar; 36 g L^{-1} mycosel agar [BD, Franklin Lakes, NJ, USA], 20 g L^{-1} Ox bile, 36 g L^{-1} malt extract [Oxoid, Basingstoke, UK], 2 mL L^{-1} glycerol, 2 mL L^{-1} oleic acid, and 10 mL L^{-1} Tween 40) [15] unless stated otherwise.

Growth and viability of cells in the presence of lipids

Two loops of cells from 7-day-old mDixon agar grown colonies were suspended in 5 mL sterile water, of which 3 ml was added to 27 mL mDixon broth (36 g L^{-1} malt extract [Oxoid], 10 g L^{-1} peptone [BD], 20 g L^{-1} Ox bile, 2 mL L^{-1} glycerol, 2 mL L^{-1} oleic acid, and 10 mL L^{-1} Tween 40) or 27 mL minimal medium (MM; containing per liter 10 mL K-buffer pH 7.0 [200 g L^{-1} K2HP04 and 145 g L^{-1} KH2PO4], 20 mL 30 g L^{-1} MgSO4.7H20, 15 g L^{-1} NaCl, 1 mL 1% CaCl2.2H2O [w/v], 10 mL 20% glucose [w/v], 10 mL 0.01% FeSO4 [w/v], 2.5 mL 20% NH4NO3 [w/v]), and 5 mL spore elements [100 mg L^{-1} ZnSO4.7H2O, 100 mg L^{-1} CuSO4.5H2O, 100 mg L^{-1} H3BO3, 100 mg L^{-1} MnSO4.H2O, l00 mg L^{-1} Na2MoO4.2H2O] containing 4.2 mM oleic acid (Carlo Erba, Val-de-Reuil, France) and / or 4.2 mM palmitic acid (Merck, Darmstadt, Germany) supplemented with 1% Brij-58. After 3 days of growth at 180 rpm, 0.3 mL culture was used to inoculate 29.7 mL of fresh mDixon broth or MM containing 4.2 mM oleic acid and / or 4.2 mM palmitic acid supplemented with 1% Brij-58. In addition, MM (i.e. in the absence of fatty acids) was inoculated with 0.3 ml pre-culture that had been washed 10 times with MM with intermediate 5-min centrifugation steps at 2,432 g. Viability and number of cells in the culture was monitored for 7 days by colony forming units (CFUs) formed on mDixon plates. All culturing was performed at 33°C. Assays were performed using biological triplicates

Detection of lipid droplets

Aliquots of cultures were washed 3 times with PBS with intermediate centrifugation at 5,000 g for 5 min. Cells were fixed with 4 % formaldehyde for 2 h at room temperature (RT) and washed 3 times with PBS (8 g L^{-1} NaCl, 2.56 g L^{-1} Na2HPO4.7H2O, 0.2 g L^{-1} KCL, 0.2 g L^{-1} KH2PO4, pH 7.4) with intermediate 5 min centrifugation steps at 1,248 g. Cells were resuspended in 1 mL 4 % buffered paraformaldehyde (136 g L^{-1} K2HP04, 6 M NaOH) containing 1 ng Nile red. Cells were incubated for 20 min at RT at 52 rpm using a rotary table and washed twice with PBS with intermediate centrifugation at 1,248 g for 1 min. Images were taken using an Olympus FV1000 laser scanning confocal microscope with a 60X / 1.42 NA oil immersion objective, a 488 nm laser for excitation, and a 500-600 nm spectral detector. LD size and numbers were determined using the Fiji image processing package of ImageJ (www.fiji.sc). Normality and homoscedasticity of data was evaluated with R using the Shapiro-Wilk test and Bartlett's test, respectively [16], after which data was analyzed by 2-factor ANOVA.

Lipidomic analysis

M. furfur was grown in 30 mL mDixon broth in which oleic acid and Tween 40 were replaced for either 10 mL L^{-1} Tween 40, 10 mL L^{-1} Tween 80, or 2 mL L^{-1} oleic acid. After 4 days of growth cells were washed 3 times with PBS with intermediate centrifugation at 1,248 g for 10 min. Cells were resuspended in 10 mL PBS and disrupted with a sonicator (Sonic Vibra Cell, Newtown, CT, USA) at 40 % amplitude during 10 times 1 min with intermediate cooling on ice for 30 sec. The disrupted cells were centrifuged at 4 ºC at 5,000 g for 10 min and the pellet was washed with deionized water and centrifuged using the same conditions. The pellet was resuspended in 10 ml 85 % sucrose and centrifuged at 8,000 g for 45 min at 4 ºC. The floating layer that was enriched in LDs was collected from the top of the gradient and resuspended in 10 ml 85 % sucrose. LDs were further purified by 2 additional gradient centrifugation steps at 4 ºC at 8,000 g for 45 min. LD fractions were washed 50 times with ultrapure, deionized water (Milli-Q, Millipore) with intermediate centrifugation at 4 ºC and 5,000 g for 5 min to remove contaminants like associated cytoplasmic membrane vesicles. The pellet was lyophilized and used for lipidomic analysis [17]. To this end, lyophilized LD preparations were extracted with chloroform / methanol according to Bligh and Dyer [18]. The apolar chloroform phase containing lipids were collected and dried *in vacuo*. Lipids were analyzed by the West Coast Metabolomic Center (University of California at Davis) using an Agilent 1290 Infinity LC system. MassHunter software was used for data processing and lipids were identified based on MS/MS fragmentation patterns using in-house software (Lipidblast). Lipid peak heights were normalized and expressed as relative concentration. Lipidomics analyses resulting from quintuple biological replicates were plotted in R Using the ggplot library [16].

Genomic analysis

Malassezia genes involved in neutral lipid synthesis were identified by blasting *Sacharomyces cerevisiae* genes against the genome of *M. furfur* (DDBJ/EMBL/GenBank with accession number LMYL00000000).

Results

Presence of LDs in *M. furfur*

M. furfur was grown in mDixon broth or MM containing palmitic acid (C16:1) and / or oleic acid (C18:1) (Figure 1A). In all cases cells maintained the common cylindrical to ovoid morphology (Figure 1C) [15]. Nile red staining revealed the

presence of LDs immediately at the moment of inoculation and after 72 and 168 h of growth (Figure 1C) LDs localized primarily at the budding base when cells had been grown in mDixon or MM with palmitic acid and / or oleic acid (Figure 1C). No differences in LD size or number were observed between the different culture conditions.

LD dynamics and cell viability was monitored upon transferring *M. furfur* cells that had been pre-grown in MM supplemented with palmitic acid and / or oleic acid to MM without supplemented fatty acids. CFUs strongly decreased upon lipid starvation irrespective of the type(s) of fatty acid present in the pre-culture (Figure 1 B). No CFUs were found after 160 h. Decrease in viability was accompanied by cell lysis as observed by cell counts (data not shown). Interestingly, cells observed in the 3 cultures after 160 h still contained LDs with distribution and morphology indistinguishable form those at the 72 h time point (Figure 1D). Yet, they were less abundant as compared to those observed at t = 0 h (Figure 1C panels d, g and j versus Figure 1D).

Lipid composition of lipid droplets

Lipidomics was performed on LD fractions of *M. furfur* cells that had been grown for 4 days in mDixon or in mDixon in which oleic acid and Tween 40 had been replaced by either Tween 80 or oleic acid. TGs, phosphatidylcholine (PC) and lyso-PC (LPC) were found to be the main lipids in the LD fractions (Figure 2).

The composition of LDs varied depending on the type of fatty acid that was supplemented in the medium. TG species 52:4, 54:3 54:4 and 54:5 were unique in cultures grown in Tween 80 or oleic acid (both donors of C18:1) when compared to growth in presence of Tween 40. The TG species in Tween 80 or oleic acid grown cells were more enriched in unsaturated C18:1 fatty acids (Table 1). In the case of Tween 40 (donor of C16:0) TG species 50:1 and 52:2 were unique and shown to contain more saturated fatty acids (Table 1). PC species content appeared less variable. PC 34:1 and 36:4 were unique for LDs derived from Tween 40 cultures, while PC species 34:2, 36:2 and 36:3 were present in LDs from all cultures (Figure 2). LPC 18:1 was only detected in LDs derived from oleic acid and Tween 40 cultures. Interestingly, SE was not found in any of the LD isolations. In contrast, several unknown compounds were found (Data no shown) and CSH_posESI #027 was present in a relative high amount especially in the cultures with oleic acid and Tween 80 (Figure 2).

In silico neutral lipid biosynthesis pathway analysis

All enzymes involved in TG and SE synthesis were detected in the genome of *M. furfur* CBS1878 (Table 2; Figure 3). Despite the absence of steryl esters in

