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Alcohol dehydrogenase 1 participates in the Crabtree effect and connects fermentative and oxidative metabolism in the Zygomycete *Mucor circinelloides*[§]

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Mucor circinelloides **is a dimorphic Zygomycete fungus that produces ethanol under aerobic conditions in the presence of glucose, which indicates that it is a Crabtree-positive fungus. To determine the physiological role of the alcohol dehydrogenase (ADH) activity elicited under these conditions, we obtained and characterized an allyl alcohol-resistant mutant that was defective in ADH activity, and examined the effect of *adh* mutation on physiological parameters related to carbon and energy metabolism. Compared to the Adh$^+$ strain R7B, the ADH-defective (Adh$^-$) strain M5 was unable to grow under anaerobic conditions, exhibited a considerable reduction in ethanol production in aerobic cultures when incubated with glucose, had markedly reduced growth capacity in the presence of oxygen when ethanol was the sole carbon source, and exhibited very low levels of NAD$^+$-dependent alcohol dehydrogenase activity in the cytosolic fraction. Further characterization of the M5 strain showed that it contains a 10-bp deletion that interrupts the coding region of the *adh1* gene. Complementation with the wild-type allele *adh1*$^+$ by transformation of M5 remedied all the defects caused by the *adh1* mutation. These findings indicate that in *M. circinelloides*, the product of the *adh1* gene mediates the Crabtree effect, and can act as either a fermentative or an oxidative enzyme, depending on the nutritional conditions, thereby participating in the association between fermentative and oxidative metabolism. It was found that the spores of *M. circinelloides* possess low mRNA levels of the ethanol assimilation genes (*adl2* and *acs2*), which could explain their inability to grow in the alcohol.**

Keywords: *Mucor circinelloides*, ADH1 enzyme, Crabtree effect, fermentative and oxidative metabolism

Introduction

Zygomycetes are saprophytes that are ubiquitous in natural environments, and contribute to the transformation of organic matter. In recent years, Zygomycetes have attracted interest in various areas, such as biotechnology and the health sector. These organisms have been studied and used to produce a wide range of metabolic products, including organic acids, enzymes, fatty acids, and biofuels; furthermore, the biomass of Zygomycetes contains proteins, lipids, amino acids, chitin, and chitosan, and is considered useful for the production of animal feed, human food, and chitosan (Ferreira *et al*., 2013; Karimi and Zamani, 2013). Regarding their interest to the health sector, some Zygomycetes have been described as opportunistic pathogens in humans because they can cause mucormycosis. The reported species include *Absidia trapeziformis*, *Cunninghamella* spp., *Mucor* spp., *Rhizomucor* spp., and *Rhizopus* spp. (Chayakulkeeree *et al*., 2006; Ibrahim and Spellberg, 2006; Mendoza *et al*., 2015).

The yeast–hyphae dimorphism of some *Mucor* species has been considered important for industrial applications such as the production of heterologous proteins (Wolff and Arnau, 2002), or metabolites and fuel ethanol in large-scale fermentation plants (Ferreira *et al*., 2013; Karimi and Zamani, 2013). In aspects related to pathogenicity, for instance, the hyphae of *Mucor* species have been associated with infected tissues in humans (Khan *et al*., 2009), and in a murine mucormycosis system (Lee *et al*., 2013).

Depending on the environmental conditions in which dimorphic species of *Mucor* are cultivated, the germination of spores produces vegetative hyphae cells (a mycelium) or spherical budding cells (yeast) (Bartnicki-García, 1963; Sypherd *et al*., 1978); the production of a mycelium or yeast

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cells can occur under aerobic or anaerobic conditions, according to the carbon source and/or the addition to the medium of morphogenetic compounds. Mycelial cells are capable of adopting an oxidative or fermentative metabolism, depending on the cultivation conditions. In contrast, the production of yeast cells requires the presence of hexoses, and such cells adopt a fermentative metabolism, producing high levels of ethanol after growth under various conditions; yeast cells can grow by anaerobiosis or aerobiosis in the presence of morphogenetic compounds, such as dibutyryl cyclic-AMP, certain amino acids, and phenethyl alcohol (PEA) (Orlowski, 1991). Metabolic studies have shown that when *Mucor circinelloides* is incubated under aerobic conditions in the presence of glucose, it produces a mycelium that exhibits an oxidative metabolism, although it also produces appreciable levels of ethanol; these observations indicate that *M. circinelloides* is a Crabtree-positive microorganism (McIntyre *et al*., 2002). The ethanol produced by the fungus under these conditions is consumed after the glucose has been exhausted. However, yeast cells produced under anaerobic conditions do not utilize ethanol because they require hexoses as a carbon source (Lübbehüsen *et al*., 2004).

In *M. circinelloides*, the mRNA of *adh1* gene is expressed in spores (Valle-Maldonado *et al*., 2015), hyphae, and yeast cells, and produces a cytoplasmic enzyme that appears to be the major alcohol dehydrogenase (ADH) in the fungus; during mycelial growth under aerobic conditions, the level of expression of the *adh1* gene is correlated with the concentration of glucose in the culture medium (Rangel-Porras *et al*., 2005). Kinetic characterization of purified ADH1 suggests that it mainly works as a fermentative enzyme and converts acetaldehyde to ethanol in an $NADH+H^+$-dependent reaction (Rangel-Porras *et al*., 2005); furthermore, adh1 mRNA accumulates in higher levels in yeast cells than in hyphae or spores (Valle-Maldonado *et al*., 2015). In the yeast *Saccharomyces cerevisiae*, the fermentative enzyme ADH1 participates in the production of ethanol, whereas the oxidative enzyme ADH2–in conjunction with the enzymes aldehyde dehydrogenase (ALD1/ALD2) and acetyl-CoA synthetase (ACS2)-participates in the utilization of ethanol as a carbon source (Wills, 1990; de Kok *et al*., 2012).

To determine the physiological function of the ADH1 enzyme in *M. circinelloides*, we obtained and characterized an allyl alcohol-resistant mutant that was defective in ADH activity; this mutant had an *adh1* gene with an altered open reading frame (ORF), and produced a protein lacking the NAD^+-binding domain. It therefore produced a protein with abolished ADH activity. This mutation was examined for its effect on physiological parameters related to carbon and energy metabolism.

Materials and Methods

Fungal strains and cultivation conditions

We used the leucine-requiring *Mucor circinelloide*s strain R7B (ATCC90608; Roncero, 1984) as the wild-type reference strain throughout this study; the M5 strain is a spontaneous allyl alcohol-resistant ($Ally^r$) mutant derived from R7B (see below). For the growth experiments, we used yeast-peptone-glucose (YPG) complete medium (Bartnicki-García and Nickerson, 1962) and Lee's minimal medium (LMM) (Lee *et al*., 1975), modified as described by Acevedo-Aguilar *et al*. (2006) and containing 0.25% KH_2PO_4, 0.20% $MgSO_4$, 0.50% $(NH_4)_2SO_4$, 0.5% NaCl, and glucose or ethanol at 2% as the carbon source. When necessary, LMM was supplemented with 20 mg/L leucine as a requirement for the auxotrophy of R7B and its derivative M5. The strains were maintained, and spores were obtained after growth in YPG medium as described (Bartnicki-García and Nickerson, 1962). To obtain aerobic mycelia, we grew liquid cultures (600 ml) in 2-L Erlenmeyer flasks containing YPG medium inoculated with spores at a final cell density of 5×10^5/ml, and incubated them in a shaken water bath at 28°C for the indicated period. In some experiments, we inoculated the cultures with swollen spores; these cells were obtained by inoculating YPG medium with intact spores and incubating the cultures under aerobic conditions at 28°C for 4 h. The obtained cells were centrifuged at 3,500 × *g*. The resulting pellets were thoroughly washed by centrifugation in sterile distilled water, and then transferred to a solid or liquid medium, as indicated. For growth in the solid medium under anaerobic conditions, we used LMM with 2% glucose, with or without leucine. After inoculating the medium with spores of the indicated strains, Petri dishes containing the inoculated medium were incubated in an anaerobic jar using the GasPak system (Becton Dickinson). Self-anaerobic growth was facilitated using 125-ml flasks filled completely with culture medium and sealed with rubber stoppers provided with a needle, to allow the exit of excess of CO_2 (Salcedo-Hernández and Ruiz Herrera, 1993). Self-anaerobic cultures containing 125 ml of YPG medium were inoculated with 1×10^6 spores/ml of *M. circinelloides*; this condition causes the spores to grow and produce budding yeast cells, as previously described (Salcedo-Hernández and Ruiz-Herrera, 1993). The germination percentage under aerobic or anaerobic conditions was calculated from the number of germinules or budding yeast cells in at least 100 cells.

For the transition experiments, swollen spores were incubated in YPG medium for 4 h under aerobic conditions or yeast cells in YPG medium for 12 h under self-anaerobic conditions, and then centrifuged the obtained cells at 3,500 × *g*. The harvested pellets were washed five times with 40 ml of sterile distilled water, transferred to LMM containing glucose or ethanol as the sole carbon source, and incubated at 28°C while shaking at 150 rpm.

