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Differences in protein profiles between *Malassezia pachydermatis* strains obtained from healthy and infected dogs

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ABSTRACT

Malassezia pachydermatis causes infections of the skin and mucous membranes, especially in individuals with metabolic, hormonal, and immunological disorders. The search for *M. pachydermatis* properties that differentiate isolates from healthy and infected animals may result in the identification of typically commensal and potentially pathogenic strains within the entire species. We aimed to determine and compare protein profiles of *M. pachydermatis* strains isolated from 30 dogs with clinical symptoms of otitis externa and 34 dogs without symptoms of any disease. Two-dimensional gel electrophoresis was applied, and proteins distinguishing the two groups of strains were identified by liquid chromatography coupled with tandem mass spectrometry. Significant differences were found between potentially pathogenic and commensal isolates. The most significant finding was the presence of nicotinamide adenine dinucleotide phosphate (NADP)-dependent mannitol dehydrogenase and ketol-acid reductoisomerase among *M. pachydermatis* strains obtained from dogs with otitis externa. Nevertheless, it is not clear whether they are associated directly with the pathogenicity or they play the role of fungal allergen. On the basis of these findings, we can conclude that there may be two distinct groups of *M. pachydermatis* strains—one typically commensal and the other with properties that enhance the infection process. These results may be used for more precise diagnosis and identification of potentially pathogenic strains in the future.

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Characteristic proteins; otitis externa; two-dimensional electrophoresis; yeasts

INTRODUCTION

Malassezia pachydermatis is a widely distributed commensal of the skin and mucous membranes of animals, mainly dogs. As an opportunist, it is isolated from 30% to 80% of cases of otitis externa and 30% cases of seborrheic and atopic dermatitis in dogs (Marshall et al. 1974; Kumar et al. 2002; Ziółkowska and Nowakiewicz 2004; Czyżewska et al. 2018b). Screening tests showed that members of this species also inhabit the skin of 20–80% of healthy animals (Grono and Frost 1969; Nakano et al. 2005). Otitis externa (inflammation of the external auditory canal) is a common disease, especially in cats and dogs at all ages. It manifests itself as pathological skin lesions in the external auditory canal, which may be caused by various etiological factors, among which *M. pachydermatis* occupies an important place (Świącicka et al. 2015). Rare cases of *M. pachydermatis* isolation from healthy human skin were explained by its transient presence in humans. Nonetheless, *M. pachydermatis* may be a good example of a zoophilic opportunist, which is increasingly associated

with some cases of human diseases (Theelen et al. 2018). Apart from skin lesions, it may cause systemic infections in cases when the host's immune system is impaired (Gueho et al. 1987, 1998; Chang et al. 1998; Dworecka-Kaszak 2004; Al-Sweih et al. 2014).

Currently, we should determine whether there are any specific features, in addition to the physiological condition of the host, that support the development of fungal infection. This problem is particularly important for opportunistic infections, where it is possible to assume the existence of typically commensal and potentially pathogenic strains. This issue is of particular importance: when mycoses affect an increasing percentage of people and animals, their treatment becomes more difficult, and the diseases are often recurrent (Zawirska and Adamski 2004). In addition, in the case of yeast from the genus *Malassezia*, the number of available drugs is limited (Cafarchia et al. 2012; Watanabe et al. 2014), and species considered to be typically zoophilic are surprisingly isolated from both

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sick and healthy people (Chang et al. 1998; Prohic and Kasumagic-Halilovic 2009).

