Cyclophilin and enolase are the most prevalent conidial antigens of Lomentospora prolificans recognized by healthy human salivary IgA and cross-react with Aspergillus fumigatus

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Abstract

Lomentospora (Scedosporium) prolificans is an emerging pathogen which causes high mortality diseases in immunosupressed patients. However, this fungus rarely infects immunocompetent individuals and, consequently the study of their immune response may provide important information that can improve the therapeutic efficacy against this multiresistant fungus. So, as *L. prolificans* penetrates into a host, above all, through their airways, the aim of this study was to identify the most prevalent conidial antigens of *L. prolificans* recognized by healthy human IgA, and to study their expression and cross-reactivity with other fungal species. To achieve this, ten proteins recognized by more than 50% of saliva were detected by 2D immunoblotting and identified by LC-MS/MS. Among these, cyclophilin and enolase were the most prevalent, recognized by 85 and 80% of the samples, respectively. These enzymes were also identified on the cell-wall surface of this fungus and on the immunomes of *Scedosporium apiospermum* and *S. aurantiacum.* Additionally, it is worth highlighting the fact that they showed cross-reactivity with the commonest pathogenic filamentous fungus *Aspergillus fumigatus*. In conclusion, these results show that the immunocompetent immune response might offer a pan-fungal recognition of conserved antigens such as enolase and cyclophilins, making them potential candidates for study as therapeutic targets.

Statement of significance of the study

In the last few decades the incidence of invasive mycosis has increased considerably, killing about one and a half million people every year. Of particular concern is the rise in the number of clinical cases produced by *Lomentospora prolificans* and the high mortality rates associated with them, mainly due to the intrinsic resistance of this fungus to the currently available antifungal medicines. There is therefore, an urgent need for effective new therapeutic tools and in consequence, we studied the humoral response in saliva from immunocompetent people. These individuals are not affected by this group of infections in spite of being in contact with the fungus frequently. Concretely, cyclophilin and enolase were identified as the most prevalent antigens, which are located on the fungal cell surface and therefore are easily accessible to the immune system and to treatment with drugs. Moreover, they were also identified in related fungi, *Scedosporium apiospermum* and *S. aurantiacum*, and more importantly, showed cross-reactivity with *Aspergillus fumigatus*. Consequently, this study is a step forward in the search for alternative ways of fighting these devastating diseases. We therefore state that the conserved enzymes mentioned above have significant potential as therapeutic targets or as immunogens for the development of vaccines. Furthermore, they may be able to protect not only against *L. prolificans* but also against other fungal species.

1 Introduction

Lomentospora prolificans is a filamentous fungus considered as an emerging pathogen owing to the increase in the number of clinical cases over the past decades [1]. This trend is associated with the rise in life expectancy and in the number of immunosupressed patients susceptible to these infections. According to the latest epidemiologic survey carried out in Spain [2] species of the *Scedosporium* genus, in which *L. prolificans* was included until last year [3], are at this moment in time the second most common cause of infection by filamentous fungi behind *Aspergillus*.

Within the *Microascaceae* family, *Scedosporium apiospermum*, *Scedosporium aurantiacum* and *L. prolificans* are the most clinically relevant species. Of these, *L. prolificans* is associated, above all, with disseminated disease, while the others typically with near-drowning syndrome and mycetoma formation [3,4]. In addition, most of the infections caused by these species worldwide are due to *S. apiospermum*, but in countries such as Australia and Spain the infections caused by *L. prolificans* and *S. aurantiacum* are on the rise [2,5]. However, the most important difference lies in the high mortality rates, up to 87.5% in the case of disseminated infections caused by *L. prolificans* [6], primarily due to its intrinsically antifungal resistance [7,8] and the kind of infections caused [3,6,9,10].

Studies into its environmental distribution indicate that *L. prolificans* is commonly spread in humanized areas [11], where conidia are likely be inhaled. Therefore, consistent with the fact that infections are almost exclusively suffered by immunocompromised people, it seems that the immune system of immunocompetent individuals is able to control the fungus. Consequently, taking into account the limited success of antifungal drugs-based therapy [6], the study and understanding of the

immune response of healthy people from the countries where these fungi show high prevalence could be crucial in the design of new treatments, such as vaccines or antibodies, to increase therapeutic efficacy. Hence, over the last few years interest in monoclonal antibodies to treat infections caused by fungal pathogens, such as *Cryptococcus neoformans*, *Candida albicans,* and *S. apiospermum,* has increased [12–16].

In addition to therapeutic problems, diagnosis of these infections is also difficult, classical methods such as fungal culture, direct microscopy, and histopathology are still currently employed. Furthermore, the promising molecular methods developed have not yet been validated for clinical use [17,18]. Therefore, an improvement in diagnostic tools, resulting from the identification of new markers or antigens, may be essential to establish a proper treatment and reduce the very high morbimortality caused by this fungus.

