The role of polyamine metabolism in dimorphism of *Yarrowia lipolytica*

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We have devised a convenient procedure to induce the yeast-to-mycelium transition of *Yarrowia lipolytica* in conditions which avoid the occurrence of the reverse process during the period of study. Yeast cells in late exponential phase were resuspended in water and cooled down to 4 °C for at least 15 min, then heat-shocked by inoculation into a pre-warmed (30 °C) medium containing *N*-acetyl-D-glucosamine. Under these conditions, yeast cells developed into large branching filaments which continued elongating for more than 24 h. Further, ornithine decarboxylase (ODC) activity and polyamine cell pools increased compared to those of cells maintained in glucose medium, which continued yeast-like growth. Addition of ODC inhibitors blocked mycelial development, but only if added during a critical initial period after which they had no effect. At effective concentrations, ODC inhibitors had no significant effect on cell growth. Comparative studies of intact and permeabilized cells suggest that this selective effect is probably due to the location of ODC in more than one cell compartment, one of them being inaccessible to the drugs. Blocking of the morphological transition by ODC inhibitors was specifically reversed by putrescine, and by growing the cells in the presence of 5-azacytidine. It is suggested that the effect of the latter compound is related to its capacity to inhibit DNA methylation, indicating a relationship between polyamines and DNA methylation at the onset of the differentiation process.

Introduction

Several species of fungi are characterized by dimorphism, which is the capacity for mycelial or yeast-like growth depending on the environmental conditions. This property is not restricted to specific taxonomic groups, but is shared by organisms representative of Basidiomycetes, Ascomycetes, Zygomycetes and Imperfect Fungi. Interestingly, many pathogenic fungal species are dimorphic, presenting different morphologies in the host and during saprophytic existence (Szániszlo, 1985). In *Candida albicans*, dimorphism appears to be related somehow to virulence (Soll, 1985; Odds, 1988). Dimorphism has been considered an interesting model for the study of cell differentiation (Bartnicki-Garcia, 1963).

Yarrowia lipolytica is a dimorphic organism which grows as a mixture of yeast-like and short mycelial cells in solid and liquid media. Study of environmental factors involved in the regulation of the dimorphic transition (Rodríguez & Domínguez, 1984) indicated that when *N*-acetylglucosamine (GlcNAc) was used as the carbon source, cell growth was mostly mycelial, whereas in the presence of glucose it was yeast-like. *Y. lipolytica* appears to be a useful model for the study of dimorphism in fungi, since in contrast to other dimorphic species, it has a sexual cycle (Wickerham *et al.*, 1970) and can be used as a subject for genetic manipulation (Gaillardin *et al.*, 1973; Ogrydziak *et al.*, 1978); and transformation (Gaillardin *et al.*, 1976; Cheng & Ogrydziak, 1987).

Studies from our laboratory on cell differentiation in fungi have demonstrated the importance of polyamine metabolism in these phenomena, including the dimorphic transitions of *Mucor rouxii* (Martínez-Pacheco *et al.*, 1989) and *C. albicans* (Martínez *et al.*, 1990). Polyamines play an important role in cell growth and development of almost all organisms (Heby, 1981; Tabor & Tabor, 1984, 1985; Smith, 1985), although their precise mode of

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Abbreviations: AC, 5-azacytidine; DAB, 1,4 diamino-2-butanone; DFMO, α -difluoromethyl ornithine; GlcNAc, *N*-acetyl-D-glucosamine; MO, α -methyl ornithine; ODC, ornithine decarboxylase.

action remains unknown. We have investigated the importance of polyamines in the dimorphic transition of *Yarrowia*, using some inhibitors of poly biosynthesis, as a first approach to studying dimorphism in this organism.

