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Analysis of major intracellular proteins of *Aspergillus fumigatus* by MALDI mass spectrometry: Identification and characterisation of an elongation factor 1B protein with glutathione transferase activity ☆

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Abstract

Aspergillus fumigatus is a recognised human pathogen, especially in immunocompromised individuals. The availability of the annotated *A. fumigatus* genome sequence will significantly accelerate our understanding of this organism. However, limited information is available with respect to the *A. fumigatus* proteome. Here, both a direct proteomic approach (2D-PAGE and MALDI-MS) and a sub-proteomic strategy involving initial glutathione affinity chromatography have been deployed to identify 54 proteins from *A. fumigatus* primarily involved in energy metabolism and protein biosynthesis. Furthermore, two novel eukaryotic elongation factor proteins (eEF1Bγ), termed ElfA and B have been identified and phylogenetically confirmed to belong to the eEF1Bγ class of GST-like proteins. One of these proteins (ElfA) has been purified to homogeneity, identified as a monomeric enzyme (molecular mass = 20 kDa; pI = 5.9 and 6.5), and found to exhibit glutathione transferase activity specific activities (mean ± standard deviation, $n = 3$) of 3.13 ± 0.27 and 3.43 ± 1.0 μmol/min/mg, using CDNB and ethacrynic acid, respectively. Overall, these data highlight the importance of new approaches to dissect the proteome of, and elucidate novel functions within, *A. fumigatus*.

Keywords: Aspergillosis; Proteomics; 2D-PAGE; Oxidative stress; Protein synthesis; GST; Glutathione

The field of fungal proteomics is gathering pace as a result of the simultaneous occurrence of fungal genome sequence availability and advances in high sensitivity protein mass spectrometry. Indeed, the recent publication of a two-dimensional protein reference map for, and the analysis of stress-induced changes in the proteome of, *Schizosaccharomyces pombe* clearly illustrates this point [1,2]. Proteomic analysis of the filamentous fungus, *Trichoderma harzianum*, has also yielded insights into the mitochondrial proteome of this mycoparasitic organism [3].

Many *Aspergillus* species have merited attention from a biotechnological standpoint, as a consequence of a secreted proteome rich in industrially useful enzymes [4]. Until recently, however, little information was available with respect to either the technical requirements for protein extraction or the proteome content of most *Aspergillus* species. However, dissection of the secreted proteome (secretome) of *Aspergillus flavus* has demonstrated the ability of this organism to adapt to altered conditions such as the presence of various carbon sources (e.g., rutin) [5].

With respect to the human pathogenic fungus, *A. fumigatus*, Bruneau et al. adopted a proteomic approach to identify glycosylphosphatidylinositol-anchored proteins which are involved in the organisation of the fungal cell wall [6]. More recently, we have undertaken the identification of nonribosomal peptide synthetases in *A. fumigatus* using a combined proteomic and molecular approach [7]. Along with *Candida albicans*, *A. fumigatus* is a significant opportunistic pathogen of immunocompromised individuals,

☆ *Abbreviations:* CDNB, 1-chloro-2,4-dinitrobenzene; 2D-PAGE, two-dimensional electrophoresis.

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indeed approximately 5% of all hospital based nosocomial deaths are due to *Aspergillus* infections [8].

The recent completion of the sequencing of the *A. fumigatus* genome and the availability of the in silico annotated genome at http://www.cadre.man.ac.uk [9] should enable detailed molecular dissection of the biology of this organism. Moreover, both unannotated and annotated genome data can be interrogated to further our knowledge of the *A. fumigatus* proteome and the organismal response to altered environmental conditions.

For instance, a greater understanding of the role played by glutathione transferases (GSTs) in mediating resistance to oxidative stress and antifungal drugs or xenobiotics is required and we have recently identified, characterised, and cloned three GST genes from *A. fumigatus* (*gstA-C*) [10]. However, GST-like domains have also been detected in distinct proteins such as Ure2p, Mak16, and EF1Bγ (a subunit of the EF1B complex) [11]. Furthermore, although GST activity has recently been ascribed to the elongation factor protein complex EF1B isolated from rice and trypanothione *S*-transferase activity detected in EF1B complex from *Leishmania major*, no detection of GST activity in native EF1Bγ has been possible [12]. In an attempt to purify the GST proteins (GstA-C) from *A. fumigatus*, glutathione (GSH)–Sepharose affinity chromatography was employed. Although GstA, B or C were undetectable, a number of other proteins were detected, one of which was identified as a eukaryotic elongation factor 1Bγ. This manuscript represents one of the first detailed descriptions of a proteomic approach to identify intracellular proteins in *A. fumigatus* and also the use of a sub-proteomic approach, involving affinity chromatography and MALDI mass spectrometry to identify new biochemical functions in fungal species.

Materials and methods

Protein extraction. Aspergillus fumigatus (ATCC 26933) was cultured in Sabouraud's media (500 ml cultures), at 37 °C with shaking at 200 rpm. Mycelia were harvested, filtered under pressure, and washed with PBS and resuspended in lysis buffer (100 mM Tris–HCl, 50 mM NaCl, 20 mM EDTA, 10% (v/v) glycerol, 30 mM DTT, 1 mM PMSF, and 1 μg/ml pepstatin A, pH 7.5; 5 ml of lysis buffer per gram of mycelia). Lysis was accomplished by grinding in liquid N_2 followed by brief sonication. Mycelial lysates were centrifuged (10,000*g*; 30 min) to remove cell debris and the subsequent supernatant analysed by 2D-PAGE following TCA/acetone precipitation [13].