Isolation and sequencing of genomic clones of the M5 *adh1* gene

The sequencing strategy was based on the polymerase chain reaction (PCR) amplification of fragments of the ORF of the *adh1* gene from the M5 mutant strain using the following oligonucleotides: adh-xhoF (5′ CTC GAG ATG TCT GAA GAA ACT TTC ACT GCC TGG G 3′); adh-notR (5′ GCG GCC GCG TTC ATG ACG ACA GCT CTG TAA CG 3′); adh31F (5′ GAC AAC GCT ACC AAG GAC AAG ACC 3′); and adh32R (5′ TTG TAG TGA GGC TCC ATT TGC TCT 3′). These oligonucleotides were designed based on the sequence from the *adh1* gene of the wild-type strain R7B as described in our previous study (Rangel-Porras *et al*., 2005). The oligonucleotides adh-xhoF and adh-notR correspond to

regions at the 5′ and 3′ ends of the *adh1* gene, respectively, and contain *Xho*I and *Not*I restriction sites (underlined in the oligonucleotides sequences). Oligonucleotides adh31F and adh32R amplify an internal fragment of the central region of the *adh1* gene. The nucleotide sequence of the genomic and cDNA versions of the wild-type R7B *adh1* gene are available at GenBank under the Accession nos. AY702961 and AY702962, respectively. The nucleotide sequence of the genomic version of the M5 mutant *adh1* gene is available at GenBank under the accession number MH130382.

The amino acid sequence of the ADH1 protein predicted from the sequenced *adh1* gene of the M5 mutant was aligned with the corresponding ADH1 protein predicted by the *adh1* gene of the wild-type R7B strain (Rangel-Porras *et al.*, 2005) using DNASTAR software.

Total RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The biological samples of *M. circinelloides* were: 1) spores, grown for 4 h under aerobic conditions to produce swollen spores (SS), 2) spores grown under self anaerobic conditions for 12 h to produce anaerobic swollen spores (ASS) (these cells were produced by the mutant M5, which, unlike the wild-type strain R7B, is unable to grow in the presence of low oxygen tension, which is why it fails to develop and grow as yeast cells), 3) mycelium grown for 12 h (M), and 5) yeast cells grown for 12 h (Y); in all cases, the growth was performed in YPG medium. The cells were collected by filtration (using Whatman No. 6 filter paper), and washed with sterile distilled water. RNA was isolated as described previously (Valle-Maldonado *et al.*, 2015). *M. circinelloides* gene-specific oligonucleotides and hydrolysis probes for *ald2* (aldehyde dehydrogenase) and *acs2* (acetyl-CoA synthetase) genes were designed using biosearch technologies software (http://www.biosearchtech.com) to ensure the specificity of all detections during the qRT-PCR assays (Supplementary data Table S1). The qRT-PCR assays, amplifications, and PCR efficiency determinations; the relative expression level calculations and the data analyses; and the use of hydrolysis probes and primers for the *adh1* (alcohol dehydrogenase) and *tfc1* (subunit of the transcription factor TFIIIC from RNA polymerase III) genes, all were carried out according to a previously described method (Valle-Maldonado *et al.*, 2015).

Northern blot analyses

Total RNA was extracted from aerobic mycelium of strains R7B and M5 cultured in YPG medium for 13 h and Northern blot analysis was performed as described by Sambrook *et al.* (1989); the incubation time selected was used because under these conditions the wild-type strain R7B shows the optimum level of ADH activity (Rangel-Porras *et al.*, 2005). The hybridization was performed using ^{32}P-labelled DNA probes, consisting of a 475 bp fragment of the *adh1* gene cDNA or a 774 bp fragment of the *leuA* gene, both obtained from R7B strain.

Construction of plasmid vector pEUKA7/adh1 and fungal transformation

For the complementation experiments, the 1,044-bp DNA fragment corresponding to the ORF of the *adh1* gene was amplified by PCR using the wild-type R7B strain genomic DNA (gDNA) and the oligonucleotides adh-xhoF and adh-notR. The amplified product was directionally cloned into the plasmid pEUKA 7.1 (Wolff and Arnau, 2002) using *Xho*I and *Not*I restriction sites, thereby generating the plasmid pEUKA7/adh1. In this vector, *adh1* gene expression is under the control of the promoter and the transcriptional terminator of the *M. circinelloides gpd1* gene. This construct was introduced into protoplasts of the *adh1*-deficient mutant strain M5. Leu$^+$ prototroph transformants were selected and purified by monosporic isolation, as described previously (Wolff and Arnau, 2002).

Preparation of cell-free extracts

Aerobic mycelia cells were disrupted in an MSK cell homogenizer (Braun) and the crude extract or the cytosolic fraction (164,500 × *g* supernatant) was prepared according to a previously described procedure (Rangel-Porras *et al.*, 2005).

ADH activity assay

NAD$^+$ or NADP$^+$-dependent ADH activity was determined spectrophotometrically in crude extracts or in the cytosolic fraction. All enzyme assays were conducted in a final volume of 2 ml, and the ADH activity was assayed in the oxidative direction, according to the method described by Bergmeyer (1983). The assays were performed in a reaction mixture containing 50 mM Tris-HCl (pH 8.5), 1.9 mM NAD$^+$ or NADP$^+$, cell-free extract (100–200 µg protein), and 0.8 M ethanol. The reaction was started by adding ethanol, and a reduction of NAD$^+$ or NADP$^+$ was monitored by the increase in absorbance at 340 nm. One unit of enzyme activity was defined as the amount required to reduce 1 µmol of NAD$^+$ or NADP$^+$ per min at 25°C. The specific ADH activity was expressed as units (U) per mg of protein.

The NAD$^+$-dependent ADH activity of the crude extracts was also determined by zymography, which was performed following electrophoresis on non-denaturing 10% polyacrylamide gels (Nikolova and Ward, 1991), as previously described (Torres-Guzmán *et al.*, 1994). The image shown in Fig. 8 comes from an electrophoretic separation in which the crude extracts of the indicated strains were loaded in the same gel, separated between them by an empty lane, to avoid cross-contamination. ADH activity was revealed in the complete gel and then the gel was photographed. The photographic image of the gel was cut to remove the empty lanes of the gel; Fig. 8 shows the image of the lanes in which the samples of extracts of the strains were loaded.

Determination of the ethanol content of the culture

Mycelium biomass (1 g) obtained from cultures grown under aerobic conditions in LMM with 2% glucose for 24 h was transferred to the same medium, and incubated under aerobic conditions. Subsequently, 1-ml samples were obtained from the culture after 12, 24, and 48 h of incubation, fil-

tered through 0.22-μm filters, and used for determination of ethanol content. The ethanol content in the supernatants was determined by gas chromatography with a flame ionization detector (GC-FID, Agilent Technologies 7890B) using an Agilent J&W HP-Innowax 19091N-136 column (60 m × 250 μm × 0.25 μm) with nitrogen as the mobile phase supplied at a flow rate of 1 ml/min. We injected 0.5 μl of each sample using an auto-sampler in split 20:1 mode at 240°C, whereas for the flame ionization detector the temperature was 260°C. The column temperature was held at 50°C for 2 min, increased to 240°C at 5°C/min, and held for 15 min. Quantification was based on a calibration curve using ethanol as a standard and 2-pentanol as the internal standard (both from Sigma-Aldrich) (López-Álvarez *et al*., 2012).

The values shown in the results are the mean from three independent experiments conducted with duplicate determinations. The error bars represent standard deviations. The one-way analysis of variance (ANOVA) post hoc Bonferroni test was used to analyse the data regarding ethanol production and the ADH activity levels in the cytosolic fraction of the *M. circinelloides* strains. We carried out all the tests using Statistica 11 software, and set a significance level of 0.05 (indicated by letters in the figures) for the analyses.

Results

Isolation and characterization of a mutant deficient in ADH activity

In a previous study, we observed marked expression of the *M. circinelloides adh1* gene in cultures incubated under aerobic conditions in a medium containing glucose (Rangel-Porras *et al*., 2005). Therefore, these conditions were chosen to select spontaneous mutants that were deficient in ADH activity using allyl alcohol resistance. This procedure has been used to elucidate the physiological role of *adh* gene-encoded enzymes in microorganisms, plants, and animals (Lutstorf and Megnet, 1968; Wills and Phelps, 1975; Freeling and Bennett, 1985; Jacobs *et al*., 1988; Williamson *et al*., 1991; Zheng *et al*., 2009). For this purpose, we incubated spores of strain R7B under aerobic conditions in solid YPG medium containing 2% glucose and supplemented with 100 mM allyl alcohol (a sufficient concentration to inhibit the growth of R7B spores). One resistant colony appeared after 7 days of incubation, and the isolate was purified twice by single-spore colony formation in the same selective medium used to produce the spores, which were able to grow in a medium containing allyl alcohol. The strain was named M5.

