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RESEARCH ARTICLE

Olaf Kniemeyer · Franziska Lessing · Olaf Scheibner
Christian Hertweck · Axel A. Brakhage

Optimisation of a 2-D gel electrophoresis protocol for the human-pathogenic fungus *Aspergillus fumigatus*

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Abstract *Aspergillus fumigatus* is the most important airborne fungal pathogen causing life-threatening infections in immunosuppressed patients. One of the important questions concerning *A. fumigatus* is the identification of pathogenicity determinants. To obtain a comprehensive overview about the proteins produced at different physiological conditions that are related to the infectious process a proteomic approach has been applied. Here, 2-D gel electrophoresis for filamentous fungi was optimised concerning removal of interfering compounds, protein extraction and separation methods. A trichloroacetic acid-based precipitation method of proteins with their subsequent solubilisation by the use of a combination of CHAPS with a second sulfobetaine detergent gave the best results. The optimised protocol was evaluated by the analysis of the proteomes of *A. fumigatus* grown on two different carbon sources, i.e., glucose and ethanol. Carbon catabolite repression has not been studied in detail at the protein level in *A. fumigatus* yet. In addition, growth on ethanol leads to activation of the glyoxylate cycle which was shown to be essential for pathogenesis in bacteria and fungi. In *A. fumigatus*, differential patterns of enzymes of the gluconeogenesis, glyoxylate cycle and ethanol degradation pathway during growth on glucose and ethanol were observed.

Keywords *Aspergillus fumigatus* · Proteome analysis · 2-D gel electrophoresis · Carbon catabolite repression · Glyoxylate cycle

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O. Kniemeyer · F. Lessing · A. A. Brakhage
Department of Microbiology and Molecular Biology, Friedrich-Schiller-University, Jena, Germany

O. Kniemeyer · F. Lessing · A. A. Brakhage (✉)
Department of Molecular and Applied Microbiology, Leibniz Institute for Natural Product Research and Infection Biology (HKI), Beutenbergstrasse 11a, 07745 Jena, Germany
E-mail: Axel.Brakhage@hki-jena.de
Tel.: +49-3641-656601
Fax: +49-3641-656603

O. Scheibner · C. Hertweck
Department of Biostructure Chemistry, Leibniz Institute for Natural Product Research and Infection Biology (HKI), Beutenbergstrasse 11a, 07745 Jena, Germany

Introduction

In the last decades, *A. fumigatus* has become the most important airborne fungal pathogen of humans. Diseases caused by *A. fumigatus* can be divided into three categories: allergic reactions and colonisation with restricted invasiveness are observed in immunocompetent individuals while systemic infections with high mortality rates occur in immunocompromised patients (Ellis 1999; Brakhage and Langfelder 2002; Brakhage 2005; Brakhage and Liebmann 2005). Due to the improvement in transplant medicine and the therapy of haematological malignancies the number of invasive aspergillosis has increased. Specific diagnostics are still limited, as are the possibilities of therapeutic intervention, leading to a high mortality rate of 30–98% for invasive aspergillosis [reviewed in Latgé (1999)]. The infectious agent of *A. fumigatus* is conidia, which are inhaled during routine daily activities (Braude 1986).

The genome of the *A. fumigatus* isolate Af293 has been fully sequenced (http://www.tigr.org) and the annotation has been almost completed. The genome consists of eight chromosomes with a total size of 29.2 Mb, of which 9,922 protein-encoding sequences were identified (Pain et al. 2004; Niermann et al. 2005). With the genome data available the regulation of genes and proteins of *A. fumigatus* on a global scale can be analysed, also under conditions that are related to infection. 2-D gel electrophoresis introduced by O'Farrel (1975) is still the most established method for studying properties of proteins in parallel (proteomics). Not many proteomic data are available for filamentous

fungi, and no extensive study from any *Aspergillus* proteome has been published yet (Bruneau et al. 2001; Nandakumar and Marten 2002; Nandakumar et al. 2003). Preparation of protein samples for 2-D gel electrophoresis is a critical step and essential for reproducible results. This is also influenced by the presence of a cell wall which makes up the majority of the cell mass in filamentous fungi. The cell wall is exceptionally robust (Latgé et al. 2005; Shimizu and Wariishi 2005). Thus, cell lysis is an important step in sample preparation for 2-D gel electrophoresis. An often used method to remove contaminants which can interfere with the 2-D gel electrophoresis separation is the classical trichloroacetic acid (TCA)/acetone precipitation. This method has often been applied to the preparation of extracts from plants (Damerval et al. 1986; Carpentier et al. 2005) and fungi (Wildgruber et al. 2002; Nandakumar et al. 2003). However, until now this method has not been compared with methods employing commercial kits for protein precipitation. Another critical step during 2-D gel electrophoresis is the solubility of proteins during isoelectric focusing. Many proteins tend to aggregate or precipitate at their isoelectric point. Increased solubility was obtained by the use of thiourea (Rabilloud 1998) and new zwitterionic detergents (Rabilloud 1996; Rabilloud et al. 1997; Molloy et al. 1999; Luche et al. 2003).

Here, a comparison of several protein precipitation methods and lysis buffers helped to optimise a 2-D gel electrophoresis protocol for the human pathogenic fungus *A. fumigatus*. Furthermore, the optimised protocol was applied to analyse the proteomes when *A. fumigatus* was grown with glucose or ethanol as the carbon source. In saprophytic filamentous fungi, the expression of catabolic pathways involved in the utilisation of carbon compounds strongly depends on available nutrients in the environment. Energetically favourable carbon sources are used preferentially to less readily metabolisable carbon sources. The synthesis of the enzymes for the utilisation of the latter is reduced and the corresponding genes are repressed. This mechanism known as carbon catabolite repression has been intensively studied in the filamentous fungus *A. nidulans* (see review Flipphi and Felenbok 2004; Kelly 2004). Until now, little is known about carbon catabolite repression and ethanol metabolism in *A. fumigatus*. Homologous genes coding for the transcriptional regulators involved in *A. nidulans*, such as CreA, FacB and AlcR are present in the genome of *A. fumigatus*, showing 78, 65 and 43% amino acid identity to the proteins in *A. nidulans*, respectively. Therefore, it is likely that similar regulation patterns of carbon catabolite repression exist in both fungi *A. nidulans* and *A. fumigatus*. Moreover, previously the glyoxylate cycle was shown to be essential for pathogenesis of bacteria and fungi, i.e., *Mycobacterium tuberculosis* (McKinney et al. 2000) and *Candida albicans* (Lorenz and Fink 2001), respectively. Therefore, it is of considerable interest to analyse the proteome of *A. fumigatus* grown under glyoxylate cycle-activating conditions, e.g., when the fungus was grown with ethanol as the sole carbon source.

Here, a 2-D gel electrophoresis protocol for *A. fumigatus* was established that resolved more than 1,000 proteins on a large format gel with a broad pH range. By comparison of the protein pattern of *A. fumigatus* grown on glucose versus ethanol the method was verified and a first proteome map was obtained.

