MINIREVIEW

Fungal proteomics: from identification to function

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Abstract

Some fungi cause disease in humans and plants, while others have demonstrable potential for the control of insect pests. In addition, fungi are also a rich reservoir of therapeutic metabolites and industrially useful enzymes. Detailed analysis of fungal biochemistry is now enabled by multiple technologies including protein mass spectrometry, genome and transcriptome sequencing and advances in bioinformatics. Yet, the assignment of function to fungal proteins, encoded either by *in silico* annotated, or unannotated genes, remains problematic. The purpose of this review is to describe the strategies used by many researchers to reveal protein function in fungi, and more importantly, to consolidate the nomenclature of 'unknown function protein' as opposed to 'hypothetical protein' – once any protein has been identified by protein mass spectrometry. A combination of approaches including comparative proteomics, pathogen-induced protein expression and immunoproteomics are outlined, which, when used in combination with a variety of other techniques (e.g. functional genomics, microarray analysis, immunochemical and infection model systems), appear to yield comprehensive and definitive information on protein function in fungi. The relative advantages of proteomic, as opposed to transcriptomic-only, analyses are also described. In the future, combined high-throughput, quantitative proteomics, allied to transcriptomic sequencing, are set to reveal much about protein function in fungi.

Introduction

Fungal proteomics research, especially that related to filamentous fungi, has progressed dramatically over the past 5 years. This has been due to the availability of multiple fungal genome sequences, the advent of next-generation nucleic acid sequencing and the availability of powerful proteomics technologies, especially tandem LC-MS (Martin *et al.*, 2008; Braaksma *et al.*, 2010; Costa *et al.*, 2010). Combined, these technological advances have enabled high-throughput protein identification and functional assignment that was not even considered possible up to 10 years ago. The requirement to further understand the clinical consequences of opportunistic fungal infection, especially in immunocompromised patients, as well as the plant pathogenic nature of fungi, allied to the biotechnological potential of fungal enzymes for biofuel production, have also driven this intense activity (Taylor *et al.*, 2008; Dagenais & Keller, 2009; Schuster & Schmoll, 2010). Consequently, proteomics, by virtue of its capacity to yield definitive information on protein identity, localization, posttranslational modification and the accuracy of *in silico* gene model prediction in fungi, has become an integral component of all large-scale 'omic' and systems approaches to understanding the rich complexity of fungal biochemistry (Table 1).

The lack of information that existed with respect to fungal proteomes has meant that significant recent research has focused on developing methodologies compatible with optimal protein extraction from fungi, and establishing basic data on the types and relative abundances of proteins present in fungi (Lakshman *et al.*, 2008). Much effort has also been directed at cataloguing mycelial, organellar and secreted proteins (secretome) across a range of fungal species (Bouws *et al.*, 2008; Kim *et al.*, 2008). These approaches have used both individual protein identification following SDS-PAGE or 2D-PAGE fractionation or 'shotgun' proteomics, where total protein digests of fungal origin are analysed by tandem LC-MS to generate constituent protein data sets (Carberry *et al.*, 2006; Braaksma *et al.*, 2010). More recently, the dynamic nature of fungal proteomes has been investigated, whereby the effects of carbon sources, antifungal drugs and gene deletion have been

Table 1. Selected examples of recent reviews in the fungal proteomics area

Area	Coverage	Reference
Animal fungal pathogens	*Aspergillus* immunoproteomics for biomarker identification	Kniemeyer *et al*. (2009)
	Proteomics of filamentous fungi	Kim *et al*. (2007)
Fungal plant pathogens	Impact of genomics, proteomics and metabolomics on fungal phytopathology.	Tan *et al*. (2009)
	Discusses the research tools available for studying plant fungal pathogens	González-Fernández *et al*. (2010)
Industrial proteomics	Role of proteomics in protein discovery and uses.	de Oliveira & de Graaff (2011)
Various	Microbial environmental proteomics (fungi and bacterial proteomics)	Keller & Hettich (2009)

