Differentially expressed proteins of pathogenic *Penicillium marneffei* in yeast and mycelial phases

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Penicillium marneffei is a dimorphic fungus endemic in southeast Asia. The incidence of *P. marneffei* infection has increased greatly in this region with the spread of human immunodeficiency virus, but the infection routes and pathogenic mechanisms of *P. marneffei* remain poorly understood. *P. marneffei* is an opportunistic human pathogen exhibiting a temperature-dependent dimorphic switch. At 25 °C it grows as filamentous hyphae, whilst at 37 °C it forms uninucleate yeast cells and divides by fission. Dimorphic fungal pathogenicity is frequently associated with the dimorphic switch, but the mechanism that regulates the switch has remained obscure. In this report, two-dimensional difference gel electrophoresis was used to investigate the proteins expressed differentially in the yeast and mycelial phases of a wild-type isolate of *P. marneffei*. Among thousands of protein molecules displayed, more than 500 showed differential expression between the two phases. In particular, 26 proteins were identified using matrix-assisted laser desorption/ionization time-of-flight MS. Expression of catalase-peroxidase, isocitrate lyase, Hsp90, binding protein and cytochrome P-450 increased significantly in the yeast phase, whereas levels of poly(A) polymerase and SNF22 were reduced.

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INTRODUCTION

Penicillium marneffei is a dimorphic fungus endemic in southeast Asia (reviewed by Li & Yeoh, 1992). Prior to the widespread AIDS epidemic, penicilliosis *marneffei* was limited to sporadic case reports. However, with the spread of human immunodeficiency virus in this region, opportunistic infection with *P. marneffei* has increased significantly and has been described as the cause of deep-seated infection in the pre-AIDS era (Supparatpinyo *et al.*, 1994). The infection routes and pathogenic mechanisms of *P. marneffei*, however, remain poorly understood and the host immune response against *P. marneffei* is yet to be clarified. A likely route of infection in most cases is through inhalation of *P. marneffei* conidia and it is believed that the ability of conidia to persist within the host might be essential for the establishment of infection (Hamilton *et al.*, 1999).

Dimorphic fungi are able to switch from non-pathogenic mycelia in soil to pathogenic yeast after spores have been

inhaled and incubated at an elevated temperature. Following entry into the host, they adapt to the host environment via mechanisms that remain obscure. It has long been believed that the phase transition from mycelia to yeast is key to the pathogenicity, but the mechanism that regulates the switch remains unknown (Leberer *et al.*, 1997; Rooney *et al.*, 2001; Sebgathi *et al.*, 2000). Borneman *et al.* (2000) found that two genes that showed homology to the *brlA* and *abaA* genes were required for the asexual development of *P. marneffei*. The *stlA* gene has also been shown to be important for the dimorphic switch of a variety of fungi, but has no detectable effect on vegetative growth, asexual development or dimorphic switching in *P. marneffei* (Borneman *et al.*, 2001). Another gene that is possibly involved in regulating the dimorphic transition is *tupA*, as both yeast and spore development are repressed when it is deleted (Todd *et al.*, 2003). As the dimorphic transition of *P. marneffei* might involve a variety of unknown proteins, a more systemic analysis of the proteins involved might help to clarify the molecular mechanism of the pathogenicity of *P. marneffei*.

In this report, we used two-dimensional difference gel electrophoresis (2D DIGE) to investigate proteins expressed differentially in the yeast and mycelial phases of a wild-type

Abbreviations: 2D, two dimensional; 2D DIGE, two-dimensional difference gel electrophoresis; Hsp, heat-shock protein; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

isolate of *P. marneffei*. Our study indicated that, among thousands of protein molecules displayed, more than 500 showed differential expression between the two phases. In particular, 26 proteins were identified using MS.

METHODS

Strains and culture conditions. *P. marneffei* strain SUMS0152 was isolated from the blood of a 2-year-old patient diagnosed with penicilliosis marneffei, characterized by fever, haemophthysis and weight loss (Xi *et al.*, 2004). The SUMS0152 isolate was confirmed to be *P. marneffei* by sequence analysis of the large subunit rRNA gene internal transcribed spacer region (Xi *et al.*, 2004) and is maintained at the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Japan (collection no. IFM52703).

