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Genotyping and characterisation of the secretory lipolytic enzymes of *Malassezia pachydermatis* isolates collected from dogs

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ABSTRACT

Introduction: *Malassezia* species are commensals of normal skin microbial flora of humans and animals. These may become pathogenic under certain conditions such as those associated with atopic dermatitis or otitis externa in dogs.

Material and methods: Isolates of *Malassezia pachydermatis* were obtained from 27 dogs with healthy external ears and 32 dogs with otitis externa. Isolates were characterised on the basis of their first internal transcribed spacer (ITS) and internal spacer 1 (IGS1) sequences. Their extracellular lipase and phospholipase activity were also analysed. Three types of phospholipase inhibitor were used to identify the subclasses of phospholipase associated with otitis externa.

Results: The clinical isolates were classified into three ITS and three IGS1 sequence types. No significant differences in pathogenicity were detected among the ITS or IGS1 genotypes, and all of the isolates exhibited similar levels of lipase activity. The isolates derived from the dogs with otitis externa showed significantly higher phospholipase activity than those obtained from the dogs with healthy external ears. A phospholipase D inhibitor reduced the phospholipase activity of the isolates obtained from the dogs with otitis externa.

Conclusions: This study did not show any significant differences in pathogenicity among the ITS or IGS1 genotypes but does suggest that phospholipase D might be one of the virulence factors involved in the inflammation of the external ear caused by *M. pachydermatis*.

INTRODUCTION

Malassezia species are recognised as opportunistic yeasts and have been isolated from normal skin of both humans and animal. However, in the presence of certain predisposing factors, *Malassezia* species can cause various dermatological conditions, including seborrheic dermatitis, dandruff, atopic dermatitis and pityriasis versicolor (Ashbee and Evans 2002). To date, 14 species of *Malassezia* have been identified, and all of them, except *Malassezia pachydermatis*, are known to be lipid dependent (Hort and Mayser 2011). It has also been demonstrated that the cell walls of *Malassezia* species, which mainly consist of lipids, influence their pathogenicity (Mittag 1995). Therefore, exogenous lipids, especially fatty acids, are considered to be important for the growth of *Malassezia* species and their associations with several diseases. Moreover, *Malassezia* species are able to hydrolyse lipids and integrate the resultant fatty acids into their cells, and these processes are essential for their growth and contribute to their pathogenicity.

Phospholipases mainly hydrolyse glycerophospholipids, resulting in the release of free fatty acids (FFAs). In general, phospholipases are classified into five subclasses: A (PLA), A2 (PLA2), B (PLB), C (PLC), and D (PLD), depending on the specific ester bond they target (Ghannoum 2000). *M. pachydermatis* is isolated from dogs more often than from humans, and the phospholipase activity of *M. pachydermatis* was found to be associated with its virulence in dogs (Cafarchia and Otranto 2004, Cafarchia and others 2008). It has been reported that *M. pachydermatis* can be brought into intensive care wards on the hands of healthcare workers after it has colonised their pet dogs (Chang and others 1998).

On the other hand, lipases hydrolyse neutral lipids such as triacylglycerols, which also results in the release of FFAs. Lipase activity is considered to be a factor in the pathogenicity of numerous bacteria and fungi (Mancianti and others 2001). Recently, FFAs were suggested to cause skin inflammation, and *Malassezia globosa*, which is an important pathogenic species for several

human skin diseases, was demonstrated to exhibit high lipase activity (DeAngelis and others 2007). It was reported that a strain of *M. pachydermatis* displayed greater lipase activity than a *M. globosa* strain (DeAngelis and others 2007). However, other researchers found that *M. globosa* exhibits the highest lipase activity of all *Malassezia* species and that *M. pachydermatis* also displays strong lipase and phospholipase activity (Juntachai and others 2009).

In previous studies in which *M. pachydermatis* isolates from dogs with healthy skin and dogs with atopic dermatitis were molecularly characterised based on DNA sequencing of the intergenic spacer 1 region (IGS1), most of the isolates from the dogs with atopic dermatitis were found to belong to the same IGS1 subtype (Kobayashi and others 2011, Koike and others 2013). Although accumulating evidence suggests that lipase and phospholipase are important for virulence in *Malassezia* species, the specific types of phospholipase associated with pathogenicity and the differences between the lipase activity levels of each type have not been determined.

In the present study, the authors investigated the genomic and enzymatic characteristics of *M. pachydermatis* isolates obtained from dogs in order to identify factors that influence the pathogenicity of this species.