The early diagnosis of opportunistic infections and identification of their etiological factors are still complicated. The increasing number of different dermatoses and issues with their diagnosis and treatment lead to the conclusion that the meaning of opportunistic fungi is still not understood and must be clearly analyzed. Naturally, some studies have reported about the differences among *M. pachydermatis* strains depending on the source of samples (Cafarchia et al. 2008; Nowakiewicz and Ziółkowska 2013; Park et al. 2013). Moreover, a few attempts were made to compare protein profiles of *M. pachydermatis*, which indicated significant differences. Denaturing electrophoresis demonstrated eight unidentified proteins that are characteristic only for the strains isolated from sick animals (Nowakiewicz and Ziółkowska 2013). On the basis of these data as well as the differences in lipid profiles between the two groups (Czyzewska et al. 2016), we assume the existence of typically commensal and potentially pathogenic strains within the species. Moreover, our recent results of random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) typing and internal transcribed spacer 1 (ITS1) sequencing have shown the formation of distinct clades and strong correlation between genotype and putative virulence of *M. pachydermatis* (Czyzewska et al. 2018a). All the data mentioned above support the hypothesis of the existence of typically commensal and potentially pathogenic strains within opportunistic species.

The aim of this research was (i) to determine and compare protein profiles using two-dimensional (2D) gel electrophoresis and (ii) to identify specific proteins (by tandem mass spectrometry) of *M. pachydermatis* strains isolated from dogs with otitis externa and dogs without symptoms of this disease to determine any distinguishing features. This could bring new insight into the diagnosis and treatment of *M. pachydermatis*-borne diseases, which is important from both cognitive and utilitarian point of view. This study provides important information that could be used to establish the method of detection of potentially pathogenic strains of *M. pachydermatis* as an example of opportunistic species.

MATERIALS AND METHODS

Yeast strains.—The strains of *M. pachydermatis* used in the present study were obtained from the Department of Preclinical Sciences (Warsaw University of Life Sciences), Department of Cytobiochemistry (University of Białystok), and Institute of Biological Bases of Animal Diseases (University of Life Sciences in Lublin). Among 64 strains

used in this study, 30 strains were isolated from dogs with clinical symptoms of otitis externa (dogs with active *M. pachydermatis* infection without any other disease diagnosed at the sampling time in the animal clinic) and the remaining 34 strains were isolated from healthy dogs (animals from animal shelter that did not have any symptoms of otitis externa during sampling and other diagnosed diseases for at least 1.5 years after isolation). For isolation of yeast, all swabs were grown in a solid Sabouraud medium with chloramphenicol (0.05 mg/mL) for 10 d at 37 °C. The identification of strains was confirmed on the basis on macroscopic (color, shape, and morphology of the colonies) and microscopic (shape and size of cells) properties and genotypic features determined using internal transcribed spacer restriction fragment length polymorphism (RFLP), according to Gupta et al. (2000). For the amplification, we used a combination of the following primers: ITS-1F (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-1R (5'-TCCTCCGCTTATTGATATGC-3'). The amplicons underwent enzymatic restriction with *EcoRI* endonuclease (Thermo Fisher Scientific, Waltham, Massachusetts). Restriction products were separated on agarose gels, stained with ethidium bromide, visualized with ChemiDoc XRS+ system (Bio-Rad, Hercules, California), and compared with reference band patterns to confirm initial identification. Finally, *M. pachydermatis* strains were stored in liquid yeast extract–peptone–D-glucose (YPD) medium (2% peptone, 2% glucose, 1% yeast extract) with 10% glycerol at –80 °C.

Protein extraction.—For protein extraction, all *M. pachydermatis* strains were grown in solid YPD medium for 7 d at 37 °C. The yeast cells were then harvested, homogenized by triple pulverization with liquid nitrogen, and stored in a solution (7 mol L⁻¹ urea, 2 mol L⁻¹ thiourea, 4% [w/v] CHAPS, 2% [v/v] immobilized pH gradient (IPG) buffer, 40 mmol L⁻¹ dithiothreitol [DTT]) at 7 °C for 24 h (1 mg of cells/1 µL) for protein extraction. Then, the suspensions were centrifuged for 15 min at 12 000 × g. Subsequently, proteins were purified using 2-D Clean-Up Kit (GE Healthcare, Chicago, Illinois), according to the manufacturer's instructions. After precipitation and centrifugation, each pellet was dried and resuspended in 50 µL of rehydration buffer (7 mol L⁻¹ urea, 2 mol L⁻¹ thiourea, 2% [w/v] CHAPS, 0.2% [v/v] IPG buffer, 18 mmol L⁻¹ DTT). For each sample, the amount of protein in relation to the fresh weight of yeast was determined. Protein concentrations (µg/µL) in the obtained supernatants were estimated using the 2-D Quant Kit (GE Healthcare).