Material and methods

Strains and culture conditions

The *Aspergillus fumigatus* wild-type strain ATCC 46645 was used for this study. *A. fumigatus* was cultivated at 37°C in *Aspergillus* minimal medium (AMM) as previously described (Weidner et al. 1998). The medium contained 50 mM glucose or 50 mM ethanol as the sole carbon source. One hundred millilitres of medium was inoculated to give a spore concentration of 3×10^6 conidia/ml. The glucose-grown cultures were incubated on a rotary shaker for 15 h with 200 rpm, the ethanol-grown cultures for 30 h. The glucose concentration of the medium was monitored enzymatically using glucose oxidase, peroxidase and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as described in Brock and Buckel (2004). The ethanol concentration was determined using ethanol dehydrogenase (Beuther 1988) at the end of cultivation. After harvesting, the mycelium was rinsed with tap water, pressed to remove any liquid and the dried mycelium was frozen in liquid nitrogen. Protein extracts were prepared as described below.

Sample preparation

Extraction protocol

Frozen mycelium was ground in a precooled mortar with a pestle in the presence of liquid nitrogen. About 50 mg of homogenate were directly dissolved in lysis buffer (described below). Alternatively, the homogenate was purified by TCA precipitation or by the application of the "Clean Up Kit" (GE Healthcare Bio-Sciences, Freiburg, Germany) with or without prior extraction of the proteins with an SDS lysis buffer.

TCA/acetone precipitation TCA/acetone precipitation was carried out as described by Damerval et al. (1986) with some modifications. Briefly, 50 mg of homogenate were precipitated over night with 500 µl acetone/13.3% (w/v) TCA/0.093% (v/v) 2-mercaptoethanol. After centrifugation for 15 min at 20,000*g* at 4°C the supernatant was removed and the pellet was rinsed twice in ice-cold acetone containing 0.07% (v/v) 2-mercaptoethanol. The suspension was centrifuged again and the pellet was air-dried for 5 min at room temperature, resuspended in 300 µl lysis buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.8% (v/v) ampholytes (Serva), 20 mM Tris and 20 mM DTT] and incubated for 1 h at room

temperature. To improve the solubilisation of the proteins the samples were sonicated for 10 min in an ultrasonic bath. After centrifugation at 20,000*g* for 30 min at 16°C, the supernatant was collected.

''Clean Up Kit'' Fifty milligrams of homogenate were resuspended in 300 µl solubilising solution consisting of 2% (w/v) SDS, 40 mM Tris base and 60 mM DTT. The extract was heated at 95°C for 5 min and insoluble debris was removed by centrifugation for 10 min at 20,000*g*. The supernatant was transferred to another microcentrifuge tube and three volumes of precipitant of the ''Clean Up Kit'' were added. The following steps were performed according to the protocol B of the manufacturer's manual (GE Healthcare Bio-Sciences). The air-dried pellet was resuspended in 300 µl lysis buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.8% (v/v) ampholytes (Serva), 20 mM Tris and 20 mM DTT].

In an alternative approach, 50 mg of homogenate was directly resuspended in 300 µl precipitant without prior SDS solubilisation. The subsequent steps were performed as described above.

Evaluation of different lysis buffers

Five different lysis buffers for protein extraction were compared. The ground mycelium was purified by TCA precipitation with the ''Clean Up Kit'' (GE Healthcare Bio-Sciences). Fifty milligrams of homogenate were directly resuspended in 300 µl precipitant solution of the ''Clean Up Kit''. After washing with acetone the dried pellet was suspended in 300 µl of the following lysis buffers: buffer I [9 M urea, 4% (w/v) CHAPS, 0.8% (v/v) ampholytes, 20 mM DTT, 20 mM Tris], buffer II [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.8% (v/v) ampholytes, 20 mM DTT, 20 mM Tris], buffer III [7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 1% (w/v) Zwittergent 3–10, 0.8% (v/v) ampholytes, 20 mM DTT, 20 mM Tris], buffer IV [7 M urea, 2 M thiourea, 1% (w/v) ASB 14, 0.8% (v/v) ampholytes, 20 mM DTT, 20 mM Tris] and buffer V (equal mixture of buffer III and buffer IV).

Protein quantification

The protein concentration was determined according to the method of Bradford (1976) using BIO-RAD protein assay (BIO-RAD Lab., Hartfordshire, USA).

2-D gel electrophoresis

The protein sample was applied via anodic cup loading to 2-D gel electrophoresis. The amount of 50 µg protein was loaded for comparison of precipitation methods, the amount of 250 µg protein for comparison of the different lysis buffers, whereas 300 µg protein was applied for comparison of ethanol and glucose-grown mycelia. The strips of 7 and 24 cm covering a non-linear pH range of pH 3–11 or 7–11 (GE Healthcare Bio-Sciences) were rehydrated over night in 150 or 450 µl rehydration buffer, respectively. The rehydration buffer contained the following: 7 M urea, 2 M thiourea, 2% (w/v) CHAPS and/or 1% of another zwitterionic detergent in accordance with the lysis buffer, 0.002% (w/v) bromophenol blue, 0.5% (v/v) IPG buffer, 1.2% (v/v) DeStreak Reagent (Olsson et al. 2002) (GE Healthcare Bio-Sciences). Isoelectric focusing was carried out using the IPGphor II with a manifold ceramic tray (GE Healthcare Bio-Sciences) at 20°C with a current of 50 μA/strip. For 7 cm strips the following protocol was applied: 5 h at 300 V (gradient), 4 h at 600 V (gradient), 3 h at 1,000 V, 2 h at 5,000 V and 10,000 V h at 5,000 V. Isoelectric focusing of 24 cm strips was carried out according to the following protocol: 3 h at 300 V (gradient), 3 h at 600 V (gradient), 4 h at 1,000 V (gradient), 3 h at 8,000 V (gradient) and 24,000 V h at 8,000 V (step). The amount of 500 µg protein was loaded for the separation of basic protein (pH 7–11). The focusing time was extended to 4 h at 600 V and the last step at 8,000 V to 32,000 V h. Prior to the start of separation by the second dimension the strips were equilibrated for 15 min in 10 ml equilibration buffer containing 1% (w/v) DTT and subsequently for 15 min in 10 ml equilibration buffer containing 2.5% (w/v) iodoacetamide. The separation in the second dimension was realised on a Minigel-Twin System for the 7 cm strips (Biometra, Göttingen, Germany) or on the Ettan DALT System (GE Healthcare Bio-Sciences) with lab cast 1.0 mm SDS polyacrylamide gels [12.5% (w/v)] containing Rhinohide as a gel strengthener (Molecular Probes, Leiden, NL): 20 mA/gel for minigels and 30 min 30 W, 4 h 100 W for six Ettan Dalt gels. Protein standard Mark 12 was purchased from Invitrogen (Karlsruhe, Germany).

