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Genetic evidence for independence between fermentative metabolism (ethanol accumulation) and yeast-cell development in the dimorphic fungus *Mucor rouxii*

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Abstract. Three allyl-alcohol-resistant mutants were isolated in the dimorphic fungus *Mucor rouxii* and characterized with regard to their alcohol dehydrogenase (ADH) activity in vitro and in vivo as well as their ability to execute the morphological alternatives of dimorphism under different environmental stimuli, either in the absence or in the presence of oxygen. These studies indicated that fermentation and yeast-cell development are independent events and that ADH activity is essential for growth of the fungus in the absence of oxygen. Heterokaryon construction and analysis indicated that in the three mutant strains the corresponding genetic alterations are recessive nuclear mutations which behave as allelic in complementation tests.

Key words: Alcohol dehydrogenase – Fermentative metabolism – *Mucor* – Allyl-alcohol-resistant mutant – Aerobic and anaerobic growth

Introduction

Several species of the fungus *Mucor* exhibit a yeast-mycelium dimorphism; that is, they are capable of vegetative growth as either filamentous (mycelium) or as spherical (yeast) cells, depending on environmental conditions (Bartnicki-Garcia 1963; Sypherd et al. 1978). Most of the research on *Mucor* morphogenesis has dealt with the carbon and energy metabolism changes accompanying the dimorphic transition. In this sense, many studies have indicated a relationship between the metabolic mode and morphogenesis. The general suggestion from these studies is that yeast development requires hexoses and invariably correlates with a fermentative metabolism, whereas the mycelial phase depends on the expression of an oxidative metabolism and can occur with several carbon sources (see reviews by Inderlied et al. 1985; Ruiz-Herrera 1985; Orlowski 1991). Regarding the yeast phase, it is known that its correlation with ethanol fermentation holds true under different growth conditions, such as anaerobiosis (Bartnicki-Garcia 1963; Inderlied and Sypherd 1978; Phillips and Borgia 1985) or growth under aerobic conditions in media containing morphogenetic compounds such as phenethyl alcohol (PEA) (Terenzi and Storck 1969), dibutyryl cyclic AMP (dbcAMP) (Larsen and Sypherd 1974; Paveto et al. 1975), or certain amino acids (Leija et al. 1986). However, it seems that fermentative metabolism is not unique to the yeast morphology, since it has been observed that some *Mucor* species produce hyphal cells as fermentative as the yeast phase when grown anaerobically (Bartnicki-Garcia 1963; Inderlied and Sypherd 1978; Phillips and Borgia 1985).

As a genetic approach to test the relationship between the fermentative mode and yeast development, we isolated mutants of *Mucor rouxii* defective in alcohol dehydrogenase activity (Adh^-), the enzyme catalysing the last step of fermentation in *Mucor*, and investigated their capacity to carry out the morphogenetic alternatives of dimorphism. Our results indicate that yeast development and fermentation are independent processes and that Adh activity is essential for anaerobic growth of the fungus.

Materials and methods

Strains. As wild-type organisms we used strains G1, a Met auxotroph, and G2, an auxotrophic mutant requiring leucine, isoleucine and valine. These strains were derived from the standard wild-type IM-80 (ATCC 24905) of *M. rouxii* by mutagenesis with N-Methyl-N-Nitro-N-Nitrosoguanidine (NTG) (Salgado-Rodriguez et al. unpublished results). G1 and G2 were used in this work as parental strains to isolate mutants resistant to 2- propenol-1-ol or allyl alcohol (AA) by mutagenesis with NTG (strain G1) or with 2-methoxy-6-chloro-9-[3-(ethyl-2-chloroethyl)] amino-propylamino acridine · 2HCl (ICR-170) (strain G2) (see below).