MATERIALS AND METHODS

Isolation and identification of the *M. pachydermatis* isolates

The fungal strains used in this study are listed in Table 1. In total, 59 strains of *M. pachydermatis* and 5 strains of other fungi isolates were obtained from 31 dogs with healthy external ears (H) and 33 dogs with otitis externa (M) in a veterinary clinic. The authors used the BLAST database analysis program in GenBank to determine homology relationships of the internal transcribed spacer (ITS)1 and IGS1 region among reference 12 strains of *M. pachydermatis*, 3 strains of other *Malassezia* species. All clinical isolates were first cultured in Sabouraud dextrose agar (1 per cent peptone, 1 per cent glucose and 1.5 per cent agar) (Oxoid) at 30°C for four days, and then CHROMagar *Malassezia* medium that had not been supplemented with lipids was used for the isolation process (Kaneko and others 2007). The *M. pachydermatis* isolates were identified based on their phenotypic and morphological characteristics and by their ability to grow on medium that had not been supplemented with lipids. They were stored in brain heart infusion broth containing 15 per cent glycerol and 10 per cent dimethyl sulfoxide (Wako) at −80°C until needed for further analysis.

Isolation of genomic DNA and PCR amplification of the ITS1 and IGS1 regions

Isolates of *M. pachydermatis* were grown on Sabouraud dextrose agar. Colony samples were harvested from each culture plate, and genomic DNA was extracted from them using a FastPrep FP100A and a QIA quick PCR purification kit (Qiagen) (Kumeda and others 2004).

The ITS regions (ITS1-5.8S-ITS2) (737 bp) and IGS1 regions (504–840 bp) were amplified from the genomic DNA by PCR using the following primers: ITS regions (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′); IGS1 (5′-TCGATTTATCGAACCACTTCTCTCT-3′) and IGS5 (5′-GATGTGTGTGTGTGGGTGAT-3′). Genomic DNA (1 µl) was added to the PCR reaction mixture (29 µl), which contained 3 µl of 10× reaction buffer, 2.5 mM dNTP, 1 µl of 10 µM of each primer and HS Taq (Hokkaido System Science). The PCR was performed in a thermal cycler (Gene Amp PCR System 9700, PerkinElmer) at 95°C for 15 minutes, followed by 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 75°C for 30 seconds, before a final step of 10 minutes at 72°C. The PCR products were purified using the Montage PCR centrifugal filter (Millipore) and sequenced using an ABI PRISM 3130 genetic analyser (Applied Biosystems). The products' sequences were compared with the *M. pachydermatis* strains and other fungi sequences held in the GenBank database.

Clinical isolates of *M. pachydermatis,* other fungi and additional other *Malassezia* species were classified into six ITS groups (I–VI) according to their ITS sequences. ITSI homologous groups of 59 clinical isolates of *M. pachydermatis* were classified into three ITSI-identical groups (Fig 1 and Table 1). Reference 12 strains and 45 isolates of *M. pachydermatis* were also classified into three IGS1 genotypes (Table 1).

Phylogenetic analysis

The isolates' nucleotide sequences were aligned using the Clustal W program. A phylogenetic tree with 1000 bootstrap replications was constructed using the neighbour-joining method. Genetic distances were calculated using the Kimura two-parameter method, and the resultant tree was drawn with the MEGA (V.5.0) software.

Production of extracellular protein extracts

The *M. pachydermatis* isolates were cultured on YPD agar (1 per cent yeast extract, 2 per cent Bacto peptone, 2 per cent glucose and 2 per cent agar) supplemented with 0.1 per cent Tween 40, 0.1 per cent Tween 80 and 0.5 per cent olive oil at 30°C for six days to obtain sufficient amounts of extracellular protein as previously reported (Juntachai and others 2009).

Phospholipase assay

Phospholipase production was assessed using two methods: (1) the egg yolk plate method and (2) a method based on the ability of the isolates to hydrolyse L-α-phosphatidylcholine dimyristoyl (L-α-PCD) (a glycerophospholipid) (Wako). In the first method, the isolates were grown on Sabouraud dextone agar and then suspended in 3 ml of saline. The resultant solutions

TABLE 1: Isolates of *Malassezia pachydermatis* (N=59 clinical isolates and 12 other isolates), three strains of other *Malassezia* species and additional five clinical isolates with ITS1 and IGS1 genotypes