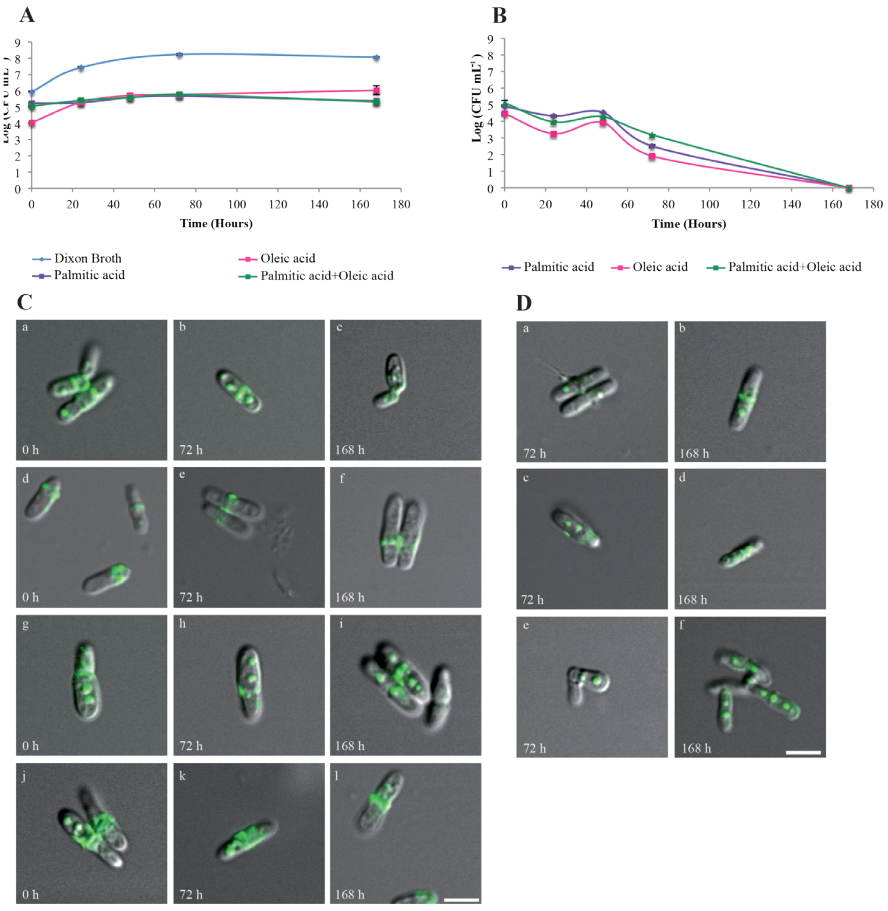

Figure 1. Growth curves (A, B) and localization of LDs (C, D) of *M. furfur* CBS1878 in mDixon and / or MM with palmitic acid and / or oleic acid (A, C) or after transfer to MM without fatty acids (B, D). LDs were localized in cells by Nile Red staining 0, 72, and 168 h after culturing in mDixon (a-c) or in MM supplemented with PA (d-f) OA (g-i), or PA+OA (j-l) (C). Alternatively, LDs were localized in cells that had been grown for 168 h in MM with PA and / or OA and that were subsequently depleted for PA (a-b), OA (b-c) or PA+OA (d-e) (D). Bar represents 5 µm

the lipidome, homologues of the yeast *Are1* and *Are2* genes that encode acyl-CoA:cholesterol acyltransferase (EC 2.3.1.26) were present. These transferases are predicted to catalyze the acylation of ergosterol. The phosphatidate phosphatase gene *PAH1* and the acylation gene *DGA1* that contribute to the synthesis of TG are also present in *M. furfur* (Table 2; Figure 3). In contrast, the additional phosphatase genes *DPP1, APP1,* and *LPP1* were absent [19]. In addition, a homologue of the alcohol O-acetyltransferase 1 gene *ATF2* was absent. This gene encodes an enzyme that, together with *SAY1,* form a sterol acetylation/deacetylation cycle (Figure 3) [20].

Discussion

LDs in *Malassezia* may play a key role in maintaining lipid homeostasis in this yeast, in particular in survival when external lipid sources become exhausted. They may also neutralize lipotoxicity in *Malassezia*, which might be crucial to survive the lipid-rich niches of host skin [21].

LDs in *S. cerevisiae* are covered by a phospholipid monolayer that contains

Table 1. Predicted combinations of fatty acids in triglycerides and phospholipids identified in lipidomic analysis. Triglycerides (TG), Phosphatidylcholine (PC), Lysophosphatidylcholine (LPC). Adapted from [17]

TG species	Possible fatty acids
TG (50:1)	16:0–16:1–18:0; 16:0–16:0–18:1
TG (50:2)	16:0–16:1–18:1; 16:1–16:1–18:0; 16:0–16:0–18:2
TG (50:3)	16:1–16:1–18:1; 16:0–16:1–18:2
TG (52:2)	16:0–18:1–18:1; 16:1–18:0–18:1; 16:0–18:0–18:2
TG (52:3)	16:1–18:1–18:1; 16:1–18:0–18:2; 16:0–18:1–18:2
TG (52:4)	16:0–18:2–18:2
TG (54:3)	18:1–18:1–18:1; 18:0–18:1–18:2
TG (54:4)	18:1–18:1–18:2; 18:0–18:2–18:2
TG (54:5)	18:1–18:2–18:2
Phospholipids species	**Possible fatty acids**
PC (34:1)	16:0–18:1; 16:1–18:0
PC (34:2)	16:1–18:1; 16:0–18:2
PC (36:1)	18:0 –18:1
PC (36:2)	18:1- 18-1
PC (36:3) A	18:1 – 18:2
PC (36:4) A	18:1- 18:1, 18:2
LPC (C18:1)	18:1

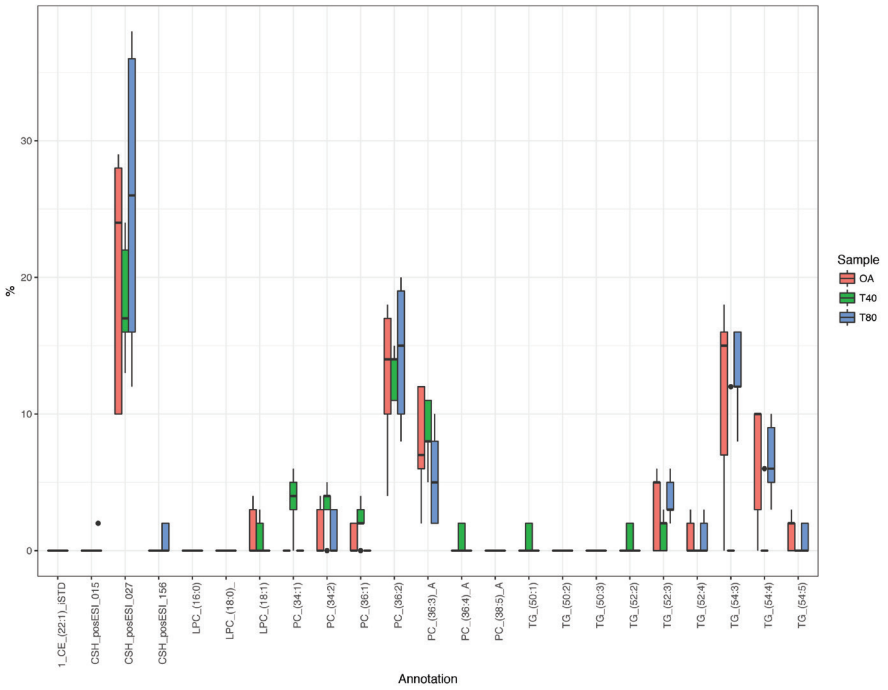

Figure 2. Triglycerides (TG), phosphatidylcholine (PC), and lysophosphatidylcholine (LPC) profiles of LDs isolated from *M. furfur* CBS1878 cultures grown in mDixon broth containing oleic acid (red bars), Tween 40 (green bars) or Tween 80 (blue bars). Error bars indicate standard deviation

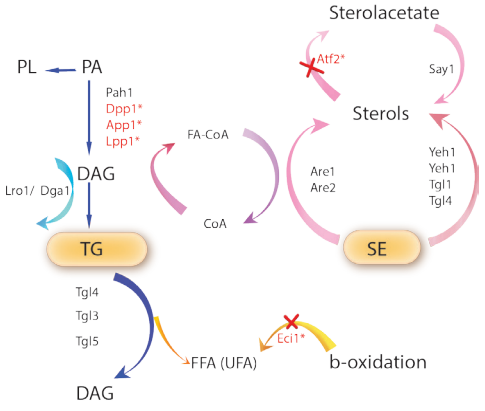

Figure 3. Biosynthesis and degradation of triglycerides (TGs) and steryl esters (SEs). PA: phosphatidic acid; DAG, diacylglycerol; FFA: free fatty acids; UFA: unsaturated fatty acids. Pah1, App1, Lpp1 represent phosphatidate phosphatase, Dga1 acyl-CoA:DAG acyltransferase, Are1 and Are2 acyl-CoA:sterol acyltransferases, Lro1 phospholipid:DAG acyltransferase, Tgl1, Tgl3, Tgl4, and Tgl5 TAG lipases, Yeh1 and Yeh2 yeast steryl ester hydrolase, Atf2 acetyl transferase, Say1, steryl Acetyl hydrolase FA-CoA fatty acyl-CoA, CoA coenzyme A, Eci1, $\Delta^{3,2}$-enoyl-CoA isomerase. *Absent in *M. furfur*

Table 2. Predicted *Malassezia* homologues of enzymes involved in neutral lipid pathway in *Sacharomyces cerevisiae*. MF: *Malassezia furfur* CBS1878. C: cytoplasm; DAG: diacylglycerol; DHAP: dihydroxyacetone phosphate; EC number: enzyme commission number; ER: endoplasmic reticulum; FFA: free fatty acids; G-3-P: glycerol-3-phosphate; LP: lipid particles; Per: peroxisomes; PM: plasma membrane; PA: phosphatidic acid; SE: steryl ester; TAG: triacylglycerol. Y and N represent presence and absence, respectively

EC number	Function	Substrate	Product	Localization	Gene (S.cerevisiae)	MF
EC 6.2.1.3	Long-chain fatty acyl-CoA synthetase	FFA	Acyl-CoA	PM, LP, Per	*FAA1, FAA2, FAA3, FAA4, FAT1*	**Y**
EC 2.3.1.15 EC 2.3.1.42	Glycerol-3-phosphate/ dihydroxyacetone phosphate sn-1acyl-transferase	G-3-P DHAP	1-acyl-G-3-P 1-acyl-DHAP	LP, ER	*GPT2 (GAT1), SCT1 (GAT2)*	**Y**
EC 1.1.1.101	1-Acyl-DHAP reductase	1-Acyl-DHAP	1-Acyl-G-3-P	ER, LP, Per	*AYR1*	**Y**
EC 2.3.1.51	1-acyl G-3-P acyltransferase	lyso-PtdOH	PtdOH	ER, LP	*SLC1/ SLC4*	**Y**
EC 2.3.1.23	1-Acyl G-3-P acyltransferase (lyso-phospholipid acyltransferase)	Lyso-PtdOH (Lyso-PL)	PtdOH/(PL)	ER	*ALE1*	**Y**
EC 2.7.7.41	Phosphatidate cytidylyltransferase	Cystidine triphosphate and phosphatidate	CDP-diacylglycerol	ER	*CDS1*	**Y**
EC 2.7.8.8	CDP-diacylglycerol--serine O-phosphatidyltransferase	CDP-diacylglycerol + L-serine	CMP + (3-sn-phosphatidyl)-L-serine	ER, Per	*CHO1*	**Y**
EC 2.1.1.17	Phosphatidylethanolamine N-methyltransferase	Phosphatidylethanolamine	Phosphatidyl-N-methylethanolamine	ER	*CHO2*	**Y**
EC 2.1.1.71	Phosphatidyl-N-methylethanolamine N-methyltransferase	S-adenosyl-L-methionine + dipalmitoylphosphatidyl-N, N-dimethylethanolamine	S-adenosyl-L-homocysteine + dipalmitoylphosphatidylcholine	ER	*OPI3*	**Y**
EC 3.1.3.4	Phosphatidate phosphatase	PA	DAG	C, LP	*PAH1*	**Y**
EC 2.7.1.107	Diacylglycerol kinase	DAG	PtdOH	ER	*DGK1*	**Y**
EC 3.1.4.4	Phospholipase D	Phospholipid	PtdOH	ER	*SPO14*	**Y**
EC 3.1.4.11	Phospholipase C	Phospholipid	DAG	ER	*PLC1*	**Y**
EC 2.3.1.15 EC 2.3.1.42	Diacylglycerol acyltransferase	DAG	TAG	ER,LP	*DGA1*	**Y**

