

Genotyping of *Malassezia pachydermatis* isolates from canine healthy skin and atopic dermatitis by internal spacer 1 (IGS1) region analysis

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Conflict of Interest

No conflicts of interest have been declared.

Abstract

Isolates of *Malassezia pachydermatis* from healthy dog skin and from dogs with atopic dermatitis were molecularly characterized using internal spacer 1 (IGS1) region analyses, and their phospholipase A2 activity and pH growth profiles were then characterized *in vitro*. The percentage of isolates from healthy dogs that had the following IGS1 subtypes (isotype, %) were as follows: 1A, 6%; 1B, 27%; 1C, 11%; 2A, 6%; 2B, 6%; 3A, 11%; 3C, 3%; and 3D, 24%. In contrast, 9% of isolates from dogs with atopic dermatitis were isotype 1B and 91% were isotype 3D, indicating that isolates of subtype 3D were the most prevalent in dogs with atopic dermatitis. Production of phospholipase A2 was statistically higher in isolates of subtype 3D than in the other subtypes. The subtype 3D isolates showed enhanced growth on alkaline medium compared with non-3D subtype isolates. The main clinical sign of canine *Malassezia* dermatitis is waxy exudates on the skin, which predispose the patient to development of a yeast overgrowth of the subtype 3D. Increased phospholipase A2 production may be involved in the inflammatory process associated with *Malassezia* dermatitis.

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Introduction

The lipophilic yeast *Malassezia pachydermatis* belongs to the resident skin flora of animals and is associated with various skin diseases of dogs.^{1–3} In particular, dogs with atopic dermatitis are predisposed to secondary *Malassezia* dermatitis.^{1–3} However, the exact mechanism by which *M. pachydermatis* causes inflammation is still unknown.

In previous studies, molecular typing of this organism was used for epidemiological studies.^{4–6} Concordance in genotypic classification of *M. pachydermatis* from canine skin was identified for the large subunit (LSU), internal transcribed spacer (ITS-1) and chitin synthase 2 (CHS2) genes.^{4–6} The relationship between genotype and virulence of *M. pachydermatis* is not well established. Sugita *et al.* investigated DNA sequence diversity of the intergenic spacer 1 (IGS1) region of *M. pachydermatis* from healthy canine skin and from the ears of dogs with otitis externa. Based on their study, *M. pachydermatis* was divided into three major groups (1–3) with 10 subtypes (A–D).⁷ No relationship was found between specific IGS genotypes, disease types or sites of fungal colonization.⁷ Since the IGS region shows remarkable intra-species diversity, examination of a large number of isolates may reveal a relationship between specific IGS genotypes and disease types, as well as the factors that underlie such a relationship.⁷ Sugita *et al.* reported that a specific IGS genotype of *M. globosa* may play a significant role in human atopic dermatitis.⁸

Phospholipases produced by pathogenic fungi are known to damage host cell membranes. It has been proposed that phospholipase production by *M. pachydermatis* is associated with its growth on skin lesions.⁹ To date, there is no evidence to directly link the pathogenicity of genetic variants of the IGS region of *M. pachydermatis* and their phospholipase production.

Clinical signs of canine *Malassezia* dermatitis usually include waxy exudates on the skin, which may enhance the growth of this yeast.^{2,3} The waxy exudates from abnormal canine skin are more alkaline than those from normal canine skin.¹⁰ Therefore, it was hypothesized that the alkaline pH of canine atopic dermatitis might selectively enhance the growth of *M. pachydermatis*.

The goals of this study were to compare the genotypes of *M. pachydermatis* isolates from dogs with healthy skin and from dogs with atopic dermatitis, to assess genotype-specific phospholipase A2 production, and their *in vitro* growth on media at various pH levels.