Media and culture conditions. YPG complete (Bartnicki-Garcia and Nickerson 1962 a) and minimal media (Bartnicki-Garcia and Nickerson 1962 b) were used for cultivation of the fungus. YPG contained 0.3% yeast extract, 1% bacto peptone and 2% glucose. Min-

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imal medium contained the specified amounts of glucose as carbon source and was supplemented, when necessary, with 0.2% of casein hydrolysate or the appropiate auxotrophic requirements at 50 μg/ml. Media were solidified by the addition of 2% agar. Sporangiospore production and propagation of cultures were as previously described (Bartnicki-Garcia and Nickerson 1962 b). To obtain aerobic mycelium, spores were inoculated in YPG or in minimal medium containing 2% glucose and the cultures incubated aerobically (Bartnicki-Garcia and Nickerson 1962 a). Anaerobic mycelium and yeast cells were obtained by sparging sterile N_2 through minimal medium containing 0.5% and 8% glucose, respectively (Bartnicki-Garcia 1968). The anaerobic yeast phase was also obtained in YPG medium sparged with a sterile mixture of N_2:CO_2 (70:30 v/v) (Bartnicki-Garcia and Nickerson 1962 a). Aerobic yeasts were obtained in minimal medium containing yeast extract supplemented with 0.22% PEA (Terenzi and Storck 1969) or 3 mM dbcAMP (Larsen and Sypherd 1974) and incubating the cultures under aerobic conditions.

Mutagenesis and isolation of mutants. Sporangiospores were suspended in 100 mM phosphate buffer, pH 7.0, and mutagenized with NTG, as described previously (Vazquez-Marrufo et al. 1992), or with acridine ICR-170 at a final concentration of 40 μg/ml. The treatment of spores with NTG and ICR-170 gave a survival rate of 10% and 5%, respectively. Mutagenized spores were allowed to complete a whole vegetative cycle in YPG medium; the spores produced were used for the selection of allyl-alcohol-resistant mutants on YPG plates containing 0.6% (v/v) of allyl alcohol.

Heterokaryon construction. Heterokaryotic mycelia were produced by fusing spheroplasts obtained by treatment of germlings with partially-purified chitosanase and commercial chitinase (Sigma) according to the procedure described by Lasker and Borgia (1980). Chitosanase was assayed and partially purified from culture filtrates of *Mixobacter* AL-1 following the procedure described by Hedges and Wolfe (1974) until the step of washings with sodium citrate buffer: The supernatants containing chitosanase activity were pooled and concentrated using an Amicon ultrafiltration system (Amicon, USA) equipped with a UM-2 membrane.

Preparation of cell-free extracts. Aerobic mycelium or yeast cells were suspended in 100 mM of Tris-HCl buffer, pH 7.5, and homogenized with glass beads (0.45–0.50 mm diameter) in a Braun MSK cell homogenizer. The suspension was centrifuged at 1000 g for 5 min, the pellet was discarded and the supernatant (crude extract) used as the source for the assay of ADH activity (see below). In some experiments this supernatant was partially purified by precipitation with acetone as follows: the crude extract was mixed with an equal volume of acetone, the mixture was incubated at 4 °C for 1 h and then centrifuged at 5 000 rpm for 30 min. The pellet was resuspended in 1.5 ml of Tris-HCl buffer, pH 7.5. The suspension was centrifuged at 20 000 rpm for 20 min and the supernatant was retained and used for activity determinations or electrophoretic studies.

Alcohol dehydrogenase assay. ADH activity was assayed according to Bergmeyer (1983). The reaction mixture contained 100 mM Tris-HCl buffer (pH 8.3), 2 mM NAD, cell-free extract (100–200 μg protein) and 0.8 M ethanol, in a total volume of 1 ml. The reaction was started by ethanol addition, and reduction of NAD was monitored by the increase in absorbance at 340 nm. The specific activity was expressed as μmoles NADH produced per min per mg protein at 25 °C. In other experiments, ADH activity was assayed in cell-free extracts separated by non-denaturing polyacrylamide-gel electrophoresis. This was performed using a 4.5% stacking gel and a 7.5% running gel, according to Laemmli (1970) except that SDS was omitted. ADH activity-staining of the gel was done according to Nikolova and Ward (1991).