Protein visualisation, image analysis and quantification

Proteins were visualised by colloidal CBB staining (Neuhoff et al. 1988). The gels were fixed for 2 h in a fixing solution [40% (v/v) methanol, 7% (v/v) glacial acetic acid], washed twice with ultrafiltrated water for 20 min and stained over night in a staining solution [10% (v/v) methanol, 0.1% (w/v) CBB G-250, 1.6% (v/v) *o*-phosphoric acid, and 10% (w/v) ammonium sulfate]. Gels were neutralised (0.1 M Tris-base titrated to pH 6.5 with *o*-phosphoric acid) for 5 min and washed in 25% (v/v) methanol for 1 h. Stained gels were scanned and calibrated with Labscan software 5 (GE Healthcare Bio-Sciences). Images were analysed using Image Master Platinum (Melanie 5). The spots were quantified using the % volume criterion and the ratio and gap parameter. Only spots with a ratio value greater than two were regarded as significant. Three replicates of three gels for each growth condition were obtained. For matching the

detected spots a synthetic master gel was created by matching eight representative gels with each other by keeping only triplicate spots.

MS identification

Protein spots were excised manually. Tryptic digest of proteins, MALDI-TOF and MALDI TOF/TOF were carried out at TOPLAB, Martinsried, Germany. Alternatively, protein spots were digested according to the protocol of Bruker (Bruker Daltonics, Bremen, Germany) that was adapted from Shevchenko et al. (1996). MALDI TOF/TOF spectra were generated with an Ultraflex I device (Bruker Daltonics). Peptide mass fingerprint and peptide fragmentation spectra of each sample were submitted for identification using MASCOT interface (MASCOT 2.1.0, Matrix Science, London, UK) or ProFound (Genomic Solutions, USA). Hits were considered significant according to the MASCOT score ($P = 0.05$).

Results

Optimisation of protein cleanup methods

Filamentous fungi possess a robust and rigid cell wall (Latgé et al. 2005). Therefore, cell lysis is a critical step in preparation of protein extracts. As shown in Fig. 1, direct lysis of ground mycelia resulted in irreproducible gels with streaks, strong background and faint spots. In total, 516 spots were detected. TCA/acetone precipitation of proteins is a well-established technique for the purification of protein extracts, helping in the elimination of carbohydrates. The TCA/acetone precipitation from *A. fumigatus* crude extracts resulted in 690 detectable spots on a mini-gel (Fig. 1). Compared with the protein samples that had been directly dissolved, the gels loaded with TCA/acetone precipitated protein showed clear spots with less background. A few spots displayed vertical streaking. The "Clean Up Kit" from GE Healthcare is a modified TCA precipitation method for quantitative precipitation of proteins while excluding interfering substances. It was specially designed for the preparation of samples for 2-D gel electrophoresis. Direct treatment of the sample with the "2-D Clean Up Kit" resulted in gels of similar quality compared to the TCA/acetone precipitated samples. Fewer spots were detected (573), which, however, showed less vertical streaking. Compared to the TCA/acetone precipitation method, the protein yield was more than two times higher (5.6 mg/ml protein versus 2.6 mg/ml protein). Extraction of proteins with an SDS buffer containing DTT and Tris at 95°C prior to the application of the "Clean Up Kit" did not produce superior results. Although a definite protein pellet was produced, only fewer spots (532) were visible and the gel pictures looked more diffuse (Fig. 1).

Evaluation of several lysis buffers

Two-dimensional gel electrophoresis is hampered by the low solubility of proteins in the first dimension, especially when they migrate close to their isoelectric points. This leads to protein streaking and protein loss. In particular, hydrophobic proteins are difficult to study by

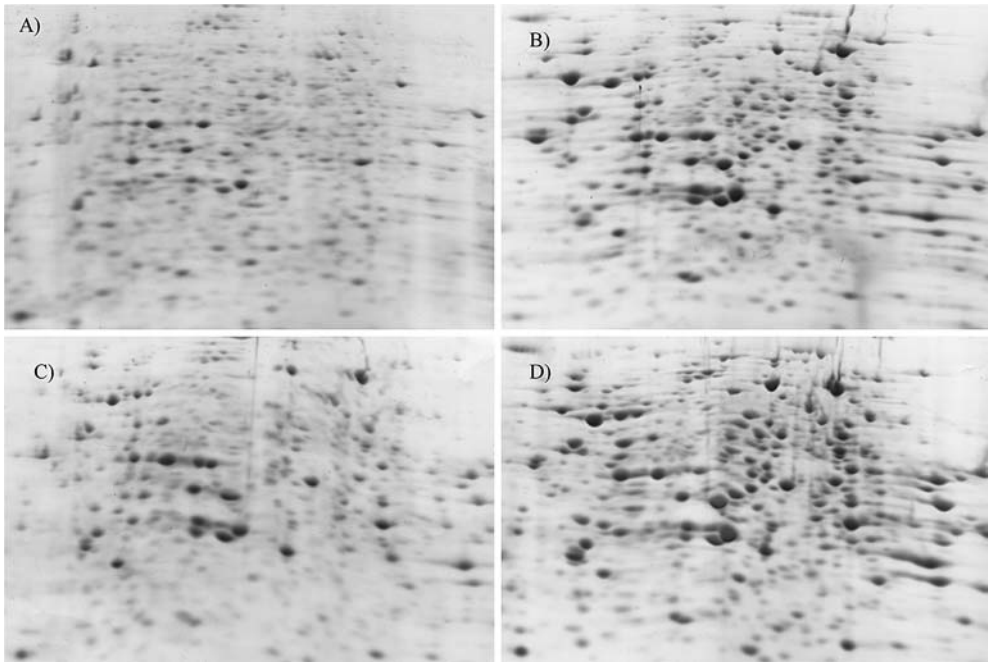


Fig. 1 Extraction of mycelia of *A. fumigatus* grown in liquid cultures with glucose as the carbon source. **a** Direct resuspension of mycelia in lysis buffer; **b** purification of protein samples with "Clean Up Kit"; **c** SDS lysis of mycelia and subsequent purification with "Clean Up Kit"; **d** purification of protein samples by TCA/acetone precipitation

2-D gel electrophoresis. These problems were in part overcome by the use of new zwitterionic detergents and of thiourea which led to an increase of resolution and capacity of 2-D gels.