EC number	Function	Substrate	Product	Localization	Gene (S.cerevisiae)	MF
EC 2.3.1.158	Phospholipid:DAG acyltransferase	DAG	TAG	ER	*LRO1*	**Y**
EC 2.3.1.26	Acyl-CoA:cholesterol acyltransferase	Sterol (DAG)	STE (TAG)	ER	*ARE1*	**Y**
EC 2.3.1.26	Acyl-CoA:cholesterol acyltransferase	Sterol (DAG)	STE (TAG)	ER	*ARE2*	**Y**
EC 2.3.1.51	Lysophosphatidic acid acyltransferase	Lyso-PA/ oleoyl-CoA	PtdOH/(PL)	ER, LP, C	*LOA1/ VPS66*	**Y**
EC 3.1.1.23	Acylglycerol lipase	monoacylglycerols	Glycerol, FAs	LP, Per, C, PM	*YJU3*	**Y**
EC 3.1.1.3	Bifunctional triacyl-glycerol lipase and LPE acyltransferase	TAG	DAG+FFA	LP	*Tgl3*	**Y**
EC 3.1.1.3	Multifunctional lipase/ hydrolase/phospholipase	TAG	DAG+FFA	LP	*Tgl4*	**Y**
EC 3.1.1.3	Bifunctional triacyl-glycerol lipase and LPA acyltransferase	TAG	DAG+FFA	LP	*Tgl5*	**Y**
EC 3.1.1.3	Serine hydrolase	TAG	DAG+FFA	LP	*LDH1*	**Y**
EC 3.1.1.13	Steryl ester hydrolase	SE	Sterol+FFA	LP	*TGL1*	**Y**
EC 3.1.1.13	Steryl ester hydrolase	SE	Sterol+FFA	LP	*YEH1*	**Y**
EC 3.1.1.13	Steryl ester hydrolase	SE	Sterol+FFA	LP	*YEH2*	**Y**
EC 3.1.3.81	Diacylglycerol pyrophos-phate phosphatase 1	DGA	PtdOH	ER	*DPP1*	**N**
EC 3.1.3.4	Phosphatidate phos-phatase	PtdOH	DAG	ER	*APP1*	**N**
EC 3.1.1.-	Lipid phosphate phos-phatase 1	PtdOH	DAG	ER	*LPP1*	**N**
EC 2.3.1.84	Alcohol O-acetyltrans-ferase 1	acetyl-CoA alcohol	CoA acetyl ester	C, ER	*ATF2*	**N**
EC 3.1.1	Steryl acetyl hydrolase 1	terols	Sterol	ER, LD	*SAY1*	**Y**
EC 2.3.1.199	Elongases	FFA C:12 -C:14	FFA C:16 – C:18	ER	*ELO1, ELO2, ELO3*	**Y**
EC 1.14.19.2	Δ9-desaturase	Stearoyl-CoA	Oleoyl CoA	ER	*OLE1*	**Y**
EC 5.3.3.8	$\Delta^{3,2}$-enoyl-CoA isomerase	(3Z)-dodec-3-enoyl-CoA	(2E)-dodec-2-enoyl-CoA	Per	*ECI1*	**N**
EC 1.3.1.34	2,4-dienoyl-CoA reductase	trans-2,3-didehydroa-cyl-CoA NADP+	trans,trans-2,3,4,5-te-tradehydroacyl-CoA NADPH H+	Per	*SPS19*	**Y**

57% phosphatidylcholine (PC), 21% phosphatidylinositol (PI), and 16% phosphatidylethanolamine (PE). Lipidomics showed that under the tested conditions LDs of *M. furfur* are covered by a monolayer of PC only [4,22,23]. Remarkably, SEs were not detected in the LDs of *M. furfur*. The fact that *M. furfur* is sensitive to terbinafine strengthens the absence of SEs because *S. cerevisiae* cells lacking these lipids have increased sensitivity to sterol synthesis inhibitors such as terbinafine [24]. Yet, *M. furfur* has all genes involved in SE synthesis. SE synthesis is strongly inhibited in *S. cerevisiae* when cells are grown in oleic acid containing medium. This is explained by competitive inhibition of *Are2p* by free oleate [25,26]. The absence of SE in *M. furfur* grown in presence of oleic acid and Tween 80 (donor of C18:1) might be explained by a similar mechanism. Possibly, Tween 40 also inhibits SE synthesis. Alternatively, absence of SE is explained by traces of stearic acid (donor of C18:0) present in Tween 40.

Sterol synthesis has to be highly regulated to avoid toxic effects of these lipids. HMG-CoA reductase (HMGR), a conserved enzyme in eukaryotes, is a key regulator of sterol synthesis that is regulated at the transcriptional, translational, and post-translational level [22,27]. The acetylation/deacetylation cycle performed by *ATF2/SAY1* is another mechanism to detoxify sterols in cells. Notably, the acetyltransferase *ATF2* was not present in *M. furfur* but a homologue of *SAY1* was shown to be present. The absence of *ATF2* would imply that this detoxifying mechanism is not operative in *M. furfur*.

TGs were shown to be present in LDs of *M. furfur*. Growth on specific fatty acids or its donors affected the composition of these neutral lipids, as was observed in other yeasts [7,25,28]. LDs remained present after prolonged growth in MM lacking fatty acids. This suggests that TGs in LDs cannot be metabolized. This, however, would provide the essential building blocks to construct new phospholipids and sphingolipids as well as energy and could thus play a major role in cellular growth [29]. The impact of the presence of glucose in the culture medium, as was done in this study, on the stability of LDs during lipid starvation is not yet known.

Tgl1, Tgl3, Tgl4 and Tgl5 present in LDs of *S. cerevisiae* take part in the degradation of TG. Their activity is important to allow the exit of the stationary cell growth [3,30,31]. The genes for these enzymes are present in *M. furfur* (Table 2) and it is expected that these genes are expressed under conditions of fatty acid starvation. Possibly, LD formed in MM with palmitic acid, oleic acid, or a combination of both fatty acids contain only a very limited set of fatty acids and recycling of the species results in imbalanced membrane composition and cell death, preventing full depletion.

Oleic acid cannot be used in β-oxidation directly by *M. furfur* due to the absence of Δ3,2-enoyl-CoA isomerase (Figure 3). In *S. cerevisiae* oleate has divers effects on cell functions, including peroxisome proliferation [26,32]. If this occurs in *M. furfur* needs to be determined, yet it would be remarkable in the context of the inability of this yeast to use oleic acid in this process. Furthermore, the excess of unsaturated fatty acids can lead to the storage in LDs as TG. One of the major functions of LDs is to prevent lipotoxicity by converting excess FAs into neutral lipids (TGs) as has been evidenced in *S. cerevisiae.* Mutants of this yeast with defects in TG synthesis become highly sensitive to unsaturated FAs, such as oleic acid and palmitoleic acid [33,34]. The lipotoxic effect of oleic acid in yeast cells has been related with morphological changes of intracellular membranes, altered phospholipid composition and formation of an additional lipid class, fatty ester ethyl esters [26]. Together, these results indicate that one of the functions of LDs of *M. furfur* is to prevent toxification by oleic acid.

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Chapter 5

Highly efficient transformation system for *Malassezia furfur* and *Malassezia pachydermatis* using *Agrobacterium tumefaciens* - mediated transformation

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Abstract

Malassezia spp. are part of the normal human and animal mycobiota but are also associated with a variety of dermatological diseases. The absence of a transformation system hampered studies to reveal mechanisms underlying the switch from the non-pathogenic to pathogenic life style. Here we describe a highly efficient *Agrobacterium*-mediated genetic transformation system for *Malassezia furfur* and *M. pachydermatis*. A binary T-DNA vector with a hygromycin B (*Hyg*) selection marker and the green fluorescent protein gene (*gfp*) was introduced in *M. furfur* and *M. pachydermatis* by combining the transformation protocols of *Agaricus bisporus* and *Cryptococcus neoformans*. Optimal temperature and co-cultivation time for transformation were 5 and 7 days at 19 °C and 24 °C, respectively. Transformation efficiency was 0.75 - 1.5 % for *M. furfur* and 0.6 - 7.5% for *M. pachydermatis*. Integration of the hygromycin resistance cassette and *gfp* was verified using PCR and fluorescence microscopy, respectively. The T-DNA was mitotically stable in approximately 80 % of the transformants after 10 times sub-culturing in the absence of hygromycin.

Introduction

Malassezia is an unusual yeast characterized by its lipid dependence [1,2]. It is associated with human skin rich in sebum production as part of their normal mycobiome, however, it has also been isolated from many other niches [3]. Currently, 17 species have been defined based on phenotypic and molecular data [2,4,5]. Dermatological diseases such as dandruff/seborrhoeic dermatitis, pityriasis versicolor, and atopic dermatitis in humans have been associated with *Malassezia globosa, Malassezia restricta, Malassezia sympodialis,* and *Malassezia furfur* [3,6–8], while *Malassezia pachydermatis* has been associated with otitis externa and dermatitis in dogs [5]. In addition, *M. furfur* and *M. pachydermatis* have been related with bloodstream infections in patients who received parenteral lipid supplementation [3,9,10]. The increasing interest in *Malassezia* as a pathogen raise the attention in the implementation of molecular tools for efficient transformation and genetic modification.