The stability of the Ally^r phenotype of the M5 mutant was tested by performing three complete vegetative cycles under non-selective conditions and inoculating the spores onto plates containing solid YPG medium. The top row of Fig. 1 shows that M5 and R7B were able to grow similarly in YPG medium when the cultures were incubated under aerobic conditions. However, under similar incubation conditions in YPG medium containing allyl alcohol, only the spores of the mutant strain M5 and not those of strain R7B were able to germinate and produce a mycelium (Fig. 1 middle row), which illustrates the Ally^r and Ally^s phenotypes of M5 and R7B, respectively. However, in cultures incubated in YPG medium under hypoxic conditions, only the spores of strain R7B and not those of M5 managed to germinate and produce a visible mycelium, which indicated that strain M5 is unable to grow under conditions of low oxygen tension (Fig. 1 bottom row). We obtained similar results for liquid YPG medium inoculated with spores and incubated under self-anaerobic conditions, defined as those stablished due to the displacement of oxygen from the cultures by the CO_2 produced during microbial metabolism. Under self-anaerobic conditions the wild-type strain R7B generated budding yeasts, as previously described (Salcedo-Hernandez and Ruíz Herrera, 1993), whereas the M5 mutant strain was unable to reach the yeast phase and produced only swollen spores (Supplementary data Fig. S1A). At difference of spores, which are metabolically dormant, swollen spores constitute a later stage in the spore germination process, characterized by isodiametric growth and by an active biosynthesis of macromolecules, formation of organelles and development of a new cell wall (Bartnicki-Garcia *et al*., 1968; Orlowski and Sypherd, 1978); for that reason, from a metabolic point of view, swollen spores are considered fully active, as vegetative cells.

Furthermore, when we transferred yeast cells of the wild-type strain R7B to LMM and incubated them under aerobic conditions with either glucose or ethanol as the carbon source, they exhibited high rates of transition to hyphae formation (approximately 90% for glucose and 75% for ethanol) (Supplementary data Fig. S1B). We carried out a complementation analysis of strain M5 to determine whether the alterations exhibited by the M5 mutant including, allyl-alcohol resistance under aerobic conditions and growth impairment under anaerobic conditions, are due to the mutation in the *adh1* gene. These complementation analysis were performed

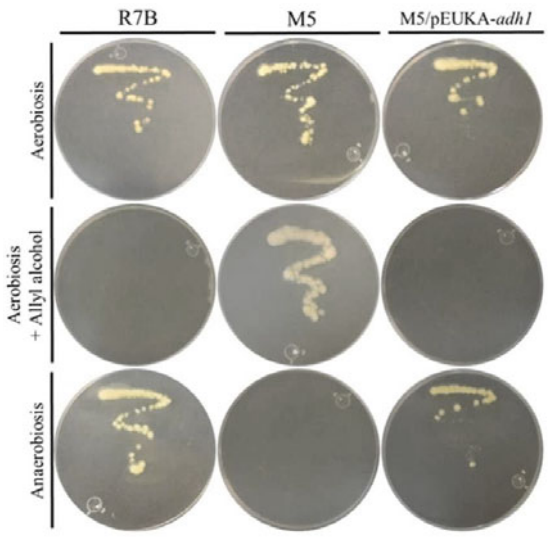

Fig. 1. Plate growth of *Mucor circinelloides* strains under various culture conditions. We inoculated plates of solid Lee's minimal medium (LMM) with or without allyl alcohol with spores of the strains, and incubated under aerobic conditions. We also incubated other inoculated plates without allyl alcohol under anaerobic conditions. In all cases, we photographed the growth of the resulting colonies over 3 days of incubation. For the growth of the wild-type R7B and M5 mutant strains, we supplemented the medium with leucine, whereas we omitted leucine from the medium used to grow complemented strain M5/pEUKA-*adh1*.

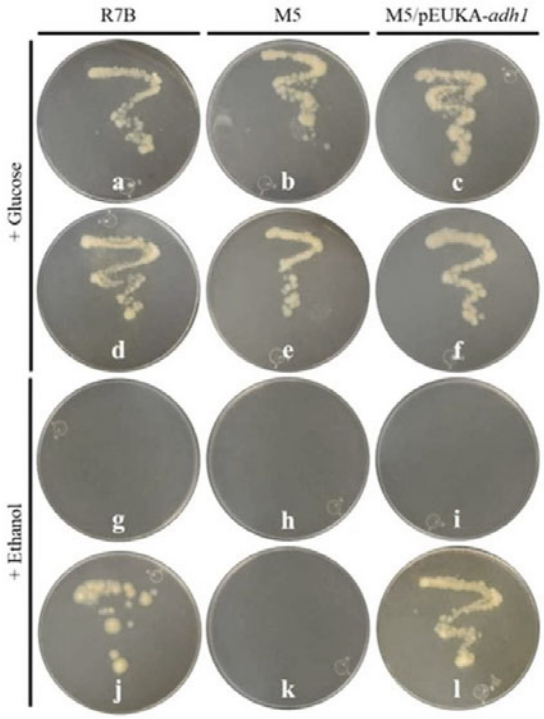

Fig. 2. Plate growth of the *Mucor circinelloides* strains under aerobic conditions in Lee's minimal medium (LMM) with 2% glucose or ethanol as the sole carbon source. We inoculated the cultures with spores (a,b,c and g,h,i) or with swollen spores (d,e,f and j,k,l), and photographed the growth of the colonies after 3 days of incubation. For the growth of the wild-type R7B and M5 mutant strains, we supplemented the medium with leucine, whereas we omitted leucine from the medium used to grow complemented strain M5/pEUKA-*adh1*.

using protoplast transformation with the pEUKA-*adh1* construct, which contains the *adh1*$^{+}$ allele from R7B under the control of the promoter and transcriptional terminator of the *M. circinelloides gpd1* gene. Selection for leucine prototrophy resulted in the M5-derived transformant M5/pEUKA-*adh1*. Figure 1 shows that, as with strain R7B, the presence of the wild-type *adh1* allele in the M5/pEUKA-*adh1* transformant caused allyl alcohol-sensitivity in this strain because it was unable to grow in medium containing allyl alcohol under aerobic conditions (Fig. 1 middle row). Furthermore, similar to the wild-type strain R7B, the transformant M5/pEUKA-*adh1* was able to grow under conditions sustaining anaerobiosis (Fig. 1 bottom row) and self-anaerobiosis (Supplementary data Fig. S1A), in which the mutant strain M5 was unable to grow.

In the next experiment, we compared the abilities of strains R7B and M5 to grow under aerobic conditions in solid LMM media with glucose or ethanol as the carbon source by inoculating the medium with spores or with swollen spores. In the medium containing glucose, the spores (Fig. 2a) and swollen spores (Fig. 2d) of strain R7B were able to grow and produce mycelial colonies; we observed similar results for the spores (Fig. 2b) and swollen spores (Fig. 2e) of the M5 mutant incubated in the same medium. In the LMM solid medium containing ethanol as the carbon source, the spores of the wild-type strain R7B (Fig. 2g) and those of the mutant M5 (Fig. 2h) failed to produce mycelial colonies. Interestingly, in the medium containing ethanol, the R7B swollen spores (Fig. 2j) managed to grow and produce mycelial colonies, but those of M5 did not (Fig. 2k). The inability of the M5 swollen spores to grow in medium containing ethanol is specifically due to *adh1* mutation as demonstrated by the

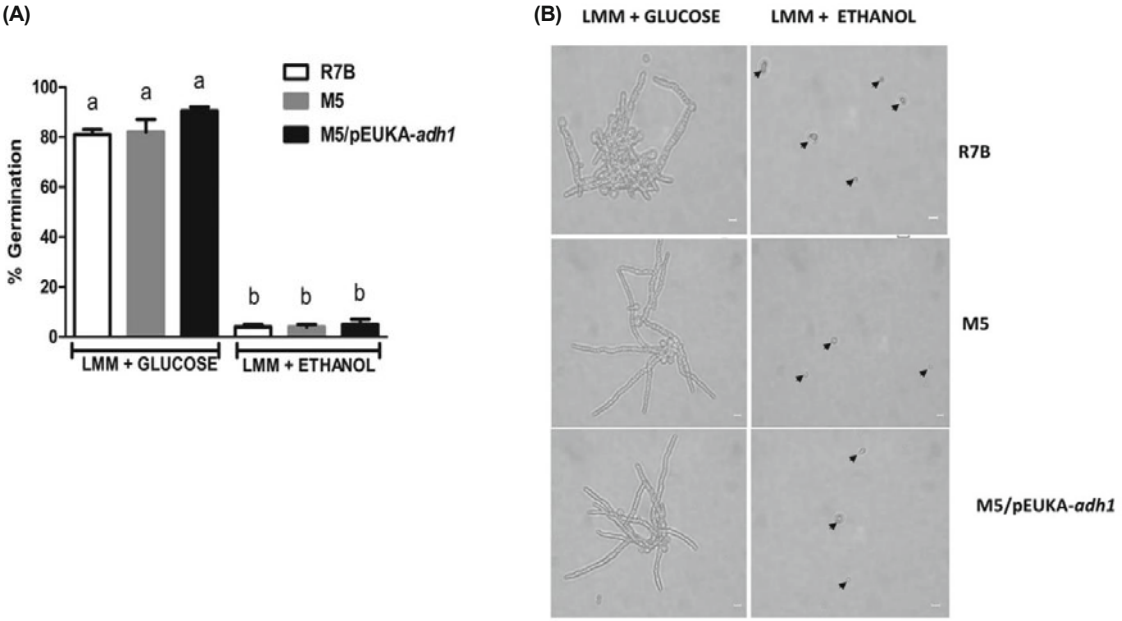

Fig. 3. Spore germination of *Mucor circinelloides* in liquid Lee's minimal medium (LMM) with glucose or ethanol as the sole carbon source. (A) Bars represent the percentage of spores germinated independently of number or size per mother cell. The data are the mean ± SD, and three independent experiments were performed for each condition. The letters denote statistically significant differences (analysis of variance (ANOVA), Fisher, $P < 0.05$). (B) Morphology of spores grown in the presence of glucose or ethanol as the sole carbon source after 12 h of incubation. The arrow heads indicate ungerminated spores; scale bar = 10 μm. For the growth of the wild-type R7B and M5 mutant strains, we supplemented the medium with leucine, whereas we omitted leucine from the medium used to grow complemented strain M5/pEUKA-*adh1*.