To obtain a mycelial culture, the SUMS0152 isolate was inoculated on Sabouraud's glucose agar (SGA; 4 % glucose, 1 % peptone, 1 % agar) and incubated at 25 °C. The mycelial colonies obtained were inoculated in Sabouraud's fluid medium (4 % glucose, 1 % peptone) and cultured with shaking at 100 r.p.m. min⁻¹ at 25 °C for 72 h. Mycelial-phase *P. marneffei* was collected by centrifugation (3000 r.p.m., 15 min, 4 °C).

To achieve the switch of *P. marneffei* from the mycelial phase to the yeast phase, mycelial colonies on SGA were transferred to brain heart infusion (BHI) agar, as described by Trewatcharegon *et al.* (2000). Repeated subculture of the fungal colonies was performed on BHI agar for 72 h at 37 °C, until yeast-like colonies appeared. This procedure took approximately 14–20 days. Thereafter, the yeast phase was maintained by subculturing at 37 °C on BHI agar. For amplification, newly grown yeast colonies were inoculated into BHI liquid medium at 37 °C and shaken at 120 r.p.m. min⁻¹ for 72 h. Cell pellets were collected by centrifugation (3000 r.p.m., 15 min, 4 °C). Mycelia and yeast pellets were stored at -80 °C.

Preparation of whole-cell protein extract and protein labelling. Cell pellets were resuspended in chilled distilled water and recovered by recentrifugation (3000 r.p.m., 15 min, 4 °C). The pellets were then resuspended in lysis buffer [7 M urea, 2 M thiourea, 4 % CHAPS buffer, 30 mM Tris/HCl (pH 6.8)] at room temperature (Hu *et al.*, 2003). Glass beads (0.4–0.6 mm; Sigma) and 1 mM PMSF were added to the cell suspension, and the mixture was vortexed for 2 min and then cooled on ice for 2 min (Choi *et al.*, 2003). This vortexing procedure was repeated five times, followed by ultrasonication three times (10 s each, with a 1 min cooling interval) (Grinyer *et al.*, 2004). The cell extract was centrifuged at 20 000 g for 30 min at 4 °C and the supernatant was kept as a crude protein sample. The protein samples were purified by a precipitation/co-precipitant method using a 2-D Clean-up kit (GE Healthcare) following the procedure recommended by the manufacturer. The protein concentration was determined by a copper iron assay using a 2-D Quant kit (GE Healthcare).

Six two-dimensional (2D) gels were prepared, comprising four analysis gels and two preparative gels. For the analysis gels, proteins were labelled with CyDye (GE Healthcare). Three fluorescent dyes, Cy2, Cy3 and Cy5, were used to label the samples using a chemical coupling method described by Friedman *et al.* (2004). In this study, four samples of the mycelial phase and four samples of the yeast phase of *P. marneffei* were used for 2D DIGE analysis. To prepare an internal standard mixture, 6.25 µg protein from each sample were pooled (50 µg in total) and labelled with 400 pmol Cy2. Protein (50 µg) taken from each yeast or mycelial protein sample was labelled with 400 pmol Cy3 or Cy5, respectively, as summarized in Table 1. The labelling reactions were quenched by the addition of 10 mM lysine for 10 min on ice in the dark. The Cy3- and Cy5-labelled samples (50 µg per sample) and

Table 1. CyDye labelling scheme

H1–4 represent four subcultures of the mycelial phase and Y1–4 four subcultures of the yeast phase of *P. marneffei* strain SUMS0152.

Gel	Cy2	Cy3	Cy5
1	Internal standard	H1	Y4
2	Internal standard	H2	Y1
3	Internal standard	Y2	H3
4	Internal standard	Y3	H4

Cy2-labelled internal standard (50 µg per sample) were subsequently pooled and applied to 2D electrophoresis gels, with a loading volume of 150 µg protein. For the two preparative gels, 62.5 µg of each of the eight samples (mycelia and yeast) were pooled to constitute a 500 µg loading volume.