Here, five different lysis buffers for the solubilisation of proteins from the filamentous fungus *A. fumigatus* were tested. Buffer 1 was composed of urea and the zwitterionic detergent CHAPS, ampholytes, Tris buffer and DTT. Thiourea was additionally added to buffer 2 while buffer 3 contained the zwitterionic sulfobetaine Zwittergent 3–10 and CHAPS. The zwitterionic detergent ASB 14 was added to buffer 4. Finally, buffer 5 consisted of an equal mixture (v/v) of buffer 4 and 5. The use of buffers 1 and 4 resulted in gels with the lowest number of detectable spots, i.e., 756 ± 81 spots and 714 ± 88 spots, respectively (Fig. 2). Buffer 1 led to the production of gels with horizontal streaking, whereas gels loaded with protein samples obtained by the use of buffer 4 showed clear spots (Fig. 2). However, when lysis buffer 4 was applied, it was difficult to reproduce the quality of gels. The application of buffers 2, 3 and 5 resulted in gels of almost the same quality and with an approximate number of spots of 828 ± 13 for buffer 2, 861 ± 51 for buffer 3 and 827 ± 64 for buffer 5. With buffer 5, more horizontal streaking was observed. Compared with lysis buffer 2, additional spots were detected when buffers 3 or 5 were used (see Fig. 2).

Proteomes of *A. fumigatus* grown on glucose or ethanol

The combination of protein precipitation with "Clean Up kit" and subsequent solubilisation with lysis buffer 3 containing CHAPS and Zwittergent 3–10 showed the best results. To evaluate the optimised method of proteome analysis, the global response of *A. fumigatus* at the proteomic level by cultivating the fungus on 50 mM ethanol or 50 mM glucose was analysed. The experiments were carried out in triplicate and 18 gels were analysed. After spot detection, the gels were matched to each other with the help of the landmark function of the Image Master Platinum software (see Materials and Methods). When 300 µg protein was loaded, on average $1{,}235 \pm 159$ spots per gel were detected (Fig. 3a). 980 spots were detected which matched with a spot in the reference gel and were assigned

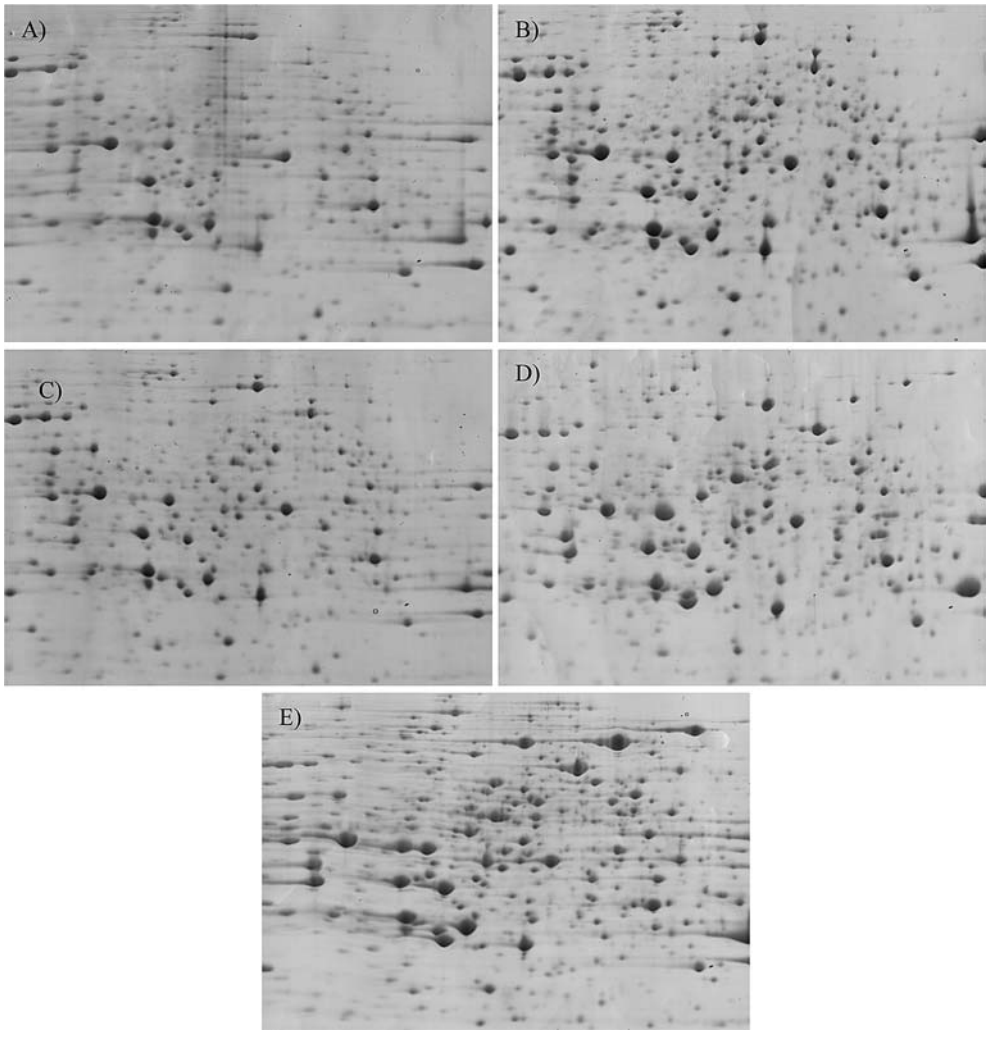


Fig. 2 Test of different lysis buffers for the solubilisation of *A. fumigatus* proteins. **a** Lysis buffer 1 (9 M urea, 4% CHAPS, 0.8% ampholytes, 20 mM DTT, 20 mM Tris); **b** lysis buffer 2 (7 M urea, 2 M thiourea, 4% CHAPS, 0.8% ampholytes, 20 mM DTT, 20 mM Tris); **c** lysis buffer 3 (7 M urea, 2 M thiourea, 2% CHAPS, 1% Zwittergent 3–10, 0.8% ampholytes, 20 mM DTT, 20 mM Tris); **d** lysis buffer 4 (7 M urea, 2 M thiourea, 1% ASB 14, 0.8% ampholytes, 20 mM DTT, 20 mM Tris); **e** lysis buffer 5 (equal mixture of buffer III and buffer IV)