Taking all these factors into account as well as the fact that salivary immunoglobulins A (IgA) are one of the most important defence mechanisms against microorganisms whose principal route of infection is the airways, the main objective of this project was to identify the most prevalent conidial antigens of *L. prolificans* recognized by immunocompetent human salivary IgA from healthy donors resident in the Basque Country (Spain). Moreover, the immunome of this fungus was compared with the phylogenetically related fungi *S. apiospermum* and *S. aurantiacum*, and finally a study was made into the cross-reactivity of these antigens with the commonest filamentous fungus *Aspergillus fumigatus*.

2 Materials and methods

2.1 Strains and collection of conidiospores

Lomentospora (Scedosporium) prolificans strain CECT 20842, isolated from a patient with a disseminated infection at the Hospital Marques de Valdecilla (Santander, Spain) was used in this study. The comparative study was performed with the strains *Scedosporium apiospermum* UPV 93-251 and *Scedosporium aurantiacum* CBS 116910, and for the cross-reactivity study *Aspergillus fumigatus* strain Af293.

All the strains were cryopreserved at -80°C and sub-cultured on Potato Dextrose Agar (PDA) (Pronadisa, Madrid, Spain) at 37°C for 7 days before use. To obtain conidiospores, plates were washed twice with sterile saline (0.9% NaCl) and the suspension filtered by gauze and centrifuged. The concentration was adjusted with a hemocytometer to inoculate 5 x 10^5 conidiospores/ml into Potato Dextrose Broth (Pronadisa) and incubated at 37°C for 7 days. Finally, the collection of conidiospores was performed through gauze filtration and centrifugation.

2.2 Human saliva sample collection and preparation

Twenty salivary samples for the antigenic study and forty for the ELISA were collected from healthy immunocompetent volunteers resident in the Basque Country. After collection, the saliva samples were centrifuged to eliminate cell debris. For the antigenicity study, 1 ml of each sample was mixed to obtain a pool of saliva. Finally, all saliva samples were conserved at -20°C until used. The procedures involved were approved by the Ethics Committee of the University of the Basque Country (UPV/EHU).

2.3 Protein extraction

Whole cell protein extraction was performed as previously described, with minor modifications [19]. Briefly, conidiospores were disrupted in PBS containing 1% (v/v) 2-mercaptoethanol and 1% (v/v) ampholytes pH 3-10 (GE Healthcare, Freiburg, Germany) by bead beating using the MillMix 20 bead beater (Tehtnica, Slovenia) for 20 min at 30 Hz. Cell debris was removed by centrifugation and supernatant was stored at -20ºC until use. On the other hand, cell wall-associated protein extraction was carried out following the protocol of Pitarch *et al.* (2002) [20]. Shortly, conidiospores were maintained for 10 min at 100°C in an extraction buffer (50 mM Tris-HCl pH 8.0, 0.1 M EDTA, 2% (w/v) SDS, 10 mM DTT). They were then centrifuged and supernatant collected. Once cell wall-associated proteins had been extracted conidiospores were stained with white calcofluor (40 ng/ml) and propidium iodide (10 µg/ml) for 30 min in the dark and visualized with an epifluorescence microscope so as to verify that conidiospores were intact and, consequently, cytoplasmic proteins had not been released. Positive control was carried out with autoclaved cells (121°C, 30 min) [21].

Protein concentration was determined using the Pierce 660 nm Protein Assay Reagent (Thermo Fisher Scientific, Rockford, USA). Subsequently, the precipitation of proteins was carried out in four volumes of acetone containing 10% (w/v) trichloroacetic acid and 0.07% (v/v) 2-mercaptoethanol, for 1 h at -20°C. Then they were centrifuged for 20 min at 14,100g. Finally, pellets were washed with acetone, air-dried and re-suspended in a rehydratation buffer (7 M urea, 2 M thiourea, 20 mM Tris, 4% (w/v) CHAPS, 1% (v/v) ampholytes, 1% (v/v) 2-mercaptoethanol, 0.002% (w/v) bromophenol blue).

2.4 Two-dimensional electrophoresis (2DE) and detection of antigens recognized by human saliva

For isoelectric focusing (IEF) 180 µg of proteins were loaded into 7-cm-long Immobiline DryStrip pH 3-10 (GE Healthcare). Thereafter, IEF was carried out in the Ettan IPGphor (GE Healthcare) with the following protocol: 10 h rehydratation, 500 V for 2,000 Vhr, 1,000 V for 3,000 Vhr, 5,000 V for 10,000 Vhr and 5,000 V for 40,000 Vhr. Once the IEF was completed strips were incubated twice for 15 min in equilibration buffer (6 M urea, 75 mM Tris-HCl pH 6.8, 25.5% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue), first with 1% (w/v) DTT and then with 2.5% (w/v) iodoacetamide. Finally, the second dimension was carried out in a Mini-PROTEAN II system (Bio-Rad, Hercules, CA, USA) at 70 mA using 12.5% polyacrylamide gels. Precision Plus Protein™ Standards (Bio-Rad) were used as molecular weight markers.