Other assays. Ethanol was determined as described by Cornell and Veech (1983). Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Results

Isolation and biochemical characterization of ADH-deficient mutants

For the isolation of mutants of *M. rouxii* deficient in ADH activity the selection technique for resistance to allyl alcohol was followed, as originally described for yeasts (Megnet 1967; Lutstorf and Megnet 1968). Mutagenized spores were inoculated in YPG medium containing 0.6% allyl alcohol, since pilot experiments indicated that this concentration inhibits spore germination of wild-type strains G1 and G2. Three independent mutants, designated G3, G13 and G14, whose spores germinated on selective plates to form visible colonies, were isolated. Of these, strain G3 was derived from wild-type G1 by mutagenesis with NTG whereas mutants G13 and G14 were obtained from wild-type G2 by treatment with ICR-170.

Table 1 shows that, as described for other cell types, allyl-alcohol-resistance in *M. rouxii* is associated with a drastic loss of ADH activity: all of the three allyl-alcohol-resistant (Aly^r) mutants showed a sharp reduction of this activity as compared with the respective allyl-alcohol-sensitive (Aly^s) parental strain. Table 1 also shows that the

Table 1. ADH activity and ethanol production in mycelial cells of Aly^r mutants of *M. rouxii*

Strain	Relevant phenotype	ADH specific activity[a]	Ethanol production[b]
G1	Aly^s	14.30	1.40
G3	Aly^r	0.08	0.20
G2	Aly^s	8.10	1.90
G13	Aly^r	0.009	0.10
G14	Aly^r	0.02	0.07

Mycelial cells were obtained by aerobic germination of spores in YPG medium at 28 °C for 12 h; ADH activity was assayed in partially-purified cell extracts. Culture filtrates were used for the determination of the ethanol produced

[a] μmoles/mg protein/min

[b] μmoles per ml

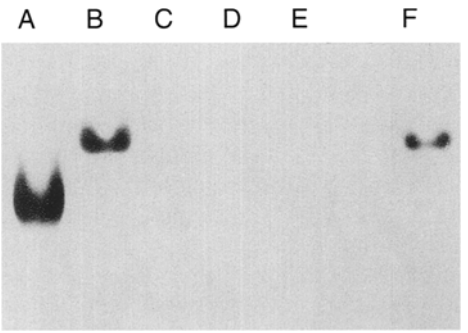

Fig. 1. ADH zymogram band pattern from cell-free extracts of *M. rouxii* Aly^s and Aly^r strains. Crude extracts of aerobic mycelia grown in YPG medium for 12 h were electrophoresed and stained as described in Materials and methods. *A*, commercial yeast ADH (Sigma Chemical Co., St. Louis, Mo., USA); *B*, strain G1; *C*, strain G3; *D*, strain G13; *E*, strain G14; *F*, strain G2

adh mutation present in each mutant strain clearly affects the fermentative pathway in the growing aerobic mycelium, leading to significant reductions in the amount of ethanol produced. Figure 1 shows the zymogram for ADH activity in cell-free extracts of the aerobic mycelium from wild-type and mutant strains. One activity band was clearly observed in extracts from wild-type strains G1 and G2 (lanes B and F, respectively), which showed an electrophoretic migration different from that shown by the ADH of *S. cerevisiae* (lane A). In contrast, no activity bands were observed in cell extracts from the three mutants (lanes C, D and E, respectively); the same result was obtained when approximately 10–20-fold more protein from the extracts was applied to the gel.

Morphogenetic responses of Adh⁻ mutants to different environmental stimuli

Table 2 shows that, when incubated under aerobic conditions, the wild-type strains G1 and G2 grew only as mycelial cells, whereas under an atmosphere of N_2:CO_2 they developed exclusively into the yeast phase, as has been described for wild-type strains of different dimorphic species of *Mucor* (Orlowski 1991). On the other hand, all the Adh⁻ mutants showed the ability to produce mycelial cells under aerobic conditions but with a complete impairment in the development of yeast cells under an atmosphere of N_2:CO_2, with the mutants remaining in the spore stage.

