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Analysis of the regulation of the *Ustilago maydis* proteome by dimorphism, pH or *MAPK* and *GCN5* genes

José L. Martínez-Salgado[a], Claudia G. León-Ramírez[b], Alberto Barrera Pacheco[a], José Ruiz-Herrera[b,*], Ana P. Barba de la Rosa[a,**,1]

[a]IPICyT, Instituto Potosino de Investigación Científica y Tecnológica. Camino a La Presa San José No. 2055, Lomas 4ª Sección, 78216, San Luis Potosí, SLP, Mexico

[b]Centro de Investigación y de Estudios Avanzados del IPN, Unidad Irapuato, Apdo. 629, 36500 Irapuato Gto., Mexico

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ABSTRACT

Ustilago maydis is a dimorphic corn pathogenic basidiomycota whose haploid cells grow in yeast form at pH 7, while at pH 3 they grow in the mycelial form. Two-dimensional gel electrophoresis (2-DE) coupled with LC-ESI/MS-MS was used to analyze the differential accumulation of proteins in yeast against mycelial morphologies. 2-DE maps were obtained in the pH range of 5–8 and 404 total protein spots were separated. From these, 43 were differentially accumulated when comparing strains FB2*wt*, constitutive yeast CL211, and constitutive mycelial GP25 growing at pH 7 against pH 3. Differentially accumulated proteins in response to pH are related with defense against reactive oxygen species or toxic compounds. Up-accumulation of CipC and down-accumulation of Hmp1 were specifically related with mycelial growth. Changes in proteins that were affected by mutation in the gene encoding the adaptor of a MAPK pathway (CL211 strain) were UM521* and transcription factors Btf3, Sol1 and Sti1. Mutation of *GCN5* (GP25 strain) affected the accumulation of Rps19-ribosomal protein, Mge1-heath shock protein, and Lpd1-dihydrolipoamide dehydrogenase. Our results complement the information about the genes and proteins related with the dimorphic transition in *U. maydis* and changes in proteins affected by mutations in a *MAPK* pathway and *GCN5* gene.

1. Introduction

Ustilago maydis is a pathogenic Basidiomycota fungus that causes common smut in maize (*Zea mays*) and its probable ancestor, *Zea mays* subsp. *parviglumis* [1]. *U. maydis* exhibits a dimorphic switch from yeast to filamentous growth in response to mating interactions and environmental conditions. Haploid cells can form filaments *in vitro* in response to acidic pH [2], or use of fatty acids as carbon source [3]. In nature, the yeast form corresponds to the saprophytic stage of the life cycle, and exists as haploid cells that divide by budding, whereas the filamentous form is the product of mating of sexually compatible yeasts, and corresponds to the dikaryotic and pathogenic stage.

The genome-wide analysis of *U. maydis* annotated at the Munich Center for Protein Sequence (MIPS) (http://mips.gsf.de/genre/proj/ustilago/), has revealed that *U. maydis* is more closely related to humans than to budding yeast, and numerous proteins are shared only by *U. maydis* and *Homo sapiens* [4]. The reported genome also has opened new fields of research on the molecular bases of the dimorphic yeast-to

* *Corresponding author.*
** *Corresponding author.* Tel.: +52 4448342000; fax: +52 4448342010.
E-mail addresses: jruiz@ira.cinvestav.mx (J. Ruiz-Herrera), apbarba@ipicyt.edu.mx (A.P.B. de la Rosa).
[1] We dedicate this work to the memory of Dr. G. Mendoza.

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mycelium transition studies. SSH and SAGE libraries have been used for the identification of up- and down-regulated genes in filamentous cells [5–7]. However, not all changes in the expression level are necessarily the reflection of alterations occurring at the transcriptional level. Accordingly analyses at the level of protein composition of both forms are necessary for our understanding of the dimorphic phenomenon.

Two-dimensional polyacrylamide gel electrophoresis (2-DE) is a tool for the analysis of complex protein mixtures and for examination of the levels of protein expression in a tissue or cell type at different stages, conditions or treatments. This technique has been used in the studies of changes in protein expression during the dimorphic transition of different fungi, including *Candida albicans* [8,9], *Penicillium marneffei* [10], and *Candida glabrata* [11]. Also, the proteome reference map for *U. maydis* has been reported, as well the dimorphic transition induced by two independent regimes; either by overexpression of the bW2/bE1-heterodimer or by overexpression of the small GTP binding protein Rac1 [12]. However, the ability to obtain mycelial growth both in liquid and solid media [2] has opened the possibility to analyze *in vitro* the protein changes involved in the yeast-to-mycelium transition.