To detect antigens recognized by IgA, 2DE gels were electrotransferred to Hybond-P PVDF membranes (GE Healthcare) using Trans-Blot SD (Bio-Rad) at 400 mA for 20 min, and Western blot (WB) was performed following the previously optimized protocol [19]. For *L. prolificans* antigens prevalence study one replica was carried out for each of the twenty salivary samples analyzed, and for the interspecific comparative study, experiments were performed in triplicate for each of the species. Thus, the immunoreactive proteins were detected using ECL Plus (GE Healthcare) in G:BOX Chemi system (Syngene, Cambridge, United Kingdom). The analysis of 2DE and WBs was carried out by image analysis using ImageMaster 2D Platinum Software (GE Healthcare), and the relative volume of the antigens was represented on a heatmap using the MeV program (Multiple Experiment Viewer).

2.5 Immunogenic protein identification

Immunoprevalent proteins were manually extracted from CBB G-250 (Sigma-Aldrich) stained gels and identified by LC-MS/MS in the proteomics facility of general services (SGIker) of the UPV/EHU.

Extracted gel pieces were swollen in digestion buffer (50 mM NH_4HCO_3, 12.5 ng/µl trypsin (Roche, Basel, Switzerland)) and incubated at 37°C overnight. After that the peptides were extracted, first with 25 mM NH_4HCO_3 and ACN, and secondly with 0.1% (v/v) trifluoroacetic acid and ACN. The recovered supernatants were pooled and dried in a SpeedVac (Thermo Electron, Waltham, MA, USA) and then dissolved in 10 µl of 0.1% (v/v) formic acid (FA) and sonicated for 5 min.

LC-MS/MS was carried out on a SYNAPT HDMS mass spectrometer (Waters, Milford, MA, USA) interfaced with a nanoAcquity UPLC System (Waters). To do this 8 µl from each sample was loaded into a Symmetry 300 C18, 180 µm x 20 mm precolumn (Waters) and washed with 0.1% (v/v) FA at a flow rate of 5 µl/min for 3 min. The precolumn was connected to a BEH130 C18, 75 µm x 200 mm, 1.7 µm (Waters), equilibrated in 3% (v/v) ACN and 0.1% (v/v) FA. Peptides were eluted with a 30 min linear gradient of 3–60% (v/v) ACN directly onto a NanoEase Emitter (Waters). Capillary voltage was set to 3,500 V and data-dependent MS/MS acquisitions performed on precursors with charge states of 2, 3, or 4 over a survey m/z range of 350–1990.

The spectra processing obtained was performed using a VEMS [22], and searched on the NCBI non-redundant database restricted to Fungi (version 20150309) using the

online MASCOT server (Matrix Science Ltd., London; http://www.matrixscience.com). For protein identification the carbamidomethylation of cysteines was carried out as a fixed modification and oxidation of methionines as a variable modification. Up to one missed cleavage site was permitted, and values of 50 ppm for peptide were fixed along with 0.1 Da for fragment mass tolerances. Protein identification was performed by comparison with orthologous proteins from other fungi because the *L. prolificans* genome sequence is not yet completely annotated.

2.6 Analysis of localization and function of *Lomentospora prolificans* antigens by bioinformatics tools

Antigen localization and function analysis were carried out by bioinformatic analysis of the sequences. Concretely, TargetP (http://www.cbs.dtu.dk/services/TargetP/) and SecretomeP 2.0 (http://www.cbs.dtu.dk/services/SecretomeP/) software were used to predict localization and secretory pathway. Results were considered positive, when the score was ≥ 0.5 for TargetP and ≥ 0.6 for SecretomeP 2.0. Moreover, to predict adhesins FaaPred (http://bioinfo.icgeb.res.in/faap/) was used, using a score threshold of -0.8. In addition the PEDANT web server (http://pedant.gsf.de) was used to study protein functionality according to the Functional Catalogue (FunCat).

2.7 Detection of salivary IgA by ELISA

The ELISA test was carried out, in triplicate, with 40 salivary samples using whole conidia or conidial protein extract as an antigen. So, 8 x 10^5 conidia or 5 µg of protein were added to ELISA plate wells and incubated at 4°C overnight. Then these wells were blocked and incubated for 1 h with TBS containing 5% (w/v) milk powder and 0.05%

(v/v) Tween 20. After that, saliva samples diluted 1/4 on TBS containing 0.05% (v/v) Tween 20 (TBST) were incubated for 1 h. Subsequently, cells were washed 3 times with TBST and incubated for 30 min with anti-human IgA-HRP diluted 1/50,000 in TBST. Afterwards, wells were washed again in triplicate with TBST and incubated with TMB (Sigma-Aldrich) for 30 min. Finally, the reactions were stopped by addition of 1 N H_2SO_4 and plates were read at 450 nm using the BioTek Synergy HT plate reader (BioTek Instruments, Winooski, VT). The differences between the absorbance values obtained for the three species were analyzed by SPSS 20 software using one way ANOVA with a confidence interval of 95%.