Isolates	Isolate groups*	ITS genotype	GenBank accession no.	IGS1 genotype	GenBank accession no.
Malassezia pachydermatis	H1	I-3		G3	AB724313
	H2	I-1			
	H3	I-1		G1	AB724314
	H4	I-3		G3	AB724315
	H6	I-3		G1	
	H8	I-3			
	H11	I-3	AB724299	G3	AB724316
	H12	I-2		G2	AB724317
	H17	I-1		G2	AB724318
	H18	I-3			
	H19	I-3	AB724300	G3	AB724319
	H20	I-3	AB724301	G3	AB724320
	H21	I-1		G1	AB724321
	H22	I-1			
	H23	I-1		G1	AB724322
	H24	I-1		G1	AB724323
	H26	I-1		G1	AB724324
	H27	I-3		G3	AB724325
	H29	I-3		G3	AB724326
	H30	I-3			
	H31	I-1		G1	
	H33	I-1		G1	
	H34	I-3	AB724302	G3	AB724327
	H35	I-3	AB724303	G3	AB724328
	H37	I-3	AB724304	G3	AB724329
	H46	I-3		G3	AB724330
	H138	I-3		G3	
	M1	I-1		G1	AB724331
	M3	I-3	AB724305	G3	AB724332
	M4	I-3		G3	AB724333
	M5	I-3			
	M6	I-3		G3	AB724334
	M7	I-3	AB724306	G3	AB724335
	M8	I-1		G1	AB724336
	M9	I-1			
	M10	I-3	AB724307	G3	AB724337
	M11	I-3		G3	AB724338
	M13	I-3		G3	AB724339
	M15	I-1			
	M16	I-3			
	M17	I-3		G3	AB724340
	M18	I-3	AB724308	G3	
	M20	I-3			
	M22	I-3			
	M23	I-1			
	M26	I-1		G3	
	M29	I-3			
	M30	I-3	AB724309	G1	
	M31	I-3		G3	AB724341
	M33	I-3		G3	AB724342
	M38	I-3			
	M43	I-3		G3	AB724343
	M45	I-3		G3	AB724344
	M48	I-3	AB724310	G3	AB724345
	M50	I-3	AB724311	G3	AB724346
	M51	I-3		G3	AB724347

Continued

TABLE 1: Continued

Isolates	Isolate groups*	ITS genotype	GenBank accession no.	IGS1 genotype	GenBank accession no.
	M54	I-3	AB724312	G3	AB724348
	M55	I-3		G1	AB724349
	M56	I-3		G3	AB724350
	IFM52750			G3	AB118597
	IFM52753	I-1	AB118938	G1	AB118600
	IFM52754			G1	AB118601
	IFM52758			G3	AB118604
	IFM52760			G2	AB118606
	IFM52761			G1	AB118607
	IFM52769			G3	AB118615
	IFM52775			G3	AB118621
	IFM52776			G3	AB118622
	4103	I-2			
	CBS1885	I-2	AY387141		
	CBS1879	I-3	AY387139		
Mahonia japonica		II	JN882323		
M. furfur		II	JQ699098		
M. restricta		III	AY387145		
Meyerozyma	H157	IV			
Pichia anomala	H134	IV			
Candida albicans	H46	V			
C. albicans	M27	V			
Meyerozyma	H135	VI			

*Isolates:
M. pachydermatis isolates:
H, isolates from dogs with no otitis externa (healthy ears); M, isolates from dogs with otitis externa; IFM, Institute of Food Microbiology; 4013, isolate obtained from human atopic dermatitis; CBS, Centraalbureau voor Scimmelcultures
Other isolates:
M. japonica, reference strain in the GenBank database for each locus; *M. furfur*, reference strain in the GenBank database for each locus; *M. restricta*, reference strain in the GenBank database for each locus; *Meyerozyma*, isolate came from dogs in this study; *Pichia anomala*, isolate came from dogs in this study; *Candida albicans*, isolate came from dogs in this study
IGS, internal spacer; ITS, internal transcribed spacer

exhibited equivalent turbidity to a 3.0 McFarland standard. Also, 10 µl samples of the suspensions were then transferred to 5 per cent egg yolk Sabouraud dextone agar and incubated at 38°C for 12 days. After the incubation period, the formation of precipitation zones around the colonies was considered to be indicative of enzyme production. Phospholipase production (Pz) was assessed as the ratio of colony diameter to the total diameter of the colony plus the precipitation zone (Porro and others 1976). Hence, the higher the Pz value, the lower the phospholipase production. Each strain was tested in duplicate, and Pz values are shown as mean values with sds (Coutinho and Paula 2000).

In the second method, to quantify and evaluate the extracellular phospholipase activity of each isolate based on their ability to hydrolyse L-α-PCD, the concentration of extracellular proteins in the extracted solution was adjusted to 0.5 mg/ml. Then, L-α-PCD was dissolved in 100 per cent ethanol, and the concentration of the solution was adjusted to 2.5 mM. A reaction mixture consisting of 20 mM acetate buffer (pH 5.0), 0.25 mM L-α-PCD and 20 µg/ml extracellular protein was made up and was allowed to react at 30°C for one hour. The reaction was terminated by adding 1 ml of methanol. The fatty acids released during the reaction period were extracted according to the method of Bligh and Dyer (1959). The FFA concentration of the final solution was measured using a colorimetric method; that is, using a FFA half micro test kit (Roche) and palmitic acid as the standard.