explored at the proteomic level (Fernández-Acero *et al*., 2010; Cagas *et al*., 2011). To date, ascomycete proteomic studies exceed those carried out using basidiomycetes, primarily due to the preponderance of the former involved in causing animal disease, and also because many ascomycetes are so-called 'cell factories' for protein secretion [e.g. *Aspergillus niger* (Adav *et al*., 2010)]. One of the most surprising aspects of fungal proteomic research has been the occurrence of either 'predicted proteins' or 'hypothetical proteins,' which pepper all fungal data sets obtained from investigations of the ascomycete, or more especially basidiomycete, Kingdoms (Martin *et al*., 2008; Ferreira de Oliveira *et al*., 2010). Of course, the identification of the actual protein means that the classification should always be upgraded to that of 'unknown function protein' (UFP), because the protein is no longer 'hypothetical' – it exists! Assigning function to the multitude of UFPs represents one of the major challenges in fungal proteomics and the purpose of this review is, in part, to indicate strategies for such investigations.

Detailed descriptions of protein mass spectrometry techniques and protocols have been described elsewhere (Shevchenko *et al*., 2006; Brewis & Brennan, 2010); hence, this review will focus primarily on the relevant and generic strategies used to identify the function of fungal proteins, particularly those for which no orthologues have been identified to date (Fig. 1).

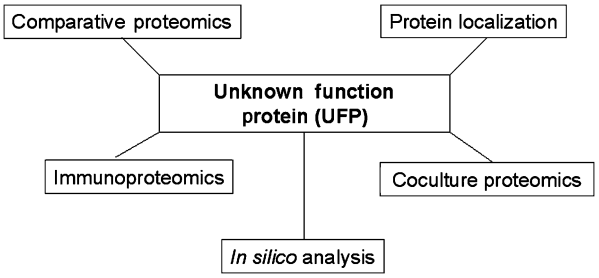

Fig. 1. Five strategies to identify the biological role of UFPs in fungi. Comparative proteomics includes global proteome analysis following exposure to chemical or naturally occurring toxins, wild type vs. mutant proteome comparison and the effects of redox perturbants. Protein localization can be achieved by immunolocalization, affinity partner analysis or subcellular fractionation (subproteomics). Co-culture proteomics involves the assessment of altered protein expression during co-culture between host–pathogen, host–symbiont or likely environmental partners. Immunoproteomics yields valuable information about protein immunogenicity and potential involvement in virulence. *In silico* analysis includes, but is not limited to, signal peptide identification, posttranslational modification motifs and structural motif/domain searching.

Comparative proteomics

Gene deletion strategies have been deployed extensively to characterize gene function in filamentous fungi (e.g. *Neurospora crassa* and *Aspergillus fumigatus*) (Dunlap *et al*., 2007; Dagenais & Keller, 2009). Comparative phenotypic analysis, following exposure to various physical and chemical stressors [e.g. hydrogen peroxide, antifungal drugs, mycotoxins, cell wall perturbants and redox-active species (e.g. dithiothreitol)], of wild-type and mutant organisms is then carried out to facilitate the identification of the consequences of gene loss. Microarray and *in silico* analysis has been especially useful in characterizing altered global gene expression in fungal mutants, compared with the wild type (Sheppard *et al*., 2006). However, comparative proteomic analysis of mutant vs. wild-type strains has been deployed recently, as a complementary technique, to further investigate the effects of gene deletion (Sato *et al*., 2009). In *Aspergillus nidulans*, the deletion of a glutathione reductase gene (*glrA*) resulted in the acquisition of a temperature-sensitive phenotype, decreased intracellular glutathione and reduced resistance to oxidative stress. Proteomic analysis enabled the identification of > 600 proteins from both *A. nidulans* wild type and Δ*glrA*. Comparative image analysis, following 2D-PAGE, revealed increased ($n = 13$) and decreased ($n = 7$) protein expression in the *A. nidulans* mutant compared with the wild type at a cut-off of greater than twofold expression difference. Matrix-assisted laser desorption ionization – time of flight (MALDI-ToF) MS identified all upregulated proteins including a thioredoxin reductase (TrrA), cytochrome *c* peroxidase and catalase B, in addition to a number of peroxiredoxins. It was concluded that elevated TrrA expression was consistent with an activated thioredoxin (Trx) system and that cross talk between