2.8 Cross-reactivity of salivary IgA against *Aspergillus fumigatus* and *L. prolificans*

Conidia of *A. fumigatus* were recovered using sterile saline containing 0.02% Tween 20 (v/v) from PDA incubated 7 days at 28°C. Afterwards, 10^{10} conidia were incubated for 1 h in 40 ml of a saliva pool at room temperature. Subsequently, this suspension was centrifuged at 4,500g for 20 min, saliva was then discarded and conidia washed three times with 20 ml of TBS. To release cell-bound antibodies, conidia were incubated for 3 h with 20 ml of a 0.1 M glycine-HCl buffer (pH 2.5). Finally, supernatant was collected by centrifugation at 4,500g for 20 min and dialyzed against TBS. The cross-reactivity between *A. fumigatus* and *L. prolificans* was studied in triplicate by 2-D WB. This was achieved by using electrotransferred membranes from *L. prolificans* conidia extracts to detect antigens recognized by purified anti-*A. fumigatus* IgAs.

3 Results

3.1 Prevalence and reactivity study of the *Lomentospora prolificans* antigens recognized by salivary IgA from healthy donors

The detection of immunogenic proteins of *L. prolificans* by human salivary IgA was performed by 2DE and WB. The immunomes obtained using the 20 saliva samples were compared to the one obtained using the pool of them. Thus, 104 antigenic spots were studied, most of them located between 4-7 p*I* and higher than 25 kDa. Of these, 10 spots were detected by more than 50% of the saliva, the most prevalent being cyclophilin and enolase, which were detected in 85 and 80% of the WBs, respectively (Table 1; Figure 1A-B). In addition, cyclophilin was identified in two spots with nearly the same *M*r but different p*I.*

Furthermore, the relative volume of each protein was represented as a heatmap, which shows a high level of differences between individuals. Moreover, although there does not seem to be a clear relationship between the relative volume and the prevalence of the spots, cyclophilin and enolase were, as expected, among the antigens that exhibited the highest relative volume values (Figure 2A-B).

3.2 Study of the localization and functionality of the *Lomentospora prolificans* antigens

Two methods were used to study the localization of the most prevalent antigens of *L. prolificans* identified in this study: bioinformatical and empirical.

The bioinformatics-based analysis showed that, except for malate dehydrogenase (Mdh) which showed adhesin-like properties and non-classical secretion, none of the proteins seemed to be secreted. In fact, the cyclophilins, the dihydrolipoyl

dehydrogenases and the heat shock protein 70 (Hsp70), were predicted to have mitochondrial localization, and the other four antigens to contain neither mitochondrial targeting nor secretory signal peptides (Figure 2C).

On the other hand, the characterization of the proteome and immunome of cell wall-associated proteins showed 119 spots, up to 34 being recognized by IgAs (Figure 1C-D). Among them, two spots were detected at the same *M*r and p*I* as enolase and the most prevalent cyclophilin in the whole conidia extract analyzed previously. Consequently, their identities were corroborated by LC-MS/MS (Table 1).

The analysis of antigen functionality was carried out by the PEDANT web server, which showed that most of proteins identified were related to metabolism, protein fate, compound transport and, cell rescue, defense and virulence (Figure 2D).

Regarding the most prevalent salivary antigens, cyclophilin is related to protein modification, folding and stabilization, apoptosis, as well as stress response. On the other hand, enolase is involved in glycolysis and gluconeogenesis, and in the metabolism of diverse substances.

3.3 Comparative analysis of the recognition of salivary IgA against *Lomentospora prolificans*, *Scedosporium apiospermum*, and *Scedosporium aurantiacum* among a healthy population

Since *L. prolificans* is phylogenetically closely related to the *Scedosporium* genus, the salivary IgA recognition against *L. prolificans*, *S. aurantiacum* and *S. apiospermum* among the population was compared by ELISA using conidia or protein extract as antigens. Both studies showed similar signal levels between the three species and high dispersion of the data (Figure 3A). Furthermore, it is worth mentioning the fact that in

the ELISA carried out against total protein extracts, much higher signals were obtained than in the one against whole conidia cells.

On the other hand, a further study into whether the most prevalent antigens of *L. prolificans* are also present on *S. apiospermum* and *S. aurantiacum* was also carried out. The results showed that their proteomic and immunomic profiles are very different, with greater similarity between the two species from the *Scedosporium* genus. Nevertheless, a similar common pattern was observed in the three species at 4-6 pH with M*r* over 50 kDa (Figure 3B-G).

In the immunome study, at least 3 antigens common to the three species were found, all of which corresponded to the enolase and cyclophilins by comparing the Mr and p*I* with the immunome of *L. prolificans* (Figure 3E-G). Two of them, enolase and the most prevalent cyclophilin, were identified to make sure that they were the same on the three species analyzed (Table 1).

3.4 Cross-reactivity study between *Aspergillus fumigatus* and *Lomentospora prolificans*

With the purpose of detecting cross-reactivity between *A. fumigatus* and *L. prolificans*, the salivary IgAs that specifically bind to *A. fumigatus* conidia were purified and utilized to detect *L. prolificans* antigens.

The results obtained showed that 16 antigens of *L. prolificans* are recognized by cross reactivity with *A. fumigatus* antigens. From these, the antigens with the highest prevalence against human salivary IgA, enolase and cyclophilins, were also detected. (Figure 4).