Two-dimensional gel electrophoresis.—To perform isoelectric focusing, 24-cm-long IPG strips (pH gradient 3–10; GE Healthcare) were hydrated at room temperature for 10 h. An equal amount of protein was applied to each strip (120 µg per strip). Isoelectric focusing was performed using the Ettan IPGphor system (GE Healthcare), with the following parameters: 500 V for 1 h and gradually increased to 1000 V over 7 h. Next, the voltage was gradually increased to 8000 V over 3 h and then maintained at 8000 V for 5 h 36 min. Finally, IPG strips were equilibrated in buffer (6 mol L⁻¹ urea, 75 mmol L⁻¹ Tris-HCl buffer, pH 8.8, 24% [v/v] glycerol, 2% [w/v] sodium dodecyl sulfate [SDS]) containing 1% DTT for 15 min, and then for next 15 min in buffer containing 2.5% iodoacetamide.

Denaturing electrophoresis was performed in 10% polyacrylamide gel in the Ettan DALTsix vertical electrophoresis system (Bio-Rad). After the isoelectric focusing process, the gel strips were placed on the top of the polyacrylamide gels and then were flooded with 0.5% agarose solution with 0.002% bromophenol blue. Laemmli buffer was used as the electrode buffer (25 mmol L⁻¹ Tris base, 192 mmol L⁻¹ glycine, 0.1% [w/v] SDS). The electrophoresis was carried out with the following parameters: 2 W/gel for 45 min and then 17 W/gel for 4 h. Subsequently, the gels were placed in a fixing solution (50% methanol with 10% acetic acid) for 12 h.

To visualize individual proteins, gels were stained with 0.12% colloidal Coomassie blue and scanned using VersaDoc XRS+ system (Bio-Rad). The densitometric analysis of the obtained protein profiles was performed using the software Quantity One 4.1 (Bio-Rad). Finally, for the comparison of the protein profiles of *M. pachydermatis* strains from dogs without otitis externa (FIG. 1A) and from dogs with otitis externa (FIG. 1B), 21 spots were selected for densitometric analysis (FIG. 2). All analyzed spots were marked appropriately, and their area (mm²) and intensity/mm² ratio were then determined using Quantity One 4.1. Both these parameters were multiplied to obtain the intensity of each analyzed spot.

Protein identification by mass spectrometry.—For liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis, we chose spots present only in one group of tested isolates or ones that showed quantitative differences in the amount of protein between the tested groups. All analyzed spots were cut from gels obtained from both groups of tested strains. In cases where no specific protein was found in one of the studied groups of strains, a gel fragment corresponding to the position of this protein in a gel derived from the group where its presence was found was collected for analysis.