Lipase assay

To quantify the lipase enzyme activity, p-nitrophenyl palmitate (p-NPP) (Sigma) was dissolved in 2-propanol at a concentration of 5 mM before being used in the lipase assay. The assay mixture consisted of 0.3 ml of 0.5 per cent Triton X-100, 100 mM citrate buffer (pH 5.6), 0.5 mM p-NPP and 10 µg of the extracted extracellular proteins. The assay mixture was incubated at 30°C for one hour. To terminate the reaction and adjust the pH of the reaction mixture, two volumes of 1 M Tris-HCl buffer (pH 8.0) were added to the reaction mixture. Then, the absorbance of the reaction mixture was measured at 405 nm. The increase in absorption is indicative of the amount of p-nitrophenol (p-NP) released by p-NPP hydrolysis, which in turn is a measure of lipase activity.

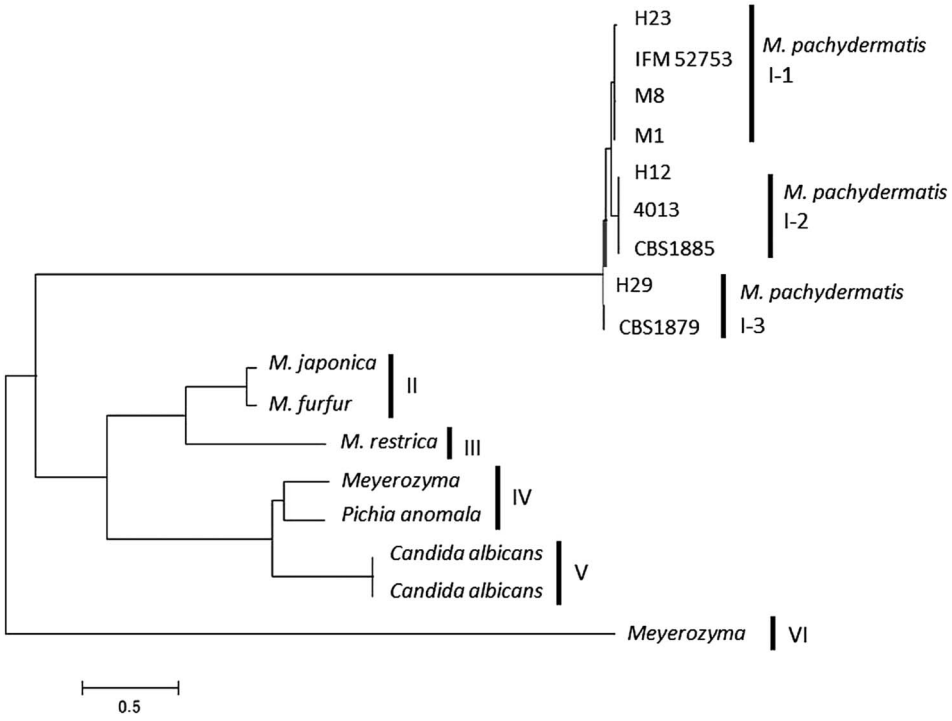

FIG 1: Cluster analysis of *Malassezia pachydermatis* and other *Malassezia* species based on their internal transcribed spacer (ITS) DNA sequences. The phylogenetic tree was constructed from ITS sequence data for *Malassezia* species (Table 1). The tree with 1000 bootstrap replications was drawn using the neighbour-joining method

Effects of phospholipase inhibitors

The *M. pachydermatis* strains that displayed the lowest and the highest phospholipase activity were used in this experiment. Aristolochic acid (Sigma), an inhibitor of phosA2, and hydrochloride hydride (5-fluoro-2-indolyl deschlorohalopemide (FIPI)) (Sigma), an inhibitor of phosD, were dissolved in dimethyl sulfoxide (Su and others 2009), and O-tricyclo [5.2.1.$0^{2,6}$]dec-9-yldithiocaribonate potassium (Sigma), an inhibitor of phospholipase C (phosC), was dissolved in distilled water (Zhao and others 2013). Extracted protein from *M. pachydermatis* H20 and M30 mixed in phospholipase inhibitor were reacted at 30°C for one hour. The released FFA of each strain was measured using FFA half micro test kit described above.

Statistical analysis

The data were analysed using Student's *t* test or two-way analysis of variance, differences being considered significant if $P<0.05$. Statistical analyses were performed using GraphPad Prism 5 software.