Two-dimensional electrophoresis. Protein separation by 2D-PAGE was as follows: extracts containing 250 μg of protein were resuspended in 8 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (v/v) Triton X-100, 10 mM Tris–HC1, 65 mM dithiothreitol (DTT), and 0.8% pH 4–7 carrier ampholytes, and loaded onto Immobiline Dry strips (IPG strip; Amersham) in the pH range 4–7. Gels underwent active rehydration at 50 V for 10 h, followed by a further 10 h focusing with a total of 26250 V applied. Following IEF, gels were equilibrated in reducing buffer (50 mM Tris–HCl, 6 M urea, 2% (w/v) SDS, 30% (v/v) glycerol, and 2% (w/v) DTT, pH 6.8) for 20 min followed by equilibration in alkylation buffer (50 mM Tris–HCl, 6 M urea, 2% (w/v) SDS, 30% (v/v) glycerol, and 2.5% (w/v) iodoacetamide, pH 6.8) for a further 20 min. The IPG strips were placed on homogeneous 12% SDS–PAGE gels and electrophoresed for 20 h at 100 V using the ProteanXi-II Cell (Bio-Rad Laboratories). Resulting gels were stained with Coomassie brilliant blue R and scanned using a Typhoon Trio Variable Mode Imager (Amersham Biosciences (Europe) GmbH, Freiburg, Germany).

Chromatographic procedures. Aspergillus fumigatus cell lysates were centrifuged as above and the supernatant collected while the pellet was discarded. Ammonium sulphate solution (100% (w/v)) was then added to the supernatant to yield 65% ammonium sulphate (65% solution) which was stirred at 4 °C overnight. This suspension was then centrifuged, the supernatant removed, and the pellet resuspended in a minimum volume of lysis buffer. Resuspended material was extensively dialysed against 2×50 volumes of 25 mM Tris–HCl, 50 mM NaCl, pH 7.4 (buffer A), and the dialysate was centrifuged at 10,000*g* for 10 min and then filtered (0.45 μM) prior to chromatography. A GSH–Sepharose column (3 ml volume) was equilibrated with buffer A and following dialysate application and washing, was eluted with 50 mM Tris–HCl, 15 mM GSH, pH 9.2 (buffer B). Fractions (0.5 ml) were collected. SDS–PAGE analysis of neat or TCA precipitated fractions was carried out as previously described [13]. GSH–Sepharose column fractions were subsequently pooled (350 μg/10 ml), applied to a Q-Sepharose resin (1.5×8 cm; flow rate: 1 ml/min), and 0.5 ml fractions collected following elution with a 25 ml linear gradient of 0–0.5 M NaCl in 25 mM Tris–HCl, pH 8.0. Peak fractions containing were identified by SDS–PAGE and enzymatic activity analysis (glutathione conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) and ethacrynic acid) [14]. Native molecular mass determination was undertaken by size exclusion chromatography using an ÄKTA Purifier 100 system (Amersham Biosciences, UK) whereby a Superose 12 column (10×300 mm) was equilibrated in PBS at a flow rate of 0.4 ml/min. The concentrated material from Q-Sepharose was loaded on the column and 0.5 ml fractions collected.

MALDI mass spectrometry. Mass spectrometry was carried out using an Ettan MALDI-ToF mass spectrometer (Amersham Biosciences (Europe) GmbH, Freiburg, Germany). Protein samples for peptide mass determination were excised from 2D-PAGE gels using an automated spot cutter (Amersham Biosciences (Europe) GmbH, Freiburg, Germany), digested with trypsin and deposited (1 μl) with 1 μl α-cyano-4-hydroxycinnaminic acid (4-HCCA; 5 mg/200 μl of 50% (v/v) acetonitrile in 0.1% (v/v) aqueous trifluoroacetic acid) onto mass spectrometry slides, and allowed to dry prior to delayed extraction, reflectron ToF analysis at 20 kV [10]. Internal calibrants, Angiotensin III (Sigma–Aldrich) and ACTH fragment 18–39 (Sigma–Aldrich), were used to calibrate all spectra. Protein identification was carried out either by *m/z* data interrogation of (i) the NCBI nr database available as part of the mass spectrometer Evaluation Software Version 2.01 or (ii) a local FASTA version of the annotated *A. fumigatus* genome available at http://www.cadre.man.ac.uk. The annotation system employed on this website (i.e., AfuNg where N and g define the chromosome and gene number, respectively) and described by Mabey et al. [9] has been adopted in this manuscript. Tandem mass spectrometry was performed by commercial arrangement (Taplin Biological Mass Spectrometry Facility, Harvard Medical School, MA, USA) using an LCQ DECAXP Plus ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA).

Enzyme activity determination. GST assays were carried out as described by Habdous et al. [14]. Briefly, assays were carried out in a total volume of 200 μl including 20 μl of protein sample with CDNB/ethacrynic acid, GSH in 0.1 M potassium phosphate (pH 5.8) at final concentrations of 0.5/0.2 and 2.5 mM, respectively. Reactions were initiated by the addition of the protein sample and a blank was created by the addition of 20 μl of 0.1 M potassium phosphate (pH 5.8) instead of the protein samples. The increase in absorbance was measured at 340 nm (corresponding to the enzymatic conjugation of CDNB/ethacrynic acid to GSH by GST).

Phylogenetic analysis. Sequence alignments and bootstrap neighbour joining phylogenetic trees were generated using the Clustal X 1.81 program available at ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/, using the program default parameters. A bootstrapping value of 1000 was used, with bootstrapping values noted at tree branch points. Trees were visualised using Treeview (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

Results

Aspergillus fumigatus proteome analysis

A total of 180 spots were excised from 2D-PAGE gels (Fig. 1A) which resulted in the identification of 50 distinct proteins (in 65 individual spots), by MALDI-MS (Table 1). Significant expectation values were not achieved for the remaining 115 spots to give positive identification. The percentage sequence coverage obtained ranged from 9.4% (peroxisome biogenesis factor) to 50.5% (enolase) and observed molecular masses ranged from 75 kDa to less than 25 kDa. Several proteins exhibited different observed molecular mass to the theoretical mass suggesting the occurrence of post-translational modification.