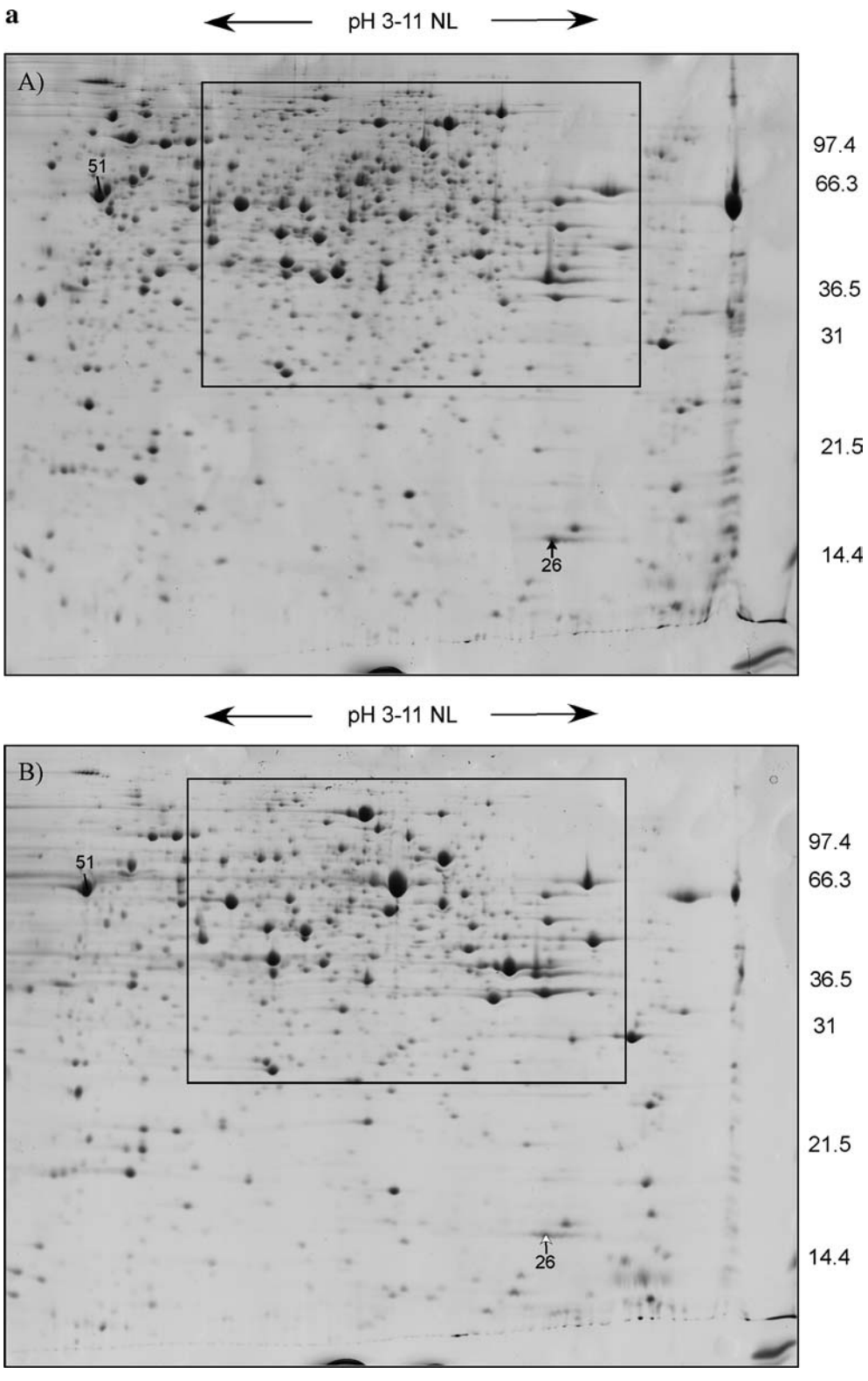
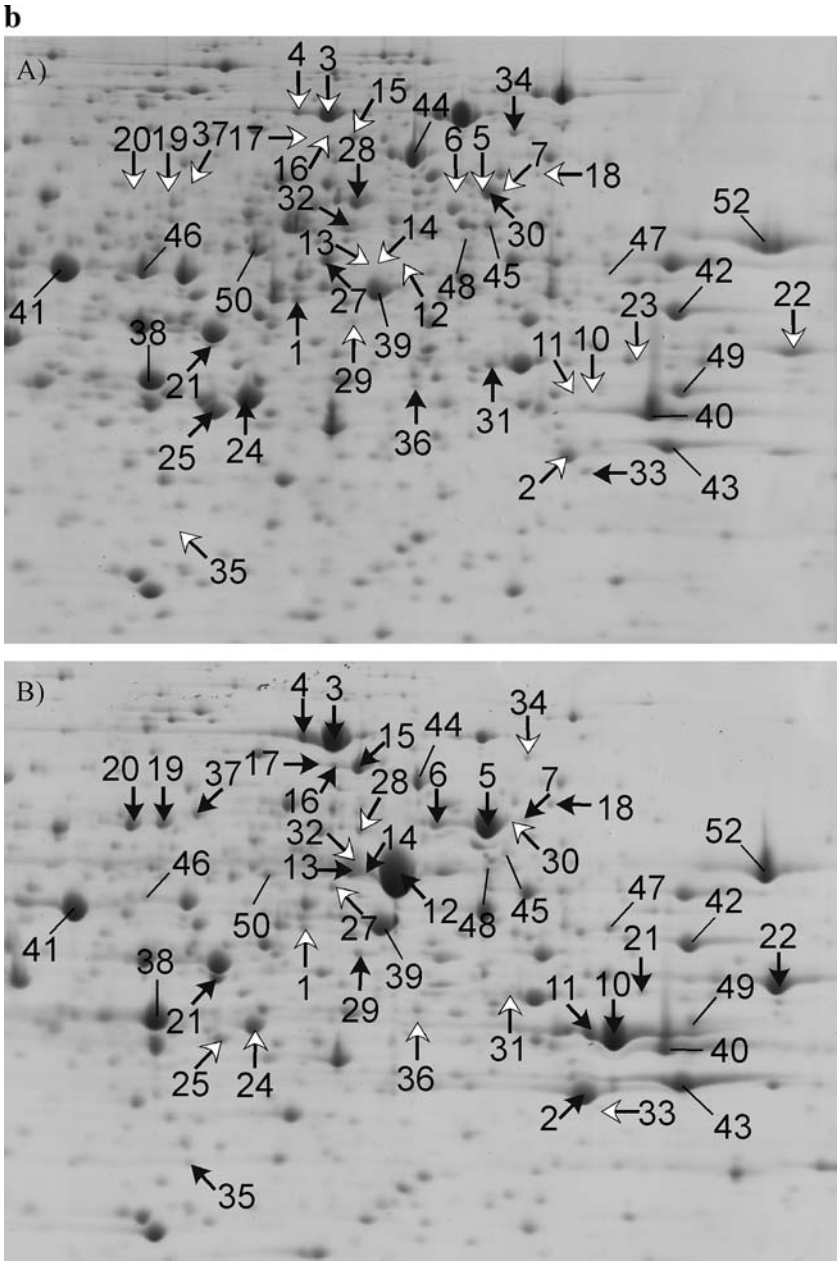


Fig. 3 **a** 2-D gel electrophoresis of proteins extracted from *A. fumigatus* grown in AMM with glucose (*A*) or ethanol (*B*) as the sole carbon source. The orientation of the IEF gel is indicated. Mr standards are indicated on the right. The numbers refer to proteins induced during growth on glucose or ethanol (see Table 1). *Black arrow* upregulation of the protein, *white arrow* downregulation of the protein. **b** shows a section of gel *A* and *B* of Fig. 3a indicated by the *black frame*

to a group number (see below). For the analysis of changes in the protein pattern, mean quantitative values and SDs were calculated. Spots representing the same protein on different gels were defined as groups. Only spot groups with a ratio higher than 2 were considered as significant change. The ratio value comprises the lower limit of one class interval and the upper limit of the other class interval. In Table 1, the fold changes of the raw central tendency and the gap value [maximum difference between the range of the current class (either glucose or ethanol) and the range of one of the other classes (either glucose or ethanol)]

Fig. 3 (Contd.)



are displayed. Negative values indicate overlapping, positive values non-overlapping class ranges.