RESULTS

ITS and IGS1 subtypes of the isolates collected from dogs with otitis externa and healthy dogs

A total of 64 isolates were identified: 59 isolates of *M. pachydermatis* and 5 isolates of other fungi. *M. pachydermatis* isolates were obtained from 59 dogs. The ITS1 sequences of all isolates were identified and classified into three ITS genotypes. ITS genotype I-1 and I-3 were more prevalent rather than ITS genotype I-2 in both groups (Fig 1 and Table 2). The isolates were also classified into three genotypes based on the lengths of their IGS1 regions. Although IGS1 genotype III was isolated most frequently, no relationship was detected between the isolates' IGS1 genotypes and their Pz values. Isolates displaying the IGS1 genotype II were collected from two

TABLE 2: Frequency of isolation as number (%) of each type of ITS sequence among *Malassezia pachydermatis* isolates from healthy dogs and dogs with otitis externa and the phospholipase activity of each ITS1 groups expressed as mean of the Pz value with the sd values according to the egg yolk method

	I-1		I-2		I-3		Total	
Group	No. isolations (%)	Mean Pz (sd)	No. isolations (%)	Mean Pz (sd)	No. isolations (%)	Mean Pz (sd)	No. isolations (%)	Mean Pz (sd)
Dogs with healthy ears (N=27)	10 (37.0)	0.67 (0.09)	1 (3.7)	0.51 (ND*)	16 (59.3)	0.81 (0.07)	27 (100)	0.75 (0.11)
Dogs with otitis externa (N=32)	6 (18.8)	0.51 (0.09)	0		26 (81.3)	0.49 (0.09)	32 (100)	0.50 (0.09)
Total (N=59)	16 (27.1)	0.61 (0.12)	1 (1.7)	0.51 (ND*)	42 (71.2)	0.61 (0.18)	59 (100)	0.61 (0.16)

*Not determined as only one isolate
ITS, internal transcribed spacer

TABLE 3: Phospholipase activity and lipase activity of the extracellular proteins produced by typical *Malassezia pachydermatis* isolates

Isolate	Phospholipase activity (μM/mg protein)*	Lipase activity (mg/μg protein)
H8	0.067±0.003	0.227±0.038
H19	0.070±0.003	0.167±0.064
H20†	0.043±0.005	0.148±0.013
H27	0.056±0.008	0.215±0.025
H33	0.047±0.002	0.215±0.075
M15	0.111±0.009	0.193±0.018
M17	0.101±0.004	0.254±0.063
M26	0.122±0.013	0.240±0.051
M30†	0.242±0.005	0.193±0.035
M38	0.147±0.008	0.286±0.088

*The values are presented as mean±sd. Isolates collected from the dogs with otitis externa (M) indicate significantly higher phospholipase activity than those obtained from the healthy dogs (H). Phospholipase activities of M group var. H group were statistically significant different by Student's *t* test (P<0.05)
†Two *M. pachydermatis* isolates that displayed the lowest and the highest phospholipase activity (H20 and M30, respectively) were used to evaluate the effects of phospholipase inhibitors

of the healthy dogs (data not shown). The ITS and IGS1 sequencing classified results for *M. pachydermatis* were similar (Table 1).

Phospholipid and lipolytic enzyme activity of *M. pachydermatis*

According to the Pz values obtained using the egg yolk method, the isolates from the dogs with otitis externa exhibited higher phospholipase activity (mean Pz value=0.50±0.09, n=32) than those from healthy dogs (mean Pz value=0.75±0.11, n=27, P<0.01, Table 2). Conversely, no significant differences were detected among ITS1 genotype 1(mean Pz value=0.61, n=16), 2 (Pz value=0.51, n=1) or 3 (mean Pz value=0.61, n=42, P=0.122, Table 2). Based on the second method, mean extracellular phospholipase activity was significantly higher for the isolates collected from five dogs with otitis externa compared with those obtained from five healthy dogs (P<0.01, Table 3). Conversely, no differences in lipase activity were detected between the isolates collected from the dogs with otitis externa and those obtained from the healthy dogs (P=0.18, Table 3).

Effects of lipolytic enzyme inhibitors on the extracellular phospholipase activity of *M. pachydermatis*

The two *M. pachydermatis* strains that displayed the lowest and the highest phospholipase activity (H20 and M30, respectively, Table 3) were used to test three types of inhibitor (phosA2, phosD and phosC) were used. PhosD inhibitor interfered with the activity of M30 and inhibited it most effectively at concentration of 0.5 mM (data not shown). Thus, the authors use this concentration of other inhibitors (phosA2 and phosC). None of the inhibitors had a significant inhibitory effect on the phospholipase activity of strain H20, which was derived from a healthy dog (Fig 2a). However, hydrochloride hydride (FIPI) significantly inhibited the phosD activity of M30, which was isolated from a dog with otitis externa (P<0.05). Both phosA2and phosC inhibitors did not exhibit effects to phospholipase activity (Fig 2b).