The majority of proteins identified appear to be involved in energy production, with proteins characteristic of the glycolytic pathway, citric acid pathway, pentose phosphate pathway, fatty acid metabolism, and oxidative phosphorylation clearly evident (Fig. 1B and Table 1). Other classes of proteins identified include structural proteins, signalling proteins, heat shock and heat shock chaperone proteins, transcription factors, and two hypothetical proteins.

A number of proteins (i.e., catalase 1, HSP 70 chaperone HscA, alanine aminotransferase, thiamine biosynthesis protein Nmt1, 14-3-3 family proteins, and uridine diphosphate-glucose-4 epimerase) (Table 1) were individually present in more than one spot, at identical molecular mass, which indicates subtle charge differences possibly due to side-chain deamination. Most proteins exhibited molecular mass and p*I* values close to the predicted values, except for NAD-dependent formate dehydrogenase p*I*-observed = 7.7; p*I*-predicted = 8.7. Moreover, protein Afu5g14680 (unknown function) presented at p*I* 4.7, 4.9, and 5.2 with molecular masses all below the theoretical 25 kDa value. Analysis of this sequence by BLAST search indicated hypothetical proteins in *Aspergillus nidulans*XM_656639 and *Gibberella zeae* XM_390618 with similarity to Afu5g14680. The theoretical and actual mass of hypothetical protein Afu2g10030 differed by 7 kDa and BLAST analysis showed similarity to hypothetical proteins in *A. nidulans* XM_658516 and *Botrytis cinerea*

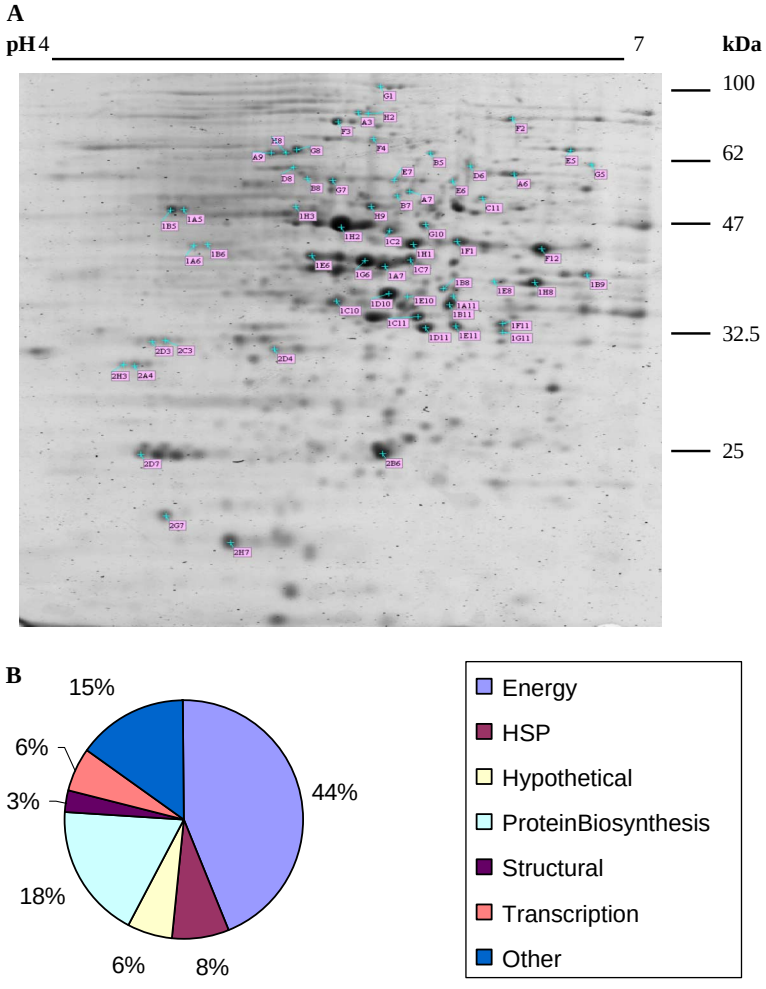

Fig. 1. (A) 2D-PAGE separation of whole protein extract from *A. fumigatus*. Whole protein extracts (250 μg) were subjected to isoelectric focussing on Immobiline Dry strips (pH range 4–7) followed by SDS–PAGE and Coomassie brilliant blue staining. (B) Functional classification of all *A. fumigatus* proteins identified by MALDI-MS whereby >60% of proteins were involved in either energy generation or protein biosynthesis. HSP, heat shock protein.

Table 1
A. fumigatus proteins ($n = 50$) present after culture in Sabouraud media following identification by 2D-PAGE and MALDI-MS (Fig. 1)