the GSH and Trx dependent was evident in the absence of *glrA*. Moreover, depleted H_2O_2 levels in *A. nidulans* Δ*glrA* were due to the observed elevation in catalase B and cytochrome *c* peroxidase levels. Significant upregulation of an elongation factor 1β (ElfA; 2.5-fold) and a glutathione *s*-transferase (GstB; 2.6-fold) was also observed in *A. nidulans* Δ*glrA*. Relevantly, orthologues of both of these proteins had previously been shown to be present and upregulated in response to oxidative stress in *A. fumigatus* (Burns *et al.*, 2005; Carberry *et al.*, 2006). Thön *et al.* (2010) observed that the deletion of *hapC*, a component of the transcriptional regulator AnCF that senses the cellular redox status and coordinates the oxidative stress response, resulted in an impaired oxidative stress response. Characterization of the *A. nidulans* Δ*hapC* proteome identified upregulation of a range of redox-active proteins including thioredoxin, peroxiredoxin A and glutathione, compared with the wild type. Pusztahelyi *et al.* (2011) investigated the *A. nidulans* proteome, compared with transcriptomic alterations, during long-term exposure to menadione to further exploit the power of comparative proteomics for cellular redox investigations.

Lessing *et al.* (2007) used comparative proteomics to explore the effect of H_2O_2 on, and the deletion of a potential transcription factor Afyap1, involved in the oxidative stress response, from *A. fumigatus*. Differential gel electrophoresis (DIGE) analysis, followed by MALDI-ToF/ToF MS identified 27 and 17 proteins, respectively, whose expression was up- and downregulated ($>$ 1.5-fold cut-off) following *A. fumigatus* exposure to H_2O_2 (2 mM). Predominant among upregulated proteins were the Allergen Asp f3 ($\times$ 10-fold), a mitochondrial peroxredoxin, Prx1 ($\times$ 3.7) and Cu,Zn superoxide dismutase (SOD; $\times$ 1.2–2.7). The authors proposed that given the classification of Asp f3 and Prx-1 as thioredoxin peroxidases, an elevation in thioredoxin system activity in response to oxidative stress is of significant importance in *A. fumigatus*. The altered expression of a range of metabolic enzymes was also evident and some proteins appeared in more than one gel spot, at identical M_r, but with an altered p*I* and amount. Lessing and colleagues speculated that this was due to either posttranslational modification or isoenzyme occurrence; either way, it revealed a type of information that can only be derived from proteomic, and not microarray, expression analyses. Of three unclassified proteins, or UFPs, the expression of two was downregulated (an NAD-dependent dehydrogenase and a UGP-1 protein), while one, a GMC oxidoreductase, was significantly upregulated ($\times$ 6.2). The expression of most of the proteins whose expression was upregulated following exposure to H_2O_2 was found to be downregulated in *A. fumigatus* Δ*yap1*. This provided strong evidence that the expression of these proteins (29 in total) was regulated by *yap1* in *A. fumigatus*. The expression of four UFPs was downregulated in *A. fumigatus* Δ*yap1* following exposure to H_2O_2 and future gene deletion studies will be required to dissect the function of these proteins. Finally, the authors observed that although *yap1* was important in *A. fumigatus* for protection against reactive oxygen intermediates, via the regulation of catalase 2 levels and activity, it was dispensable for virulence in a murine infection model.

The identification of resistance mechanisms to antifungal drugs such as amphotericin B and caspofungin (an echinocandin) in *A. fumigatus* has been investigated by determining the fungal proteomic response to drug exposure (Gautam *et al.*, 2008; Cagas *et al.*, 2011). Differential expression (at least a twofold difference in expression) of 85 proteins (76 upregulated and nine downregulated) was detected, compared with normal growth conditions, when *A. fumigatus* was exposed to amphotericin B. These were identified by MALDI-ToF/ToF MS as cell stress proteins, transport proteins and enzymes involved in ergosterol biosynthesis (a key amphotericin B target). Concomitant microarray analysis of the fungal response to amphotericin B was also undertaken and the expression of 295 genes was found to be differentially expressed, whereas that of 165 genes was upregulated and 130 downregulated. It is notable that 142/265 genes encoded hypothetical proteins, and that few of these were detected by proteomic analysis. This points to the usefulness of integrated genomic and proteomic strategies, where possible, for such studies – which may be facilitated in future by RNaseq, as opposed to microarray technology (Sheppard *et al.*, 2006). Expressions of three genes, a Rho-GDP dissociation inhibitor, a secretory-pathway GDI and Mn SOD, were detectable at both microarray and proteomic levels. An unexpected alteration in the enzyme levels involved in protein secretion was evident; however, the biological significance of this finding requires further study.