DISCUSSION

In this study, the relationship between the genotype and pathogenicity of *M. pachydermatis* was analysed using isolates collected from healthy dogs and dogs with otitis externa. The *M. pachydermatis* isolates were classified into three ITS groups and three IGS1 groups based on their

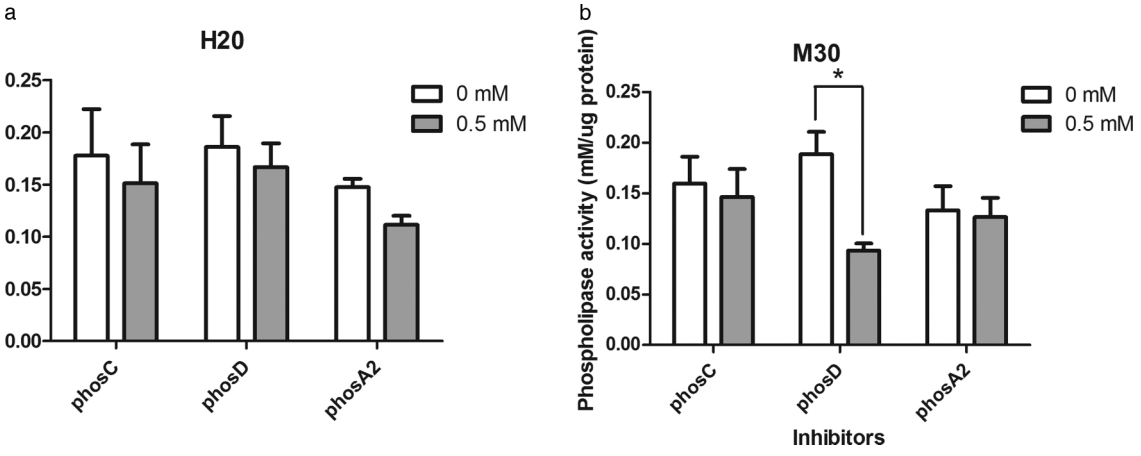

FIG 2: Effect of phospholipase inhibitors on extracellular phospholipase activity of two *Malassezia pachydermatis* isolates: (a) H20 and (b) M30. The values on the y-axis represent phospholipase activity as the concentration of the free fatty acid (FFA) released from L-α-phosphatidylcholine dimyristoyl (L-α-PCD). The x-axis represents three experiments using different phospholipase inhibitors (phosC, phosD and phosA2) with the bars showing the means±sd. Phospholipase inhibitor (phosD) interfered with the activity of M30 and inhibited it most effectively at concentration of 0.5 mM (data not shown). Each experiment compared activity in the absence of phospholipase inhibitors (0 mM) and at a concentration of 0.5 mM. *P<0.05

sequences, respectively. The ITS and IGS1 sequencing results were similar and no significant differences in pathogenicity were detected among the ITS or IGS1 genotypes. Although both ITS and IGS1 sequencing is comparatively quick and specific, the regions were designed from ribosomal DNA and conservative in numbers of *M. pachydermatis*.

It has been proposed that the growth of *M. pachydermatis* on skin lesions is influenced by the fungus's phospholipase production (Cafarchia and Otranto 2008). The authors measured phospholipase activity using (1) the egg yolk method and (2) a method based on the ability of each isolate to hydrolyse L-α-PCD, a glycerophospholipid. The measurement of phospholipase activity (Pz) using the egg yolk method was successful, and the method based on the hydrolysis of L-α-PCD also proved to be a useful way of assessing phospholipase activity. In both assays, the *M. pachydermatis* isolates collected from the dogs with otitis externa demonstrated higher phospholipase activity than those from the healthy dogs. Thus, phospholipase activity might play a pathogenic role in otitis externa caused by *M. pachydermatis* and could also contribute to the virulence of *M. pachydermatis*. The authors did not detect any difference in lipase activity between the *M. pachydermatis* isolates collected from dogs with otitis externa and those obtained from healthy dogs, and they consider this to be a novel finding.

