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 between 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 Mays 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′-TCCGTAGGTAACCTGCGG-3′) and ITS4 (5′-TCCT CCGTATTGATATGC-3′); IGS1 (5′-CCAGTTATCGGAAATCACCTC-3′) and IGS5 (5′-GATGTGTTGATCTGGTG-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. IGS1 homologous groups of 59 clinical isolates of *M. pachydermatis* were classified into three IGS1-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 dextrose agar and then suspended in 3 ml of saline. The resultant solutions...
| 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
exhibited equivalent turbidity to a 3.0 McFarland standard. Also, 10μl samples of the suspensions were then transferred to 5 per cent egg yolk Sabouraud dextrose 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.
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 [5.2.1.02,6]dec-9-yldithiocarbonate 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 ITS 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

| Group                          | No. isolations (%) | Mean Pz (sd) | No. isolations (%) | Mean Pz (sd) | No. isolations (%) | Mean Pz (sd) | Total 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                  | 0.49 (0.09) | 26 (81.3)          | 0.61 (0.18) | 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*
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 phosA2 and 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

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**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                            | 0.146±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.

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**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 FIP1 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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