Agrobacterium tumefaciens-mediated transformation (AMT) is based on the capacity of this bacterial-plant pathogen to transfer DNA (T-DNA) into a host cell. This method combines the use of a binary vector system with a plasmid containing the T-DNA and a plasmid containing the virulence genes that are involved in the transfer of the T-DNA to the host [11,12]. This methodology was first described in fungi for *Saccharomyces cerevisiae* [13]. Since then, it has been implemented successfully in yeasts and filamentous fungi including the pathogens *Candida* spp, *Paracoccidiodes brasiliensis*, *Cryptococcus neoformans*, *Coccidiodes immitis*, and *Trichophyton mentagrophytes* [14–18]. Recently AMT was also reported in *Malassezia* yeast showing that the introduction of exogenous DNA and direct gene manipulation are feasible in this genus [19].

In this study, we have adapted AMT from the protocols reported for *A. bisporus* and *C. neoformans* [15,20] to transform *M. furfur* and *M. pachydermatis*. We tested different co-cultivation parameters, including temperature and time. We used hygromycin B as a selection marker and evaluated the use of GFP as a reporter protein in this yeast. The improvements we obtained when compared to the published transformation system (Ianiri et al., 2016) will enable molecular studies to reveal mechanisms underlying pathogenicity of *Malassezia*.

Materials and Methods

Chemicals

Chemicals were purchased from Sigma (St Louis, MO, USA) unless otherwise indicated.

Strains and culture conditions

Frozen stocks of *M. furfur* CBS 1878 and *M. pachydermatis* CBS 1879 were reactivated for 4 to 5 days at 33 °C on modified Dixon agar (mDixon; 36 g L^{-1} mycosel agar [BD], 20 g L^{-1} Oxbile, 36 g L^{-1} malt extract [Oxoid, Basingstoke, UK], 2 mL L^{-1}glycerol, 2 mL L^{-1} oleic acid, and 10 mL L^{-1} Tween 40) (Boekhout, 2010; Guého et al., 1996). For liquid shaken cultures, *Malassezia* was grown in 150 ml Erlenmeyers at 180 rpm and 33 °C using 150 mL mDixon broth (36 g L^{-1} malt extract [Oxoid], peptone 6 g L^{-1} [Oxoid], 20 g L^{-1} Oxbile, 2 mL L^{-1} glycerol, 2 mL L^{-1} oleic acid, and 10 mL L^{-1} Tween 40.

To determine the minimum concentration of hygromycin B that prevented yeast growth, 100 µl *Malassezia* suspension (10^6 yeast mL^{-1}) was incubated in triplicate for 7 days at 33 °C on mDixon agar supplemented with 6.25-100 ug mL^{-1} antibiotic. The minimal hygromycin B concentration was 25 and 50 µg mL^{-1} for *M. furfur* and *M. pachydermatis,* respectively. This assay was performed with each new hygromycin batch.

Transformation vectors

Plasmid pBHg (kindly provided by Peter Romaine, Pennsylvania State University) contains the hygromycin B phosphotransferase (*hph*) gene from *E. coli* under the control of the *A. bisporus* glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter [20]. Vector pBH-GFP-ActPT was constructed to express the green fluorescent protein gene *gfp* from *Aequorea victoria* under the control of the regulatory sequences of the actin gene (*act*) of *A. bisporus*. To this end, primers 1 & 2 and 3 & 4 (Table 1) were used to amplify the *act* promoter and terminator, respectively. The products were cloned in pGEMt [Promega, Madison, WI, USA] and reamplified with primers 5 & 6 and 7 & 8. The fragments were cloned in *PacI* / *AscI* [Thermo Scientific, St Peters, MO, USA] digested pBHg-PA [23] using InFusion cloning [Clontech, Mountainview, CA, USA], resulting in plasmid pBHg-ActPT that contains *PacI* and *AscI* sites between the *act* promoter and terminator. Gene *gfp* from *Aequorea victoria* [Entelechon, Bad Abbach, Germany] was amplified using primers 9 & 10, digested with PacI / AscI and inserted in *PacI* / *AscI* digested pBHg-ActPT, resulting in the 10,704 bp pBH-GFP-ActPT plasmid.

AMT of *Malassezia furfur* and *M. pachydermatis*

The transformation procedure was adapted from protocols for transformation of *A. bisporus* and *C. neoformans* (Chen et al., 2000; McClelland et al., 2005). Briefly, *A. tumefaciens* strain AGL-1 was transformed with vectors pBHg and pBH-GFP-ActPT by electroporation applying 1.5 kV with capacitance set at 25 µF (Gene

Pulser and Pulse Controller, Biorad, Watford, UK). Transformants were selected at 28 °C in Luria broth (LB) supplemented with 50 ug mL^{-1} kanamycin and 100 ug mL^{-1} hygromycin. After 2 days, transformants were transferred to minimal medium [24] supplemented with 50 ug mL^{-1} kanamycin and grown overnight on a rotatory shaker at 28 °C and 250 rpm to OD600 0.6-0.8. Cells were collected by centrifugation for 15 min at 1,248 g and resuspended in induction medium containing 200 µM acetosyringone (AS). The bacterial suspension was incubated for 3 h at 19 °C with shaking at 52 rpm. *Malassezia* cells were harvested from liquid shaken cultures by centrifugation for 5 min at 2,432 g, washed twice in milliQ H2O with Tween 80 (0.1 %), and suspended in induction medium at a density of 10^7 cells mL^{-1}. Equal volumes of yeast and *A. tumefaciens* cells were mixed and 20 mL of the mix was filtered through a 0.45 µm pore cellulose membrane (Millipore, Billerica, MA, USA) using a 13 mm diameter syringe filter holder. The membrane filters were placed on co-cultivation medium with 200 µM (AS) and incubated at 19 °C, 24 °C, or 28 °C for 3, 5, and 7 days. The membranes were washed with 0.1% Tween 80 and transferred to mDixon agar containing 50 µg mL^{-1} hygromycin B, 200 µg mL^{-1} cefatoxin, 100 µg mL^{-1} carbenicillin, and 25 µg mL^{-1} chloramphenicol to select transformants. Individual colonies were transferred to a fresh selection plate. Experiments were performed in duplo using biological triplicates.

Table 1. Primers used in this study

Primer	Primer name	Sequence 5'-3'
1	Actin prmtr F	AAGCTTAGCCGAGAGAAGATGCCCC
2	Actin prmtr R	CCATGGTTTGTTATTCGTGTGTTCG
3	Actin trmntr F	GGATCCGCTGATGGTGCTTTATGATAAATAAAGTCCTTGGG
4	Actin trmntr R	GAATTCTACTACTACCCCCAAAACCGACATCATCC
5	Act-Pr_F	CCAGGGGGATCGTTAAAGCTTAGCCGAGAGAAG
6	pBHg_ActP_R	AATTAAGAATTCAGATCTCAATTGGGCGCGCCTTTGTTATTCGTGTGTTCG
7	pBHg_ActT_F	TCTGAATTCTTAATTAAGGATCCGCTGATGGTGCTTTATG
8	Act-Ter_R	CGCCGAATTGGCGCGGAATTCTACTACTACCCCC
9	GFP-Fw	CATGATGGCGCGCCATGACCATGATTACGCCAAGC
10	GFP-Rv	CATGATTTAATTAAGGATCCTTACTTGTACAGCTCG
11	Hy-Fw	GACAGGTCGAGGCGGGAAGCTTTAAGAGGTCCGCAAG
12	Hy-Rv	CGTACGCAAAGATGGTCGGGGGATCTGGATTTTAG

Fluorescence microscopy analysis

GFP fluorescence was monitored using a confocal microscope (Leica SPE-II) with 63x ACS APO (NA=1.30) oil objective. Fluorescence was detected using the spectral band 500 – 600 nm. The Fiji image processing package of ImageJ (www.fiji.sc) was used for image analysis and processing.

Molecular analysis and evaluation of mitotic stability

Genomic DNA of wild-type strains and transformants of *M. furfur* and *M. pachydermatis* was extracted as described [25]. Presence of the hygromycin cassette was analyzed by PCR using primers Hy-Fw&Hy-Rv (Table 1). Mitotic stability of 30 transformants was assessed by sub-culturing 10 times on mDixon agar without hygromycin followed by culturing in the presence of the antibiotic.

Statistical analysis

The number of transformants obtained at the different growth conditions was analyzed by 2-factor ANOVA in order to assess the effect of temperature and days of incubation. Normality and homoscedasticity of the data was evaluated with R using the Shapiro-Wilk test and Bartlett's test, respectively [26]. The best condition for the transformation was determined using Student's *t* test between the means of the repeated experiments [27].

Results

Effect of temperature and time of co-cultivation on transformation efficiency of *M. furfur* and *M. pachydermatis*

A. tumefaciens containing the vector pBHg or pBH-GFP-ActPT was co-cultivated with *M. furfur* and *M. pachydermatis* at 19 °C, 24 °C, and 28 °C for 3, 5, and 7 days. Optimal co-cultivation time and temperature for transfer of pBHg was 5 and 7 days at 19 °C or 24°C and for the GFP construct 5 days at 19 ºC. Transformation efficiencies were 0.75 - 1.5 % (Figure 1A, B) and 0.6 - 7.5% (Figure 1C, D) for *M. furfur* and *M. pachydermatis*, respectively.