Previous studies have shown that some *Malassezia* species display lipase activity. An analysis of the amino acid sequences of the extracellular lipases secreted by *M. pachydermatis* and other species revealed that these proteins contain a conserved consensus lipase motif (Gly-X-Ser-X-Gly). Three *Malassezia* lipase genes have been cloned and characterised so far; that is, those encoding the extracellular lipase molecules secreted by *Malassezia furfur* (Brunke and Hube 2006), *M. pachydermatis* (Shibata and others 2006) and *M. globosa* (DeAngelis and others 2007). In a study in which a heterologous *Pichia pastoris*-based expression system was used to express the extracellular lipase secreted by *M. furfur*, it was suggested that the molecule performs best at a pH of 5.8 and a temperature of 40°C, which is slightly higher than body temperature. In addition, the extracellular lipase secreted by *M. pachydermatis* operated best at 30°C and pH 7.5, which is higher than the normal pH of the skin (5.4–5.9). However, MgLIP1, which encodes the lipase produced by *M. globosa*, has been detected on the human scalp, indicating that it might play a key role in the virulence of *M. globosa*.

Many previous studies have found that *M. pachydermatis* isolates collected from skin lesions exhibited higher phospholipase activity than isolates collected from healthy skin (Cafarchia and Otranto 2004, 2008; Vlachos and others 2013). A previous study detected higher extracellular phospholipase activity in *M. pachydermatis* isolates obtained from healthy dogs than in isolates collected from human skin. Thus, it was suggested that secreted phospholipases might not have significant effects on the pathogenicity of *Malassezia* species (Park and others 2013). However, the authors found that *M. pachydermatis*-induced otitis externa is associated with increased phospholipase production.

In order to identify the phospholipase subclasses that contribute to the virulence of *M. pachydermatis*, three subclasses of phospholipase inhibitor were used in a further analysis of the phospholipase activity of *M. pachydermatis*. Since FIPI significantly inhibited the PLD activity of strain M30, PLD production may be one of virulence of otitis externa associated with *M. pachydermatis* in dogs.

Based on this result, the authors suggest that PLD might make an important contribution to the pathogenicity of *M. pachydermatis*. Although PLD is involved in a range of essential cellular processes (Xie and Bikle 1997), PLD activity has also been detected in *Candida albicans* (McLain and Dolan 1997). In addition, a reduction in virulence was observed in mice that were orally infected with a PLD1-deficient *C. albicans* mutant, suggesting that PLD might play a critical role in the pathogenicity of *C. albicans* (Hube and others 2001). On the other hand, the phospholipase activity of *M. pachydermatis* was not completely inhibited by any of the phospholipase inhibitors, which makes sense as fungi are known to possess complex pathogenic mechanisms. Thus, the pathogenicity of *M. pachydermatis* might not depend on a single factor.

In this report, the authors examined the lipase and phospholipase activity of *M. pachydermatis* isolates collected from dogs that visited a veterinary clinic and found that the pathogenicity of *M. pachydermatis* might be related to PLD production. Further work is planned to identify other factors that are associated with dermatological disease and elucidate the role played by lipolytic activity in several of the skin diseases caused by *M. pachydermatis*.

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REFERENCES

Ashbee H. R., Evans E. G. (2002) Immunology of diseases associated with Malassezia species. *Clinical Microbiology Reviews* 15, 21–57

Bligh E. G., Dyer W. J. (1959) A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* 37, 911–917

Brunke S., Hube B. (2006) MfLIP1, a gene encoding an extracellular lipase of the lipid-dependent fungus Malassezia furfur. *Microbiology* 152, 547–554

Cafarchia C., Gallo S., Danesi P., Capelli G., Paradies P., Traversa D., Gasser R. B., Otranto D. (2008) Assessing the relationship between Malassezia and leishmaniasis in dogs with or without skin lesions. *Acta Tropica* 107, 25–29

Cafarchia C., Otranto D. (2004) Association between phospholipase production by Malassezia pachydermatis and skin lesions. *Journal of Clinical Microbiology* 42, 4868–4869

Cafarchia C., Otranto D. (2008) The pathogenesis of Malassezia yeasts. *Parassitologia* 50, 65–67

Chang H. J., Miller H. L., Watkins N., Arduino M. J., Ashford D. A., Midgley G., Aguero S. M., Pinto-Powell R., Von Reyn C. F., Edwards W., Mcneil M. M., Jarvis W. R. (1998) An epidemic of Malassezia pachydermatis in an intensive care nursery associated with colonization of health care workers' pet dogs. *New England Journal of Medicine* 338, 706–711

Coutinho S. D., Paula C. R. (2000) Proteinase, phospholipase, hyaluronidase and chondroitin-sulphatase production by Malassezia pachydermatis. *Medical Mycology* 38, 73–76