2D DIGE. For each gel, an immobilized DryStrip (pH 3–10 NL, 24 cm; GE Healthcare) was rehydrated with 450 µl Cy-labelled protein sample according to the manufacturer's instructions using the Ettan IPGphor IEF system (GE Healthcare). Once rehydration was complete, samples were subjected to IEF sequentially at 500 V for 1 h, 1000 V for 1 h and 8000 V for a total of 80 000 V h. The DryStrips were incubated in equilibration buffer I (6 M urea, 30 % glycerol, 2 % SDS, 50 mM Tris/HCl pH 6.8, 1 % DTT) for 15 min, followed by equilibration buffer II (6 M urea, 30 % glycerol, 2 % SDS, 50 mM Tris/HCl pH 6.8, 2.5 % iodoacetamide) for 15 min. The strips were then embedded in 0.5 % (w/v) agarose on top of 12.5 % acrylamide gels. Six gels were performed simultaneously on an Ettan DALTsix electrophoresis system (GE Healthcare). The proteins were separated at 15 °C at 12 W for 30 min, followed by 100 W until the indicator reached the bottom of the gel. The Cy2-, Cy3- and Cy5-labelled proteins of each analysis gel were imaged individually using a DIGE enabled Typhoon imager 9400 (GE Healthcare). The two preparative gels were stained using a Deep Purple total protein stain (GE Healthcare) according to the manufacturer's instruction and scanned using a Typhoon imager.

Image analysis and protein identification. DeCyder software (GE Healthcare) was used to detect and quantify images. Those obtained from gels for the same *P. marneffei* phase were matched using the software. Changes in protein spot abundance across all 12 spot maps were obtained. Student's *t*-test was used to test the hypothesis that the abundance differed between the two groups. The degree of difference in the standardized abundance between two protein spot groups was expressed as the mean ratio. We selected protein spots where the mean ratio was greater than 3-fold or less than -3-fold.

The proteins of interest were excised robotically in a 96-well plate using an Ettan spot handling workstation (GE Healthcare) for subsequent MS and database interrogation. Destaining, digestion, extraction, mass spectrometer sample preparation and spotting on target slides were performed robotically.

Peptide mass fingerprinting was carried out by matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF MS) (Ettan; GE Healthcare) operated in reflectron mode. Internal calibration was performed using the trypsin autodigestion peaks at *m/z* 842.509 and 2211.104. Proteins identified by peptide mass fingerprinting were interrogated by using the MASCOT search engine (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=PMF).

RESULTS AND DISCUSSION

A unique feature of *P. marneffei* compared with other penicillia is its thermal dimorphism (Cooper & Haycocks, 2000). Whilst several genes of this fungus, including *brlA*, *abaA* and *tupA*, are thought to be involved in asexual development, fungal morphogenesis and immunogenicity, the mechanisms that regulate the dimorphic switch remain obscure (Borneman *et al.*, 2000, 2001; Todd *et al.*, 2003). To understand further the significance of *P. marneffei* dimorphism, we compared the proteomic profiles of *P. marneffei* at both end points of the dimorphic transition. Global protein expression profiles of the mycelial phase and yeast phase were characterized using 2D DIGE. The total number of spots on four analysis gels were 4075, 3986, 3743 and 3407, respectively. In Fig. 1, the Cy2-labelled (blue) spots represent the internal standard, the Cy3-labelled (green) spots represent mycelial-phase proteins and the Cy5-labelled (red) spots represent yeast-phase proteins. Comparative analysis of the protein abundance between the two phases showed that 511 spots were expressed differentially with statistical significance.