Molecular analysis of the transformants and mitotic stability

Transformants were examined by PCR analysis to confirm integration of the T-DNA. PCR products of expected size of 1049 and 774 bp for the *hpt* and the *gfp* gene, respectively, were obtained from 30 out of 30 *M. furfur* and *M. pachydermatis* transformants (Figure 2). In no case was a fragment amplified from the wild-type strains. Sequencing of the PCR products confirmed the presence of both genes in the *Malassezia* transformants. Microscopy showed GFP fluorescence in *M.*

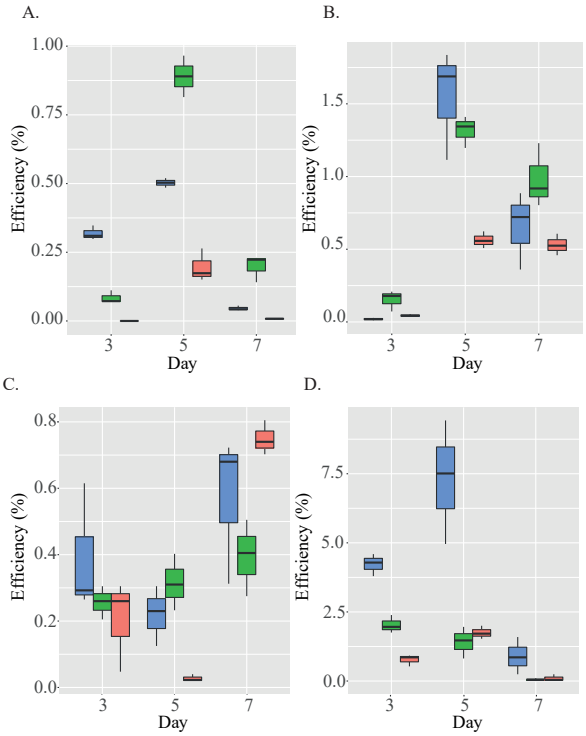

Figure 1. Transformation efficiency of *M. furfur* (A, B) and *M. pachydermatis* (C, D) transformed with pBHg (A, C) and pBHg-GFP-ActPT (B, D) at 19 °C (blue boxes), 24 °C (green boxes), and 28 °C (red boxes) after 3, 5, and 7 days of co-cultivation with *A. tumefaciens*

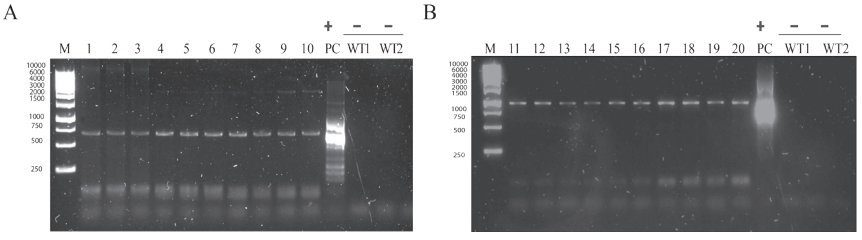

Figure 2. Gel electrophoresis of *gfp* (A) and *hpt* (B) fragments amplified by PCR from *M. furfur* (lanes 1-10) and *M. pachydermatis* transformants (lanes 11-20). Wild-type *M. furfur* (WT1) and *M. pachydermatis* (WT2) were used as negative controls; vector pBHg and pBH-GFP-ActPT were used as positive control (PC); with M representing the molecular size marker (M)

furfur and *M. pachydermatis* transformants with wild-type strains showing some background autofluorescence (Figure 3).

A total number of 30 *M. furfur* and 30 *M. pachydermatis* transformants were 10 times subcultured on mDixon plates in the absence of hygromycin. Of these transformants, 80 % were mitotically stable as shown by replating on hygromycin.

Discussion

Agrobacterium tumefaciens-mediated transformation (AMT) was recently used to transform *Malassezia furfur* and *M. sympodialis* [19]. Here, transformation procedures described for *A. bisporus* and *C. neoformans* were adapted resulting in an improved transformation system for *Malassezia furfur* and *M. pachydermatis*. In this study adaptations were introduced to optimize the transformation protocol. First, a filtration step of the mixture of *A. tumefaciens* and *Malassezia* suspension was used instead of placing the suspension directly onto induction medium or onto the filter as is usually done [11,14,15,19,28]. Possibly, filtration facilitates the contact between the bacterial and yeast cells. Secondly, minimal medium was used as co-cultivation medium. Notably, *Malassezia* spp was able to recover its growth after a co-cultivation period in this medium for 7 days despite the fact that these yeasts are lipid dependent. Thirdly, a concentration of 200 µM acetosyringone (AS) was used rather than 100 µM as was reported for basidiomycota yeast transformation. This may be the reason of the increased number of transformants. High transformation frequencies have been obtained only when sufficient AS was present during *Agrobacterium* pre-culture and during co-cultivation [12]. Finally, a mixture of 108 bacterial cells ml^{-1} and 106 *Malassezia* cells ml^{-1} resulted in the highest transformation efficiency. This ratio corresponds to 100 bacterial cells per yeast cell. A correct ratio of *A. tumefaciens* cells relative to fungal cells is important to avoid the bacterium to overgrow the fungus and to obtain optimal transformation efficiency [11,12].

An overall transformation efficiency of 0.75 - 1.5 % and 0.6 - 7.5% was obtained for *M. furfur* and *M. pachydermatis,* respectively. These efficiencies are similar to that of *C. albicans* [18] but were substantially higher than those reported by [19] or for other yeasts such as *C. neoformans* and *P. brasiliensis.* The latter yeasts were transformed with an efficiency of 0.2 % and 0.0003 %, respectively [14,15].

Others factors impacted transformation efficiency such as time and temperature of co-cultivation. The optimal temperature and co-cultivation time were 5 and 7 days at 19 °C and 24 °C, respectively, for the 2 constructs that were tested. These co-cultivation temperatures agree with those of the yeasts *C. neoformans* and *Candida albicans* [15,18] but not of *P. brasiliensis* that was most efficiently

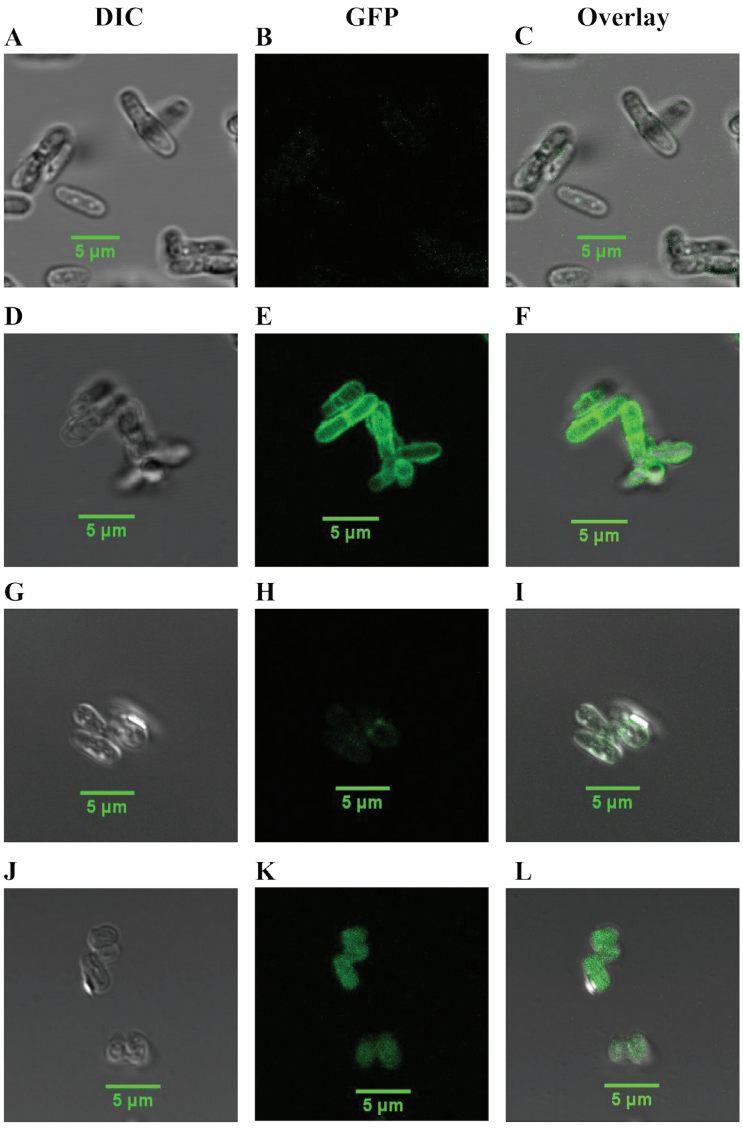

Figure 3. Fluorescence microscopy analysis of *M. furfur* (A-F) and *M. pachydermatis* (G-L). Wild-type strains show low fluorescence (A-C, G-I) when compared to strains transformed with the *gfp* expression vector pBHg-GFP-ActPT (D-F, J-L)

transformed at 28 ºC [14]. These differences have been related with the growth rate of fungi and differences in their susceptibility to *A. tumefaciens* [11].

Hygromycin resistance was mitotically stable as 80 % of the transformants remained resistant after 10 times sub-culturing in the absence of the antibiotic which is similarly to other fungi and yeasts [14,29,30]. *M. furfur* transformants showed consistent high fluorescent signals using the *act* promoter of *A. bisporus.* Signals were lower in the case of *M. pachydermatis* but still sufficient for detection. These results and those obtained with the *hpt* gene show that regulatory sequences from *A. bisporus* are active in *Malassezia.*

Conclusions

In this study, an efficient *Agrobacterium*-mediated transformation system is described for *M. furfur* and *M. pachydermatis*. The efficiency would even enable a marker free transformation. GFP was shown to be expressed in *Malassezia* enabling localization and expression studies aimed to understand the life style of these fungi.

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Chapter 6

Summary and General Discussion

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Malassezia is a fungal genus comprising opportunistic pathogens. These yeasts are part of the normal microbiota of human and animal skin but also cause superficial skin infections like dandruff/seborrheic dermatitis, pityriasis versicolor, atopic dermatitis, and folliculitis. In addition, *M. furfur* and *M. pachydermatis* can cause bloodstream infections in immunocompromised hosts [1,2]. Clearly, *Malassezia* infections have an important socio-economic impact.

Until recently, *Malassezia* comprised 14 species but 3 additional species have been reported [3–6]. This genus is typified by its dependence of external lipids for growth. Only *M. pachydermatis* that causes fungemia in humans and that is an important animal pathogen [7,8] was considered lipid independent. This was based on the finding that it can grow on Sabouraud medium without additional lipid sources [9]. Lipid dependence is a main factor determining the human and animal skin as an ecological niche for *Malassezia*. Understanding lipid metabolism of these yeasts is expected to provide new insights in their biology and ecology, and as a consequence, their commensalism and pathogenicity. Therefore, the aim of this Thesis was to study lipid dependence and metabolism of *Malassezia*.