Cagas *et al.* (2011) have quantitatively evaluated the proteomic response of *A. fumigatus* to caspofungin by subcellular fractionation (for localization) and MALDI-ToF/ToF MS identification. Postcaspofungin exposure, subcellular fractionation was achieved by differential centrifugation to yield secreted, cell wall/plasma membrane (CW/PM), microsomal and cytoplasmic fractions; however, only CW/PM and secreted fractions were subjected to quantitative proteomic analysis. In the CW/PM fraction, an altered expression of 56 proteins was evident (26 up- and 30 downregulated), 81% of the upregulated proteins were ribosomal proteins, the most highly upregulated protein was a UFP and chitinase was the most significantly downregulated protein (12-fold). In the secreted fraction, the expression of six proteins (including ATP citrate lyase, hsp60, Asp f4 and transaldolase) was elevated, while eight proteins were significantly downregulated, including the ribotoxin Asp f1 (12-fold). The authors also used an *A. fumigatus* echinocandin-resistant strain to confirm the specificity of protein identification and demonstrated that

potential biomarkers of caspofungin resistance, changing 12-fold or more, include Asp f1, a PT repeat family protein, a subunit of the nascent polypeptide-associated complex, the citrate synthase Cit1, and FKBP-type peptidyl-prolyl isomerase, a mitochondrial hypoxia response domain protein, 4-hydroxyphenylpyruvate dioxygenase and one UFP. Furthermore, parallel microarray analysis of gene expression alterations in response to caspofungin exposure provided a broadly similar response (e.g. elevation in ribosomal protein transcripts at 24 h); however, opposite gene/protein responses were observed in some cases. Ultimately, alterations in intracellular or extracellular protein expression should improve our understanding of fungal drug resistance and facilitate the development of strategies to circumvent drug resistance with concomitant efficacy potentiation of current antifungal drugs.

In an effort to identify proteins associated with yeast–hyphal transition in *Candida albicans*, which is strongly associated with the virulence potential of this organism, analysis of the acidic subproteome was undertaken (Monteoliva *et al.*, 2011). This led to the identification of 21 differentially abundant acidic proteins, 10 of which had not been found previously upon comparative 2D-PAGE/DIGE analysis and underscores the necessity for multiple comparative proteomic strategies.

Pathogen-induced expression

Candida albicans–macrophage interactions were studied using proteomics (Fernández-Arenas *et al.*, 2007). Here, a combination of 2D-PAGE and MALDI-ToF/ToF MS showed the differential expression of 132 yeast proteins upon macrophage interaction. This study was the first to explore *C. albicans*–macrophage interaction using proteomics, and identified 67 proteins that were either downregulated (carbon-compound metabolism) or upregulated (lipid, fatty acid, glyoxylate and tricarboxylic acid cycles) in expression upon co-culture.

Fusarium graminearum is a filamentous fungal pathogen of wheat, maize and grains; as such, it is a major threat to the global food supply (Kikot *et al.*, 2009). Moreover, *Fusarium* spp. are potent producers of mycotoxins, which can cause significant disease in humans. Although initial proteomic studies involving *F. graminearum* focused on altered plant protein responses to fungal exposure (Zhou *et al.*, 2006), genome availability and improvements in protein extraction techniques have meant that *Fusarium* proteomics research has intensified since 2007 (Paper *et al.*, 2007; Taylor *et al.*, 2008). Indeed, Pasquali *et al.* (2010) have produced an online video tutorial to demonstrate the intricacies of protein extraction from *Fusarium* strains. Other studies have sought to explore fungal proteins associated with plant infection. Paper *et al.* (2007) used LC-MS/MS to identify proteins secreted from *F. graminearum* after growth on culture media (*in vitro*) and *in planta* during infection of wheat heads. A total of 289 proteins were identified, and 49/120 *in planta* proteins were not found under *in vitro* conditions. Indeed, only 56% of the *in planta* proteins had predicted signal peptides, whereas virtually all proteins produced *in vitro* exhibited this motif. Fungal housekeeping enzymes, such as enolase, triose phosphate isomerase, phosphoglucomutase, calmodulin, aconitase and malate dehydrogenase, were primarily found *in planta*, which, the authors speculated, either indicated the occurrence of fungal lysis during pathogenesis or specific *in planta* release to enable the fungal–plant interaction.