To identify the most significant protein molecules displayed differentially by 2D DIGE, 245 spots were selected for further MS analysis. From these, 26 proteins were identified by peptide mass fingerprinting using the MALDI-TOF MS and the MASCOT search engine (Table 2). This analysis resulted in the identification of two known *P. marneffei* proteins: catalase-peroxidase and isocitrate lyase, which showed a 14.38-fold and 5.32-fold increase, respectively, in the yeast phase compared with the mycelial phase. Homologues of the remaining 24 proteins that were not identified in *P. marneffei* were searched for in other fungi, as shown in Table 2. *P. marneffei* homologues of Hsp90, binding protein, Hsc70, cytochrome P-450 and others demonstrated a significant increase in the yeast phase, whereas several other proteins, including poly(A) polymerase and SNF22, were found to be decreased (Fig. 2).

The differentially expressed proteins could be categorized into the following groups: heat-shock protein (Hsp) family, catalase family, enzymes in the glyoxylate bypass, amino acid metabolism-related proteins, energy metabolism-related proteins and a series of hypothetical proteins that have

Fig. 1. 2D DIGE analysis spot map image of gel 1. The pI range was 3–10 (left to right) and the molecular mass separation was from 120 to 14 kDa (top to bottom). Blue Cy2-labelled spots represent the internal standard, green Cy3-labelled spots represent mycelial-phase proteins and red Cy5-labelled spots represent yeast-phase proteins. The identified proteins are indicated with arrows and the numbers correspond to the spot numbers in Table 2 and Fig. 2.

Table 2. Differentially expressed proteins from *P. marneffe*

Category	Protein name	Spot no.*	GenBank accession no.†	Species	Mass (Da)	pI	Mean ratio‡
Catalase	Catalase-peroxidase	1297	AAN04057	<i>Penicillium marneffe</i>	82380	6.35	14.38
Glyoxylate bypass	Isocitrate lyase	1089	AAK54240	<i>Penicillium marneffe</i>	60683	7.64	5.32
HSP family	Hsp90 homologue	761	O43109	<i>Podospora anserina</i>	79331	4.90	6.93
	Hsp90	613	EAL85888	<i>Aspergillus fumigatus</i>	80647	4.95	5.29
	Binding protein	698	CAA70090	<i>Aspergillus awamori</i>	73372	4.93	4.91
	Mitochondrial Hsp70 chaperone Ssc70	791	XP_755328	<i>Aspergillus fumigatus</i>	74477	6.02	3.66
Transcription-related proteins	Hypothetical 21.3 kDa protein	2688	P38828	<i>Saccharomyces cerevisiae</i>	21357	9.40	4.51
	SNF22	1986	O94421	<i>Schizosaccharomyces pombe</i>	190308	9.19	−3.24
	PalA/RIM20	2899	Q5KEK0	<i>Filobasidiella neoformans</i>	99747	5.80	−4.21
	Poly(A) polymerase	3453	Q9UW26	<i>Candida albicans</i>	63648	8.39	−8.22
Energy metabolism-related proteins	<i>n</i> -Alkane-inducible cytochrome P-450	2122	BAA05146	<i>Candida maltosa</i>	10366	9.39	18.51
	Phosphoglucosmutase	1178	EAL86586	<i>Aspergillus fumigatus</i>	56677	5.78	4.26
	Enoyl-CoA hydratase/isomerase family protein	2764	XP_755425	<i>Aspergillus fumigatus</i>	31794	8.73	3.85
	γ -Aminobutyrate transaminase	1542	NP_011533	<i>Saccharomyces cerevisiae</i>	53369	6.34	3.54
Hypothetical proteins	Hypothetical 26.8 kDa protein	866	P39975	<i>Saccharomyces cerevisiae</i>	26969	8.25	14.08
	Soluble fumarate reductase	905	EAL85317	<i>Aspergillus fumigatus</i>	68021	7.05	11.24
	Hypothetical 80.5 kDa protein	3476	P40460	<i>Saccharomyces cerevisiae</i>	80552	8.71	8.71
	Dehydrogenase	1328	EAL91597	<i>Aspergillus fumigatus</i>	64102	5.53	4.58
	VPS	1668	AAA18831	<i>Saccharomyces cerevisiae</i>	179631	4.77	5.01
	2-Oxoisovalerate dehydrogenase complex α subunit	853	EAL88734	<i>Aspergillus fumigatus</i>	50613	5.94	4.80
	Hypothetical protein MG03239.4	1092	XP_360696	<i>Magnaporthe grisea</i>	18399	6.52	4.50
	Conserved hypothetical protein	3295	AAW45009	<i>Cryptococcus neoformans</i>	15587	8.09	−3.22
	Hypothetical protein MG02432.4	2818	XP_365730	<i>Magnaporthe grisea</i>	127663	6.18	−3.67
	Hypothetical protein CaO19_13172	2985	XP_718019	<i>Candida albicans</i>	23856	4.30	−3.29
	Hypothetical protein	2069	XP_324731	<i>Neurospora crassa</i>	274321	5.89	−4.08
	Hypothetical protein FG02875.1	2869	EAA72375	<i>Gibberella zeae</i>	47327	5.78	−6.15