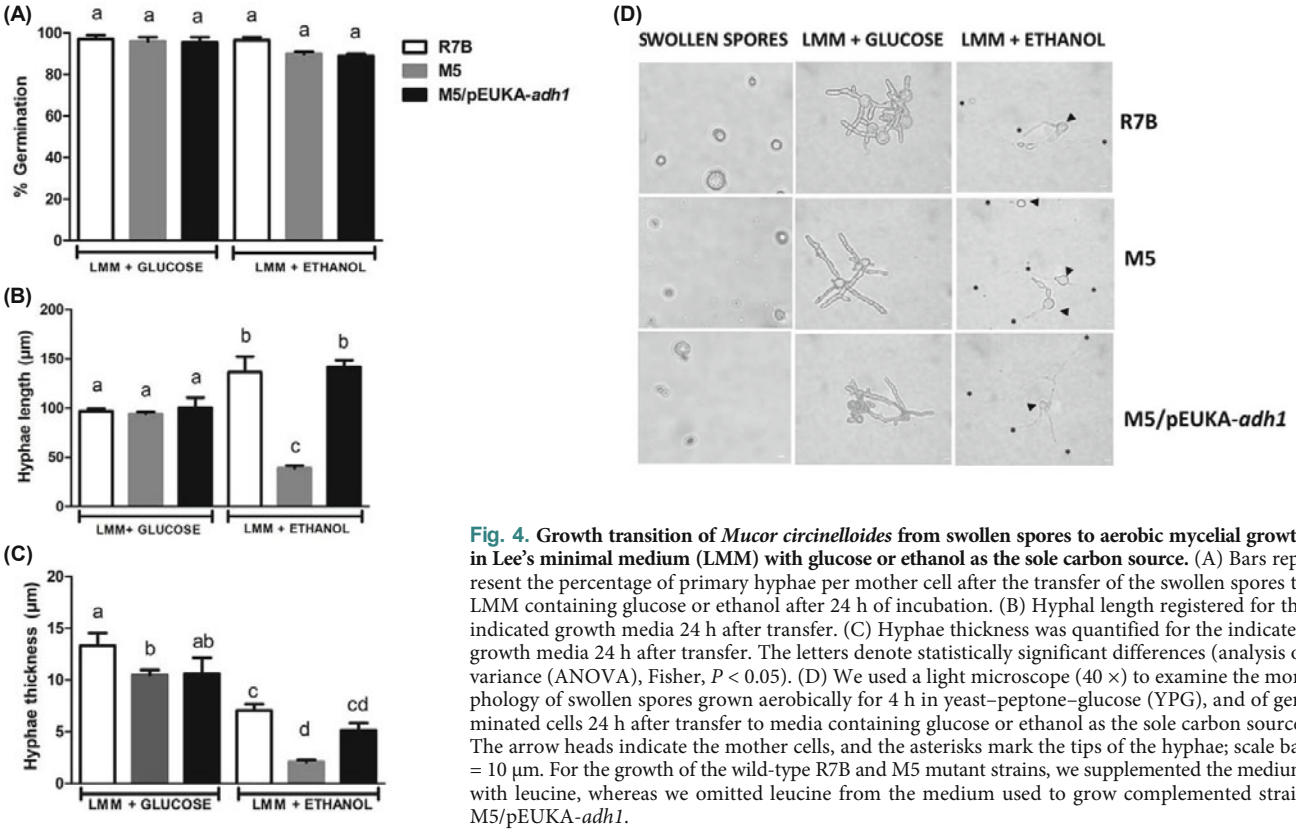

Fig. 4. Growth transition of *Mucor circinelloides* from swollen spores to aerobic mycelial growth in Lee's minimal medium (LMM) with glucose or ethanol as the sole carbon source. (A) Bars represent the percentage of primary hyphae per mother cell after the transfer of the swollen spores to LMM containing glucose or ethanol after 24 h of incubation. (B) Hyphal length registered for the indicated growth media 24 h after transfer. (C) Hyphae thickness was quantified for the indicated growth media 24 h after transfer. The letters denote statistically significant differences (analysis of variance (ANOVA), Fisher, $P < 0.05$). (D) We used a light microscope (40 ×) to examine the morphology of swollen spores grown aerobically for 4 h in yeast–peptone–glucose (YPG), and of germinated cells 24 h after transfer to media containing glucose or ethanol as the sole carbon source. The arrow heads indicate the mother cells, and the asterisks mark the tips of the hyphae; scale bar = 10 µm. For the growth of the wild-type R7B and M5 mutant strains, we supplemented the medium with leucine, whereas we omitted leucine from the medium used to grow complemented strain M5/pEUKA-*adh1*.

fact that the swollen spores of the complemented strain+ M5/pEUKA-*adh1* were able to grow and produced mycelial colonies, as readily as the R7B swollen spores, in medium containing ethanol as the only carbon source (Fig. 2i).

To confirm these observations under different conditions, we decided to grow the strains in liquid LMM containing either glucose or ethanol, using intact spores or swollen spores as inoculum and incubating the cultures under aerobic conditions. Spores from the wild-type R7B, the mutant M5, as well as those from the complemented M5/pEUKA-*adh1* strain were able to germinate at similar levels (> 80%) in medium with glucose as the carbon source, whereas spores of these three strains were unable to germinate when ethanol was the carbon source (Fig. 3A and B). Moreover, when we transferred swollen spores from R7B, M5, and the complemented M5/pEUKA-*adh1* to LMM containing either glucose or ethanol, they exhibited high germination rates of at least 90% (Fig. 4A). However, the hyphae that emerged from the swollen M5 spores incubated in LMM with ethanol were much shorter (Fig. 4B) and narrower (Fig. 4D) than those produced by R7B. These last two phenotypes were remediated by introduction of the *adh1* wild-type allele into the M5 strain as length and thickness of the complemented M5/pEUKA-*adh1* strain were comparable to those shown by the R7B wild-type strain. These results may explain, in part, why swollen M5 spores in solid LMM containing ethanol did not generate visible mycelial colonies to any appreciable extent (Fig. 2k), in contrast to the wild-type strain R7B, which shows robust colonies in this medium (Fig. 2j). In general, the development of hyphae was less extensive in

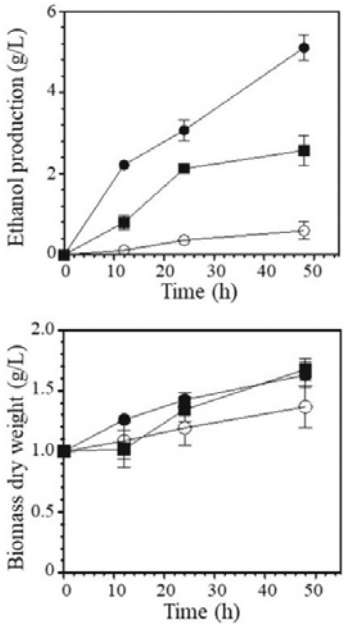

Fig. 5. Ethanol production and growth rate in *Mucor circinelloides* strains incubated in medium containing glucose under aerobic conditions. The cultures were grown in Lee's minimal medium (LMM) containing 2% glucose under aerobic conditions; at the indicated incubation times, we collected aliquots from the cultures to determine the ethanol concentration by gas chromatography with a flame ionization detector (GC-FID) (top) and growth (bottom), as described in the 'Materials and Methods' section. Wild-type R7B (●), mutant M5 (○), and complemented mutant M5/pEUKA-*adh1* (■).

swollen cells grown in ethanol than those grown in glucose, and the difference was particularly evident in the mutant strain M5 (Fig. 4D). These observations indicate that under the conditions tested, the vegetative cells, but not the spores of *M. circinelloides* grow well in ethanol as the carbon source and that this process requires a functional ADH1 enzyme.