CADRE I.D.	Spot No.	% Coverage	M_r (kDa)	p*I*	Proposed function
Afu1g07480	1C7	26.8	50.16	6.9	Coproporphyrinogen III oxidase
Afu1g10130	D6	24.7	48.47	5.8	Adenosylhomocysteinase
Afu1g10350	F12	35.3	44.74	6.5	Phosphoglycerate kinase
Afu1g10630	1A7	44.4	42.18	5.7	*S*-Adenosylmethionine synthetase
Afu1g12170	1F7	21.8	48.27	6.7	Translation elongation factor EF-Tu
Afu1g13500	E5	23.5	74.81	6.1	Transketolase
Afu1g13490	2D4	29.7	33.43	5.3	Spermidine synthetase
Afu2g00720	B7	14.4	51.92	5.6	Aldehyde dehydrogenase
Afu2g03290	2A4	25.7	29.08	4.8	14-3-3 Family protein ArtA
Afu2g04230	1F1	10.9	46.80	6.0	Fumarylacetoacetate hydrolase fahA
Afu2g07420	B5	22.4	72.46	5.8	Fimbrin
Afu2g09960	G8	30.9	74.45	5.4	Mitochondrial HSP70 chaperone Ssc
Afu2g10030	1E11	22.3	28.25	6.2	Hypothetical protein
Afu2g11150	1H3	24.6	52.26	5.3	Secretory pathway gdp dissociation inhibitor
Afu2g13240	A7	24.5	56.4	5.7	V-type ATPase subunit B
Afu3g00590	2B6	41.0	15.18	5.8	Asp-hemolysin
Afu3g01110	E6	17.2	61.41	5.8	GMP synthase
Afu3g02270	A3	27.1	79.9	5.6	Catalase 1
Afu3g02270	H2	27.1	79.9	5.7	Catalase 1
Afu3g02270	F4	23.4	79.89	5.5	Catalase 1
Afu3g05450	H9	36.0	53.0	5.5	Glutamate carboxypeptidase
Afu3g07630	2H3	26.8	19.61	4.7	Dihydropteroate synthase
Afu3g08160	1A6	15.0	45.76	5.0	Eukaryotic translation initiation factor eIF4A
Afu3g09290	G7	20.4	57.44	5.6	Phosphoglycerate mutase 2,3-biphosphoglycerate-independant
Afu3g11070	A6	31.3	62.98	6.1	Pyruvate decarboxylase PdcA
Afu3g11690	1D10	43.6	39.77	5.5	Fructose-biphosphate aldolase class
Afu3g11690	1E10	33.1	39.77	5.8	Fructose-biphosphate aldolase class II
Afu4g06620	1H1	43.9	49.35	5.8	Glutamate/leucine/phenylalanine/valine dehydrogenase
Afu4g07690	G5	13.3	65.01	6.8	Phosphoribosylaminoimidazole carboxamideformyltransferase/IMP hydrocyclase
Afu4g10200	B8	13.2	71.68	5.4	Transcription factor RfeF
Afu4g11730	1C11	16.3	36.8	6.0	Glycerol dehydrogenase GldB
Afu4g13170	1F11	45.9	34.96	6.4	Guanine nucleotide binding protein β-subunit
Afu4g13170	1G11	36.4	34.96	6.4	Guanine nucleotide binding protein β-subunit
Afu5g01030	IH8	43.4	36.12	6.5	Glyceraldehyde-3-phosphate dehydrogenase Ccg-7
Afu5g01030	1E8	39.2	36.12	6.2	Glyceraldehyde-3-phosphate dehydrogenase Ccg-7
Afu5g02470	1A11	29.5	38.3	6.0	Thiamine biosynthesis protein Nmt1
Afu5g02470	1B11	29.5	38.3	6.0	Thiamine biosynthesis protein Nmt1
Afu5g02470	1C10	19.9	38.3	5.7	Thiamine biosynthesis protein Nmt1
Afu5g04170	G1	15.4	80.6	4.9	Molecular chaperone HSP1
Afu5g09230	1D11	40.1	35.43	6.0	Transaldolase
Afu5g10550	1A5	41.4	55.6	4.8	ATPase synthase F1 subunit β
Afu5g10550	1B5	41.4	55.6	4.8	ATPase synthase F1 subunit β
Afu5g10780	1B8	39.9	40.59	6.1	UDP-glucose 4-epimerase
Afu5g10780	1C8	27.8	40.59	5.9	UDP-glucose 4-epimerase
Afu5g14680	2D7	39.6	25.42	4.7	Hypothetical protein
Afu5g14680	2G7	34.8	25.42	4.9	Hypothetical protein
Afu5g14680	2H7	34.8	25.42	5.2	Hypothetical protein
Afu6g04740	1E6	26.7	43.88	5.9	Actin
Afu6g04920	1B9	33.0	45.73	7.7	NAD-dependant formate dehydrogenase
Afu6g06750	2D3	28.0	30.05	4.7	14-3-3 Family protein
Afu6g06750	2C3	28.0	30.05	4.8	14-3-3 Family protein
Afu6g06770	1H2	50.5	47.29	5.6	Enolase
Afu6g07720	E7	15.8	66.8	5.8	Phosphoenolpyruvate carboxykinase
Afu6g07770	F3	24.6	55.12	6.2	Alanine aminotransferase
Afu6g07770	C11	18.2	55.12	6.3	Alanine aminotransferase
Afu6g07770	H10	33.1	55.12	5.9	Alanine aminotransferase
Afu6g08050	1C2	31.4	55.78	5.9	6-Phosphogluconate dehydrogenase decarboxylating
Afu6g12930	F2	16.9	85.5	6.4	Aconitate hydratase
Afu7g01860	F3	28.7	65	5.5	Heat shock protein Sti1
Afu7g05070	G10	22.5	51.25	5.7	FAD-dependent oxidoreductase
Afu7g05720	1G6	9.6	39.1	5.9	Peroxisome biogenesis factor

(*continued on next page*)

Table 1 (*continued*)

CADRE I.D.	Spot No.	% Coverage	M_r (kDa)	p*I*	Proposed function
Afu7g05720	D8	27.0	52.01	5.4	Pyruvate dehydrogenase complex dihydrolipoamide acetyltransferase component
Afu8g00230	2F4	22.3	32.65	5.6	Phytanoyl-CoA dioxygenase family protein
Afu8g03930	A9	31.3	66.96	5.3	HSP70 chaperone HscA
Afu8g03930	H8	22.8	66.96	5.4	HSP70 chaperone HscA

CADRE I.D., *A. fumigatus* gene annotation nomenclature according to Mabey et al. [9] and Nierman et al. [33].