Table 2 also shows that wild-type strains G1 and G2 grew exclusively as mycelial cells when cultured under an atmosphere of N_2 in growth medium containing 0.05% glucose, but as a mixture of yeast and hyphal cells when cultured in the same conditions in the presence of 8.0% glucose. However, under these conditions spores of all of the Adh⁻ mutants failed to germinate, regardless of the glucose concentration in the medium. It was observed that, contrary to aerobically-germinated hyphal cells from wild-type strains G1 and G2, those from the three ADH-deficient mutants failed to continue cell enlargement when transferred to an atmosphere of N_2 in growth medium containing 0.05% glucose (data not shown). These findings indicate that *adh* mutations impair the ability of the fungus to grow in the absence of oxygen.

Table 3 shows the kind of morphogenetic pattern adopted by Adh⁻ mutants when cultured in the presence of oxygen under yeast cell-promoting conditions, i.e., in growth medium supplemented with the morphogenetic compounds dbcAMP or PEA. As shown, in the parental strains G1 and G2 1 mM of dbcAMP induces the formation of about 50% and 35% of mycelial cells and 50% and 65% of yeast cells, respectively. Under these conditions, all the Adh⁻ mutant strains produced both developmental alternatives in similar proportions to those of wild-type strains. Table 3 also shows that, in the presence of PEA, strains G1 and G2 differentiated exclusively into yeast cells which, at the period of growth used (24 h), amounted to about 80% and 95%, respectively, of the cell population; the rest of the cells consisted of swollen spores. Again, under these conditions Adh⁻ mutants also showed the ability to develop into the yeast phase, reaching similar (strains G3 and G14) or slightly-reduced proportions (strain G13) of yeast cells as compared with the corresponding wild-type strains.

In cell-free extracts obtained from aerobic yeast cells of the three allyl-alcohol-resistant strains ADH activity remained at low levels relative to those present in wild-type extracts (Table 4). Table 4 also shows the results obtained when spores of wild-type and mutant strains were germinated in the presence of both PEA and allyl-alcohol. Under these conditions wild-type spores were unable to germinate, remaining as intact spores, whereas those from Adh⁻ mutant strains were able to germinate, reaching the yeast phase (Table 4). These results indicated that expression of the same ADH enzyme occurs in both mycelial and yeast cells.

Table 2. Morphological development of Adh⁻ mutants of *M. rouxii* under different environmental stimuli

Strain	Developmental stage reached in the indicated growth condition			
	O_2 atmosphere, 2.0% glucose[a]	N_2/CO_2 atmosphere, 2.0% glucose[b]	N_2 atmosphere, glucose at:	
			0.5%[b]	8.0%[b]
G1	M	Y	M	M, Y
G3	M	S	S	S
G2	M	Y	M	M, Y
G13	M	S	S	S
G14	M	S	S	S

Cultures were inoculated with spores, incubated at 28 °C for 12 or 24 h and the different types of cells (M, mycelial; S, spores; Y, yeast cells) produced were indicated. Strains G1 and G2 cultured for 24 h under an N_2 atmosphere in growth medium with 8% glucose, produced mycelial and yeast cells in about the same proportion

[a] 12-h incubation

[b] 24-h incubation

Table 3. Morphogenetic alternatives of Adh⁻ mutants of *M. rouxii* cultured aerobically under yeast-cell-promoting conditions

Strain	Percentage of cells developed into the mycelial (M) or yeast (Y) phase in growth medium supplemented with:			
	dbcAMP		PEA	
	M	Y	M	Y
G1	48	52	< 1.0	80
G2	35	65	< 1.0	93
G3	40	60	< 1.0	90
G13	31	69	< 1.0	83
G14	50	50	< 1.0	92

Spores were germinated under aerobic conditions in growth medium containing dbcAMP or PEA; after 24 h of growth the different morphological types were observed microscopically and expressed as a percentage of the total cell population

Table 4. ADH activity and allyl alcohol resistance of Adh$^-$ mutants of *M. rouxii* cultured in aerobiosis in the presence of PEA