As indicated above, *U. maydis* grows in a yeast-like form when the initial pH of the medium is close to neutrality, whereas at pH 3 mycelial forms are obtained [2]. In addition, monomorphic mutants such as the CL211 constitutive yeast deleted in a gene coding for a member of the MAPK family [13] and GP25 constitutive mycelium mutated in the gene encoding the histone acetyl transferase *GNC5* [González-Prieto and Ruíz-Herrera, unpublished] are useful tools for search the factors directly involved in the morphological transition process. The aim of this work was to use the proteomics tools to analyze the changes in proteins related to *U. maydis* FB2 wild type dimorphic transition induced by pH, and controlled by *UBC2* [13] and *GCN5*.

2. Materials and methods

2.1. Strains and growth conditions

The strains used in this study were: FB2*wt* (*a2b2*, wild type) [14], CL211 [*a2b2myc*$^-$; a yeast constitutive mutant reported by Martínez-Espinoza et al. [13], and GP25 (*a2b2 gcn5::hyg*, mycelium constitutive mutant [González-Prieto and Ruíz-Herrera, unpublished data] of *U. maydis*.

Strains were grown for 18 to 20 h in liquid complex medium (CM) [15]. The cells were recovered by centrifugation (16,000 ×*g*), washed twice with sterile distilled water and suspended in the original volume of sterile distilled water. The cell suspension was shaken at 28 °C for about 3 h, until most cells appeared unbudded. They were recovered by centrifugation, washed, suspended in sterile distilled water and maintained at 4 °C for not more than 48 h. These cell suspensions were used as experimental inoculum. Cell suspensions (5×10^6 cells ml^{-1}) were used to inoculate synthetic medium (MM) [15] maintained at 30 °C. The cultures were then incubated at 28 °C on a shaker (200 rpm) in media with an initial pH of 7.0 where the FB2*wt* strain grew as a homogeneous population of budding yeast-like cells. When the initial pH of the medium was adjusted to 3.0, almost the whole population grew in the form of mycelium. The CL211 strain grew at both pH values as budding yeast-like cells, whereas GP25 cells maintained the mycelial form at both pH values.

2.2. Light microscopy

Cells were stained with cotton blue for observation by bright field microscopy (Leica, DMRB).

2.3. Protein extraction

Cultures were centrifuged at 16,000 ×*g* for 10 min at 4 °C and the cells were suspended in 500 μl of extraction acid buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, and 80 mM citric acid at pH 4). The cells were sonicated on ice for 1.5 min with intervals of 10 s. Cells debris were removed by centrifugation at 16,000 ×*g* for 10 min at 4 °C [16]. The supernatant was collected and the proteins were precipitated with cold acetone. The protein pellets were solubilized in sample rehydration buffer (8 M urea, 2% (w/v) CHAPS, 1.8 mM dithiothreitol, 0.5% Pharmalite 4–7 (GE Healthcare Bioscience, Piscataway, NJ; USA), and 0.002% bromophenol blue. Protein concentration was estimated using the dye-binding assay according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA).

2.4. 2-DE and image analysis

Proteins solubilized in rehydration buffer were used to rehydrate 11 cm linear immobilized gradient strips (Bio-Rad) with a pH gradient from 5 to 8. Strips were rehydrated for 12 h and then focused with a pre-step of 100–150 V for 2 h in the IPGphor II (GE Healthcare) to a final 60,000 V/h. Focused strips were equilibrated for 15 min in a buffer containing 50 mM Tris–HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue and 1% (w/v) dithiothreitol. Protein separation in the second dimension was performed in 12% SDS-PAGE using a Ruby cell (GE Healthcare). Proteins were resolved at a constant current of 20 mA/gel at 4 °C.

Proteins were visualized following staining with Coomasie Brilliant Blue G-250 (Bio-Rad) and gels were digitalized in a Gel-doc (Bio-Rad). Spot detection and volume quantitation were carried out with Melanie 7 (GeneBio, Geneva, Switzerland). At least three independent experiments for each growth conditions were performed. Changes on protein expression were considered significant if the mean normalized spot volume varied at least 1.6-fold and confirmed by analysis of variance at a significant level of $P\leq0.05$.

2.5. In-gel digestion and MS

Selected spots were carefully excised from 2-DE gels and were washed successively with ultrapure water and 25 mM NH_4HCO_3. Gel pieces were dehydrated with acetonitrile (ACN) to remove contaminants and distain completely. Samples were reduced with 10 mM DTT in 25 mM NH_4HCO_3 followed by protein alkylation with 55 mM iodoacetamide. Digestion was carried out overnight at 37 °C with sequencing grade trypsin (Promega, Madison, WI, USA). The resulting tryptic fragments were extracted twice with ACN:water (3:2 v/v) containing