Thirty-seven spots were identified by MALDI-TOF/TOF showing consistent differences in intensity between growth on glucose and ethanol (Table 1 and Fig. 3b). A sequence coverage between 21 and 87% was obtained (Table 1) during MS analysis. Some proteins appeared in gels as more than one spot with the same apparent molecular mass, but with different p*I* values and abundance, presumably due to post-translational modifications or isoenzyme variation. Proteins for which several variants were identified, included aconitate hydratase, isocitrate lyase, alcohol dehydrogenase (ADH), aldehyde dehydrogenase, acetyl-CoA synthetase and phosphoenolpyruvate carboxykinase. Thirty-seven spots were sorted into six different categories (Table 1). Among the enzymes that are involved in glycolysis, pyruvate dehydrogenase changed most significantly in quantity. During growth on glucose it showed a 3.1-fold increase. During growth on ethanol some enzymes of the citrate cycle were upregulated. Aconitate hydratase, which is involved in the tricarboxylic acid cycle, revealed a 2.8-fold increase during growth on ethanol. Two different malate dehydrogenases (MDHs) exhibited a higher abundance during growth on ethanol, i.e., MDH with the locus tag Afu6g05210 increased 3.1-fold, whereas the other MDH (locus tag Afu7g05740) did not increase significantly (1.7-fold). Both enzymes possess a conserved domain characteristic of glycosomal-mitochondrial MDH, but only Afu7g05740 exhibited an

Table 1 Differentially synthesised proteins during growth of *A. fumigatus* on glucose or ethanol

Spot number	Locus tag	Description	Pi/MW[a]	Glucose	Ethanol	-fold	Gap	Sequence coverage (%)
Glycolysis								
1	Afu1g06960	Pyruvate dehydrogenase, α-SU	6.36/42	Up	Down	3.1	0.068	40
TCA cycle								
2	Afu6g05210	Malate dehydrogenase	6.77/35	Down	Up	3.1	0.50	87
3	Afu6g12930	Aconitate hydratase	6.26/86	Down	Up	2.7	0.74	43
4	Afu6g12930	Aconitate hydratase	6.26/86	Down	Up	2.8	0.11	34
Glyoxylate cycle								
5	Afu4g13510	Isocitrate lyase	6.26/60	Down	Up	415	1.31	68
6	Afu4g13510	Isocitrate lyase	6.26/60	Down	Up	Unique	0.18	26
7	Afu4g13510	Isocitrate lyase	6.26/60	Down	Up	Unique	0.035	52
8	Afu6g03540	Malate synthase	8,44/61	Down	Up	164	0.908	67
9	Afu6g03540	Malate synthase	8.44/61	Down	Up	Unique	0.156	32
Ethanol utilisation								
10	Afu7g010110	ADH I	7.04/38	Down	Up	103.4	2.06	76
11	Afu7g010110	ADH I	7.04/38	Down	Up	16.7	0.29	74
12	Afu7G01000	Aldehyde dehydrogenase	6.16/54	Down	Up	352	3.11	71
13	Afu7G01000	Aldehyde dehydrogenase	6.16/54	Down	Up	11.7	0.32	60
14	Afu7G01000	Aldehyde dehydrogenase	6.16/54	Down	Up	14.6	0.046	40
15	Afu4g11080	Acetyl-CoA-synthetase	5.99/75	Down	Up	33.1	0.18	36
16	Afu4g11080	Acetyl-CoA-synthetase	5.99/75	Down	Up	8.4	0.011	31
17	Afu4g11080	Acetyl-CoA-synthetase	5.99/75	Down	Up	5.3	0.011	21
18	Afu2g12530	Carnitine acetyl transferase	8.67/73	Down	Up	7.0	0.013	50
Gluconeogenesis								
19	Afu6g07720	PEP carboxykinase	5.79/67	Down	Up	66.6	0.23	47
20	Afu6g07720	PEP carboxykinase	5.79/67	Down	Up	28.8	0.17	55
21	Afu4g11310	Fructose-1,6-bisphosphatase	5.87/29	Down	Up	5.0	−0.015	49
Amino acid metabolism								
22	Afu4g10410	Aspartate aminotransferase	8.94/48	Down	Up	6.2	0.22	71
23	Afu2g09650	Aspartate aminotransferase	8.6/51	Down	Up	Unique	0.013	26
Other proteins								
24	Afu5g02470	Thiamine biosynthesis protein	6.04/39	Up	Down	2.2	0.50	78
25	Afu5g09230	Transaldolase	6.04/36	Up	Down	3.5	0.39	79
26	Afu5g03490	Nucleoside diphosphate-kinase	7.77/17	Up	Down	2.2	0.19	46
27	Afu3g10660	Hydroxy-methylglutaryl-CoA synthase	6.12/51	Up	Down	4.6	0.16	59
28	Afu3g11830	Phosphoglucomutase	6.29/61	Up	Down	3.2	0.13	58
29	Afu8g06080	Flavohemoprotein	8.35/48	Down	Up	4.1	0.055	27
30	Afu6g04920	Formate dehydrogenase	8.42/46	Up	Down	11.6	0.055	34
31	Afu5g07000	NAD-binding Rossmann fold OR	6.53/40	Up	Down	3.0	0.052	56
32	Afu3g12690	Conserv. hypoth. protein	5.27/61	Up	Down	17.9	0.043	43
33	Afu1g09930	Glycerol dehydrogenase	7.1/34	Up	Down	4	0.027	23
34	Afu1g08810	Glycerol-3-phosphate dehydrogenase	6.71/77	Up	Down	2.8	0.021	47
35	Afu2g00570	Acetyltransferase	5.90/29	Down	Up	Unique	0.019	32
36	Afu6g11850	Hypothetical protein	8.68/44	Up	Down	33	0.014	31
37	Afu2g08280	Malic enzyme	6.34/73	Down	Up		0.079	27

[a]Theoretical MW and p*I* according to the sequence

N-terminal signal peptide for import into the mitochondria. The upregulated MDH is therefore presumably localised in the glyoxysome or the cytosol. Enzymes of the glyoxylate cycle and the ethanol utilisation pathway showed the most significant change, e.g., isocitrate lyase increased 415-fold. Three different spots with different isoelectric points represented this key enzyme of the glyoxylate shunt (Fig. 3b). Spot No. 5 exhibited the biggest spot with a proportion of 86%. Two additional, but minor spots, which were identified as isocitrate lyase, were only detected in gels from ethanol-grown cultures (spot 6 and 7). The abundance of ADH (103- and 17-fold), aldehyde dehydrogenase (352-, 12- and 15-fold) and acetyl-CoA synthetase (33- and 8-fold) was strongly increased during growth on ethanol (Table 1). In all cases, spots of the same molecular mass but with different isoelectric points were detected. Alcohol and aldehyde dehydrogenase together represented a total of 3.8 vol% of all spots.