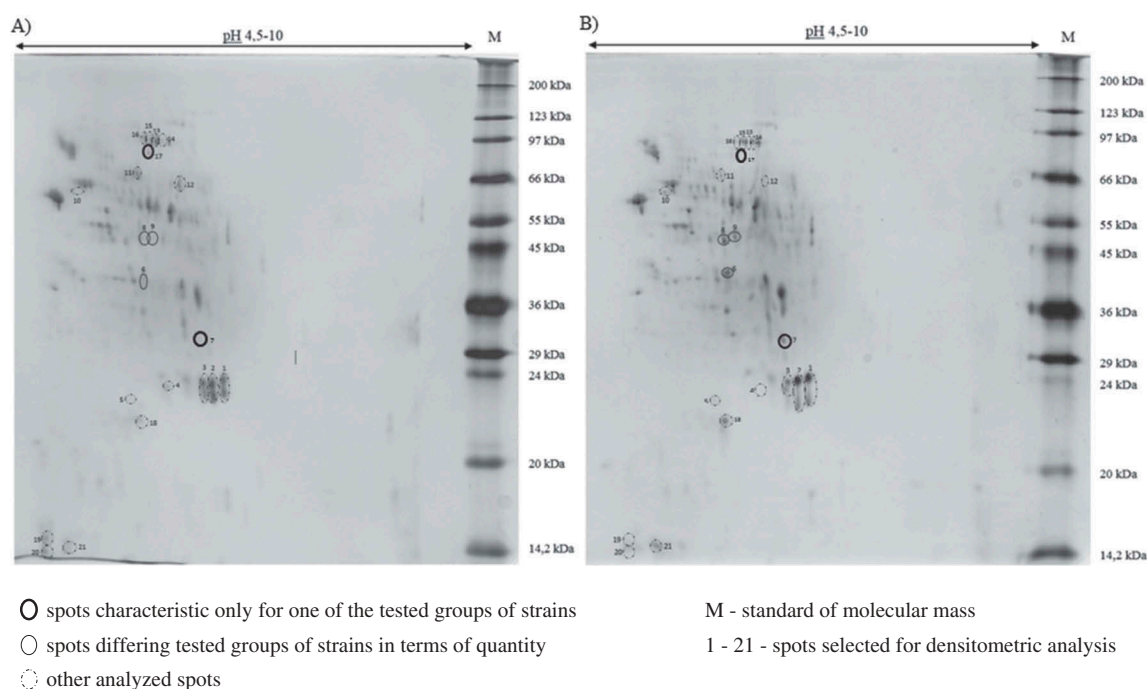


Figure 1. An example of two-dimensional electrophoresis of proteins isolated from *Malassezia pachydermatis* strains from dogs. A. Dogs without otitis externa symptoms. B. Dogs with otitis externa.