Ethanol production of M5 mutant deficient in ADH activity

We sought to evaluate the involvement of ADH1 in the production of ethanol. To this end, the ethanol levels were measured in aerobic cultures of R7B and M5 grown for 48 h on LMM with 2% glucose. The top panel of Fig. 5 shows that in cultures of R7B, ethanol production increased starting at 12 h of incubation, and reached a production level of approximately 5 g/L ethanol at 48 h. However, in the M5 cultures, the level of ethanol produced was very low (< 0.15 g/L) at 12 h and markedly low thereafter (0.5 g/L). This difference in ethanol production between R7B and M5 was statistically significant ($P < 0.05$). The presence of the wild-type *adh1*$^{+}$ gene in the complemented M5/pEUKA-*adh1* strain partially restored ethanol production (Fig. 5 top panel).

Although there was a slight difference in the production of biomass between the strains, being lower in M5, the difference in growth does not explain why the ethanol content

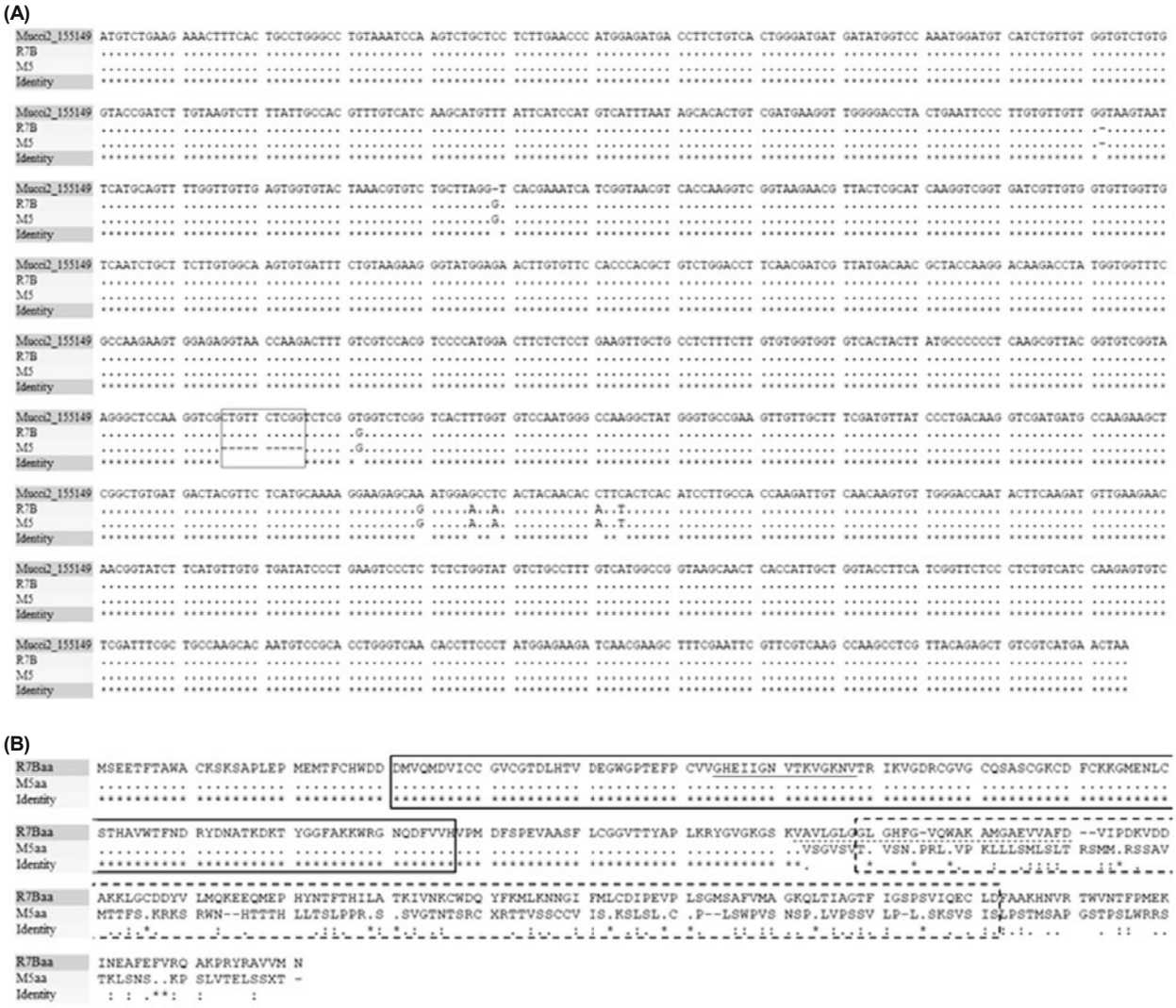

Fig. 6. Sequence analysis of the *Mucor circinelloides adh1* gene. (A) DNA sequence alignment of the *adh1* open reading frame (ORF) from the wild-type CBS277.49 strain (JGI genome Mucci_155149), the wild-type R7B strain (GenBank AY702961), and the M5 mutant strain (GenBank MH130382). A 10-bp deletion in the *adh1* gene from the M5 mutant (boxed in the figure) is indicated. A seven-nucleotide difference is also indicated between the ORFs of the *adh1* gene of strains R7B and M5, with respect to the sequence (Mucci2|155149) (gi 309952144|) of the genome of the reference strain *M. circinelloides* CBS277.49 V2. (B) Amino acid sequence alignment between the *adh1*-encoded ADH enzyme from the wild-type R7B and M5 mutant strains. The ADH N-terminal domain (31–147, IPR013154, solid line box), the ADH C-terminal domain (189–312, IPR013149, dashed line box), the ADH zinc-type conserved site (64–78, IPR002328, solid underlined), and the NAD-binding domain conserved site (182–209, IPR029752, dashed underlined) are indicated. The seven-nucleotide difference between the ORFs of the *adh1* gene of strains R7B and M5 with respect to the sequence (Mucci2|155149) (gi 309952144|) of the genome of the reference strain *M. circinelloides* CBS277.49 V2 is also indicated. All alignments were made using the ClustalW algorithm of the DNASTAR software program.

Table 1. NAD- and NADP-dependent alcohol dehydrogenase (ADH) activity in the cytosolic fraction[a] of aerobically grown mycelial cells of *Mucor circinelloides* strains

Strain	ADH-specific activity (U/mg)	
	Assay with NAD as cofactor	Assay with NADP as cofactor
R7B	13.5 ± 1.4	0.015 ± 0.003
M5	0.04 ± 0.003	< 0.001
M5/pEUKA-*adh1*	15.5 ± 1.3	0.020 ± 0.003

[a]The cytosolic fraction for the determination of ADH activity was obtained from mycelia developed in yeast-peptone-glucose medium in cultures incubated for 24 h under aerobic conditions.

produced by the M5 mutant was 10-fold lower than that by R7B strain (Fig. 5, bottom panel). These observations indicate that the mutation present in M5 negatively affected the ethanol production.

Biochemical characterization of the ADH-deficient strain M5

ADH enzymes can use NAD^+ and/or $NADP^+$ as a cofactor (Reid and Fewson, 1994). In a previous study, we observed that in the cytosolic fraction of R7B, the NAD^+-dependent ADH activity was approximately 750 times higher than the $NADP^+$-dependent ADH activity (Rangel-Porras *et al*., 2005). To determine if the mutation present in M5 affected the activity of an enzyme dependent on NAD^+, $NADP^+$, or both cofactors, we determined the ADH activity in the cytosolic fraction obtained from cell-free extracts of R7B and M5, and performed the assays in the presence of ethanol as a substrate. The results obtained (Table 1) show that in the extracts of strain R7B, the ADH activity was approximately 900 times higher when using NAD^+ as a cofactor (specific activity 13.5 U/mg) compared to $NADP^+$ (specific activity 0.015 U/mg). In the cell-free extracts of M5, the NAD^+-dependent ADH activity was approximately 338 times lower (a specific activity of 0.04 U/mg) than the corresponding activity of R7B, and the $NADP^+$-dependent ADH activity was at least 15 times lower (specific activity < 0.001 U/mg) than the corresponding activity in the cell-free extracts of R7B. The difference in NAD^+- and $NADP^+$-dependent ADH activity between R7B and M5 was statistically significant ($P < 0.05$). Cell-free extracts of the complemented strain M5/pEUKA-*adh1* exhibited levels of NAD^+- and $NADP^+$-dependent ADH activity similar to those detected in the R7B strain (Table 1), indicating the observed effects are indeed specific to *adh1*.

The expression and activity of the *Adh1* introduced into the complemented strain M5/pEUKA-*adh1* was confirmed by electrophoresis. The zymograms revealed that the electrophoretic mobility of the ADH activity band of the transformant M5/pEUKA-*adh1* was similar to that of the wild-type strain R7B (Fig. 8) and not ADH activity was detected in the M5 mutant strain. These results demonstrate that the ADH enzyme present in the cytosolic fraction of *M. circinelloides* is mainly NAD^+-dependent, and that $NADP^+$ use is very inefficient. Furthermore, our data support the model that the activity of the ADH1 enzyme is dramatically affected by the mutation present in the M5 mutant strain.