4 Discussion

Although fungal infections affect millions of individuals and cause high mortality rates, often over 50%, total spending on medical mycology research is very low compared to the funding available for the study of other infections [23]. Moreover, currently disposable treatments are highly toxic and not efficient against some fungi such as *Lomentospora prolificans* which, above all, affects immunosupressed patients. Thus, the study of the immune response among immunocompetent population, which is normally able to avoid these infections, may offer new opportunities for the development of new more effective treatments. Taking into account this fact and that human IgA recognize *L. prolificans* conidia very strongly, but hyphae poorly [19], the present study was designed to identify the most prevalent antigens of *L. prolificans* recognized by saliva from a healthy population in conidial morph. Moreover, their location in this fungus, and the cross-reactivity with other related species was studied.

In the antigenic prevalence study, cyclophilin and enolase were shown to be the most prevalent salivary antigens. In fact, all the samples used exhibited reactivity against at least one of the two proteins and therefore, antibodies against those enzymes could offer 100% cover against *L. prolificans*. The other prevalent antigens identified were ribosomal protein S18, hypothetical protein (tubulin), Mdh, dihydrolipoyl dehydrogenase, Hsp70, and putative branched-chain-amino-acid aminotransferase (BCAT) TOXF. Their high prevalence, over 50%, highlights the interest in these antigens considering the different antigenic patterns observed among the population studied.

Regarding their antigenicity, while ribosomal protein S18, tubulin, dihydrolipoyl dehydrogenase and putative BCAT have not been described as antigens on other fungi, enolase, cyclophilin, Mdh and Hsp70 are well-known antigens in fungi [24-29]. In fact,

the latter three have also been identified previously as immunodominant antigens of *L. prolificans* conidia recognized by human IgA [19] and although other antigens, such as enolase, were not detected in the study, this may be due to differences in protein extraction protocol and in spot selection criteria, since in that study antigens were identified according to their relative intensity but not prevalence. Furthermore, enolase, Mdh and Hsp70 have recently been described as *L. prolificans* antigens, showing high reactivity against sera from immunocompetent individuals with up to 70%, 90% and 100% of prevalence, respectively. Furthermore, enolase and Hsp70 were also identified as cell wall associated antigens recognized by healthy human IgG [30]. Additionally, enolase and cyclophilin were identified in the cell wall-associated subproteome. Therefore, these antigens may also be good targets because of their accessibility to the immune system. Their extracellular location is not surprising since cyclophilin has been detected previously in the wall of the pathogenic fungus *Histoplasma capsulatum* as a receptor of dendritic cells [31] and enolase has been identified as a main wall protein in *C. albicans* [32].

Furthermore, immunomic comparative studies were carried out between *L. prolificans* and the species *S. apiospermum* and *S. aurantiacum* because of their phylogenetic proximity [3] and clinical relevance [4,5]. First, an ELISA assay was performed to compare IgA concentration among healthy people. The signal obtained was higher when protein extracts were used rather than whole conidia, which might be due to different amounts of target protein on conidia and extracts, or to the fact that conidial antigens could be partly hidden or intracellularly located. However, no significant differences were found between the three species whatever. Taking into account the fact that some of the antigens identified are broadly conserved among fungal

pathogens, these results suggest that cross-reactivity between species might play a significant role in the detection of these fungi.

To complete the comparative study, the three proteomes and immunomes were analyzed. The very different patterns that resulted were not surprising since *L. prolificans* is separated from the *Scedosporium* genus [3]. Nonetheless, cyclophilin and enolase were also detected in *S. apiospermum* and *S. aurantiacum.* As already mentioned, a cross-reactivity process could explain this result as well as their high prevalence, and may well occur with other fungi. Consequently, a cross-reactivity study was performed against *A. fumigatus* since it is the most common filamentous fungus related to airborne infections worldwide [23,33]. This study showed that IgAs purified by binding to *A. fumigatus* were also able to detect *L. prolificans* enolase and cyclophilins, proving that cross-reactivity occurs among these proteins from different filamentous fungal species. This is not surprising, since cyclophilins have been described as pan-allergens across fungal species and even indirectly in humans [25], which should be studied in greater depth to avoid undesirable results if cyclophilins are to be considered for the development of vaccines in the future. It can therefore be deduced that IgA may have a protective role against these highly conserved enzymes, at least in part, against different fungal species, representing a pan-fungal immune response. The use of one of these enzymes for therapy, enolase, has been partially tested as vaccine against *C. albicans*, bringing about a decrease in fungal burden and tissue damage [34]. However, the effect against other fungi has not so far been tested.

In conclusion, due to the high prevalence and reactivity of cyclophilin and enolase of *L. prolificans* against IgA and their localization on the cell wall surface, these enzymes seem to have good potential as therapeutic targets or as immunogens for developing

protective vaccines. Furthermore, the cross-reactivity studied here shows that therapies against enolase and cyclophilins may protect against different, common fungal pathogens.

5 References

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Conflict of interest statement

The authors have declared no conflict of interest.

Figure legends

Figure 1. 2D proteome and immunome analysis of *Lomentospora prolificans* conidia. Images of the whole proteome (A) and immunome reactive against human salivary IgA (B). Antigenic spots detected by more than 50% of the samples are marked. Cell wall-associated proteome (C) and immunome (D) images, where circled spots correspond to cyclophilin (1) and enolase (2).