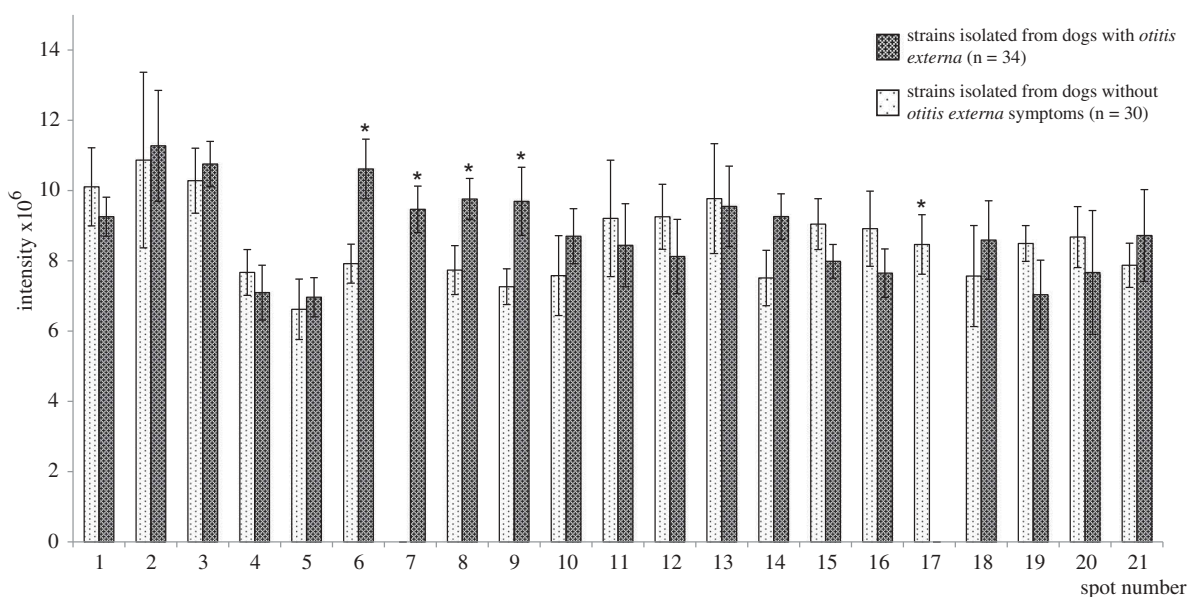


Figure 2. An example of two-dimensional electrophoresis of proteins isolated from *Malassezia pachydermatis* strains from dogs. A. Dogs without otitis externa symptoms. B. Dogs with otitis externa.

To identify the proteins in the spots visualized after SDS-polyacrylamide gel electrophoresis (PAGE), gel pieces were first manually excised, destained at 37 °C by several washes in 25% and 50% acetonitrile, reduced with 50 mmol L⁻¹ DTT at 37 °C for 1 h, and alkylated with 55 mmol L⁻¹ iodoacetamide at room temperature for 2 h in the dark. Next, gel pieces were dehydrated in 100% acetonitrile and dried twice for 15 min using SpeedVac vacuum concentrator (Thermo Fisher Scientific, Waltham, Massachusetts). Dried protein bands were digested in trypsin solution (10 ng/μL in 25 mmol L⁻¹ NH₄HCO₃, pH 8.0) at 37 °C for 18 h. After digestion, peptides were extracted by sonication, dried, and then resuspended in 10% acetonitrile with 0.1% formic acid.

For peptide analysis, LC-MS/MS was carried out on a HCT-Ultra ETD II mass spectrometer (Bruker, Bremen, Germany) coupled to an ultrahigh-performance liquid chromatography system (Dionex Ultimate 3000; Thermo Fisher Scientific, Waltham, Massachusetts). The peptides were separated on a 100 mm × 2.1 mm Accucore C18 column (particle size 2.6 μm; Thermo Fisher Scientific), with a gradient of 10–70% of 0.1% formic acid in 80% acetonitrile for 38 min. The mass spectrometer was operated in a standard MS/MS mode with simultaneous fragmentation of the most intensive precursor ions by collision-induced dissociation.

Mascot generic format (*.mgf) files were generated by preprocessing of the raw data using the software Data Analysis 4.0 (Bruker). The lists of obtained peaks

were searched against the nonredundant protein database of the National Center for Biotechnology Information (NCBI; entries: 119 007 460 sequences) or with taxonomy restriction (Fungi: 6 738 227 sequences) using a Mascot server (version 2.3.0; Matrix Science, London, UK). The following search parameters were applied: enzyme specificity: trypsin; permitted number of missed cleavages: 1; fixed modifications: carbamidomethylation (C); variable modifications: oxidation (M); protein mass: unrestricted; peptide mass tolerance: ±0.3 Da; and fragment mass tolerance: ±0.3 Da. Only the proteins identified with a Mascot protein score (defined as the sum of the highest ion score for each separate peptide sequence without the scores of duplicate matches) higher than 100 and the number of identified peptides higher than 2 were considered during analysis. The best Mascot parameters observed for a particular protein are listed in TABLE 1.

Statistical analysis.—The results of comparative densitometric analysis of selected proteins from strains representing the two groups are presented as mean value ± standard error. The normality of distribution was analyzed using the Shapiro-Wilk *W* test. The homoscedasticity of variances was analyzed using Levene *L* test. Because the data were not normally distributed, the significance of differences was determined using Mann-Whitney

Table 1. Proteins identified in selected spots in *Malassezia pachydermatis* strains.