CNS01C5R. Conserved Domain analysis [15] of Afu2g10030 indicted that it may possess an RNA Recognition Motif (RRM). The phytanol-CoA dioxygenase family protein, (Afu8g00230) which is a peroxisomal enzyme catalysing the first step of phytanic acid α-oxidation, has not been previously identified in *A. fumigatus*, however it exhibited similarity with hypothetical protein AN9227.2 in *A. nidulans*. A Conserved Domain search identified both Afu8g00230 and AN9227.2 as containing a phytanol-CoA dioxygenase domain as well as a COG5285 domain. In *Burkholderia fungorum* LB400, a protein containing a COG5285 domain is involved in biosynthesis of mitomycin antibiotics and the polyketide, fumonisin. Dihydropteroate synthase (Afu3g07630) has been associated with drug resistance in *Saccharomyces cerevisiae* and has been shown to be derived from a polycistronic gene also responsible for the production of two other functional proteins [16]. Catalase 1 (Afu3g02270) involved in oxidative stress and identified here as three separate protein spots has been implicated in *A. fumigatus* pathogenicity [17].

Almost 44% (22/50) of all proteins identified were derived from intron-containing genes. For instance, 3 peptides of *m*/*z* 2027.99, 2567.11, and 2630.31 were detected in pyruvate decarboxylase (Afu3g11070) which derive from three distinct splicing events. In addition, two peptides derived from exon–exon splicing were detected in each of seven proteins (catalase, glutamate carboxypeptidase, vacuolar synthase subunit B, enolase, secretory pathway dissociation inhibitor, F1-ATPase (β-subunit), and translation elongation factor EF-Tu) (Table 1). A single peptide, in each of the 14 remaining proteins, was identified which resulted from exon splicing (data not shown).

Aspergillus fumigatus sub-proteome analysis

GSH–Sepharose affinity chromatography was undertaken in order to selectively detect and purify glutathione binding proteins in *A. fumigatus*. Affinity column elution with 5 mM GSH (in 50 mM Tris–HCl, pH 9.2) did not result in the displacement of any bound proteins (data not shown) and all resultant GSH-binding proteins were displaced from the column in the presence of 15 mM GSH. Overall, the total protein yield was 7.4 mg/g mycelia in cell lysate supernatant and the yield of GSH-binding proteins was 5.3 μg/g mycelia which indicates that these proteins represent 0.0007% of total soluble protein in *A. fumigatus*.

The presence of three proteins was detected by SDS–PAGE analysis following GSH–Sepharose chromatography and a peptide (SVDVVEEYLQDR) was identified by Tandem-MS from the protein present in band 3 (Figs. 2A and B). Database interrogation http://www.tigr.org; http://www.cadre.man.ac.uk with this sequence identified a putative translation elongation factor 1Bγ type protein (Afu8g00580; ElfB) with 10% sequence coverage (Fig. 2B). Further analysis of GSH-binding proteins by 2D-PAGE (Fig. 2C) demonstrated that at least 10 proteins (molecular mass range: 18–40 kDa) were present following GSH–Sepharose affinity chromatography. All GSH-binding proteins were excised from 2D-PAGE gels and analysed by MALDI-MS. Four proteins were identified based on comparison to both the NCBI nr database and a FASTA version of the in silico-annotated *A. fumigatus* genome available at http://www.cadre.man.ac.uk (Table 2). Specifically, the most abundant protein (Fig. 2C-spot 3 a,b; Afu1g17120) was identified, based on 46.4% sequence coverage, as an translation elongation factor 1Bγ subunit. This protein, termed ElfA, appeared to be present as two distinct spots of identical molecular mass but different p*I* values (5.9 and 6.5, respectively) (Fig. 2C).

Anion-exchange chromatography (Fig. 3A) was subsequently used to purify ElfA to homogeneity followed by assessment of protein purity by SDS–PAGE (Fig. 3B). When the GST activity of purified ElfA was analysed, both CDNB and ethacrynic acid were recognised as substrates with specific activities (mean ± standard deviation, $n = 3$) of 3.13 ± 0.27 and 3.43 ± 1.0 μmol/min/mg, respectively. No activity was detected against DCNB and neither was any glutathione peroxidase activity detected in ElfA (data not shown). Moreover, the observed specific activities against CDNB and ethacrynic acid were much greater than those observed for recombinant *A. fumigatus* GstC expressed in *Escherichia coli* (0.01 ± 0.0009 μmol/min/mg) [10]. Size exclusion analysis identified the molecular mass of ElfA to be ~20 kDa, suggesting it is present as a monomer within the cell, unlike most forms of GST which are located intracellularly in dimeric form (data not shown). These results represent the first demonstration of GST activity associated with an elongation factor 1Bγ subunit in a fungus. Moreover, it also the first demonstration of GST activity in a native elongation factor 1Bγ subunit disassociated from other components of the EF1B complex.

Aspergillus fumigatus ElfA and ElfB were compared to 28 sequences of GST-like proteins from various species, previously analysed by McGoldrick et al. [11], in order to assess homology (Fig. 4). From this analysis, three distinct clades were observed namely EF1γ-like, Ure2, and