Taylor *et al.* (2008) sought to investigate quantitative alterations in *F. graminearum* protein expression in response to *in vitro* stimulation of biosynthesis of the mycotoxin, trichothecene. This approach was based on the rationale that mycotoxin synthesis is associated with early-stage plant infection, and that any altered protein expression seen *in vitro* should mimic that occurring during the infectious process. Quantitative protein mass spectrometry using isobaric Tags for relative and absolute quantification (iTRAQ) analysis confirmed that 130 of 435 proteins detected exhibited statistically significant expression changes. Included in this cohort were many proteins known to be involved in fungal virulence; however, of particular relevance was the number of UFPs that were also identified. Although the precise function of these proteins remains outstanding, their association with the commencement of mycotoxin synthesis and the infectious process serves to contextualize further targeted functional proteomic studies. This clearly underlines the importance of large-scale fungal proteomics for identifying the function of individual proteins. Taylor *et al.* (2008) also used Northern analysis and reverse transcriptase-PCR to confirm alterations in selected protein expression following iTRAQ and 2D-PAGE analyses, and very good agreement between both transcript and protein expression was observed. This is somewhat at variance with the observations of Cagas *et al.* (2011) with respect to caspofungin effects on *A. fumigatus*; however, it most likely reflects the specific nature of the metabolic responses in different organisms. Georgianna *et al.* (2008) also adopted a quantitative proteomic approach to study the effect of temperature on protein expression and aflatoxin production in *Aspergillus flavus*.

Losada *et al.* (2009) have speculated that competition among environmental fungi may involve the deployment of secreted mycotoxins/secondary metabolites to attenuate competitor growth. Moreover, they speculated that the operation of such systems would necessitate resistance mechanisms in secreting organisms. Using 2D-PAGE and MALDI-ToF MS analysis of *A. fumigatus* protein expression following exposure to gliotoxin, Schrettl *et al.* (2010) identified a threefold upregulation of GliT, a gliotoxin oxidoreductase and a component of the gliotoxin

biosynthetic cluster. Subsequent targeted deletion of this gene confirmed its key role in self-protection against gliotoxin toxicity in *A. fumigatus* and also established a role for *gliT* in gliotoxin biosynthesis (Scharf *et al.*, 2010; Schrettl *et al.*, 2010). Interestingly, two isoforms of GliT were detected in *A. fumigatus*; however, the biological significance of this observation remains to be established. In a comprehensive analysis of altered protein expression during *A. fumigatus* biofilm formation, Bruns *et al.* (2010) found that at 48 h in mature biofilms, the expression of genes and proteins involved in secondary metabolite biosynthesis in general, and gliotoxin biosynthesis in particular (e.g. GliT), is upregulated. This suggests a protective role for GliT, as gliotoxin was also detected in *A. fumigatus* biofilms. The expression of GliG, a glutathione *s*-transferase (GST), was also elevated; however, the recent demonstration that this gene is only involved in gliotoxin biosynthesis, and not self-protection (Davis *et al.*, 2011), underlines the key role of GliT in fungal self-protection against gliotoxin.