Strain	ADH specific activity[a]	Germination of spores and morphogenetic stage reached in the presence of PEA and AA
G1 (Wt)	32.50	–, (S)
G3	0.12	+, (Y)
G2 (Wt)	25.5	–, (S)
G13	0.01	+, (Y)
G14	0.03	+, (Y)

Yeast cells were obtained by aerobic germination of spores in the presence of PEA as indicated in Table 3; ADH activity was assayed in partially-purified cell extracts. The right column indicates the occurrence (+) or the absence (–) of spore germination as well as the morphological types (S, spore; Y, yeast) produced after 24 h in growth medium containing PEA and AA

[a] μmoles/mg protein/min

Table 5. Dominance/recessivity tests of *adh* mutations in *M.rouxii* by heterokaryon analysis

Strain	ADH activity[a] in aerobic mycelium	Spore germination in the absence of oxygen and morphology developed
G1 (Wt)	9.5	+, (Y)
G3	0.02	–, (S)
G2 (Wt)	11.0	+, (Y)
G13	0.008	–, (S)
G14	0.01	–, (S)
G2*G3	5.0	+, (Y)
G1*G13	7.0	+, (Y)
G1*G14	5.5	+, (Y)

Spores were germinated aerobically in minimal medium; after 16 h the mycelial cells produced were processed to obtain partially-purified extracts for Adh assay. Spores were germinated in minimal medium in the absence of oxygen under an atmosphere of N_2/CO_2; the occurrence (+) or the absence (–) of spore germination as well as the morphological types (S, spores; Y, yeast cells) produced after 24 h of growth are indicated

[a] μmoles/mg protein/min

The Adh$^-$ phenotype in the mutants is controlled by a recessive nuclear gene, adhI

Table 5 indicates that heterokaryons constructed between the three Adh$^-$ mutants and the appropiate wild-type strain carrying a complementary auxotrophy (heterokaryons G2*G3, G1*G13 and G1*G14) yielded mycelia which showed allyl-alcohol-sensitivity and ADH activity, which gave rise to spores with the ability to germinate in the absence of oxygen. These observations indicated recessivity of the corresponding *adh* mutations present in the mutant strains. The pattern of segregation in spores of the genetic markers present in mycelia was similar in the three heterokaryons. Thus, heterokaryon G2*G3 produced spores with the phenotypes Aly^r Met$^-$, Aly^s Leu$^-$ Ile$^-$ Val$^-$, and Aly^s prototrophs at frequencies of 0.40, 0.50 and 0.05, respectively. On the other hand, heterokaryons G1*G13 and G1*G14 produced spores with the phenotypes Aly^r Leu$^-$ Ile$^-$ Val$^-$, Aly^s Met$^-$, and Aly^s prototrophs at frequencies that varied in the range of 0.45–0.55, 0.40–0.60 and 0.05–0.20, respectively. It was observed that allyl-alcohol-sensitive prototrophic mycelia derived from all these heterokaryons gave rise to spores which produced colonies with the three different phenotypes, and in a frequency similar to the above mentioned values. In none of the cases were stable, non-segregating, prototrophic Aly^s derivatives recovered. Thus, the close co-segregation of these *adh* mutations with nuclear auxotrophic markers indicates a similar origin for them, i.e., *adh* mutations affect a nuclear gene. Complementation tests between the mutants were performed through the construction of heterokaryons. It was observed that aerobic prototrophic mycelia from heterokaryons G3*G13 and G3*G14 were deficient in ADH activity and that spores derived from them were unable to germinate in the absence of oxygen (data not shown). In addition, it was noted that the cells recovered from fusing mixtures of protoplasts of strains G13 and G14, whose heterokaryons could not be selected as prototrophic colonies, were unable to grow in the absence of oxygen. These results indicate that mutants G3, G13 and G14 contain allelic mutations, thus defining a gene which we have tentatively designated as *ADHI*.