Table 1. Malassezia strains used this study

Group I			Group II		
Strain no.	Origin	IGS1 subtype	Strain no.	Origin	IGS1 subtype
1002	Healthy dog 1	1A	M.P. 13	Atopic dermatitis dog 1	1A
1003	Mouth mucosa	1A		External ear	
1004	Healthy dog 1	1A	1074	Atopic dermatitis dog 2	1B
1005	Interdigital area	1A	1075	Mouse mucosa	1B
1006	Healthy dog 2	1B	1076	Atopic dermatitis dog 2	1B
1007	External ear	1B	1077	External ear	1B
1008	Healthy dog 3	1B	1078	Atopic dermatitis dog 3	1B
1009	Interdigital area	1B	1079	External ear	3D
1010	Healthy dog 4	1B	1080	Atopic dermatitis dog 4	3D
1011	External ear	1B	1081	Thigh	3D
1012	Healthy dog 5	1B	1082	Atopic dermatitis dog 5	3D
1013	External ear	1B	1083	External ear	3D
1014	Healthy dog 5	1B	1084	Atopic dermatitis dog 6	3D
1015	Interdigital area	1B	1085		3D
1016	Healthy dog 6	1B	1086	Atopic dermatitis dog 7	3D
1017	External ear	1B	1087	Abdomen	3D
1018	Healthy dog 7	1B	1088	Atopic dermatitis dog 8	3D
1019	External ear	1B	1089	Limb	3D
1020	Healthy dog 7	1B	1090	Atopic dermatitis dog 9	3D
1021	Interdigital area	1B	1091	Loin	3D
1022	Healthy dog 8	1B	1092	Atopic dermatitis dog 10	3D
1023	External ear	1B	1093	Abdomen	3D
1024	Healthy dog 8	1B	1094	Atopic dermatitis dog 11	3D
1025	Interdigital area	1B	1095	Axilla	3D
1026	Healthy dog 9	1C	1096	Atopic dermatitis dog 11	3D
1027	External ear	1C	1097	Abdomen	3D
1028	Healthy dog 10	1C	1098	Atopic dermatitis dog 12	3D
1029	External ear	1C	1099	Abdomen	3D
1030	Healthy dog 11	1C	1100	Atopic dermatitis dog 13	3D
1031	External ear	1C	1101	Paws	3D
1032	Healthy dog 11	1C	1102	Atopic dermatitis dog 13	3D
1033	Interdigital area	1C	1103	External ear	3D
1034	Healthy dog 12	2A	1104	Atopic dermatitis dog 14	3D
1035	Abdomen	2A	1105	External ear	3D
1036	Healthy dog 12	2A	1106	Atopic dermatitis dog 15	3D
1037	External ear	2A	1107	Paws	3D
1038	Healthy dog 13	2B	1108	Atopic dermatitis dog 15	3D
1039	Mouth mucosa	2B	1109	Mouth mucosa	3D
1040	Healthy dog 13	2B	1110	Atopic dermatitis dog 16	3D
1041	External ear	2B	1111	Abdomen	3D
1042	Healthy dog 14	3A	1112	Atopic dermatitis dog 16	3D
1043	External ear	3A	1113	Paws	3D
1044	Healthy dog 14	3A	1114	Atopic dermatitis dog 16	3D
1045	Interdigital area	3A	1115	Axilla	3D
1046	Healthy dog 15	3A	1116	Atopic dermatitis dog 17	3D
1047	External ear	3A	1117	Neck	3D
1048	Healthy dog 15	3A	1118	Atopic dermatitis dog 17	3D
1049	Inguinal region	3A	1119	Paws	3D
1050	Healthy dog 16	3B	1120	Atopic dermatitis dog 18	3D
1051	External ear	3B	1121	Abdomen	3D
1052	Healthy dog 16	3B	1122	Atopic dermatitis dog 18	3D
1053	Interdigital area	3B	1123	Neck	3D
1054	Healthy dog 17	3C	1124	Atopic dermatitis dog 19	3D
1055	External ear	3C	1125	Interdigital area	3D
1056	Healthy dog 17	3D	1126	Atopic dermatitis dog 20	3D
1057	Interdigital area	3D	1127	Abdomen	3D
1058	Healthy dog 18	3D	1128		3D
1059	External ear	3D	1129		3D
1060	Healthy dog 19	3D	1130	Atopic dermatitis dog 21	3D
1061	External ear	3D	1131	External ear	3D
1062	Healthy dog 20	3D			
1063	External ear	3D			
1064	Healthy dog 21	3D			
1065	External ear	3D			

Table 1. (Continued)