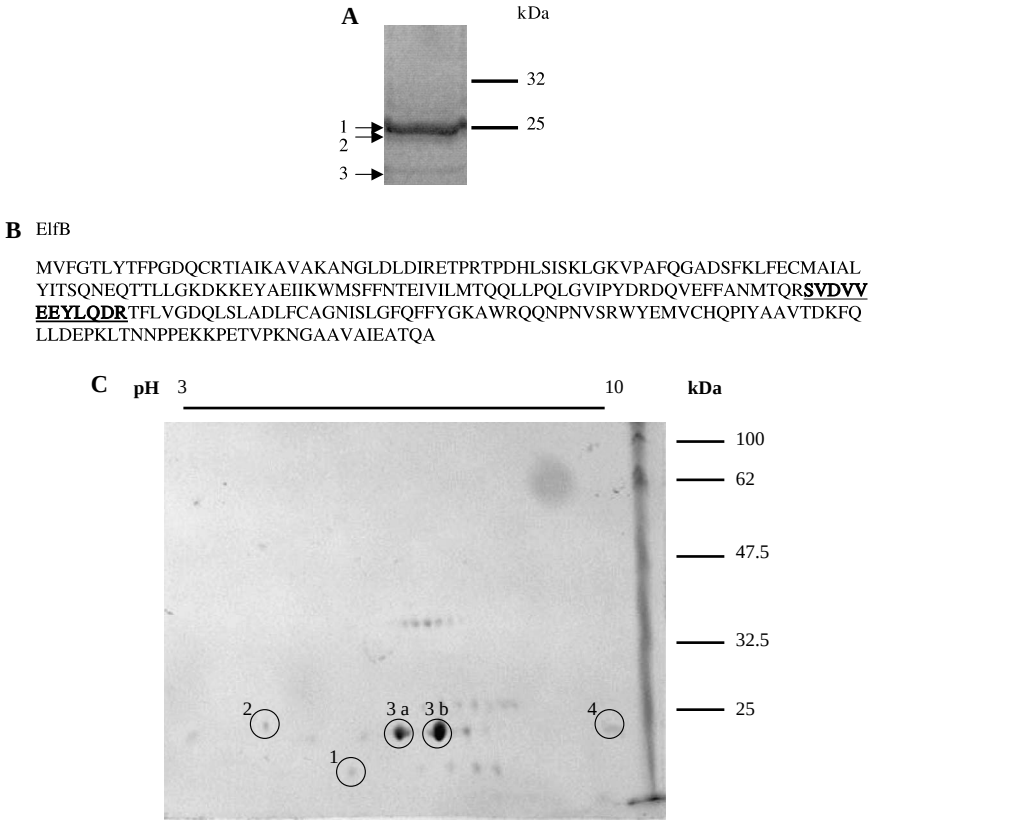

Fig. 2. (A) SDS–PAGE analysis of GSH-binding proteins from *A. fumigatus*. (B) Tandem mass spectrometry identified peptide **SVDVVEEYLQDR** which was specific to the sequence of *A. fumigatus* ElfB (CADRE I.D: Afu8g00580) (C) 2D-PAGE analysis of GSH-binding proteins. Isoelectric focussing on Immobiline Dry strips (pH range 3–10) followed by SDS–PAGE and Coomassie brilliant blue staining. Proteins in spots 1–4 were identified by MALDI-MS, one of which was ElfA (CADRE I.D: Afu1g17120; Table 2). ElfB was not detectable following 2D-analysis.

Table 2
GSH-binding proteins of *A. fumigatus* separated using 2D-PAGE and identified by MALDI mass spectrometry

Afu Code	Spot No.	% Coverage	Mol. mass (kDa)	p*I*	Proposed function
Afu5g06060	1	14.8	18.1	4	Sulphur metabolism regulator SkpA, putative
Afu1g03970	2	10	25	3.2	Mitochondrial translation initiation factor IF-2, putative
Afu1g17120	3a,b	46.4	24.3	5.9	ElfA; elongation factor 1-γ, putative
Afu4g09130	4	25.8	24	10	Mannosyltransferase, putative

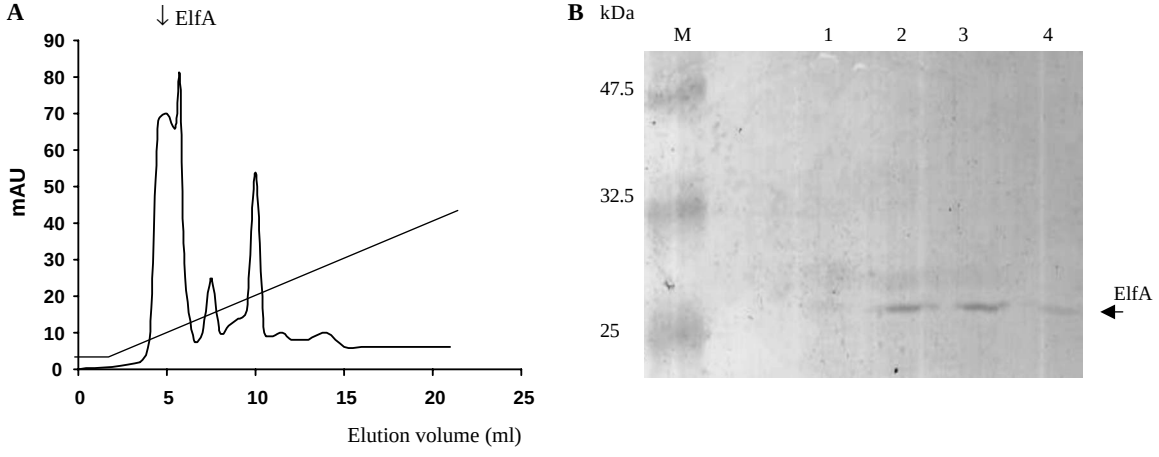

Fig. 3. (A) Purification of *A. fumigatus* ElfA by anion-exchange chromatography of GSH-binding proteins from *A. fumigatus*. GSH purified proteins were applied to a Q-Sepharose column (14 ml column volume) and eluted with an increasing salt gradient (0–0.5 M NaCl in 25 mM Tris–HCl, pH 8.0). ElfA was identified in the first peak as indicated. (B) SDS–PAGE analysis of ElfA purified by ion exchange chromatography (Q-Sepharose). Lane M, molecular mass markers; lanes 1–4, Q-Sepharose column fractions containing purified ElfA.