Group of proteins	Name of the protein (protein accession number)	Isoelectric point	Molecular weight (kDa)	Spot on the gel	Score
Characteristic for strains isolated from dogs with otitis externa	NADP-dependent mannitol dehydrogenase (XP_017991091.1)	6.12	28	7	584
	Cytochrome <i>b</i> ₅ reductase (XP_017992936.1)	8.90	37	7	261
	ATPase v1 a1 complex subunit e (XP_017993085.1)	6.69	26	7	150
	Ketol-acid reductoisomerase (XP_017990341.1)	8.63	44	8	137
	Phosphoglycerate kinase	5.81	45	9	134
Common for strains isolated from dogs	Cytochrome <i>c</i> (XP_017993716.1)	5.79	34	6	271
	Hypothetical protein Malapachy_0291 (XP_017990058.1)	5.63	35	6	163
	Aldo keto reductase (XP_017990341.1)	5.61	72	6	152
	Fructose-bisphosphate aldolase (XP_017993717.1)	5.51	40	8	241
	Ubiquinol-cytochrome <i>c</i> reductase complex core protein 2 (XP_017991257.1)	6.02	45	8	222
	NAD-malate dehydrogenase (XP_017994086.1)	6.00	35	9	485
	Glyceraldehyde-3-phosphate dehydrogenase (XP_017993503.1)	6.19	36	9	174
	Guanine nucleotide binding protein beta subunit (XP_017993476.1)	5.84	34	6	233
	ATP synthase f1 gamma (XP_017993379.1)	7.79	33	6	150
	Polyadenylate-binding protein (XP_017990376.1)	5.83	69	17	150
Characteristic for strains isolated from dogs without otitis externa symptoms	Glycyl-tRNA synthetase (XP_017992146.1)	5.70	75	17	126

U test. Values for $P < 0.01$ were considered statistically significant.

RESULTS

The comparison of protein profiles of *M. pachydermatis* strains isolated from dogs with otitis externa (9.3 ± 1.35 µg/mg of tissue) and dogs without clinical symptoms of this disease (8.1 ± 1.6 µg/mg of tissue) indicated no statistically significant differences between the samples. In both groups, the mass of most proteins ranged between 36 and 66 kDa.

By using 2D electrophoresis, we obtained specific protein profiles of the two compared groups of strains. On the basis of these results, we demonstrated group-characteristic proteins. Spot 2 was chosen as the house-keeping spot because the expected protein amount did not vary between the two groups of tested strains (FIG. 2).

From the comparison of the intensities of protein spots (FIG. 2), we noted five spots in which the amount of protein differed between the two tested groups. Strains from clinical cases of otitis externa had higher protein content in spots 6, 8, and 9 (34%, 27%, and 33%, respectively). In addition, we observed a spot (7) in this group that was not found among strains from dogs without otitis externa. The opposite situation was observed for spot 17. The proteins from all spots mentioned above had the following molecular masses: spot 6, about 35 kDa; spots 8 and 9, about 45 kDa; spot 7, about 30 kDa; and spot 17, about 72 kDa. The observed differences were confirmed by analysis of the intensity of each spot versus that of the housekeeping spot 2 (SUPPLEMENTARY TABLE 1).

By using the LC-MS/MS method, we found significant differences in protein profiles between the groups of *M. pachydermatis* strains. These results, including the characteristics of proteins for each particular group of strains, are summarized in TABLE 1.

Each of the spots tested by the LC-MS/MS method contained two to five proteins. Among the identified proteins, five were characteristic for strains from dogs with otitis externa, four were typical for isolates obtained from dogs without clinical symptoms of any disease, and the remaining seven proteins were common for both groups of strains.