When *A. fumigatus* was grown with ethanol as the sole carbon source, the amount of the key enzyme of gluconeogenesis, phosphoenolpyruvate carboxykinase, increased 67-fold, a variant of this enzyme with a different p*I*, 29-fold. Under the same conditions, malic enzyme increased 10.5-fold and fructose-1,6-bisphosphatase 5-fold. The second key enzyme of the glyoxylate cycle, malate synthase, was only detected in gels covering the pH range from 7 to 11. No significant amounts of malate synthase were detectable in glucose-grown mycelium (Fig. 4).

During growth on ethanol the abundance of two aspartate aminotransferases increased. The aspartate

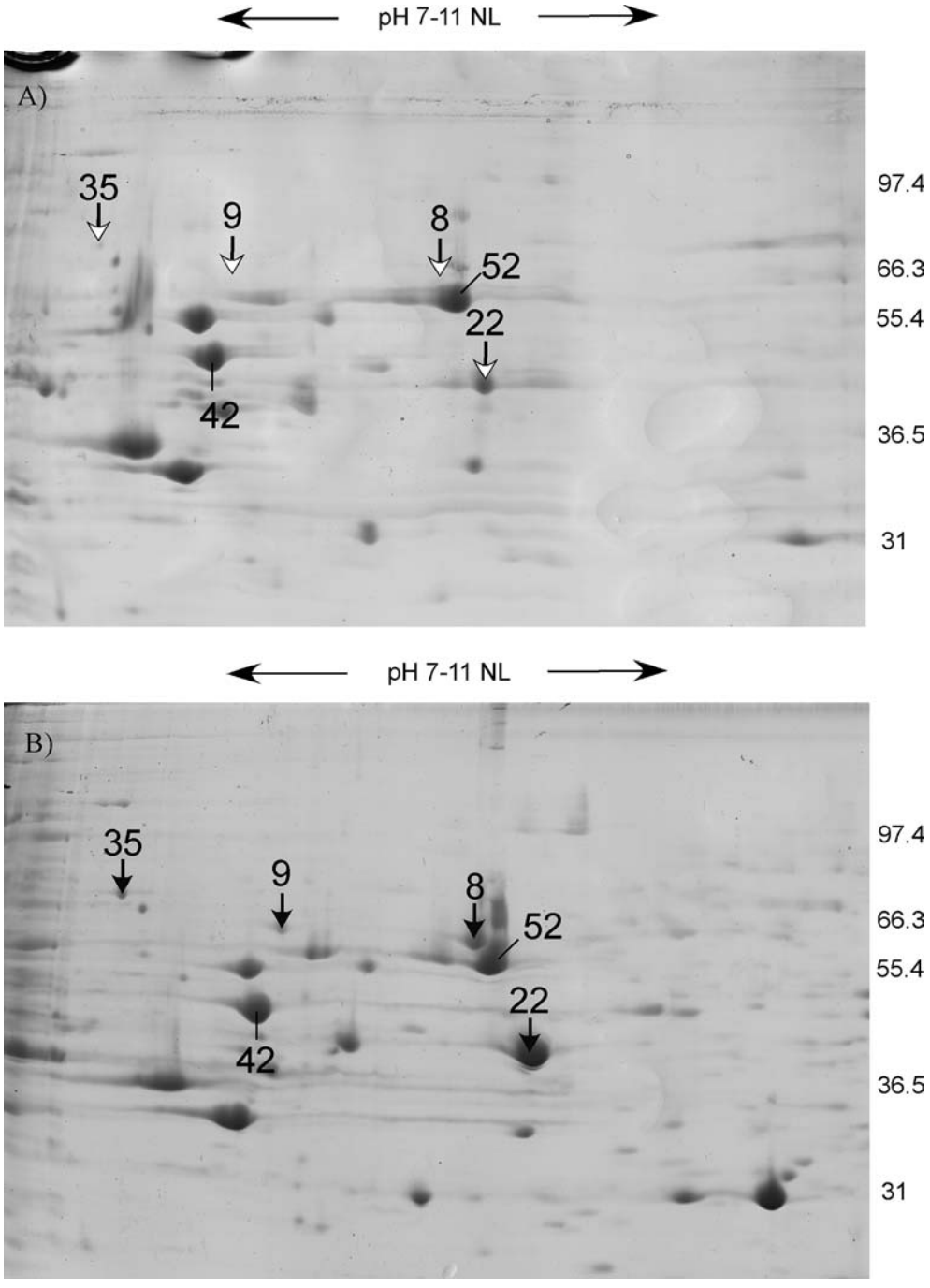


Fig. 4 Section of a 2-D gel electrophoresis gel separating basic polypeptides (p*I* 7–11) from *A. fumigatus* grown with glucose (*A*) or ethanol (*B*) as the sole carbon source. The numbers refer to proteins induced during growth on glucose or ethanol (see Table 1). *Black arrow* upregulation of the protein, *white arrow* downregulation of the protein. Spot number 8 and 9 represented the malate synthase, which could not be detected when a broad pH range from 3 to 11 was applied for separation

aminotransferase with the locus tag Afu4g10410 represented presumably the mitochondrial form with a 29 amino acid long signal peptide (6.2-fold increase) and the enzyme with locus tag Afu2g09650 the cytosolic form, because it did not show an obvious signal peptide. The protein Afu2g09650 was only detected in gels run with protein from ethanol-grown mycelia. A putative carnitine acetyl transferase showed an increased level during growth on ethanol of 7-fold. This carnitine acetyl transferase with the locus tag Afu2g12530 contains a signal peptide of 41 amino acids with a 99.4% probability for export to mitochondria calculated by Mitoprot (Claros and Vincens 1996). Additionally, several other proteins with different physiological functions changed their abundance significantly during growth of *A. fumigatus* on glucose and ethanol (Table 1; Fig. 3a, b). A flavohemoprotein (4.1-fold), an acetyltransferase and several other proteins showed a higher abundance during growth on glucose. Also, enzymes of the primary metabolism were identified which did not show a significant change. These enzymes are listed in Table 2.