Figure 2. Prevalence, relative volume, localization and function of the most prevalent antigens of *Lomentospora prolificans* recognized by IgA from healthy donors. Heatmap (A) and graphical (B) representation of prevalence and relative volume. Bioinformatic study of the cellular localization and adhesin-like properties (C), and functional analysis according to FunCat (D).

Figure 3. ELISA tests, 2D proteomes and 2D immunomes of *Lomentospora prolificans*, *Scedosporium apiospermum* and *Scedosporium aurantiacum*. Comparison by ELISA of IgA concentration against whole conidium cell and total protein extract of *L. prolificans*, *S. apiospermum* and *S. aurantiacum* using 40 saliva samples (A). Proteomes (B-D) and salivary immunomes (E-G) of *L. prolificans*, *S. apiospermum* and *S. aurantiacum*. Cyclophilins (1, 3) and enolase (2) are marked.

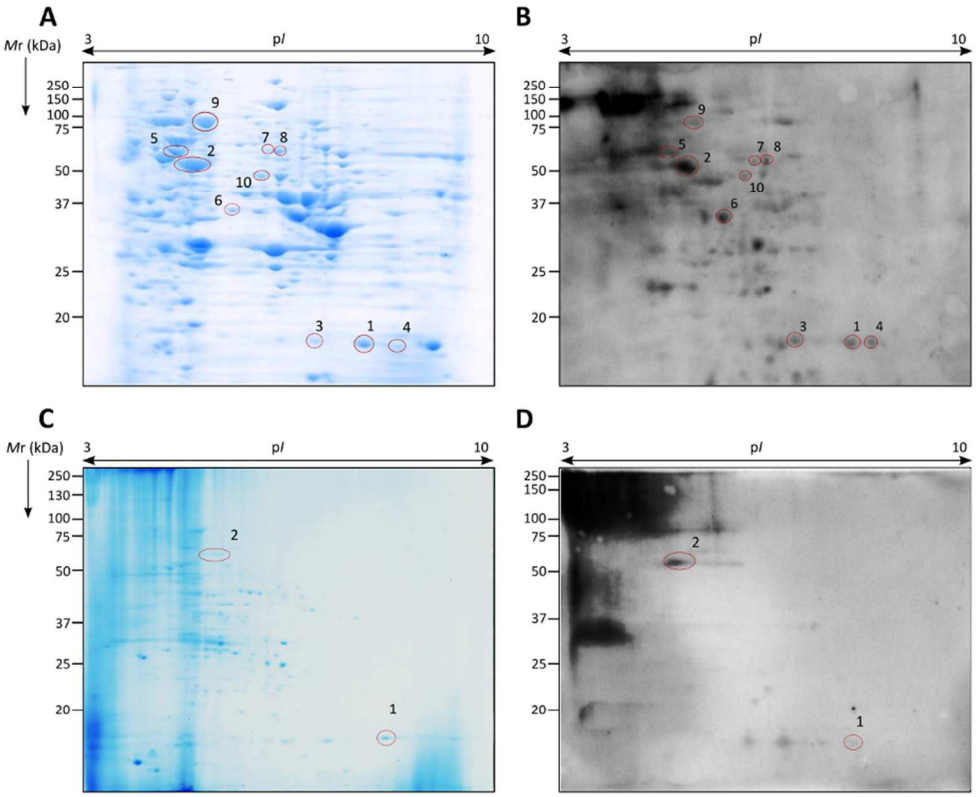
Figure 4. Study of the cross-reactivity between *A. fumigatus* and *L. prolificans* detected by salivary IgAs. *A. fumigatus* conidia were incubated in a saliva pool for 1 h at 37°C, and then harvested and incubated in Gly-HCl buffer (pH 2.5) to release *Aspergillus*-

bound antibodies (A). Finally, the purified and dialyzed anti-*A. fumigatus* IgAs were used to detect *L. prolificans* conidial proteins by WB (B). Cyclophilins (1, 3) and enolase (2) are marked in the immunoblot.

Table 1- Identification by LC-MS/MS of the most prevalent antigens of *Lomentospora prolificans* conidia against human IgA, cell wall antigens of *Lomentospora prolificans* conidia, and *S. apiospermum* and *S. aurantiacum* conidia against human IgA.