In details, spot 7 (FIG. 1B) was characteristic only for strains from infected dogs, and it was composed of three proteins: nicotinamide adenine dinucleotide phosphate (NADP)-dependent mannitol dehydrogenase (EC 1.1.1.138), cytochrome *b*₅ reductase, and e-subunit of the adenosine triphosphatase (ATPase) v1 a1 complex (EC 3.6.3.14). The densitometric analysis of electrophoregrams for these strains also confirmed quantitative differences in protein content for spots 6, 8, and 9 (FIG. 2). By using LC-MS/MS analysis, we proved that in addition to proteins present in both groups of strains (as observed for spots 6, 8, and 9), these spots are also composed of proteins characteristic only for strains from clinical cases of disease (spots 8 and 9). For example, these characteristic proteins were ketol-acid reductoisomerase (EC 1.1.1.86) in spot 8 and phosphoglycerate kinase (EC 2.7.2.3) in spot 9. Moreover, proteins typical only for putative commensal strains were also identified: polyadenylate binding protein and glycyl-tRNA synthetase (EC 6.1.1.14; spot 17, FIG. 1A).

DISCUSSION

One of the tasks of a proteome study is searching for differences that can be used to improve and facilitate clinical diagnosis. This could help to develop appropriate treatment and monitor a course of the disease (Kossowska et al. 2009). Currently, proteomics allows identifying any differences between protein profiles of microorganisms isolated from both infected and healthy individuals. The causative factor of the disease and its particular properties may be the effects of the presence of specific proteins (Nahaczewska et al. 2014). The synthesis of various proteins (including enzymes and antigens) by microorganisms is strongly correlated with their pathogenicity. In this context, protein profiling is an even more powerful tool used for this purpose than gene expression-based research, because it detects differences in final products and not only in the relative amounts of mRNA.

According to current knowledge, the development of otitis externa symptoms is correlated with the health status of an animal, including its metabolic, hormonal, or immune system disorders that predispose the host to infection (Masuda et al. 2000). However, in the context of recent findings, a hypothesis based on the properties of the fungus and assuming the existence of specialized, putative virulent strains has been recently proposed (Czyzewska et al. 2018b). Although the importance of the genus *Malassezia* in the pathogenesis of several diseases has been proved (Gaitanis et al. 2013), there are few data on the diversity of proteins in strains of different origins, especially commensals and isolates from clinical cases of dermatoses. An attempt to compare protein profiles of *M. pachydermatis* isolated from healthy dogs and those with ear inflammation by using one-way electrophoresis showed significant differences (Nowakiewicz and Ziółkowska 2013). There were eight bands found, with molecular masses ranging from 13 to 75 kDa, that were characteristic only for strains isolated from infected animals. This result may indicate some differences in the gene expression pattern of the two groups of strains or polymorphism of the genetic information possessed. The detected proteins may also be linked to the antigenic structure diversity of the two groups of strains (Zargari et al. 2003; Habibah et al. 2005). Unfortunately, the authors did not characterize proteins differentiating the two groups of strains more closely. Chen et al. (2002) detected an *M. pachydermatis* protein that caused an immune reaction in more than 50% of dogs with atopic dermatitis, whereas in healthy dogs this reaction was not observed. Similar measurements regarding the immune response in dogs caused by specific *M. pachydermatis* proteins were made by Bond and Lloyd (2002) and Kim et al. (2010).

In this study, the separation of proteins isolated from the strains obtained from dogs with otitis externa and healthy ones by 2D electrophoresis and subsequent identification by LC-MS/MS were performed to identify any potential differences between the study groups. Our approach was based on previous studies on both genetic and biochemical properties of *M. pachydermatis* from healthy dogs and from those with external ear canal inflammation. Significant differences in lipid profiles between representatives of the two groups (Czyzewska et al. 2016) were found to be in good agreement with genetic profiling using RAPD-PCR fingerprinting and ITS1 sequencing-based phylogeny reconstruction (Czyzewska et al. 2018a). The results strongly suggested that there may be two groups of strains—commensal and virulent—with distinct features and adaptations within the analyzed species.