Metarhizium spp. are important entomopathogenic fungi that have significant potential for use as alternatives to chemical insecticides for agricultural pest control (Pedrini *et al.*, 2007); however, while genome and EST sequence analyses have been published (Wang *et al.*, 2009; Gao *et al.*, 2011), few proteomic studies had been undertaken. However, recent studies are beginning to reveal the proteome of this fungus, which may have a significant impact on the future use of *Metarhizium* spp. Barros *et al.* (2010) have used 2D-PAGE to detect 1130 ± 102 and 1200 ± 97 protein spots for *Metarhizium acridum* conidia and mycelia, respectively. Approximately 35% of protein spots were common to both developmental stages, with the remainder equally occurring only in either conidia or mycelia. Of 94 proteins identified by MALDI-ToF/ToF MS, heat shock proteins and an allergen (Alt a 7) were uniquely identified in conidia, while metabolic proteins (e.g. transaldolase, protein disulphide isomerase and phosphoglycerate kinase) were primarily identified in mycelia. Barros *et al.* (2010) noted the differences in the extent of expression of identical proteins, and isoform occurrence, between conidia and mycelia. Although not discussed in detail, this observation highlights the requirement for future quantitative proteomic studies to reveal the biological significance of altered protein expression. Interestingly, most protein identifications were achieved by comparison against homologues or orthologues in related fungal species, because few *Metarhizium* sequence entries were present in the NCBInr data database when this study was undertaken; however, genome availability (Gao *et al.*, 2011) will improve the quality and extent of protein identifications in future studies. A combined enzymatic and proteomic approach has also been exploited to identify the *Metarhizium anisopliae* response to the chitin-containing exoskeleton of the cowpea weevil plant pathogen (*Callosobruchus maculatus*) (Murad *et al.*, 2006). Enhanced protein secretion (fivefold) from *M. anisopliae* was observed in the presence of *C. maculatus* exoskeleton. Specifically, elevated chitinolytic and proteolytic activities were observed and 2D-PAGE revealed the expression of seven additional proteins during exposure; however, definitive identification was not initially confirmed by protein mass spectrometry. Subsequently, Murad *et al.* (2008) identified *N*-acetyl-D-glucosamine kinase and D-glucosamine *N*-acetyltransferase in the *M. anisopliae* secretome, following exoskeleton co-incubation, by 2D-PAGE and MALDI-ToF/ToF MS. Murad and colleagues proposed that chitosan adsorption by *M. anisopliae* was facilitated, in part, by these enzymes because chitosan is more soluble, and therefore, more readily absorbed as a nutrient by *M. anisopliae*, than chitin.

Immunoproteomics

Combining mass spectrometry-based protein identification with the specificity of immunoblotting represents an emerging strategy for the identification of immunoreactive fungal antigens, some of which may be potent allergens (Doyle, 2011). This research strategy has found particular use in exploring the immunoproteome, or 'immunome', of *C. albicans*, *Cryptococcus* spp. and *A. fumigatus*.

Pitarch *et al.* (2004) detected 85 *C. albicans* proteins that were immunoreactive with systemic candidiasis patient sera, using a combination of MALDI-ToF MS and nanoelectrospray ionization-ion trap (ESI-IT) MS. Furthermore, they also observed, for the first time, that 35 of the immunoreactive proteins were targets of the human antibody response to systemic candidiasis, and that the production of antiphosphoglycerate kinase and alcohol dehydrogenase antibodies during systemic candidiasis might be linked to a differentiation of the human immune response to *C. albicans*. Increased antienolase antibody levels appeared to be associated with recovery from systemic candidiasis in this patient cohort, providing the possibility of predicting patient outcome using an immunoproteomic strategy. Pitarch *et al.* (2006) subsequently demonstrated that serum antienolase (cell wall associated) antibodies were a prognostic indicator for systemic candidiasis and that this protein, along with Bgl2p, may be candidates for *Candida* vaccine development. Recent immunoprotoemic work furthers these findings with respect to immunotherapy against invasive candidiasis (Pitarch *et al.*, 2011).

Cryptococcosis is a potentially fatal fungal disease of humans and other animals (Datta *et al.*, 2009). In particular, *Cryptococcus gattii* is especially virulent as, unlike *Cryptococcus neoformans*, which primarily infects immunocompromised individuals, it can cause disease in immunocompetent hosts (Datta *et al.*, 2009). Jobbins *et al.* (2010) have used an immunoproteomic strategy to reveal the *C. gattii* immunome