Group I			Group II		
Strain no.	Origin	IGS1 subtype	Strain no.	Origin	IGS1 subtype
1066	Healthy dog 22	3D			
1067	External ear	3D			
1068	Healthy dog 23	3D			
1069	External ear	3D			
1070	Healthy dog 23	3D			
1071	Interdigital area	3D			
1072	Healthy dog 24	3D			
1073	External ear	3D			

Materials and methods

Isolates

The strains used in this study are listed in Table 1. The *M. pachydermatis* isolates were obtained from 24 dogs without skin disease and 21 dogs with skin lesions consistent with atopic dermatitis. A total of 131 isolates (see Table 1 for location) were used in the study, and were cultured using CHROMagar *Malassezia* medium (CHROMagar, Paris, France).¹¹

The isolates were divided into two groups: group I consisted of 72 isolates collected from 24 healthy dogs without evidence of skin disease, and group II consisted of 59 isolates collected from 21 dogs with atopic dermatitis. Atopic dermatitis was diagnosed according to Patel and Forsythe.³

Isolates of *M. pachydermatis* were identified phenotypically based on morphology and on their ability to grow on medium without lipid supplementation.¹² All isolates were subjected to molecular characterization and were evaluated for phospholipase A2 activity and pH growth profiles on modified Dixon agar.

Isolation of genomic DNA and PCR amplification of the IGS region

After cultivation of the isolates on modified Dixon agar (3.6% malt extract, 0.6% peptone, 2.0% desiccated oxbile, 1.0% Tween 40, 0.2% glycerol, 0.2% oleic acid and 12% agar, pH 6.0)¹² at 32°C for 1 week, all strains were collected separately by scraping the surface of the media and were then suspended in lysis buffer [1 mg/mL of zymolyase 100T (Takara, Kyoto, Japan), 0.1 mmol/L EDTA, 1% sodium dodecyl sulfate (SDS), 10 mmol/L Tris hydrochloride and 0.3% 2-mercaptoethanol, pH 8.0] and incubated at 37°C for 14 h.

The microbial genomic DNA samples were collected using standard phenol-chloroform extraction of the lysed suspensions. The DNA was precipitated by the addition of 2.5x volume of ethanol followed by gentle mixing. The precipitated DNA was centrifuged at 10 000g for 10 min, the supernatant discarded and the pellet washed in 70% ethanol. The air-dried DNA samples were dissolved in 75 µL of distilled water.

The following primer set for PCR amplification of the IGS1 domain from rDNA was designed by Sugita *et al.* as previously described: 26S-F (5'-ATCCTTGCAGACGACTTGA-3') and Mala-R (5'-TGCTTA-CTTCGCGATCGG-3').⁷

Thirty-five cycles of PCR amplification were performed in the following conditions: denaturation for 30 s at 95°C, primer annealing for 30 s at 59°C and polymerization for 1 min at 72°C in a total reaction volume of 20 µL of an amplification mixture consisting of 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.001% gelatin, 2.5 mmol/L of each deoxynucleotide triphosphate, 1.0 U Taq polymerase (Takara, Kyoto, Japan) and 0.5 µg of each primer. The resultant amplified DNA fragments were electrophoresed on a 2% (w/v) agarose gel and were visualized using ethidium bromide.

An approximately 550–900 bp DNA band amplified from each strain was excised from the gel and was then purified using the Exo-SAP-IT® kit (USB Corp., Cleveland, OH, USA) and sequenced using

an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

To examine homology relationships between the IGS1 region of a reference strain of *M. pachydermatis* and that of the samples, we used the BLAST database analysis program in GenBank (<http://blast.ncbi.nlm.nih.gov/>).

Evaluation of phospholipase A2 production

Phospholipase A2 production was assessed using the Enzchek® Direct Phospholipase A2 Assay Kit (Invitrogen, Carlsbad, CA, USA). Briefly, five isolates of subtype 1A (1002, 1003, 1004, 1005 and M.P.13), three isolates of subtype 1B (1007, 1008 and 1078), two isolates of subtype 1C (1028 and 1029), two isolates of subtype 2A (1034 and 1035), two isolates of subtype 2B (1038 and 1039), two isolates of subtype 3A (1044 and 1048), two isolates of subtype 3B (1052 and 1053) and 15 isolates of subtype 3D (1056, 1057, 1058, 1059, 1072, 1073, 1079, 1124, 1125, 1126, 1127, 1128, 1129, 1130 and 1131) were incubated on modified Dixon agar and were then suspended in modified Dixon broth¹² (1 × 10⁵ CFU/mL) and incubated again (incubations were for 7 days at 32°C in both media). Fifty microlitres of yeast suspension were analysed for phospholipase A2 activity according to the Enzchek® phospholipase A2 assay kit user manual (Invitrogen).