M. pachydermatis is lipid dependent

Genome sequencing of *M. globosa* and *M. restricta* in 2007 and *M. sympodialis* in 2013 indicated that lipid dependence was due to the absence of genes encoding the fatty acid synthase complex [10,11]. The fact that *M. pachydermatis* was assumed to be lipid independent suggested that these genes would be present in the genome of this yeast. Its genome was sequenced (Chapter 2A) showing a genome size of 8.15 Mbp similar to that of *M. restricta* (7.2 Mbp), *M. sympodialis* (7.67 Mbp) and *M. globosa* (9.0 Mbp). Notably, genes encoding the fatty acid synthase (FAS) complex were not present in *M. pachydermatis*. In addition, the number of lipolytic enzymes (i.e. 35 lipases and 15 esterases) was higher in comparison with other *Malassezia* species [3]. These findings suggested that *M. pachydermatis* should be lipid dependent. Indeed, Sabouraud was found to contain small amounts of lipids [4]. These lipids originate from peptone that was reported to contain 0.6% lipid, of which 6 µg palmitic acid (PA) per gram of peptone. It contains also other fatty acids (FAs), albeit in lower amounts [4]. Lipid dependency of *M. pachydermatis* was studied in more detail by culturing on chemically defined medium (minimal medium, MM) (Chapters 2B and 4). *M. pachydermatis* could not grow in MM without addition of lipids. Notably, the saturated lipid PA (C16:0) had a fungicidal effect on *M. pachydermatis* when supplemented in MM (Chapter 2B). On the other hand, the unsaturated lipid oleic acid (OA) (C18:1) exhibited a fungistatic effect. Addition of both lipids to MM did sustain growth and viability indicating that the mixture alleviates

the fungicidal and fungistatic effects of PA and OA, respectively. Together, it has been demonstrated that *M. pachydermatis* is lipid-dependent and requires a mixture of FAs for growth.

Reconstruction of lipid biosynthesis and degradation pathways in *Malassezia*

Genomes of *M. furfur* CBS1878 and the atypical *M. furfur* isolate, most distantly related to other *M. furfur* strains, were sequenced and compared to other *Malassezia* strains including *M. pachydermatis* (Chapter 2A). *M. furfur* CBS1878 was shown to have a genome twice the size of previously sequenced *Malassezia* species including other *M. furfur* strains [3,4,10–12]. Notably, the atypical *M. furfur* showed to have a smaller genome of 10.38 Mbp. The variability of genome sizes in *M. furfur* strains have been reported before based on pulsed-field gel electrophoresis [12]. Phylogenetic analysis suggests that *M. furfur* strains with a double genome size result from hybridization between *M. furfur* 7982 and *M. furfur* JPLK23 [4]. This was previously suggested as indirect evidence for sexual reproduction in this yeast, which would be in line with the presence of a bipolar mating type system [13]. It is tempting to speculate that the differences in steroid biosynthesis genes between *M. furfur* and the other 4 *Malassezia* species is linked to the production of steroid-like hormones that would be required for sexual reproduction in *M. furfur* [14]. Genome duplication could be another explanation for the larger genomes of some *M. furfur* strains. Changes in ploidy have been linked to adaptation to adverse environments [15,16]. This may also be the case for *M. furfur.* This species is associated with bloodstream infections in individuals that obtain intravenous lipid emulsions. Such emulsions are enriched in linoleic acid, oleic acid, medium chain triglycerides and saturated FAs and serve as a food source [17]. A double number of particular genes may help to adapt to environments with mixtures of such complex lipid sources. However, although there are indeed protein duplications, most of the predicted proteins represent unique proteins in the genomes of *M. furfur* and the atypical *M. furfur* strains (Chapter 3).

Flux balance analysis combined with proteomics revealed that *M. furfur* and *M. sympodialis* displayed high fluxes in reactions related to degradation of CoA-activated long-chain FAs by β–oxidation. The complete β–oxidation pathway for degradation of saturated FAs is expected to be present in *M. furfur* and *M. sympodialis* as was reported for *M. globosa* [18]. However, these species lack the Δ3,2-enoyl CoA isomerase (EC 5.3.3.8) (Table 1) that is involved in catalyzing an important step in catabolism of unsaturated FAs with odd numbered double bonds as is the case in oleic acid (C18:1 cis-9) [18–20]. Indeed, *M. furfur* did

Table 1. Comparison of predicted *Malassezia* homologues of enzymes in *Sacharomyces cerevisiae*. MF: *Malassezia furfur* CBS1878, AMF: atypical *Malassezia furfur* 4DS, MP: *Malassezia pachydermatis* CBS1878, MS: *Malassezia sympodialis* CBS1878, MG: *Malassezia globosa* CBS1878Y represents presence, while N represents not presence. Only genes mentioned in Chapter 4 table 2 are indicated that are absent in some of the indicated species as compared to *S. cerevisiae*. All other genes mentioned and present in *M. furfur* are also present in the genomes of the indicated strains

Glycerolipid synthesis enzymes							
Gene (*Sacharomyces cerevisiae*)	**EC number**	**Function**	**MF**	**AMF**	**MP**	**MS**	**MG**
CHO2	EC 2.1.1.17	Phosphatidylethanolamine N-methyltransferase	**Y**	**Y**	**Y**	**N**	**Y**
OPI3	EC 2.1.1.71	Phosphatidyl-N-methylethanolamine N-methyltransferas	**Y**	**N**	**Y**	**N**	**Y**
SPO14	EC 3.1.4.4	Phospholipase D	**Y**	**Y**	**Y**	**N**	**N**
PLC1	EC 3.1.4.11	Phospholipase C	**Y**	**Y**	**Y**	**N**	**Y**
Glycerolipid precursor enzymes and transporters							
FAS1	EC 3.1.2.14	Fatty acid synthase (β subunit)	**N**	**N**	**N**	**N**	**N**
FAS2	EC 2.3.1.41	Fatty acid synthase (α subunit)	**N**	**N**	**N**	**N**	**N**
OLE1	EC 1.14.19.2	Δ9-desaturase	**Y**	**Y**	**Y**	**Y**	**N**
β-oxidation							
ECI1	EC 5.3.3.8	$\Delta^{3,2}$-enoyl-CoA isomerase	**N**	**N**	**N**	**N**	**N**
Glycerolipid turnover enzymes							
DPP1	EC 3.1.3.81	Diacylglycerol pyrophosphate phosphatase 1	**N**	**N**	**N**	**N**	**N**
APP1	EC 3.1.3.4	Phosphatidate phosphatase	**N**	**N**	**N**	**N**	**N**
LPP1	EC 3.1.1.-	Lipid phosphate phosphatase 1	**N**	**N**	**N**	**N**	**N**
Acetylation/deacetylation (Sterol acetates formation)							
ATF2	EC 2.3.1.84	Alcohol O-acetyltransferase 1	**N**	**N**	**N**	**N**	**N**

not grow in MM containing oleic acid but it had no effect on viability (Chapter 2B and 4). In contrast, *M. sympodialis* was killed in the presence of OA as sole lipid compound (Chapter 2B). Apparently, oleic acid has a fungistatic effect in *M. furfur* but is fungicidal for *M. sympodialis*. The fact that *M. furfur* remains viable in the presence of OA may be explained by the presence of an yet unknown Δ3,2-enoyl-CoA isomerase gene that is structurally distinct from those of other fungi. Alternatively, it uses another biochemical route to metabolize this unsaturated FA. Notably, while *M. furfur,* atypical *M. furfur,* and *M. pachydermatis* can grow on MM with OA and PA, *M. sympodialis* cannot. The latter species may need a mixture of more FAs to sustain growth, explaining why it can grow on mDixon.
Synthesis of unsaturated FA (UFAs) is an essential process to ensure proper functionality of membranes. Interestingly, the Δ9-desaturase gene *OLE1* (Table 1) that is involved in the desaturation of palmitic acid and stearic acid to palmitoleic acid and oleic acid, respectively, is not present in all *Malassezia* species. It is present in *M. sympodialis* [11], *M. furfur*, atypical *M. furfur,* and *M. pachydermatis* (Chapter 3). In contrast, it is absent in *M. globosa* and *M. restricta* and therefore these species cannot synthesize unsaturated FAs [19]. Thus, this species should exploit these sources from the host establishing a strong relation as an adaptation mechanism to this environment. However, alterations in the availability of these compounds can lead to disease.

FA biosynthesis is completed by elongation steps that involves (1) condensation of malonyl-CoA with a long-chain acyl-CoA primer, (2) reduction of the 3-oxoacyl-CoA to 3- hydroxyacyl-CoA, (3) dehydration of the 3-hydroxyacyl-CoA, and (4) reduction of the resulting trans -2,3-enoyl-CoA. High fluxes in reactions related to FA elongation (i.e. the conversion of hydroxyacyl-CoA to trans-2,3-enoyl-CoA) were observed in *M furfur* and *M. sympodialis* (Chapter 3). Elongases *ELO1, ELO2, ELO3* (EC 2.3.1.19) that catalyze step 1 were identified in the genome of *M. furfur* (Chapter 4) and *M. sympodialis*. Elo1P is involved in elongation of a C14:0 to C16:0 FA while Elo2p can elongate FAs up to C24. Elo3p has broader substrate specificity and is essential for the conversion of 24-carbon acids to 26-carbon species. Disruption of the *ELO2 and ELO3* genes in *S. cerevisiae* reduces cellular sphingolipid levels and results in the accumulation of the long chain base phytosphingosine [21]. FA modifications are expected to be important for *Malassezia* spp that are dependent on lipid sources from their environment.