in the Koala animal model of cryptococcosis. Extensive optimization of 2D-PAGE conditions involved the incorporation of lithium chloride into protein extraction reagents to ensure efficient high M_r protein release from *C. gattii* lysates that contain large amounts of capsular material (Jobbins *et al.*, 2010). Subsequent 2D-immunoblot analysis and corresponding protein identification from 2D-PAGE by LC-MS/MS revealed 54 protein spots (37 proteins identified) that were immunoreactive with disease-state sera. The identification of a number of proteins of the thioredoxin antioxidant system (e.g. Trx and Trr), combined with observations made elsewhere, led the authors to conclude that this system is important for *C. gattii* pathogenesis. Further, they suggest that the low M_r fungal form of Trx may represent a potential therapeutic target as it is absent from higher eukaryotes. Interestingly, Ito *et al.* (2006) identified antibodies against *A. fumigatus* Asp f3, a putative thioredoxin peroxidase (Kniemeyer *et al.*, 2009) in sera from an immunocompromised murine model of pulmonary aspergillosis using an immunoproteomic approach. These authors also demonstrated that vaccination with recombinant Asp f3, or truncated versions of this protein, induced a significant protective response to subsequent infection of immunocompromised animals with *A. fumigatus*. However, Ito and colleagues speculated that T-cell memory or restoration of macrophage functionality in the corticosteroid-suppressed animals may form the basis of this protective effect because the presence of anti-Asp f3 IgG was not essential for protection. Nonetheless, these combined findings clearly demonstrate the power of immunoproteomics to reveal potential drug targets or vaccine candidates for recalcitrant fungal diseases.

Immunoproteomic analysis of *A. fumigatus* culture supernatants, mycelia (including *Avicularia versicolor*) and conidia-associated proteins has recently been deployed to identify potential allergens or vaccine candidates, respectively (Asif *et al.*, 2006; Gautam *et al.*, 2007, 2008; Singh *et al.*, 2010a, b). Simultaneous immunoblotting and MALDI-ToF MS analysis of the protein spots from 2D-PAGE led to the identification of a total of 16 allergens, 11 of which were reported for the first time (e.g. isoforms Asp f 13 and chitosanase) (Gautam *et al.*, 2007). Individual sera from patients with Allergic Bronchopulmonary Aspergillosis (ABPA) yielded a range of reactivity against *A. fumigatus* proteins. However, three proteins (a UFP, extracellular arabinase and chitosanase) were proposed to be major allergens with specific IgE immunoreactivity in six out of eight patient sera. Immunoreactivity of these proteins observed among the patients with ABPA may be potentially useful for serodiagnosis and the future development of individual immunotherapeutics for ABPA patients (Gautam *et al.*, 2007).

Fungal immunoproteomics can be confounded by multiple antigen nomenclatures. *Aspergillus fumigatus* GliG, a GST involved in gliotoxin biosynthesis (Davis *et al.*, 2011), was previously proposed to be a fungal allergen based on *in silico* analysis (Bowyer & Denning, 2007). These authors named GliG as 'Asp f GST'. Shankar *et al.* (2005) demonstrated human antibody reactivity against GSTs from different fungal species, including *A. fumigatus*, and a recombinant GST from *Alternaria alternata* was identified as a major fungal allergen (Shankar *et al.*, 2006) (called Alt A GST or Alt A 13 in Bowyer & Denning, 2007). Alt A GST shares 76% identity with Asp f GST (i.e. *A. fumigatus* GliG; Bruns *et al.* 2010; Davis *et al.*, 2011). Thus, GliG is the same protein as Asp f GST (Bowyer & Denning, 2007) and exhibits 94.8% sequence similarity to that identified by Shankar *et al.* (2006). GliG was not identified in mycelial or conidial immunoproteomic investigations as exhibiting antibody reactivity. The absence of previous GliG detection illustrates a potential limitation of global immunoproteomic approaches, whereby differentially, or low-level, expressed – yet antigenic – proteins will not be detected.

Schrettl *et al.* (2010) observed widespread immunoreactivity in human sera against *A. fumigatus* GliT and suggested that immunoaffinity purification of antibodies from human, or animal sera, using recombinant fungal antigens could represent a valuable source of antigen-specific reagents for native protein identification in the organism. This proposal, which may potentially obviate the requirement for antisera generation, also has applications in other species, which induce generalized immune responses in animals.

Conclusions

Proteomics will play a major role in future research into the nature, and biotechnological uses, of fungi. The assignment of biological roles to both *in silico* annotated, and unannotated genes, remains a significant challenge. Allied to robust analytical strategies such as quantitative proteomics, and RNAseq for the assessment of altered gene expression, the emerging availability of online resources for functional categorization of fungal genes and proteins (Priebe *et al.*, 2011) will contribute considerably to this challenge. It has been suggested that fungal protein identification by protein mass spectrometry is reminiscent of stamp collecting. However, a better analogy may be the completion of a mega jigsaw puzzle and it is only when all the pieces are in place that the true richness and complexity of fungal proteomes will be revealed.

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