Analysis of the *adh1* gene in the Ally^r M5 mutant

In a previous study using a reverse genetics approach (Rangel-Porras *et al*., 2005), we cloned the gene encoding the ADH1 enzyme from the wild-type strain R7B (GenBank AY702961) (Supplementary data Fig. S2A). We used the deduced amino acid sequence of ADH1 in an *in silico* search for paralogous genes of the *M. circinelloides* CBS277.49 V2 genomic database (https://genome.jgi.doe.gov/Mucci2/Mucci2.home.html).

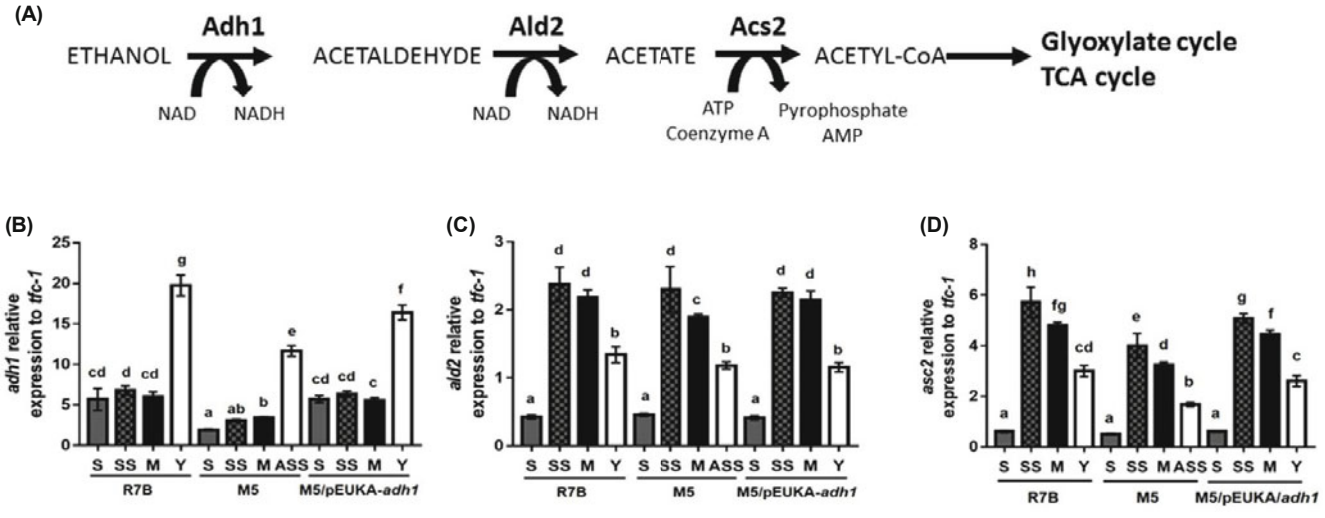

Fig. 7. Quantification of mRNA levels of genes encoding enzymes involved in ethanol consumption in spores and vegetative cells from *Mucor circinelloides*. (A) Initial steps of ethanol metabolism in *M. circinelloides* for its use as a carbon source for growth, involving the participation of alcohol dehydrogenase 1 (Adh1), aldehyde dehydrogenase 2 (Ald2) and acetyl CoA synthetase (Acs2). Ethanol is consumed to produce acetyl-CoA, which finally could be used to supply the glyoxylate or tricarboxylic acid cycles. We measured the mRNA levels by performing quantitative reverse transcription polymerase chain reaction (qRT-PCR) on: (B) *adh1*, alcohol dehydrogenase 1; (C) *ald2*, aldehyde dehydrogenase 2; and (D) *acs2*, acetyl CoA synthetase. We carried out Ct analyses to compare mRNA levels between spores (S), spores aerobically grown for 4 h (SS), mycelium grown under aerobic conditions for 12 h (M) and yeast cells (Y) cultured for 12 h under conditions of self-anaerobiosis; all the cells were grown in yeast-peptone-glucose (YPG) medium. We performed three independent experiments for each condition. The letters denote statistically significant differences (analysis of variance (ANOVA), Fisher, $P < 0.05$).

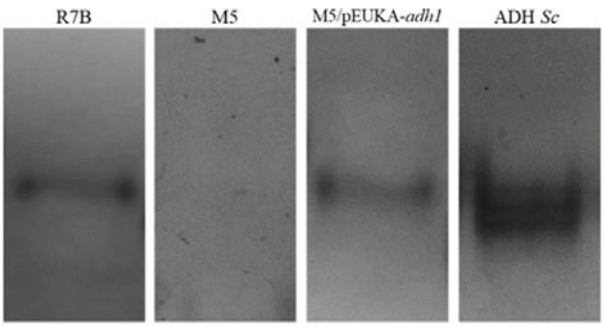

Fig. 8. Zymogram band pattern of ADH activity in cell-free extracts of the *Mucor circinelloides* strains. We electrophoresed and stained crude extracts obtained from aerobic mycelia grown in yeast-peptone-glucose (YPG) medium for 18 h, as described in the 'Materials and Methods' section. The upper part of each lane indicates the strain source of the extract or the enzyme used for the ADH activity assay: wild-type R7B, mutant M5, complemented strain M5/pEUKA-*adh1*, and commercial *Saccharomyces cerevisiae* ADH enzyme (Sigma). Note that, as described in 'Materials and Methods', all samples were analysed on the same gel, leaving an empty lane between each sample to avoid sample contamination across contiguous lanes. The empty lanes were excised-off and the gel strips containing samples were photographed.

We found 100% identity with a sequence (Mucci2|155149) (gi 309952144|) corresponding to the *adh1* gene (Supplementary data Fig. S2B), and 44–59% identity with six additional *adh* paralogous sequences (Mucci2|157439|, Mucci2|34200|, Mucci2|90838|, Mucci2|140177|, Mucci2|120424|, and Mucci2|152844|).

The northern blot analysis of the expression of the *adh1* gene in the aerobic mycelium of R7B and M5 incubated in YPG medium revealed that a 1.15-kb transcript is produced in both strains (Supplementary data Fig. S3). This observation refutes the notion that the mutation in M5 affects a region or regulatory product involved in the transcription of the *adh1* gene. The coding region of the *adh1* gene has been sequenced to determine whether the sequence of *adh1* is altered in the M5 strain. A PCR amplification strategy of overlapping DNA fragments was followed by the use of oligonucleotides adh-xhoF, adh-notR, adh31F, and adh32R, which were designed based on the sequence of the *adh1* gene of strain R7B (Rangel-Porras *et al.*, 2005). As with the wild-type *adh1* allele (Rangel-Porras *et al.*, 2005), sequence analysis of the M5 *adh1* allele revealed a non-continuous ORF resulting from the presence of two small introns at positions 142–202 and 251–306 (Supplementary data Fig. S2C). The alignment of the *adh1* allele in R7B and M5 revealed a deletion of 10 bp at position 666 of the *adh1* gene ORF in the mutant M5 (Fig. 6A). This alteration results in the deletion of three amino acids and a frameshift, which leads to the formation of a smaller predicted protein (243 amino acids with an approximate molecular weight of 28 kDa) than that encoded by the wild-type *adh1* gene (348 amino acids with a calculated molecular mass of 37 kDa) (Rangel-Porras *et al.*, 2005). Importantly, the predicted M5 ADH1 protein lacks the cofactor (NAD^+/$NADP^+$)-binding domain (Fig. 6B). These sequence-deduced alterations in the M5 ADH1 enzyme account for the defective physiological phenotype displayed by the M5 mutant.

The enzymes ADH2, ALD2, and ACS2 are involved in the utilization of ethanol as a carbon source in *S. cerevisiae* (Wills, 1990) (Fig. 7A). Using ALD2 and ASC2 as targets, we identified the corresponding homologues in the *M. circinelloides* genome with the highest identity and similarity scores with the respective yeast genes (Supplementary data Tables S2 and S3). To ascertain whether the genes involved in ethanol consumption are differentially expressed during the morphological development of *M. circinelloides*, we designed specific probes to determine the *adh1*, *ald2*, and *acs2* mRNA levels in spores or vegetative cells. In general, the levels of mRNA of the *adh1* gene in the different morphological stages, including spores, aerobic swollen spores and mycelium of the strains R7B, M5, and M5/pEUKA-*adh1* are approximately similar, being a little lower in the cells of the M5 mutant (Fig. 7B). These results agree with those from a previous study, which found similar *adh1* mRNA levels in both spores and in aerobic mycelium of the R7B strain (Valle-Maldonado *et al.*, 2015). However, the *adh1* mRNA levels were significantly higher in the yeast phase of the R7B and M5/pEUKA-*adh1* strains, and in the anaerobic swollen spores of the M5 mutant strain (Fig. 7B). In these three strains the spores show very low *ald2* (Fig. 7C) and *asc2* (Fig. 7D) mRNA levels, compared with the stages of swollen spore and aerobic mycelium, as well as in comparison with the yeast phase of R7B and M5/pEUKA-*adh1* strains and the anaerobic swollen spores of the M5 mutant. These results suggest that the low expression of the *ald2* and *asc2* genes in spores may result in the spores' inability to use ethanol as a carbon source. Finally, our observations also indicate that the *adh1* mutation does not noticeably affects the expression of the *adh1*, *ald2*, and *asc1* genes in the morphogenetic stages tested in M5 strain.