Methods

Fungal strain and growth conditions. *Yarrowia lipolytica* CX 39-74A was obtained from A. Dominguez of the University of Salamanca, Spain. It was maintained by periodic transfer to slants of YED medium containing 1% yeast extract, 2% glucose and 2% agar (all w/v) and incubating at 26 °C. Yeast cells were obtained by inoculating a loopful into 25 ml of liquid Yeast Nitrogen Base (YNB)-glucose medium and shaking (250 r.p.m.) at 28 °C. YNB medium was prepared from its components according to the formulation described in the Difco manual. Seven different solutions of the following composition (per 100 ml) were prepared: (1) biotin, 2 mg; folic acid, 2 mg; (2) pantothenic acid, 4 mg; inositol, 20 mg; *p*-aminobenzoic acid, 2 mg; pyridoxin, 4 mg; riboflavin, 2 mg; thiamin, 4 mg; niacin, 4 mg; (3) boric acid, 50 mg; CuSO₄, 4 mg; KI, 10 mg; FeCl₃, 20 mg; MnCl₂, 40 mg; Na₂MoO₄, 20 mg; ZnSO₄, 40 mg. These solutions were sterilized by filtration through Millipore filters of 0.45 µm pore diameter. (4) MgSO₄, 1.25 g; NaCl, 0.25 g; CaCl₂, 0.25 g; (5) (NH₄)₂SO₄, 5 g; KH₂PO₄, 1 g; (6) glucose, 10 g; or GlcNAc, 10 g; (7) 1 M-citric acid adjusted to pH 6.0 with saturated NaOH. Solutions were mixed in the following proportion: 1, 0.1 ml; 2, 10 ml; 3, 1 ml; 4, 40 ml; 5, 100 ml; 6, 100 ml. Citrate buffer solution (no. 7, 50 ml per l) was added only when GlcNAc was used as the carbon source. The medium was taken to 1 litre with distilled water and sterilized by Millipore filtration. Storage of solutions 1 and 2 for more than 1 month at 4 °C led to a reduction in cell growth as well as in the proportion of mycelial cells. For this reason they were used not more than one month after being prepared.

Mycelial growth. The inocula used were yeast cells grown for 12 h as described above. Cells were harvested by centrifugation at room temperature, washed with sterile distilled water and inoculated at a final density of 3×10^6 cells per ml in YNB medium which contained 0.5% GlcNAc instead of glucose, as carbon source (YNBGlcNAc, see above). Alternatively, cells were washed with ice-cold sterile distilled water, maintained at 4 °C for various periods of time (see Results), and inoculated into pre-warmed medium. Cells were incubated at 30 °C in a water bath shaken at 250 r.p.m. for various periods of time.

Determination of cell morphology. Samples of the media were withdrawn and treated with formaldehyde. Cells were counted with a haemocytometer and morphology of no fewer than 200 cells was scored using a phase-contrast microscope. Cells were considered mycelial when they had a well-defined slender germ tube longer than the large diameter of the mother cell.

Determination of cell growth. Cells were sedimented by centrifugation, washed with water and resuspended in 1 M-NaOH. Protein was measured by the Lowry method using BSA as standard. Cell number was assessed by direct count (see above) or by turbidity, using a standard curve which related OD₆₀₀ to cell numbers, measured with a haemocytometer.

Determination of ornithine decarboxylase (ODC) activity. The method described by Calvo-Mendez *et al.* (1987), which is a modification of the technique described by Sissions *et al.* (1976), was used. Whole or permeabilized cells were incubated in PDP buffer (see below) at 30 °C, and CO₂ was trapped using NaOH. Activity was expressed as nmol CO₂ evolved in one min. Specific activity was related to one mg of cell protein. When necessary, cells were permeabilized by the method

described by Adams (1972). Cells were centrifuged, washed and resuspended in 40% (v/v) DMSO in water and shaken in a Vortex mixer for 6 periods of 20 s each with intermediate cooling in an ice bath. Cells were washed three times by centrifugation with 20 mM-phosphate buffer, pH 7.3, containing 2 mM-DTT and 0.2 mM-pyridoxal phosphate (PDP buffer).

Determination of polyamine concentration. The procedure for polyamine extraction was the one described by Torrigiani *et al.* (1980), which separates the compounds into three classes: free, conjugated and bound. Determination of polyamines of each class was performed by TLC separation of the dansyl derivatives as described by Calvo-Mendez *et al.* (1987). Concentrations are expressed as nmol polyamine (mg cell protein)⁻¹.