Deangelis Y. M., Saunders C. W., Johnstone K. R., Reeder N. L., Coleman C. G., Kaczvinsky J. R., Jr, Gale C., Walter R., Mekel M., Lacey M. P., Keough T. W., Fieno A., Grant R. A., Begley B., Sun Y., Fuentes G., Youngquist R. S., Xu J., Dawson T. L., Jr (2007) Isolation and expression of a Malassezia globosa lipase gene, LIP1. *Journal of Investigative Dermatology* 127, 2138–2146

Ghannoum M. A. (2000) Potential role of phospholipases in virulence and fungal pathogenesis. *Clinical Microbiology Reviews* 13, 122–143, table of contents

Hort W., Mayser P. (2011) Malassezia virulence determinants. *Current Opinion in Infectious Diseases* 24, 100–105

Hube B., Hess D., Baker C. A., Schaller M., Schafer W., Dolan J. W. (2001) The role and relevance of phospholipase D1 during growth and dimorphism of Candida albicans. *Microbiology* 147, 879–889

Juntachai W., Oura T., Murayama S. Y., Kajiwara S. (2009) The lipolytic enzymes activities of Malassezia species. *Medical Mycology* 47, 477–484

Kaneko T., Makimura K., Abe M., Shiota R., Nakamura Y., Kano R., Hasegawa A., Sugita T., Shibuya S., Watanabe S., Yamaguchi H., Abe S., Okamura N. (2007) Revised culture-based system for identification of Malassezia species. *Journal of Clinical Microbiology* 45, 3737–3742

Kobayashi T., Kano R., Nagata M., Hasegawa A., Kamata H. (2011) Genotyping of Malassezia pachydermatis isolates from canine healthy skin and atopic dermatitis by internal spacer 1 (IGS1) region analysis. *Veterinary Dermatology* 22, 401–405

Koike A., Kano R., Nagata M., Chen C., Hwang C. Y., Hasegawa A., Kamata H. (2013) Genotyping of Malassezia pachydermatis isolates from canine healthy skin and lesional skin of atopic dermatitis in Japan, Korea and Taiwan. *Journal of Veterinary Medical Science* 75, 955–958

Kumeda Y., Asao T., Takahashi H., Ichinoe M. (2004) Erratum to "High prevalence of B and G aflatoxin-producing fungi in sugarcane field soil in Japan: heteroduplex panel analysis identifies a new genotype within Aspergillus Section Flavi and Aspergillus nomius". *FEMS Microbiology Letters* 47, 263

Mancianti F., Rum A., Nardoni S., Corazza M. (2001) Extracellular enzymatic activity of Malassezia spp. isolates. *Mycopathologia* 149, 131–135

Mclain N., Dolan J. W. (1997) Phospholipase D activity is required for dimorphic transition in Candida albicans. *Microbiology* 143(Pt 11), 3521–3526

Mittag H. (1995) Fine structural investigation of Malassezia furfur. II. The envelope of the yeast cells. *Mycoses* 38, 13–21

Park M., Do E., Jung W. H. (2013) Lipolytic enzymes involved in the virulence of human pathogenic fungi. *Mycobiology* 41, 67–72

Porro M. N., Passi S., Caprill F., Nazzaro P., Morpurgo G. (1976) Growth requirements and lipid metabolism of Pityrosporum orbiculare. *Journal of Investigative Dermatology* 66, 178–182

Shibata N., Okanuma N., Hirai K., Arikawa K., Kimura M., Okawa Y. (2006) Isolation, characterization and molecular cloning of a lipolytic enzyme secreted from Malassezia pachydermatis. *FEMS Microbiology Letters* 256, 137–144

Su W., Yeku O., Olepu S., Genna A., Park J. S., Ren H., Du G., Gelb M. H., Morris A. J., Frohman M. A. (2009) 5-Fluoro-2-indolyl des-chlorohalopemide (FIPI), a phospholipase D pharmacological inhibitor that alters cell spreading and inhibits chemotaxis. *Molecular Pharmacology* 75, 437–446

Vlachos C., Gaitanis G., Alexopoulos E. C., Papadopoulou C., Bassukas I. D. (2013) Phospholipase activity after beta-endorphin exposure discriminates Malassezia strains isolated from healthy and seborrhoeic dermatitis skin. *Journal of the European Academy of Dermatology and Venereology* 27, 1575–1578

Xie Z., Bikle D. D. (1997) Cloning of the human phospholipase C-gamma1 promoter and identification of a DR6-type vitamin D-responsive element. *Journal of Biological Chemistry* 272, 6573–6577

Zhao J. F., Chen H. H., Ojcius D. M., Zhao X., Sun D., Ge Y. M., Zheng L. L., Lin X., Li L. J., Yan J. (2013) Identification of Leptospira interrogans phospholipase C as a novel virulence factor responsible for intracellular free calcium ion elevation during macrophage death. *PLoS ONE* 8, e75652