Defects in the assimilation of PA in *M. globosa, M. sympodialis, M. pachydermatis,* and the atypical variant of *M. furfur*, but not in *M. furfur,* were also predicted in silico. These predictions were validated via physiological characterization. *M. furfur* was able to sustain its growth in FA C16:0 sources

such as PA or Tween 40. PA is normally produced as the end product of the FAS complex. Since all *Malassezia* species lack this complex (Table 1) they should obtain FAs like C16:0 from external sources and use these in lipid synthesis. It is therefore remarkable that growth on palmitic acid was not possible for *M. globosa, M. sympodialis, M. pachydermatis,* and the atypical variant of *M. furfur* but was possible for *M. furfur*. Uptake of FAs is accompanied with activation to its FA-CoA derivative which is channeled into the metabolic routes. Possibly, transport and activation of palmitic acid into palmitic acid CoA is happening efficiently in *M. furfur* but is hampered in other strains. The genes encoding the acyl-CoA synthetases (FAA1, FAA2, FAA3, FAA4, and FAT1) involved in these steps are present in all genomes. Faa1p, Faa4p, and Fat1p activate exogenously supplied FAs and free FAs that are derived from phospholipid, TG, and SE breakdown, while Faa2p is required for the activation of FAs that are directed towards β-oxidation [22,23]. Palmitic acid CoA is required for the biosynthesis of glycosphingolipids that play important roles as signaling molecules for example in regulation of growth, responses to heat stress, cell wall synthesis and repair, endocytosis and dynamics of the actin cytoskeleton [24]. Defects in the synthesis of glycosphingolipids might explain the inability of *M. globosa, M. sympodialis, M. pachydermatis,* and the atypical variant of *M. furfur* to remain viable in MM with PA. Further analysis is required to assess whether acyl-CoA synthetase genes are indeed expressed and whether PA is indeed converted to palmitic acid CoA.
Metabolism of the lipid arachidonic acid was predicted to be present in the atypical *M. furfur* strain but not in the other species. Arachidonic acid is metabolized via cyclooxygenase and lipoxygenase pathways to eicosanoids compounds (prostaglandins and leukotrienes) that act as immunomodulators and mediators of skin inflammation. Phospholipase A2 secreted by *M. furfur* could release arachidonic acid from epithelial cells inducing inflammatory response associated with the pathogenicity of this yeast. Lipooxygenase activity in *Malassezia* was linked with alterations in skin pigmentation associated with pitiriasis versicolor and recently with dandruff [25,26]. This mechanism has also been reported in other pathogenic yeasts such as *Candida albicans* and *Cryptoccocus neoformans* [26,27]. *C. neoformans* exploits arachidonic sources from the macrophages by phospholipase B1 (PLB1). It is proposed to be used to remodel its cell membrane which will be enriched in unsaturated FAs resulting in increased membrane fluidity that would promote replication and intracellular survival [28]. This mechanism should be clarified in the future for *Malassezia* species.

In addition to lipid metabolic pathways, more research is required to understand how *Malassezia* metabolizes carbohydrates and sugars which might be limiting

on the skin [4]. In this regard the predicted high fluxes of reactions involved in the degradation of maltose, fructose, and starch in the atypical *M. furfur* strain and in *M. globosa* and *M. sympodialis* are of interest. The predictive power of metabolic network reconstructions can be further optimized. Proteome analysis showed to be an important tool to validate the model proposed by flux balance analysis, since most of the proteins predicted by genome analysis were detected in the protein profiles. To optimize the prediction further it is necessary to implement high-throughput proteomic and metabolomic approaches to validate the models.

Storage of lipids in lipid droplets

Lipid droplets (LDs) of *Malassezia* were studied in (Chapter 4). Lipidomics revealed the presence of phospholipids, expected to represent the LD surface monolayer, as well as triglycerides (TGs) that are expected to be stored in the droplets. Notably, steryl esters (SEs) that are also stored in LDs of other organisms were not detected. The absence of SEs could be explained by competitive enzymatic inhibition of Are2p by free oleate as was previously reported in *S. cerevisiae* [29]. The absence of storage of SEs in LDs of *M. furfur* reduces the possibilities to keep a balance in free sterols. On the one hand, free sterols are toxic, on the other hand they are important to maintain membrane integrity. Sterol levels are controlled in a cell by regulating their synthesis or by detoxification. Synthesis is controlled at various levels (from transcription to degradation of the enzymes) as is the case for instance of HMG-CoA reductase (HMGR) that catalyzes a rate limiting step in sterol biosynthesis. Detoxification of sterols is obtained by ATF2 that acetylates sterols that are secreted as reported for *S. cerevisiae* [30,31]. Interestingly, only the gene encoding the enzyme involved in the deacetylation of acetylated sterols SAY1 (resulting in a functional sterol) was present in *M. furfur* (Chapter 4) but not the gene encoding ATF2. Apparently, *Malassezia* is lacking this detoxification step for sterols and depends therefor especially on the regulation via synthesis of ergosterol or storage of SE in LD to detoxify sterols. It will be of interest to express the *S. cerevisiae Atf2* gene in *Malassezia* to restore this detoxification route and to analyze the effects on growth.

Storage of TGs in LD is expected to be very important because *M. furfur* is not able to use unsaturated FAs in β-oxidation directly due to the absence of Δ3,2-enoyl-CoA isomerase (Chapter 3). By converting these unsaturated FAs into TGs *M. furfur* can avoid lipotoxicity. LDs are important sources for FAs, which can be used in lipid synthesis and energy generation [32,33]. The observation that LDs remained present in *M. furfur* after prolonged growth in MM without any source of FAs, albeit at lower abundance, is remarkable. Possibly, the FAs stored in the TG are metabolically converted to other acyl forms that are toxic and stored in

TG. Alternatively, the absence of FAs in the culture medium negatively affects gene expression of lipid metabolic pathways resulting in cell growth arrest and cell death. More research is required to unravel this.

Genetic modification of *Malassezia*

Genetic tools are crucial to study lipid metabolism of *Malassezia* in more detail. In Chapter 5, an *Agrobacterium tumefaciens*-mediated transformation (AMT) system is described, which showed to be highly efficient. A binary T-DNA vector with the hygromycin B phosphotransferase (*hpt*) as a selection marker and the green fluorescent protein gene (*gfp*) was efficiently introduced in *M. furfur* and *M. pachydermatis*. Previous reports using AMT to transform *M. furfur* and *M. sympodialis* exhibited a low efficiency of transformation [34]. The high efficiency described in Chapter 5 may be due to optimization steps in the protocol such as a filtration step, use of minimal medium as co-cultivation medium, a concentration of 200 μM acetosyringone (AS), and a ratio of 100 bacterial cells per yeast cell. Of interest, [34] reported targeted gene replacements via homologous recombination. The ADE2 gene for purine biosynthesis and the LAC2 gene predicted to be involved in melanin biosynthesis were deleted. I expressed GFP in *Malassezia,* which will enable protein localization and expression studies both on artificial media, skin models, and in animal studies. Together, the molecular toolbox for *Malessezia* is getting filled.

Conclusions

lipid metabolism of *Malassezia* was studied In this Thesis. Moreover, molecular tools were developed to study lipid metabolism in more detail. Such knowledge is important to understand how these yeasts live as commensals or pathogens on the human skin. Current treatments of superficial *Malassezia* infection involve mostly antifungals like azoles and terbinafine [35]. Azole resistance is an increasing problem for many fungal pathogens including *Malassezia* species [36]. Terbinafine (the brand name is for example Lamisil or Terbinex) is an allylamine antifungal and blocks ergosterol synthesis. It can have serious side effects like liver toxicity. Therefore, alternative treatments are required. Insight in lipid metabolism may result in such treatments, for example by using specific FAs that induce lipid toxicity and that do not affect the skin cells and microbiome harmony and avoids dysbiosis.

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Appendix

Nederlands Samenvatting

Malassezia is een schimmelgeslacht dat opportunistische ziekteverwerkers omvat. Deze gisten maken deel uit van de normale microbiota van menselijke- en dierlijke huid, maar veroorzaken ook oppervlakkige huidinfecties zoals roos / seborrheic dermatitis, pityriasis versicolor, atopische dermatitis en folliculitis. Daarnaast kunnen *M. furfur* en *M. pachydermatis* ook infecties van de bloedbaan veroorzaken, met name in gastheren met een verzwakt immuunsysteem. *Malassezia*-infecties hebben dus een belangrijke sociaal-economische impact.
Tot voor kort bestond het *Malassezia* geslacht uit 14 soorten, maar recent zijn er 3 nieuwe soorten gerapporteerd. Dit geslacht wordt getypeerd door zijn afhankelijkheid van externe lipiden voor groei. Alleen *M. pachydermatis,* een belangrijk dierpathogeen die ook bloedbaan infecties bij mensen kan veroorzaken, werd als lipide-onafhankelijk beschouwd. Dit was gebaseerd op het feit dat alleen deze soort in Sabouraud-medium kon groeien zonder toevoeging van extra lipiden. Afhankelijkheid van externe lipiden is een belangrijke factor die de menselijke en dierlijke huid als een ecologische niche voor *Malassezia* bepaald. Inzicht in het lipide metabolisme van deze gisten zal naar verwachting nieuwe inzichten verschaffen over hun biologie en ecologie en daarmee ook inzicht in hun commensalisme en pathogeniciteit. Het doel van het onderzoek dat beschreven is in dit proefschrift was om de lipide afhankelijkheid en het lipide metabolisme van *Malassezia* in meer detail te bestuderen.

M. pachydermatis is afhankelijk van externe lipiden

Genoom sequentiebepaling van *M. globosa* en *M. restricta* in 2007 en *M. sympodialis* in 2013 gaf aan dat de afhankelijkheid voor externe lipiden was toe te schrijven aan de afwezigheid van genen die coderen voor het vetzuursynthase complex (FAS). Omdat *M. pachydermatis* lipide onafhankelijk zou zijn, werd verwacht dat deze genen juist aanwezig zouden zijn in het genoom van deze gist. Het genoom van *M. pachydermatis* werd sequenced (hoofdstuk 2A) en de genoomgrootte van 8,15 Mbp was vergelijkbaar met die van *M. restricta* (7,2 Mbp), *M. sympodialis* (7,67 Mbp) en *M. globosa* (9,0 Mbp). De genen die voor het FAS complex coderen waren echter niet aanwezig in het genoom van *M. pachydermatis*. Bovendien was het aantal lipolytische enzymen (35 lipases en 15 esterases) hoger in vergelijking met andere *Malassezia*-soorten. Deze resultaten suggereerden dat *M. pachydermatis* wel degelijk afhankelijk is van externe lipiden. Het eerder vernoemde Sabouraud medium bleek kleine hoeveelheden lipiden te bevatten die gebruikt kunnen worden voor de groei van *M. pachydermatis*. De lipide afhankelijkheid van *M. pachydermatis* werd daarom gedetailleerd bestudeerd door te kweken op chemisch gedefinieerd

medium (minimaal medium, MM) (hoofdstukken 2B en 4). *M. pachydermatis* kon niet groeien in MM als er geen vetzuren werden toegevoegd. Toevoeging van alleen het verzadigde vetzuur palmitinezuur (PA) (C16: 0) aan het MM bleek een fungicide effect te hebben op *M. pachydermatis* (Hoofdstuk 2B). Anderzijds bleek het onverzadigde oliezuur (OA) (C18: 1) een fungistatisch effect te hebben. Toevoeging van beide vetzuren aan het MM resulteerde in groei wat aangeeft dat dit mengsel de fungicide en fungistatische effecten van respectievelijk PA en OA opheft. Hiermee heb ik aangetoond dat *M. pachydermatis* lipide afhankelijk is en een mengsel van vetzuren in het kweekmedium voor groei vereist.