Discussion

Carbon and energy metabolism have been extensively studied in dimorphic species of *Mucor* in a search for biochemical correlates with the yeast-hyphae interconversion. The general conclusion from these studies is that yeast development occurs under conditions which favour a fermentative metabolism and that hyphal cells are formed under conditions supporting an oxidative metabolic mode (for reviews see Inderlied et al. 1985; Ruiz-Herrera 1985; Orlowski 1991). However, although "aerobic" or anaerobic yeast cells are highly fermentative, with ethanol as the main product, it has been observed that, under some conditions, mycelial cells can be also wholly fermentative (Orlowski 1991). The results of the present work provide genetic evidence for the independence between yeast development and ethanol production in *M. rouxii*.

It was found that spores of Adh$^-$ mutants of *M. rouxii* were unable to germinate and yield either yeast cells or mycelia in the absence of oxygen. By contrast, when cultured under aerobic conditions, these mutants did not differ appreciably from wild-type strains in their morphogenetic capabilities. Adh$^-$ mutants produced yeast cells when incubated in growth medium supplemented with dbcAMP or PEA, efficient inducers of the production of highly fermentative yeast cells in *Mucor*. In the absence of these compounds Adh$^-$ mutants essentially produced mycelial cells, as did the wild-type strain. Thus, in spite of their reduced production of ethanol, Adh$^-$ mutants of *M. rouxii* were able to reach the yeast phase, indicating the absence of a cause-effect relationship between the fermentative metabolic mode and yeast development.

It has been observed that mutants of *S. cerevisiae* deficient in ADHI, the fermentative alcohol dehydrogenase, require an active respiratory chain, since they are unable to grow when incubated under aerobic conditions in the

presence of the uncoupler antimycin (Ciriacy and Breitenbach 1979). Similarly, mutants of the obligately-aerobic fungus *Aspergillus nidulans* affected in ADH III are unable to survive long-term incubation under anaerobic conditions (Kelly et al. 1990). Our results with Adh^- mutants of *M. rouxii* indicate that in this fungus ADH activity is essential for anaerobic growth, probably because the step catalyzed by the enzyme is a major point for the regeneration of the NAD required for the continued operation of the glycolytic pathway.

The ability of Adh^- mutants of *M. rouxii* to grow in the presence of PEA deserves further attention, since it has been suggested that in this organism PEA acts as an uncoupler of oxidative phosphorylation (Terenzi and Storck 1969) without affecting oxygen uptake and cytochrome content (Terenzi and Storck 1969; Cano-Canchola et al. 1988). Such growth of the mutants is not a consequence of the induction by PEA of another (or other) alcohol dehydrogenase(s), which could relieve the effects of the genetically-altered enzyme. This was verified by two observations: (1) cell-free extracts of "aerobic" PEA-induced yeasts of the mutants maintained low levels of ADH activity; (2) whereas wild-type spores were unable to germinate in growth medium containing PEA and allyl-alcohol, those from the three mutants were able to do so reaching the yeast phase. These observations indicate that mycelial and yeast cells of *M. rouxii* make the same ADH enzyme. Growth of Adh^- mutants of *M. rouxii* in the presence of PEA indicates the existence of an ADH-independent mechanism for the production of NAD by reoxidation of NADH, which is not operative in the absence of oxygen. Such a mechanism could involve activities with the ability to oxidize cytoplasmic NADH, such as those described in mitochondria from *S. cerevisiae* (De Santis and Melandri 1984) or *Candida utilis* (Bruinenberg et al. 1985). Recently, it has been suggested that mitochondrial preparations from *Mucor* cells can actively oxidize exogenous NADH (Salcedo-Hernández and Ruiz-Herrera 1993). At the present time it is not known if PEA needs to be metabolized in order to function as a morphogen, but the results of the present work eliminate the fermentative ADH of *M. rouxii* as being responsible for such hypothetical metabolism.

Heterokaryon studies indicated that in the three independent mutants G3, G13 and G14 the corresponding *adh* mutations reducing the fermentative ADH activity are recessive and affect the same nuclear gene (*ADHI*). Whether *ADHI* in *M. rouxii* is a regulatory or a structural gene coding for the ADH enzyme of the fungus remains uncertain.

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