Discussion

Previous studies have shown that under aerobic conditions and in the presence of glucose, *M. circinelloides* produces significant amounts of ethanol, which suggests that it is a Crabtree-positive microorganism (McIntyre *et al.*, 2002). Moreover, the ethanol produced by the aerobic mycelium of *M. circinelloides* is consumed after glucose depletion in the medium, and the yeast cells produced under anaerobic conditions cannot use ethanol owing to their requirement for hexoses as a carbon source (Lübbehüsen *et al.*, 2004).

Alcohol dehydrogenases play an important role in the metabolism of alcohols, and participate in the last steps of fermentative metabolism or in the first steps of oxidative metabolism (Reid and Fewson, 1994). In a previous study, we observed that glucose positively regulates the production of the ADH1 enzyme at the transcriptional level in *M. circinelloides*. Furthermore, the kinetic characterization of purified ADH1 indicates that it acts as a fermentative enzyme and reduces acetaldehyde to ethanol (Rangel-Porras *et al.*, 2005).

In the present study, we attempted to determine the physiological function of ADH1 in connection with its role in the Crabtree effect and in ethanol metabolism in *M. circinelloides*. We obtained and characterized the allyl alcohol-resistant strain M5 for this purpose. Compared with the reference strain R7B, in addition to allyl alcohol resistance,

M5 exhibited deficiencies in several physiological parameters related to carbon and energy metabolism: 1) an inability to grow in a medium containing glucose in the absence of oxygen; 2) a failure to utilize ethanol as a carbon source for growth under aerobic conditions; and 3) a reduced ability to produce ethanol from glucose under aerobic conditions. In agreement with these characteristics, M5 exhibited dramatically reduced levels of NAD-dependent ADH activity in the cytosolic fraction.

Sequence analysis of the *adh1* allele from M5 showed that it contains a deletion of 10 bp at position 666 of the coding sequence of the *adh1* gene. This sequence alteration causes a deletion of three amino acids and a frameshift that results in a modification of the downstream sequence, which leads to the formation of a smaller predicted protein that lacks the cofactor (NAD^+/$NADP^+$)-binding domain. This alteration affects the enzymatic activity of the ADH1 protein and provides an explanation for the physiological alterations exhibited by M5. The reintroduction of the wild-type allele *adh1* into M5 allowed us to verify that in the resultant transformant (M5/pEUKA-*adh1*), the physiological and biochemical characteristics of R7B were restored in the M5 background. This observation indicates that the product of the *M. circinelloides adh1* gene mediates the Crabtree effect in the fungus, and functions as either a fermentative or an oxidative enzyme, depending on the culture conditions.

The *in silico* analysis performed in this work indicated that the *M. circinelloides adh1* gene (Rangel-Porras *et al*., 2005) corresponds to sequence (Mucci2|155149) in the *Mucor circinelloides* CBS277.49 V2 genomic database, although the existence of additional sequences encoding paralogous ADH proteins with a significant degree of identity to the Adh1 enzyme (i.e., Mucci2|152844, 59% identity; Mucci2|120424, 48% identity; Mucci2|140177 and Mucci2|90838, 47% identity) was noted. However, it seems that the physiological replacement of the ADH1 enzyme by another paralogous ADH enzyme is eliminated based on the pronounced phenotype of the M5 mutant in relation to its complete inability to grow under anaerobic conditions or to use ethanol as a carbon source. This is corroborated by the absence of other bands of ADH activity in the zymogram activity patterns of the wild-type strain R7B and the M5 mutant. The altered phenotype of M5 indicates that ADH1 is active in R7B from the germination of the spores and in vegetative cells obtained under aerobic or anaerobic conditions in the presence of glucose, or under aerobic conditions in the presence of ethanol.

Our findings suggest that *M. circinelloides* spores are less prone to assimilate ethanol as the sole carbon source than vegetative cells, because the mRNA levels of genes involved in the initial steps of ethanol metabolism for its use as a carbon source, such as *ald2* and *acs2*, are notably lower in spores than in vegetative cells. These findings explain why spores grow poorly in ethanol as the only carbon source, both in solid and liquid media. The observation that the germination of swollen spores in LMM containing ethanol was practically the same in M5, R7B, and in the complemented strain M5/pEUKA-*adh1*, and that there was an obvious difference in the degree of hyphae elongation between the M5 mutant and the other strains that contained the $adh1^+$ allele, could indicate that ADH activity is required after the germination of the spores, possibly in sustaining the growth of the hyphae. A previous study revealed that the spores of the dimorphic zygomycete *M. racemosus* contain pre-transcribed mRNA, which is packed into the spores (Linz and Orlowski, 1982). Thus, the finding in this work of the presence in the spores of *adh1*, *ald2*, and *acs2* mRNAs, may derive from packing these mRNAs into the spores, rather than from transcription in the spores. We find that the lack of ADH activity in the M5 mutant does not affect its development from spore to swollen spore and swollen spore to hypha, although the hyphae formed fails to growth. These results support the interpretation that ADH1 activity is not required for the breakdown of the spore's dormant state nor the passage to the swollen cell stage and the polarization of growth to form hyphae, but rather, ADH1 activity is required to support hyphal growth.

When the filamentous ascomycete *Fusarium oxysporum* is grown in the presence of glucose, ethanol production is low under aerobic conditions and high under anaerobic conditions (Panagiotou *et al*., 2005; Corrales-Escobosa *et al*., 2011), which indicates that this fungus is a Crabtree-negative organism. Adh1 participates in the production of ethanol in *F. oxysporum*. Furthermore, ethanol is used as a carbon source by conidia and mycelia, and Adh1 participates in this capacity and is required for full expression of virulence by the fungus (Corrales-Escobosa *et al*., 2011).

The ascomycete yeast *Saccharomyces cerevisiae* is a Crabtree-positive organism that, in the presence of oxygen and a high concentration of glucose, uses glycolysis and fermentation pathways to obtain energy for growth (Pronk *et al*., 1996). In *S. cerevisiae* and other Crabtree-positive yeasts, glucose is completely depleted when the ethanol concentration reaches a maximum and begins to be utilized as a carbon source, which constitutes a close connection between fermentative and respiratory metabolism (Hagman *et al*., 2013). It has been proposed that in *S. cerevisiae*, the duplication of the genome–in particular the duplication of the *ADH* gene (to generate ADH1 and ADH2) and the duplication of hexose transporter coding genes-constitutes the molecular background for the development of the Crabtree effect (Hagman *et al*., 2013; Pfeiffer and Morley, 2014). In *S. cerevisiae*, ADH1 regenerates NAD and converts acetaldehyde into ethanol, whereas ADH2 is responsible for the oxidation of ethanol as one of the initial steps in the use of this alcohol as a carbon source. Although ADH1p and ADH2p exhibit a high degree of identity at the nucleotide level of the encoding genes and amino acids, the difference in their physiological roles lies in the regulation of their production according to the culture conditions (de Smidt *et al*., 2008). Because there is no paralog enzyme exhibiting a high degree of identity to the ADH1 protein in the *M. circinelloides* genome, our finding that ADH1 may act as either a fermentative or oxidative enzyme, depending on the nutritional conditions, indicates the different characteristics of the elements involved in determining the activity of ADH with regard to the connection between fermentative and oxidative metabolism, and the operation of the Crabtree effect in the fungus. A perspective of interest is the determination of the role of paralogous ADH1 enzymes in ethanol metabolism, and their possible participation in the connection between the two types of metabolism in *M. circinelloides*.

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Conflicts of Interest

The authors declare no conflicts of interest.

References

Acevedo-Aguilar, F.J., Espino-Saldaña, A.E., León-Rodríguez, I.L., Ávila-Rodríguez, M., Wrobel, K., Wrobel, K., Lappe, P., Ulloa, M., and Gutiérrez-Corona, J.F. 2006. Hexavalent chromium removal *in vitro* and from industrial wastes, using chromate-resistant strains of filamentous fungi indigenous to contaminated wastes. *Can. J. Microbiol.* **52**, 809–815.

Bartnicki-García, S. 1963. III. Mold-yeast dimorphism of *Mucor*. *Bacteriol. Rev.* **27**, 293–304.

Bartnicki-Garcia, S., Nelson, N., and Cota-Robles, E. 1968. Electron microscopy of spore germination and cell wall formation in *Mucor rouxii*. *Arch. Mikrobiol.* **63**, 242–255.

Bartnicki-García, S. and Nickerson, W.J. 1962. Nutrition, growth, and morphogenesis of *Mucor rouxii*. *J. Bacteriol.* **84**, 841–858.

Bergmeyer, H.U. 1983. Reagents for enzymatic analysis, pp. 139. *In* Bergmeyer, H.U. (ed.), Methods of enzymatic analysis. Verlag Chemie, Weinheim, Germany.

Chayakulkeeree, M., Ghannoum, M., and Perfect, J. 2006. Zygomycosis: the re-emerging fungal infection. *Eur. J. Clin. Microbiol. Infect. Dis.* **25**, 215–229.