Spot no.	Prevalence (%)	Accession no.	Protein name	Orthologous to	Matching peptides (no.)	Sequence coverage (%)	MASCOT score	Theor. pI/Mr (KDa)	Exper. pI/Mr (KDa)
Prevalent antigens of *L. prolificans* conidia (Figure 1A)									
1	85	gi|33357681	Putative mitochondrial cyclophilin 1	*Botrytis cinerea*	2	13	150	9.14/24.27	8.22/19.11
2	80	gi|666868085	Enolase	*Scedosporium apiospermum*	23	28	747	5.19/47.50	5.38/55.33
3	60	gi|302924262	Cyclophilin type peptidyl-prolyl cis-trans isomerase/CLD	*Colletotrichum fioriniae* PJ7	4	12	149	9.73/25.52	7.49/18.32
4	60	gi|154270985	40S ribosomal protein S18	*Histoplasma capsulatum* NAm1	2	14	152	10.56/18.00	8.76/16.24
5	60	gi|666868431	Hypothetical protein (tubulin)	*Scedosporium apiospermum*	13	15	417	4.94/50.70	4.63/53.19
6	60	gi|685397755	Malate dehydrogenase	*Gaeumannomyces graminis var. tritici* R3-111a-1	14	21	463	5.87/34.67	5,89/36,02
7	60	gi|930140064	Dihydrolipoamide dehydrogenase	*Grosmannia clavigera* kw1407	5	9	444	6.66/54.99	6,42/60,10
8	55	gi|367032352	Hypothetical protein MYCTH_2309210 (dihydrolipoyl dehydrogenase)	*Myceliophthora thermophila* ATCC 42464	3	8	254	7.64/54.41	6,63/56,11
9	55	gi|666864429	Heat shock 70 kDa protein	*Scedosporium apiospermum*	34	23	947	5.82/ 72.38	5.25/69.84
10	55	gi|666870338	Putative branched-chain-amino-acid aminotransferase TOXF	*Scedosporium apiospermum*	4	12	275	5.84/42.01	6.08/43.97
Cell wall proteins of *L. prolificans* conidia (Figure 1C)									
1	-	gi|10179991	Cyclophilin	*Magnaporthe grisea*	1*	8	81	8.67/17.89	8.22/16.60
2	-	gi|74661880	Enolase	*Cryphonectria parasitica*	4	14	333	5.25/ 47.39	5.38/52.01
***Scedosporium apiospermum* conidia (Figure 3C)**									
1	-	gi|573974808	Peptidyl-prolyl cis-trans isomerase(cyclophilin)	*Cordyceps militaris* CM01	8	17	145	8.65/19.63	8.28/16.84
2	-	gi|666868085	Enolase	*Scedosporium apiospermum*	58	60	1882	5.19/47.50	5.32/48.71
***Scedosporium aurantiacum* conidia (Figure 3D)**									
1	-	gi|827056251	Cyclophilin type peptidyl-prolyl cis-trans isomerase/CLD	*Colletotrichum graminicola* M1.001	11	20	490	9.69/25.75	8.04/16.13
2	-	gi|666868085	Enolase	*Scedosporium apiospermum*	40	34	910	5.19/47.50	5.24/52.59

* An interpreted MS spectrum for this peptide is supplied as supporting information.



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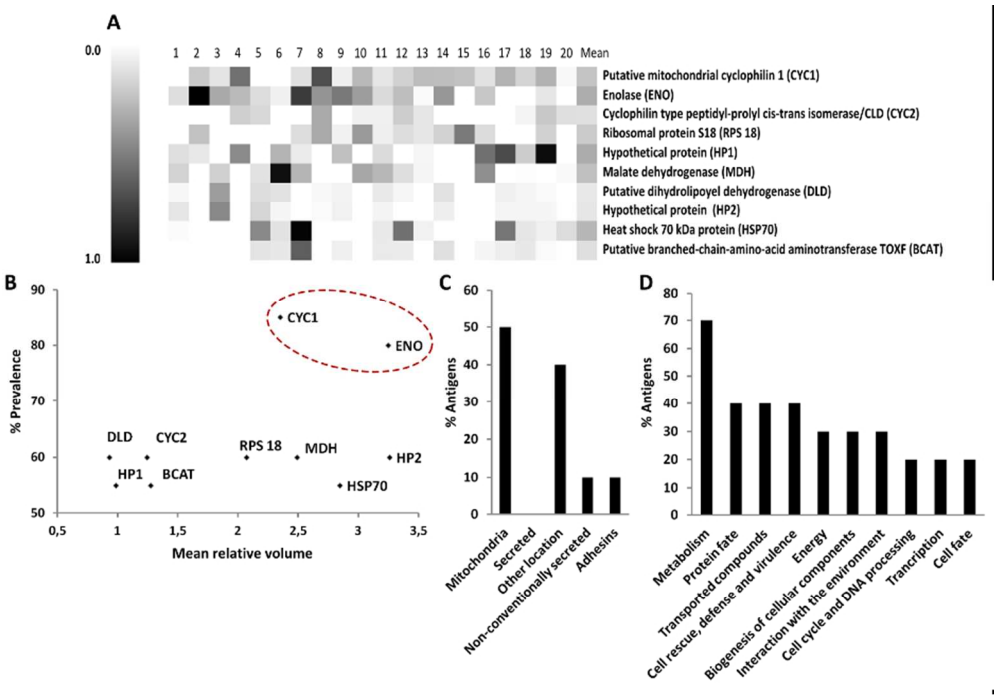