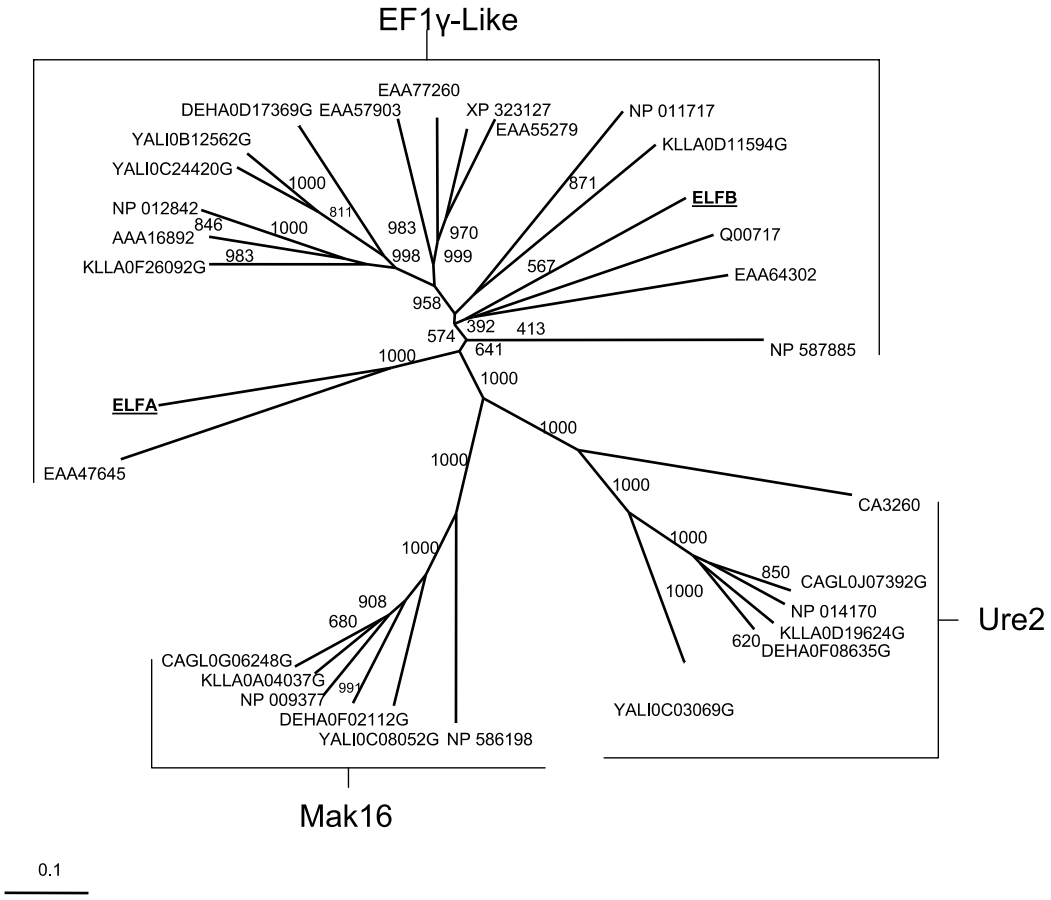

Fig. 4. Phylogenetic analysis of ElfA and B from *Aspergillus fumigatus* along with 28 predicted GST proteins from a range of fungal species. It is clear that both ElfA and B cluster in the EF1Bγ-like group of GST-like proteins. EF1Bγ-like proteins comprise: EAA47645 (*Magnaporthe grisea*); Q00717 (*Aspergillus nidulans*); EAA77260 (*Gibberella zeae*); EAA55279 (*Magnaporthe grisea*); NP_011717 (*Saccharomyces cerevisiae*); KLLA0D11594G (*Kluyveromyces lactis*); KLLA0F26092G (*Kluyveromyces lactis*); AAA16892 (*S. cerevisiae*); NP_012842 (*S. cerevisiae*); YALI0C24420G (*Yarrowia lipolytica*); YALI0B12562G (*Yarrowia lipolytica*); DEHA0D17369G (*Debaryomyces hansenii*); EAA57903 (*A. nidulans*); XP_323127 (*Neurospora crassa*); EAA64302 (*A. nidulans*); NP_587885 (*Schizsaccharomyces pombe*). Ure 2 proteins comprise: KLLA0D19624G (*K. lactis*); NP_014170 (*S. cerevisiae*); CAGL0J07392G (*Candida glabrata*); DEHA0F08635G (*D. hansenii*); YALI0C03069G (*Yarrowia lipolytica*). MAK 16 proteins comprise: NP_009377(*S. cerevisiae*); NP_586198(*Encephalitozoon cuniculi*); CAGL0G06248G (*C. glabrata*); KLLA0A04037G (*K. lactis*); DEHA0F02112G (*D. hansenii*); YALI0C08052G (*Yarrowia lipolytica*).

MAK16. Both ElfA and ElfB appear in the EF1γ-like clade of GSTs showing homology to EF1γ, both clearly distinct from the Ure2 and MAK16 clades. ElfA and EAA47645 (56% similarity) appear to be orthologues, as do ElfB and Q00717 (59% similarity), confirming that these proteins contain GST-like domains (Fig. 4).

Discussion

Here we present data on the identification of 54 intracellular proteins from the pathogenic fungus *A. fumigatus* using a combined 2D-PAGE/sub-proteomic/MALDI-MS approach. In addition, conclusive evidence is presented for the first time that a eukaryotic elongation factor 1Bγ protein of molecular mass 20 kDa exhibits glutathione transferase activity.