*Spot numbers correspond to those in Figs 1 and 2.

†GenBank protein accession number according to the MASCOT search engine.

‡Mean ratio of protein expression abundance between the two groups.

not been named (Table 2). It is of note that the only protein reported previously to be expressed differentially in the two phases of *P. marneffe*, catalase-peroxidase, was confirmed in our analysis. Differential expression levels of the other proteins identified in our study have not been reported previously. Catalase-peroxidase and isocitrate lyase have

been reported to be related to pathogenicity in other fungi (Lorenz & Fink, 2001; Paris *et al.*, 2003). Catalase-peroxidase shows bifunctional activity of both catalase and peroxidase to transform intracellular reactive oxygen species into harmless products in *Aspergillus fumigatus* and *Aspergillus nidulans* (Kawasaki & Aguirre, 2001; Paris *et al.*,

Fig. 2. Enlargement of sections of 2D DIGE gel of *P. marneffei* mycelia and yeast proteins; the spot numbers indicate the identified proteins and correspond to those in Table 2 and Fig. 1. The green spot map represents Cy3-labelled mycelial-phase proteins and the red spot map represents Cy5-labelled yeast-phase proteins. Arrows indicate protein spots that are significantly differentially expressed between the two phases.

2003; Zamocky, 2004). It was found that the catalase-peroxidase mRNA level was elevated in the yeast but not in the mycelial phase (Pongpom *et al.*, 2005). Our study demonstrated an increase in the protein level of catalase-peroxidase in the yeast phase of more than 14-fold compared with that in the mycelial phase (Fig. 2). As an intracellular pathogen, *P. marneffei* survives as yeast inside phagocytes, protecting itself from the host defence machinery. It will be of great interest to investigate whether catalase-peroxidase serves as a virulence factor of *P. marneffei* that counteracts the oxidative defence reactions of the host phagocytes. Another significantly altered protein, isocitrate lyase, is the key rate-limiting enzyme in the glyoxylate bypass, a metabolic pathway supplementary to the tricarboxylic acid cycle when the micro-organism needs to survive in mammalian hosts (Lorenz & Fink, 2002). The glyoxylate bypass is required for the pathogenicity of intramacrophage *Mycobacterium tuberculosis*, *Candida albicans* and *Paracoccidioides brasiliensis* (de Voss *et al.*, 2000; Goldman *et al.*, 2003; Lorenz & Fink, 2001; McKinney *et al.*, 2000). As *P. marneffei* is also an intramacrophage pathogen, the pathogenic role of isocitrate lyase and the glyoxylate bypass in *P. marneffei* is under investigation in our laboratory.

A number of genes have been found previously to be transcribed differentially in the yeast compared with the mycelial phase in *P. marneffei*, such as catalase-peroxidase, *brlA*, *abaA* and *tupA*. However, with the exception of the catalase-peroxidase gene, most of these genes did not show remarkable changes at the protein level in our study. The reason for this may be that the proteins of interest selected in our study were selected on the basis of the mean ratio being higher than 3-fold or lower than -3-fold. The biological and pathological significance of the 24 proteins identified in our current report requires further study.

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