The effect of pH on the growth of *M. pachydermatis*

Five isolates of subtype 1A (1002, 1003, 1004, 1005 and M.P.13), five isolates of subtype 1B (1008, 1016, 1018, 1074 and 1075), four isolates of subtype 1C (1026, 1027, 1028 and 1029), three isolates of subtype 2A (1034, 1035 and 1036), four isolates of subtype 2B (1038, 1039, 1040 and 1041), four isolates of subtype 3A (1042, 1044, 1046 and 1048), three isolates of subtype 3B (1050, 1052 and 1053) and 20 isolates of subtype 3D (1058, 1060, 1062, 1066, 1070, 1072, 1079, 1082, 1083, 1084, 1094, 1096, 1098, 1100, 1102, 1106, 1108, 1120, 1122 and 1125) were suspended in modified Dixon broth (5 × 10⁶ CFU/mL), and 10 µL of yeast suspension were cultured on modified Dixon agar at four different levels of pH (pH 6.0, 7.0, 8.0 and 9.0). After incubation for 7 days at 32°C, the growth of each organism was determined by counting the number of colonies on each plate.

Statistical analyses

The statistical significance of the differences between phospholipase A2 activity and growth at different levels of pH between the 3D and non-3D groups was analysed using Student's unpaired *t*-test (Excel Statistical Program File YSTAT2002; Igaku Tosho Shuppan, Tokyo, Japan). Differences were considered statistically significant at *P* < 0.05.

Results

IGS1 subtypes of isolates from dogs with atopic dermatitis and healthy dogs

Based on the length of the IGS1 region of *M. pachydermatis* isolates, which was obtained using PCR, and

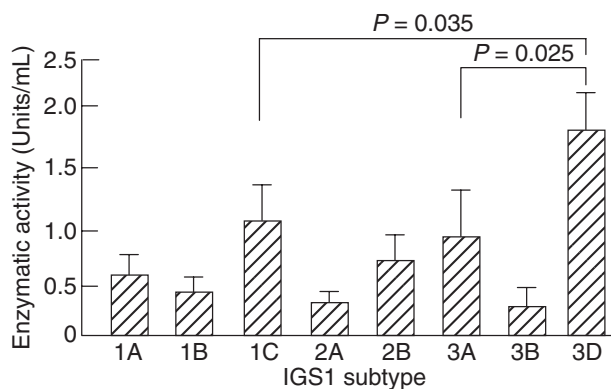


Figure 1. Phospholipase A2 production by IGS1 subtypes of *Malassezia pachydermatis* isolates. The mean phospholipase A2 concentration produced by each IGS1 subtype was assayed using a commercial kit. The phospholipase production of subtype 3D isolates was significantly higher than that of the 3A and 1C subtypes.

ranged in size from 552 to 898 bp, the isolates were classified into nine previously described genotypes.⁷

The percentages of each isolate subtype from healthy dogs were 6% of 1A, 27% of 1B, 11% of 1C, 6% of 2A, 6% of 2B, 11% of 3A, 3% of 3B, 3% of 3C and 24% of 3D (Figure 1); those from dogs with atopic dermatitis were 2% of 1A, 8% of 1B and 90% of 3D. Isolates of subtype 3D were the most prevalent in dogs with atopic dermatitis.

Phospholipase A2 production of the IGS1 isolate subtypes

The mean phospholipase A2 production of each subtype is shown in Figure 1. Phospholipase A2 production of isolates of subtype 3D was significantly higher than that of isolates of the other subtypes (Figure 1).

Effect of pH on the growth of isolates

The colony growth of 3D and non-3D subtype isolates on modified Dixon agar at three different levels pH is shown in Figure 2. The subtype 3D isolates grew better at alkaline pH than the subtype non-3D isolates (Figure 2).