Table 2 Further detected proteins of the primary metabolism of *A. fumigatus* during growth on glucose or ethanol showing no significant change

Spot number	Locus tag	Description	Pi/MW	Glucose	Ethanol	-fold	Gap	Sequence coverage (%)
Glycolysis								
38	Afu3g11690	Fructose bisphosphate aldolase	5.55/40	Similar	Similar	1.0	−0.919	76
39	Afu1g10350	Phosphoglycerate kinase	6.31/45	Similar	Similar	1.1	−0.131	79
40	Afu5g01970	Glyceraldehyde-3-phosphate dehydrogenase	6.06/36	Up	Down	1.4	−0.277	70
41	Afu6g06770	Enolase	5.39/47	Similar	Similar	1.05	−0.595	64
TCA cycle								
42	Afu5g04230	Citrate synthase	8.69/52	Down	Up	1.2	−0.087	38
43	Afu7g05740	Malate dehydrogenase	9.08/36	Down	Up	1.7	−0.659	80
Pentosephosphate cycle								
44	Afu1g10350	Transketolase	6.13/75	Up	Down	2.5	0.112	60
45	Afu3g08470	Glucose-6-phosphate dehydrogenase	6.55/58	Up	Down	2.4	−0.0024	33
46	Afu6g08050	6-Phosphogluconate dehydrogenase	5.86/56	Up	Down	2.2	−0.126	57
Methylcitrate cycle								
47	Afu6g03590	Methylcitrate synthase	8.95/51	Down	Up	3.4	0.0081	41
48	Afu6g03730	2-Methylcitrate dehydratase	8.62/63	Down	Up	4.7	−0.0055	27
Others								
49	Afu5g06240	ADH, putative	7.55/35	Up	Down	1.8	−0.145	59
50	Afu6g10660	ATP-citrate lyase	5.88/53	Up	Down	1.3	−0.223	52
51	Afu5g10590	ATP synthase F1, beta-subunit	5.3/56	Similar	Similar	1.1	−0.859	66
52	Afu8g05320	ATP synthase F1, alpha subunit	9.7/60	Down	Up	1.3	−0.564	31

Discussion

Here, we optimised a 2-D gel electrophoresis protocol for *A. fumigatus* which is based on the purification of protein samples with "Clean Up Kit" in combination with a lysis buffer containing CHAPS and Zwittergent 3–10. Lysis buffer 3 was further evaluated in experiments resulting in the first proteome maps of *A. fumigatus* grown on different carbon sources, i.e., on a glycolytic (glucose) and a gluconeogenic substrate (ethanol). Ethanol-grown mycelia of *A. fumigatus* showed a strong up-regulation of enzymes required for the glyoxylate cycle, gluconeogenesis and ethanol oxidation (see Table 1, Fig. 3b). Isocitrate lyase was the most up-regulated enzyme with a 415-fold increase. Post-translational modification of isocitrate lyase or very similar isoforms may be the reason for the occurrence of three different spots with different isoelectric points, but only a single isocitrate lyase sequence is present in the genome of *A. fumigatus* (De Lucas et al. 1994). The second enzyme of the glyoxylate cycle, malate synthase, was only detected in gels with a pH range from 7 to 11. The application of such a narrow pH range improved the spatial resolution of proteins, resulting in the finding that spot No. 52 in a gel covering a pH range from 3 to 11 represented two spots, namely spot 52 and 8 (Fig. 4).

All key enzymes of gluconeogenesis, especially PEP carboxykinase and fructose-1,6-bisphosphatase, were upregulated during growth of *A. fumigatus* on ethanol. In addition, malic enzyme showed a higher abundance during growth on ethanol. Consistently, in *A. nidulans* malic enzyme which converts malate to pyruvate was shown to have higher activity during growth on ethanol (Kelly and Hynes 1981). Besides the PEP carboxykinase, this enzyme is essential for growth on C2 compounds (Hondmann and Visser 1994).

As expected, the ethanol utilisation enzymes were strongly upregulated in *A. fumigatus* during growth on ethanol. In *A. nidulans*, they are under the control of the AlcR protein. Moreover, in *A. nidulans* growth on ethanol resulted in high levels of both alcohol and aldehyde dehydrogenase (up to 1% of total protein) (Felenbok et al. 2001). As shown here, similarly, in *A. fumigatus* both enzymes represented a total of 3.8 vol% of all spots. Interestingly, the genome of *A. fumigatus* contains more than 30 putative ADHs, but only the ADH with the locus tag Afu7g010110 represents the ethanol-oxidising enzyme. This ADH also shows high similarity to ADH I from *A. nidulans*, the enzyme required for the utilisation of ethanol as sole carbon source (Gwynne et al. 1987). Furthermore, carnitine acetyltransferase was found to be inducible by acetate in *A. nidulans* (Stemple et al. 1998) and, in *S. cerevisiae*, by ethanol (Claus et al. 1982). This protein is required for the shuttle of acetyl groups between intracellular compartments. The detected carnitine acetyl transferase Afu2g12530 presumably represents the protein localised in both mitochondria and peroxisomes. The second annotated, but not detected carnitine acetyl transferase Afu1g12340 possesses no obvious signal peptide and shows a high degree of similarity to the cytosolic carnitine acetyl transferase FacC of *A. nidulans* (Stemple et al. 1998). Furthermore, aconitate hydratase and MDH revealed an increase in quantity during growth on ethanol. This

can be explained by the necessary replenishment of the glyoxylate cycle by the activity of citrate synthase and aconitase as shown for *A. nidulans* (Sims et al. 2004). Furthermore, two MDHs were detected. The up-regulated MDH identified here is presumably located either in glyoxysomes or the cytoplasm. In contrast to *S. cerevisiae* (Steffan and Mc Alister-Henn 1992), there is no evidence for three MDH isoenzymes (mitochondrial, glyoxysomal, cytosolic) in *A. fumigatus*. Although the genome of *A. fumigatus* encodes two additionally annotated MDHs with the locus tags Afu2g13800 and Afu7g02420, these ORFs were presumably misinterpreted as MDHs because convincing similarity to MDHs is missing.

Gluconeogenesis requires metabolite transport between mitochondria, the glyoxysomes and the cytosol. During growth on C_2 compounds, oxaloacetate has to be transported from the mitochondria to the glyoxysomes via the MDH route or, alternatively, aspartate aminotransferase route (Bakker et al. 2001). The latter would explain the higher abundance of aspartate transaminase during growth on ethanol.

During growth on glucose less proteins showed an increase in abundance. Not surprisingly, enzymes of the glycolysis, such as pyruvate dehydrogenase were upregulated. Presumably due to the higher growth rate on glucose, transaldolase and transketolase of the pentose phosphate cycle (Hondmann and Visser 1994), phosphoglucomutase involved in cell wall biosynthesis and trehalose production (Hoffmann et al. 2000), and the glycerol-synthesising glycerol dehydrogenase were upregulated (Schuurink et al. 1990).

In summary, most of the key enzymes for gluconeogenic growth were detected in ethanol-grown mycelia. The obtained results helped to improve functional assignments to the annotated ORFs of the *A. fumigatus* genome. Future experiments will reveal the role of the key enzymes for the metabolism of C_2 carbon compounds during infection. It is interesting to note that in *C. albicans*, the genes encoding isocitrate lyase, carnitine acetyl transferase and other genes involved in β-oxidation were induced after phagocytosis by macrophages. The encoded proteins were shown to play an important role in microbial pathogenesis (Lorenz and Fink 2001; Prigneau et al. 2003; Prigneau et al. 2004).

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