Corrales-Escobosa, A.R., Rangel-Porras, R.A., Meza-Carmen, V., Gonzalez-Hernandez, G.A., Torres-Guzman, J.C., Wrobel, K., Wrobel, K., Roncero, M.I.G., and Gutierrez-Corona, J.F. 2011. *Fusarium oxysporum* Adh1 has dual fermentative and oxidative functions and is involved in fungal virulence in tomato plants. *Fungal Genet. Biol.* **48**, 886–895.

de Kok, S., Kozak, B.U., Pronk, J.T., and van Maris, A.J.A. 2012. Energy coupling in *Saccharomyces cerevisiae*: selected opportunities for metabolic engineering. *FEMS Yeast Res.* **12**, 387–397.

de Smidt, O., du Preez, J.C., and Albertyn, J. 2008. The alcohol dehydrogenases of *Saccharomyces cerevisiae*: a comprehensive review. *FEMS Yeast Res.* **8**, 967–978.

Ferreira, J.A., Lennartsson, P.R., Edebo, L., and Taherzadeh, M.J. 2013. Zygomycetes-based biorefinery: Present status and future prospects. *Bioresour. Technol.* **135**, 523–532.

Freeling, M. and Bennett, D.C. 1985. Maize *Adh1*. *Annu. Rev. Genet.* **19**, 297–323.

Hagman, A., Säll, T., Compagno, C., and Piskur, J. 2013. Yeast "Make-Accumulate-Consume" life strategy evolved as a multi-step process that predates the whole genome duplication. *PLoS One* **8**, e68734.

Ibrahim, A.S. and Spellberg, B. 2006. Zygomycetes as agents of infectious disease in humans, pp. 429–440. *In* Heitman, J., Filler, S.G., Edwards, Jr.J.E., and Mitchell, A.P. (eds.), Molecular principles of fungal pathogenesis. American Society for Microbiology Press, Washington, D.C., USA.

Jacobs, M., Dolferus, R., and Van den Bossche, D. 1988. Isolation and biochemical analysis of ethyl methanesulfonate induced alcohol dehydrogenase null mutants of *Arabidopsis thaliana* (L.) Heynh. *Biochem. Genet.* **26**, 105–122.

Karimi, K. and Zamani, A. 2013. *Mucor indicus*: biology and industrial application perspectives: a review. *Biotechnol. Adv.* **31**, 466–481.

Khan, Z.U., Ahmad, S., Brazda, A., and Chandy, R. 2009. *Mucor circinelloides* as a cause of invasive maxillofacial zygomycosis: an emerging dimorphic pathogen with reduced susceptibility to posaconazole. *J. Clin. Microbiol.* **47**, 1244–1248.

Lee, K.L., Buckley, H.R., and Campbell, C.C. 1975. An aminoacid liquid synthetic medium for the development of mycelial and yeast forms of *Candida albicans*. *Sabouraudia* **13**, 148–153.

Lee, S.C., Li, A., Calo, S., and Heitman, J. 2013. Calcineurin plays key roles in the dimorphic transition and virulence of the human pathogenic zygomycete *Mucor circinelloides*. *PLoS Pathog.* **9**, e1003625.

Linz, J.E. and Orlowski, M. 1982. Stored mRNA in sporangiospores of the fungus *Mucor recemosus*. *J. Bacteriol.* **150**, 1138–1144.

López-Alvarez, A., Díaz-Pérez, A.L., Sosa-Aguirre, C., Macías-Rodríguez, L., and Campos-García, J. 2012. Ethanol yield and volatile compound content in fermentation of agave must by *Kluyveromyces marxianus* UMPe-1 comparing with *Saccharomyces cerevisiae* baker's yeast used in tequila production. *J. Biosci. Bioeng.* **113**, 614–618.

Lübbehüsen, T.L., Nielsen, J., and McIntyre, M. 2004. Aerobic and anaerobic ethanol production by *Mucor circinelloides* during submerged growth. *Appl. Microbiol. Biotechnol.* **63**, 543–548.

Lutstorf, U. and Megnet, R. 1968. Multiple forms of alcohol dehydrogenase in *Saccharomyces cerevisiae*. I. Physiological control of ADH-2 and properties of ADH-2 and ADH-4. *Arch. Biochem. Biophys.* **126**, 933–944.

McIntyre, M., Breum, J., Arnau, J., and Nielsen, J. 2002. Growth physiology and dimorphism of *Mucor circinelloides* (syn. *racemosus*) during submerged batch cultivation. *Appl. Microbiol. Biotechnol.* **58**, 495–502.

Mendoza, L., Vilela, R., Voelz, K., Ibrahim, A.S., Voigt, K., and Lee, S.C. 2015. Human fungal pathogens of mucorales and entomophthorales. *Cold Spring Harb. Perspect. Med.* **5**, a019562.

Nikolova, P. and Ward, O.P. 1991. Production of L-phenylacetyl carbinol by biotransformation: product and by-product formation and activities of the key enzymes in wild-type and ADH isoenzyme mutants of *Saccharomyces cerevisiae*. *Biotechnol. Bioeng.* **20**, 493–498.

Orlowski, M. 1991. Mucor dimorphism. *Microbiol. Rev.* **55**, 234–258.

Orlowski, M. and Sypherd, P.S. 1978. Regultion of macromolecular synthesis during hyphal germ tube emergence from *Mucor racemosus* sporangiospores. *J. Bacteriol.* **134**, 76–83.

Panagiotou, G., Villas-Boas, S.G., Christakopoulos, P., Nielsen, J., and Olsson, L. 2005. Intracellular metabolite profiling of *Fusarium oxysporum* converting glucose to ethanol. *J. Biotechnol.* **115**, 425–434.

Pfeiffer, T. and Morley, A. 2014. An evolutionary perspective on the Crabtree effect. *Front. Mol. Biosci.* **1**, 17.

Pronk. J.T., Yde Steensma, H., and Van Dijken, J.P. 1996. Pyruvate metabolism in *Saccharomyces cerevisiae*. *Yeast* **12**, 1607–1633.

Rangel-Porras, R.A., Meza-Carmen, V., Martínez-Cadena, G., Torres-Guzmán, J.C., González-Hernández, G.A., Arnau, J., and Gutiérrez-Corona, J.F. 2005. Molecular analysis of an NAD-dependent alcohol dehydrogenase from the zygomycete *Mucor circinelloides*. *Mol. Genet. Genomics* **274**, 354–363.

Reid, M.F. and Fewson, C.A. 1994. Molecular characterization of microbial alcohol dehydrogenases. *Crit. Rev. Microbiol.* **20**, 13–56.

Roncero, M.I.G. 1984. Enrichment method for the isolation of auxotrophic mutants of Mucor using the polyene antibiotic N-glycosyl-polifungin. *Carlsberg Res. Commun.* **49**, 685–690.

Salcedo-Hernández, R. and Ruiz-Herrera, J. 1993. Isolation and characterization of a mycelial cytochrome aa_3-deficient mutant

and the role of mitochondria in dimorphism of *Mucor rouxii*. *Exp. Mycol.* **17**, 142–154.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular cloning: a laboratory manual (2nd ed.). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Sypherd, P., Borgia, P.T., and Paznokas, J.L. 1978. Biochemistry of dimorphism in the fungus *Mucor*. *Adv. Microb. Physiol.* **18**, 67–104.

Torres-Guzmán, J.C., Arreola-García, G.A., Zazueta-Sandoval, R., Carrillo-Rayas, T., Martínez-Cadena, G., and Gutiérrez-Corona, F. 1994. Genetic evidence for independence between fermentative metabolism (ethanol accumulation) and yeast-cell development in the dimorphic fungus *Mucor rouxii*. *Curr. Genet.* **26**, 166–171.

Valle-Maldonado, M.I., Jácome-Galarza, I.E., Gutiérrez-Corona, F., Ramírez-Díaz, M.I., Campos-García, J., and Meza-Carmen, V. 2015. Selection of reference genes for quantitative real time RT-PCR during dimorphism in the zygomycete *Mucor circinelloides*. *Mol. Biol. Rep.* **42**, 705–711.

Williamson, V.M., Long, M., and Theodoris, G. 1991. Isolation of *Caenorhabditis elegans* mutants lacking alcohol dehydrogenase activity. *Biochem. Genet.* **29**, 313–323.

Wills, C. 1990. Regulation of sugar and ethanol metabolism in *Saccharomyces cerevisiae*. *Crit. Rev. Biochem. Mol. Biol.* **25**, 245–280.

Wills, C. and Phelps, J. 1975. A technique for the isolation of yeast alcohol dehydrogenase mutants with altered substrate specificity. *Arch. Biochem. Biophys.* **167**, 627–637.

Wolff, A.M. and Arnau, J. 2002. Cloning of glyceraldehyde-3-phosphate dehydrogenase-encoding genes in *Mucor circinelloides* (Syn. *racemosus*) and use of the *gpd1* promoter for recombinant protein production. *Fungal Genet. Biol.* **35**, 21–29.

Zheng, Z., Luo, S., Li, X., Wu, X., Pan, L., and Jiang, S. 2009. Screening of allyl alcohol resistant mutant of *Rhizopus oryzae* and its fermentation characterization. *Afr. J. Biotechnol.* **8**, 280–284.