The spectroscopic studies revealed proteins present in only one of the groups as well as proteins that exhibit differences in quantity between the two groups. The presence of unique protein characteristic only for strains from infected animals supports the hypothesis of the existence of potentially pathogenic strains among commensals within *M. pachydermatis*. A question arises as to whether the identified proteins can be considered to be markers for pathogenicity. Our interest was aroused mainly by the presence of NADP-dependent mannitol dehydrogenase (EC 1.1.1.138) in strains from dogs with otitis externa. This enzyme plays an important role in the mannitol cycle, catalyzing the oxidation reaction of D-mannitol to D-fructose, which is involved in the regeneration of NADPH in cells of numerous fungi. It has also been proved that this enzyme exists as a single isoform. The characteristics of this protein in *Aspergillus parasiticus* were reported by Niehaus and Dilts in 1982. It is important to note that this protein may be associated with the pathogenicity of microorganisms. Simon-Nobbe et al. (2006) proved that this is the main allergen of *Cladosporium herbarum*, an important etiological factor of allergy in almost all climatic zones. They also revealed that NADP-dependent mannitol dehydrogenase is recognized by immunoglobulin E (IgE) antibodies in approximately 57% of all patients suffering from allergies associated with *C. herbarum*. Moreover, NADP-dependent mannitol dehydrogenase is an important allergen of *Alternaria alternata*, which is an opportunistic pathogen of over 380 host plant species, causing leaf spots as well as rots and blights on many plant parts. In humans, *A. alternata* is an important cause of inhalation allergy worldwide, with sensitization rates ranging from 2% to 30% among the allergic population (Schneider et al. 2006).

Another important finding was related to ketol-acid reductoisomerase (EC 1.1.1.86), which was present only among putative virulent isolates. This enzyme is involved in the branched-chain amino acid biosynthesis that occurs in various organisms, including bacteria, fungi, and higher plants, but not in animals. Liu et al. (2014) characterized the ketol-acid reductoisomerase from *Fusarium graminearum*, a facultative parasite of many plant species, from cereals to tropical crops. They also proved (by using mutants with the deletion of the gene encoding the protein) that this enzyme is necessary for the complete virulence of *F. graminearum*. Moreover, this enzyme is also one of the essential enzymes in *Aspergillus* for the biosynthesis of leucine, isoleucine, and valine; therefore, it is considered to be a potential target for antifungal drugs. Furthermore, the disruption of leucine, isoleucine, and valine biosynthetic pathways may affect the survival of *Aspergillus* spp. under the limitation of threonine. Thus, targeting this enzyme will not alter the amino acid metabolism in humans with simultaneous inhibition of numerous metabolic pathways in pathogenic organisms (Morya et al. 2012). On the basis of these findings, we may hypothesize that ketol-acid reductoisomerase could be important in the infection process caused by *M. pachydermatis*.

In conclusion, we mention that besides the recognized role of host health condition in the etiology of otitis externa, there may be fungus-related properties enhancing its pathogenic potential. In particular, NADP-dependent mannitol dehydrogenase and ketol-acid reductoisomerase may be important in the disease symptom development, and consequently they may be regarded as potential virulence markers and treatment targets.

Thus, our results may find practical application in more precise diagnosis and identification of potentially pathogenic strains. Selected proteins may be used as the markers for strain pathogenicity and could be used in appropriate immunoassays. Such tests can help to assess whether the carrier is at risk for the disease and, possibly, take appropriate preventive measures even before the onset of symptoms.

The obtained results may be a promising starting point for more detailed analyses in the future using genetic techniques and biological tests. They may be the basis for gene deletion or overexpression experiments in combination with the animal model—a condition necessary to fulfill Koch's molecular postulates for any candidate proteins. We can hypothesize that variations in protein expression can be associated with general strain-specific differences and can cause different virulence potentials. However, they could also be the result of natural selection during the lifetime of fungi in an inflammatory environment (otitis externa), rather than the cause of disease.

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