Archer and Dyer [18] have noted that proteomics will play an important role in future investigations into the biology and pathogenicity of *A. fumigatus*. Yet, it is quite surprising that reliable methods for the extraction of proteins from filamentous fungi did not emerge until the early 2000s. Since then, we, and others, have reported essentially similar strategies for mycelial lysis involving vigorous physical disruption (bead-beating [19], grinding in liquid N_2 [7,20], sonication or the use of French press technology [21]). Despite the availability of efficient approaches to cell wall disruption, it is only recently that a strategy for the identification of intracellular proteins from *A. fumigatus* has been forthcoming [22]. Indeed, 28 of the 50 proteins identified in our study have not previously been reported in *A. fumigatus*. The percentage sequence coverage range (9.4–50.5%) obtained here for MALDI-MS identification of *A. fumigatus* proteins is consistent with that recently reported by Medina et al. for the identification of rutin-induced secreted proteins from *A. flavus* (7–43% sequence coverage) and Grinyer et al. who mapped mitochondrial proteins from *Trichoderma harizium* (8–54% sequence coverage) [3,5].

Many of the proteins identified were shown to be present in several spots following 2D-PAGE, including

HSP70 chaperone HscA, alanine aminotransferase, thiamine biosynthesis protein, UDP-glucose 4 epimerase, catalase, and glyceraldehyde-3-phosphate dehydrogenase. Glyceraldehyde-3-phosphate dehydrogenase was also identified from several protein spots in *Candida albicans* [23]. The expression of three previously unidentified proteins in *A. fumigatus* (pyruvate decarboxylase, glyceraldehyde-3-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase) has been previously reported to be up-regulated in *C. albicans* in response to the presence of the antifungal azole agent, ketoconazole [23]. Interestingly, pyruvate decarboxylase, along with two other proteins (HSP90 and enolase), was identified as a fungal antigen expressed during invasive aspergillosis [24]. A number of identified proteins have been shown to be located in the cell wall of other fungi, included HSP90 and HSP70 in *C. albicans* [25], enolase and glyceraldehyde-3-phosphate dehydrogenase [26]. Others, such as catalase, ATP synthase (subunit β), and phosphoglycerate mutase were recently shown to be secreted by *A. flavus* [5]. Overall, it is clear that a more systematic and focused approach is required to further elucidate the functional rationale governing the unexpected localisation, and dynamic nature, of these fungal proteins which were previously thought to be predominantly of cytoplasmic origin.

In an attempt to move towards a consistent nomenclature for *A. fumigatus* proteins, the annotation described by Mabey et al. and employed on the CADRE website at http://www.cadre.man.ac.uk has been adopted [9]. The initial availability of the unannotated genome of the *A. fumigatus* [http://www.tigr.org] and subsequently that of thein silico annotated version at http://www.cadre.man.ac.uk have been critical to the success of the work presented here with respect to protein identification. Moreover, the availability of the *A. nidulans* genome sequence [http://www.broad.mit.edu/annotation/fungi/aspergillus/] has also been of great assistance in protein annotation and identification. However, it should be noted that proteomic data also serve to validate the predictive bioinformatic tools (e.g., Artemis software [27] and GlimmerM [28,29]) used, in part, to predict intron–exon splice sites in fungal genes. Consequently, at least 29 peptides from 22 proteins have been identified as arising from mRNA formed as a consequence of intron excision. Notably, 22 of 50 proteins identified in this work derive from intron-containing genes in *A. fumigatus* and represent biochemical validation of bioinformatic gene prediction data.

The eukaryotic elongation factor 1B complex (eEF1B) plays a key role in the elongation step of protein synthesis and is comprised of three subunits termed α, β, and γ, in order of increasing molecular mass [30]. The identification of an EF1Bγ protein (ElfA) in *A. fumigatus*, which exhibits GST activity, significantly extends the role of *S*-transferase activity beyond glutathionyl conjugation to xenobiotic compounds and advances experimental and bioinformatic evidence for the presence of GST-like domains in eEF1Bγ subunits [11,30,31]. In fact, a recent study has demonstrated an *S*-transferase activity in the eEF1Bγ subunit of the parasite *Crithidia fasciculata*, capable of utilising trypanothione (N^1,N^8-bis(glutathionyl)spermidine), but not glutathione, as a substrate for CDNB conjugation [32]. No glutathione peroxidase activity was detectable in ElfA preparations. Interestingly, Vickers et al. [12] have shown that the intact eEF1B complex is necessary for peroxidase activity found in the eEF1Bγ subunit of *Leishmania major* eEF1B. Microarray analysis of *A. fumigatus* gene expression has revealed that ElfA (termed elongation factor-1γ) expression is up-regulated twofold within 1 h of a temperature shift (30–48 °C) which suggests a role for ElfA in protecting against heat shock [33].

Five clusters of GST-like proteins were previously identified, three of which included EF1γ, Ure2, and MAK16 [11]. Recreating a similar phylogenetic tree revealed that both ElfA and ElfB from *A. fumigatus* clustered and shared homology to GST-like proteins with EF1γ similarity. These data suggest that both ElfA and ElfB are eEF1Bγ proteins containing GST-like domain and that this GST-like domain is conserved in various eukaryotes. These genes could possibly have a wide range of functions including protection from oxidative stress, controlling translation in response to oxidative stress [34] or regulating protein folding in a chaperone like manner [35]. Unlike the high affinity of ElfA for GSH-affinity media, the eEF1Bγ subunits/eEF1B complexes identified by Kobayashi et al. and Kamiie et al. did not exhibit affinity for glutathione-affinity media, although eEF1Bγ subunits purified from rice and *Bombyx mori* were amenable to purification by GSH-affinity chromatography [36,37] and GST activity was present in the purified EF1B complex preparations. Identification of the complete enzymatic and functional repertoire of ElfA will have to await eEF1B complex studies in *A. fumigatus*.

In summary, our findings represent one of the first detailed identifications of the most abundant intracellular proteins of *A. fumigatus*, identifies the importance of subproteomic studies in fungal biology, as exemplified by the identification and characterisation of the eEF1Bγ protein, ElfA and B, and will facilitate future studies into the dynamic proteome of *A. fumigatus*.

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