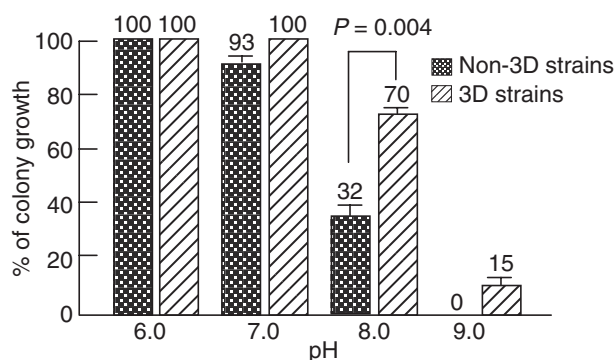


Figure 2. The pH dependence of the growth of *Malassezia pachydermatis* isolates. Colony growth of subtype 3D and nonsubtype 3D isolates on modified Dixon agar at a variety of pH levels was evaluated. The growth of subtype 3D isolates was significantly higher than that of the other subtypes at pH 8.0 but not at other levels of pH.

Discussion

The genotypes of *M. pachydermatis* isolates from canine atopic dermatitis were analysed to determine the relationship between genotype and virulence for canine skin. In this study, subtype 3D isolates of *M. pachydermatis* were isolated more frequently from canine atopic dermatitis skin than from healthy canine skin. The results of the present study support the hypothesis that the pathogenicity of *M. pachydermatis* isolates is related to the level of phospholipase A2 production.¹³

Machado *et al.* predicted that the genotypes of *M. pachydermatis* isolates from canine skin would vary depending on the predisposition of the host to diseases.¹³ In addition, the distribution pattern might be related to an interaction between *Malassezia* and factors in the skin microenvironment (e.g. bacterial flora, pH, salts, immune response, biochemistry and physiology).¹³ In the present study, each isolate subtype was cultured on modified Dixon agar at four different levels of pH. The subtype 3D isolates grew actively on alkaline medium when compared with the non-3D subtype isolates, indicating that the growth of specific subtypes of this yeast depends significantly on the pH of the medium. These findings suggest that subtype 3D isolates of *M. pachydermatis* grow selectively on skin with a higher pH. The pH of normal canine skin has been reported to range between 5.5 and 7.2.¹³ However, the pH of canine skin with activated apocrine secretion was reported to be more alkaline than normal canine skin, ranging from 8.2 to 9.0.¹⁰ Clinical signs of canine *Malassezia* dermatitis usually include waxy exudates, which enhance the growth of *M. pachydermatis*. Therefore, alkaline waxy exudates from abnormal skin might selectively enhance the growth of subtype 3D isolates of *M. pachydermatis*, thereby worsening the dermatitis due to the high production of phospholipase A2 by these isolates. Atopic dogs predisposed to recurrent *Malassezia* overgrowth may benefit from bathing in shampoos or use of topical agents that lower the pH.

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Résumé Des isolats de *Malassezia pachydermatis* de chiens sains et de chiens atteints de dermatite atopique ont été caractérisés d'un point de vue moléculaire par l'analyse de région IGS1 (internal spacer 1); l'activité de leur phospholipase A2 et leur profil de croissance de pH ont été déterminés *in vitro*. Les pourcentages d'isolats de chiens sains ayant les sous-types IGS1 suivants (isotype, %) étaient : 1A, 6%; 1B, 27%; 1C, 11%; 2A, 6%; 2B, 6%; 3A, 11%, 3C, 3% et 3D, 24%. En revanche, 9% des isolats de chiens atopiques étaient isotype 1B et 91% étaient isotype 3D, indiquant que ces isolats de sous-type 3D étaient plus fréquents chez les chiens atteints de dermatite atopique. La production de phospholipase A2 était statistiquement plus élevée pour les isolats de sous-type 3D que pour les autres sous-types. Les isolats de sous-type 3D ont montré une croissance augmentée sur milieu alcalin par rapport aux isolats de sous-type non-3D. Le signe clinique principal de dermatite à *Malassezia* canine est la présence d'un exsudat cireux sur la peau qui prédispose le patient au développement d'une croissance excessive de levures du sous-type 3D. La production accrue de phospholipase A2 pourrait être impliquée dans le processus inflammatoire associé à la dermatite à *Malassezia*.