Reconstructie van lipide biosynthese en afbraakwegen in *Malassezia*

De genomen van *M. furfur* CBS1878 en het atypische *M. furfur* isolaat, een stam die genetisch minder gerelateerd is aan andere *M. furfur* stammen, werden gesequenced en vergeleken met andere *Malassezia* stammen, waaronder die van *M. pachydermatis* (Hoofdstuk 2A). *M. furfur* CBS1878 bleek een genoom te hebben dat tweemaal zo groot was als die van andere *Malassezia* soorten, waaronder andere *M. furfur* stammen. De atypische *M. furfur* stam bleek juist een iets kleiner genoom te hebben namelijk van 10,38 Mbp. De variabiliteit van genoomgroottes in *M. furfur* stammen is eerder gerapporteerd op basis van pulse-field gelelektroforese en eerder gepubliceerde fylogenetische analyses suggereerden dat *M. furfur* stammen met een dubbele genoomgrootte voort zouden zijn gekomen uit hybridisatie tussen *M. furfur* 7982 en *M. furfur* JPLK23. Dit werd eerder voorgesteld als indirect bewijs voor seksuele reproductie.

Via een in silico reconstructie van het metabole netwerk van het lipide metabolisme op basis van de genoom sequenties en op grond van proteomics en flux-balans analyse bleek dat *M. furfur* en *M. sympodialis* hoge fluxen vertoonden in reacties met betrekking tot de afbraak van CoA-geactiveerde langketen vetzuren via β-oxidatie (Hoofdstuk 3). De volledige β-oxidatieweg voor de afbraak van verzadigde vetzuren is naar verwachting aanwezig in *M. furfu*r en *M. sympodialis* zoals eerder is gerapporteerd voor *M. globosa*. Deze soorten missen echter het $\Delta^{3,2}$-enoyl CoA isomerase (EC 5.3.3.8) dat betrokken is bij de afbraak van onverzadigde vetzuren met oneven genummerde dubbele bindingen, zoals in het geval van OA (C18: 1 cis-9). Dit komt overeen met mijn vinding dat *M. furfur* niet kon groeien in MM met OA. Het had echter geen invloed op levensvatbaarheid van de stam (Hoofdstuk 2B en 4). Echter, *M. sympodialis* ging wel dood in MM met OA als enige vetzuur in dit medium (Hoofdstuk 2B). Blijkbaar heeft OA een fungistatisch effect op *M. furfur* terwijl het een fungicide is voor *M. sympodialis*. Synthese van onverzadigde vetzuren is een essentieel proces voor cellen en onder

andere nodig om de integriteit en functie van membranen te waarborgen. Het OLE1 Δ9-desaturase genproduct dat betrokken is bij de omzetting van verzadigde vetzuren tot onverzadigde vetzuren is echter niet aanwezig in alle *Malassezia*-soorten. Het OLE1 gen bleek aanwezig in *M. sympodialis*, *M. furfur*, atypische *M. furfur* en *M. pachydermatis* (Hoofdstuk 3). Het gen is echter afwezig in *M. globosa* en *M. restricta*. Daarom kunnen deze soorten geen onverzadigde vetzuren maken en moeten zij deze vetzuren via hun gastheer verkrijgen.
De in silico analyse van het lipide metabolisme voorspelde ook defecten in de assimilatie van PA in *M. globosa*, *M. sympodialis*, *M. pachydermatis* en de atypische variant van *M. furfu*r, maar echter niet in *M. furfur* (hoofdstuk 3). Deze voorspellingen werden gevalideerd via het kweken in MM met vetzuren. *M. furfur* bleek inderdaad in staat te groeien in MM met C16: 0 bronnen zoals PA of Tween 40, dit in tegenstelling tot de andere vier stammen. PA wordt normaal gesproken geproduceerd als het eindproduct van het FAS-complex. Omdat alle *Malassezia* soorten dit complex ontbreken, zouden ze dit vetzuur uit externe bronnen moeten verkrijgen en gebruiken in de synthese van lipiden. Het is dan ook opmerkelijk dat juist de groei op PA niet mogelijk was voor *M. globosa*, *M. sympodialis*, *M. pachydermatis*, en de atypische variant van *M. furfur* maar wel voor *M. furfur*.

Opslag van lipiden in lipide druppels

In hoofdstuk 4 van mijn proefschrift toon ik aan dat er lipide druppels (LD's) in *Malassezia* aanwezig zijn. Analyse van deze LD's via lipidomics toonde aan dat deze fosfolipiden, die de LD-oppervlakmonolaag kunnen vormen, en triglyceriden (TG's) bevatten. Echter, sterylesters die ook in LD's van andere organismen zijn opgeslagen, werden niet gevonden. De opslag van TG's in LD is belangrijk om vetzuren te ontgiften, zoals bijvoorbeeld OA welke *M. furfur* niet kan gebruiken in de β-oxidatie door de afwezigheid van $\Delta^{3,2}$-enoyl-CoA-isomerase (Hoofdstuk 3). LD's zijn ook belangrijk voor vetzuur opslag en daarmee een belangrijke bron voor energie en voor vetzuren voor cellen, zeker wanneer *Malassezia* groeit in afwezigheid van externe vetzuren. Mijn waarneming dat LD's nog steeds aanwezig zijn in *M. furfur* cellen na langdurige groei in MM zonder enige bron van vetzuren is dan ook zeer opmerkelijk.

Genetische modificatie van *Malassezia*

Genetische modificatie is van cruciaal belang om het lipide metabolisme van *Malassezia* in meer detail te gaan bestuderen. In hoofdstuk 5 beschrijf ik een nieuw en zeer efficiënt transformatiesysteem voor *Malassezia* op basis van *Agrobacterium tumefaciens*. Met behulp van deze bacterie en een binaire T-DNA-vector werd selectie gen tegen het hygromycine en het gen dat codeert voor het

GFP eiwit, een groen fluorescerende eiwit, succesvol geïntroduceerd in *M. furfur* en *M. pachydermatis*. Hiermee is de gereedschapskist voor het bestuderen van *Malassezia* verder uitgebreid.

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Curriculum vitae

Adriana Marcela Celis Ramírez was born November 26,1974 in Neiva (Huila), Colombia. She moved to Bogotá in June 1991, there she attended the Colegio Mayor de Cundinamarca where she obtained her bachelor's degree in Bacteriology and Clinical Laboratorist in December 1997. Later, she worked in Neiva as a bacteriologist for two years. In 2000, she began her Master studies with emphasis in Medical Mycology in the Biological Sciences Department of Universidad de Los Andes. She obtained a research grant from the governmental institute for science Colciencias, allowing her to follow her research project focused on the study of the epidemiology and molecular characterization of *Malassezia* yeast under the supervision of MSc Maria Caridad Cepero de García. During her master's program, Adriana worked as teaching assistant in fungal biology. She obtained her master's degree in 2003, and began teaching microbiology and industrial microbiology in Universidad de Los Andes. Adriana was hired as a Lab Manager for the Biological Science Department in the same university; a permanent position that she combined with the teaching of medical microbiology for medicine, biology and microbiology students, and with her research assistantship associated to Laboratory of Mycology and Phytopathology of the Los Andes University (LAMFU). Currently, Adriana is the head of the medical mycology research in LAMFU with a special interest in the study of host-fungal pathogen interactions focused on yeast specimens such as *Malassezia* spp, *Candida* spp, and mold fungus *Fusarium* spp. She is researching to understand aspects related with their ecology, epidemiology, virulence, pathogenicity, disease, antifungal resistance, molecular characterization, phylogenetic relationship, and new antifungal molecules as therapeutic alternatives. These approaches have been done using conventional tools of identification, as well as using new Omic´s tools, combined with confocal microscopy, phylogenetic analysis and genetic transformation. In 2014, the Netherlands Fellowship Programme (NFP) granted Adriana funds to finance her PhD within the Molecular Microbiology group of the Department of Biology at Utrecht University under the supervision of Prof. Dr. Han Wösten and Dr. Hans de Cock. During her PhD, she was studying and unraveling lipid metabolism in lipid-dependent pathogenic *Malassezia* yeasts. Her research results are described in this thesis.

List of Publications

1. Sergio Triana, Hans de Cock, Robin A. Ohm, Giovanna Danies, Han A. B. Wösten, Silvia Restrepo, Andrés Fernando Gonzalez Barrios and **Adriana Celis**. 2017. Lipid metabolic versatility in *Malassezia* spp yeasts studied through metabolic modeling. Front. Microbiol. doi: 10.3389/fmicb.2017.01772.
2. **Celis AM**, Triana S, Ibarra H, Cardona J, Restrepo S, González, A, Wösten HAB, de Cock H. 2017. Identification and characterization of lipid droplets in *Malassezia furfur.* Submitted.
3. **Celis, A.M.,** Vos, A.M., Triana, S., Medina, C.A., Escobar, N., Restrepo, S., Wösten, H.A.B., Cock, H. De, 2017. Highly efficient transformation system for *Malassezia furfur* and *Malassezia pachydermatis* using *Agrobacterium tumefaciens* -mediated transformation. J Microbiol Methods. 2017, 134:1-6. doi:10.1016/j.mimet.2017.01.001.
4. Olave MC, Vargas-Zambrano JC, **Celis AM,** Castañeda E, González JM. Infective capacity of *Cryptococcus neoformans* and *Cryptococcus gattii* in a human astrocytoma cell line. Mycoses. 2017, 60(7):447-453. doi: 10.1111/myc.12619
5. Galvis-Marín JC, Rodríguez-Bocanegra MX, Pulido-Villamarín AD, Castañeda-Salazar R, **Celis-Ramírez AM**, Linares-Linares MY. In vitro antifungal activity of azoles and amphotericin B against *Malassezia furfur* by the CLSI M27-A3 microdilution and Etest® methods. Rev Iberoam Micol. 2017, S1130-1406(16)30056-0. doi: 10.1016/j.riam.2016.05.004.
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