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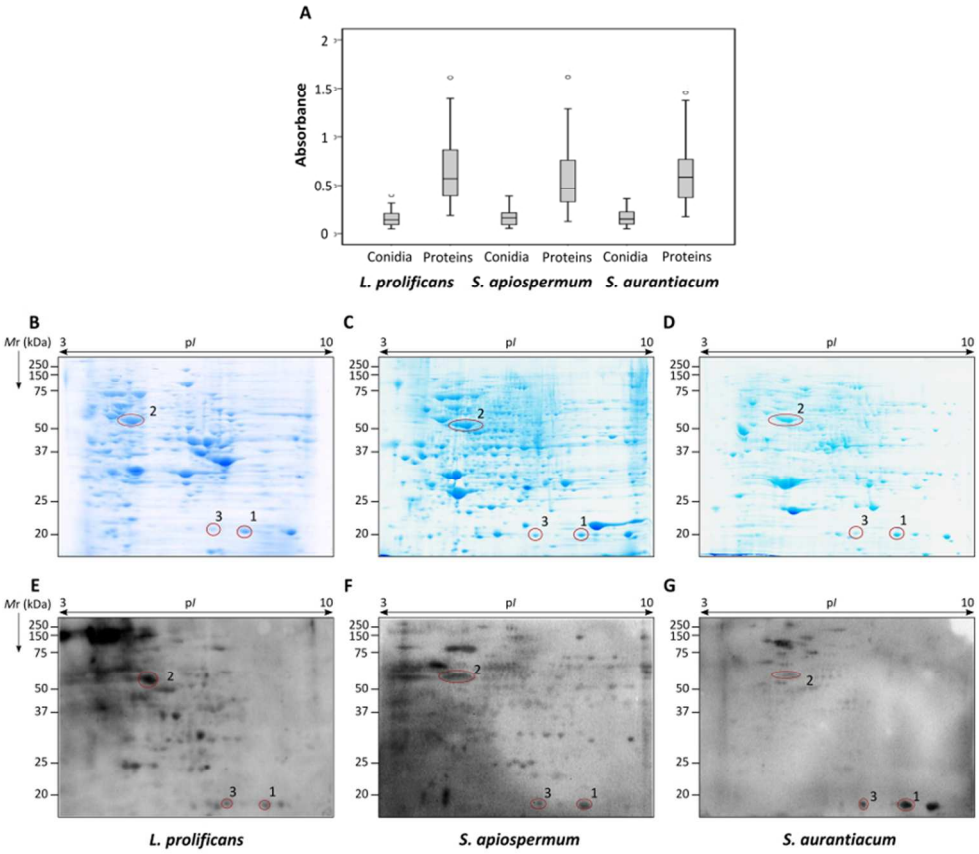

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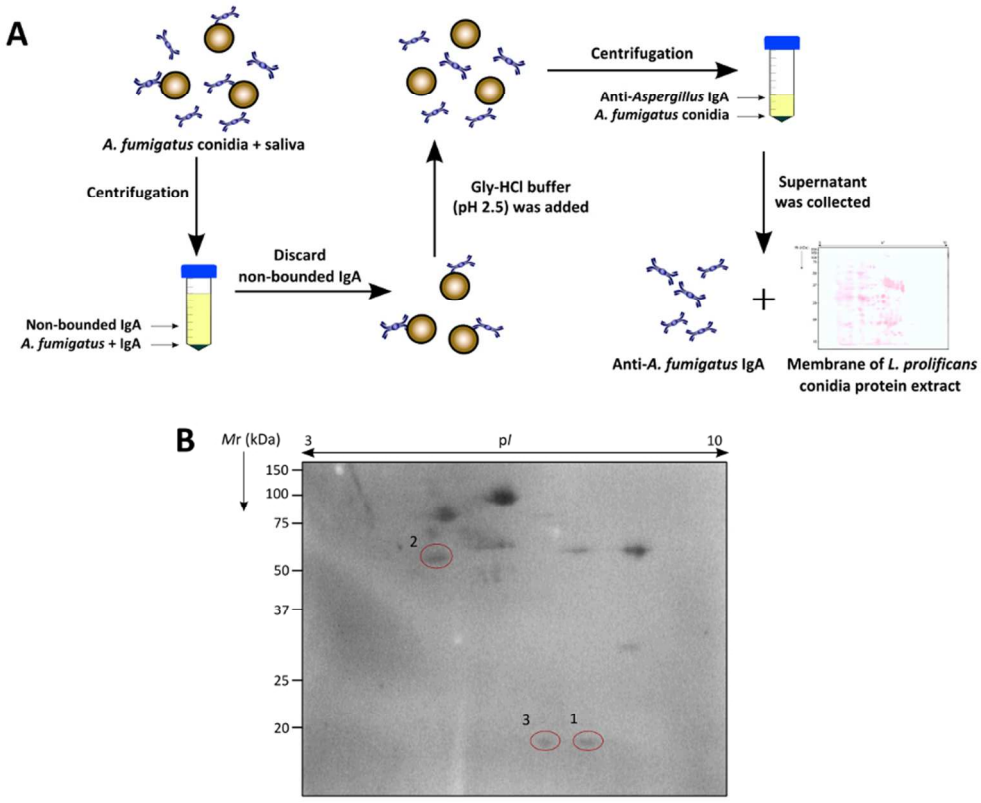

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