Resumen Aislados de *Malassezia pachydermatis* de piel de perros sanos y de perros con dermatitis atópica se caracterizaron a nivel molecular utilizando análisis de la región del espaciador interno 1 (IGS1) y su actividad fosfolipasa A2 y los perfiles de crecimiento a diferente pH se caracterizaron *in vitro*. El porcentaje de aislados de perros sanos que tenían los siguientes subtipos de IGS1 (% del isotipo) fueron: 1A, 6%; 1B, 27%; 1C, 11%; 2A, 6%; 2B, 6%; 3A, 11%; 3C, 3% y 3D, 24%. Por el contrario, 9% de los aislados de perros con dermatitis atópica fueron del isotipo 1B y 91% fueron del isotipo 3D, indicando que aislado de subtipo 3D eran los mas prevalentes en perros con dermatitis atópica. La producción de fosfolipasa A2 fue estadísticamente mayor en aislados del subtipo 3D que en los otros subtipos. Los aislados del subtipo 3D mostraron mayor crecimiento en medio alcalino, comparado con subtipos no 3D. El principal signo clínico en casos de dermatitis por *Malassezia* son exudados ceruminosos en la piel, lo cual predispone a desarrollar crecimiento de levaduras del subtipo 3D. El incremento de la producción de fosfolipasa A2 puede estar involucrado en el proceso inflamatorio asociado con la dermatitis por *Malassezia*.

Zusammenfassung Isolate von *Malassezia pachydermatis* aus gesunder Hundehaut und von Hunden mit atopischer Dermatitis wurden mittels Internal Spacer 1 (IGS1) Regionsanalyse molekular charakterisiert und ihre Phospholipase A2 Aktivität und pH Wachstumsprofile dann *in vitro* charakterisiert. Der Prozentsatz der Isolate von gesunden Hunden, die dem folgenden Subtyp entsprachen (Isotyp %) waren: 1A, 6%; 1B, 27%; 1C, 11%; 2A, 6%; 2B, 6%; 3A, 11%; 3C, 3% und 3D, 24%. Im Gegensatz dazu waren 9% der Isolate von Hunden mit atopischer Dermatitis vom Isotyp 1B und 91% waren vom Isotyp 3D, was darauf hindeutet, dass die Isolate vom Subtyp 3D am häufigsten bei Hunden mit atopischer Dermatitis vorkamen. Die Produktion von Phospholipase A2 war statistisch höher bei den Isolaten vom Subtyp 3D als bei den anderen Subtypen. Die Isolate der 3D Subtypen zeigten erhöhtes Wachstum auf alkalischem Medium im Vergleich zu Isolaten, die nicht-3D Subtypen entsprachen. Das hauptsächliche klinische Symptom von *Malassezia pachydermatis* ist ein schmieriges Exsudat auf der Haut, welches beim Patienten zur Entstehung einer Überwucherung durch Hefepilze des Subtyps 3D prädisponiert. Eine erhöhte Phospholipase A2 Produktion kann beim Entzündungsprozess, der mit *Malassezia pachydermatis* im Zusammenhang steht, involviert sein.

要約 健康犬とアトピー性皮膚炎の犬の皮膚から分離した *Malassezia pachydermatis* の株を internal spacer 1 (IGS1) 領域分析を用いて、分子的特徴を明らかにした。また、同時に試験管内においてホスホリパーゼ A2 活性と pH による増殖能の変化を明らかにした。健康犬から得られた IGS1 のサブタイプの割合は 1A:6%、1B:27%、1C:11%、2A:6%、2B:6%、3A:11%、3C:3%、3D:24%であった。一方でアトピー性皮膚炎の犬から得られた株の 9%は 1B であり、91%が 3D であった。このことから 3D 株がアトピー性皮膚炎において最も多く存在していることが示された。ホスホリパーゼ A2 の産生は他の株と比較してサブタイプ 3D 株で統計的に高かった。3D 株は他のサブタイプに比べてアルカリ性の培養液内で増殖が促進されることがわかった。犬のマラセチア皮膚炎の主な臨床症状は皮膚の脂漏であり、これが 3D 株の過剰増殖の好発要因となっている。ホスホリパーゼ A2 の産生が増加していることもマラセチア皮膚炎にかかわる炎症過程に関与している可能性がある。