Results

Mycelial growth of *Yarrowia lipolytica* can be induced by a simple change in the carbon source (Rodriguez & Dominguez 1984). Whereas cultivation in a glucose medium leads to yeast growth, the substitution of GlcNAc induces mycelial growth (Fig. 1). Yeast cells of *Y. lipolytica* are rather elongated and a clear differentiation from mycelial cells can be ascertained only after germ tubes are rather long, a property attained after several hours of growth in GlcNAc-containing medium. However, as already noted by Rodriguez & Dominguez (1984), after some time of incubation the proportion of mycelial cells in YNBGlcNAc medium started to decrease (Fig. 2). This decrease was not due to the disappearance of mycelial cells or to mycelial-to-yeast conversion, but to the active reproduction of those yeasts which had not originally responded to the morphogenetic stimulus. This effect causes confusion whenever the effect of environmental conditions on dimorphism is studied. We therefore looked for conditions which avoided this phenomenon. Starving the cells in distilled water at 4 °C for variable periods of time induced mycelial growth when they were inoculated into medium containing glucose as carbon source. This effect was dependent on growth temperature and was maximal at 30 °C. At higher temperatures both cell growth and yeast-mycelial transition decreased (not shown). However, the mycelial proportion never exceeded 55% of the total population. Combined use of both starvation and growth on GlcNAc gave optimal results. About 90% of the cell population germinated as mycelium, and their proportion remained constant for 24 h (Fig. 2, see also Fig. 1). Continued incubation up to 48 h gave rise to extremely long and branched filaments without an increase in yeast numbers (results not shown). This result suggests that the remaining yeast cells must be dead since they are unable to bud.

Further studies revealed that the starvation period was not necessary to induce mycelial growth. The mere cooling of cells (the minimum period tested was 15 min)

Fig. 1. Morphology of *Yarrowia lipolytica* grown as yeast in YNBGlc medium (*a* and *c*), or as mycelium in YNBGlcNAc medium, after a heat-shock (*b* and *d*), after 12 h (*a*, *b*) or 24 h (*c*, *d*) incubation. Bar, 10 μ m.

Fig. 2. Dimorphic transition of *Yarrowia lipolytica* in YNBGlcNAc medium. Filled circles: yeast cells grown for 12 h in YNBGlc medium, harvested at room temperature, inoculated (3×10^6 cells ml^{-1}) into YNBGlcNAc medium and incubated with shaking at 28 °C. Open circles: cells grown for 12 h in YNBGlc medium, harvested at 4 °C, washed with ice-cold distilled water and maintained at 4 °C for 15 min, then inoculated into warm (30 °C) medium (3×10^6 cells ml^{-1}) and incubated with shaking at the same temperature. At intervals, samples were withdrawn and cell morphology was determined. Data are means of three different experiments; bars indicate standard deviations.

and their further resuspension into medium maintained at 30 °C gave almost identical results. The heat shock, and not starvation, appears to cause the induction of mycelial growth.

Transfer of yeast cells after a heat shock to GlcNAc-containing medium gave rise to a sudden increase in ornithine decarboxylase (ODC) activity. This increase was almost absent when cells were recovered at 24 °C and transferred to a medium with glucose as the carbon source (Fig. 3).

Accompanying the increase in ODC levels, there was also a build-up in polyamines (mainly free and conjugated) as compared to inoculum cells (Table 1). Levels of bound polyamines on the other hand, decreased. More total polyamines were synthesized by mycelial than

Fig. 3. ODC activity during the growth of *Yarrowia lipolytica*. Yeast cells were grown in YNBGlc medium at 28 °C for 12 h. One set of samples was maintained at this temperature; the others were kept at 4 °C for 15 min. Cells (3×10^6 ml^{-1}) were then inoculated into YNBGlc or YNBGlcNAc media, respectively, and incubated at 30 °C. At intervals, duplicate 50 ml samples were removed, and the cells were centrifuged, washed with PDP buffer and resuspended in PDP buffer. ODC activity was measured as described in Methods. Open circles: cells incubated in YNBGlcNAc medium. Filled circles: cells incubated in YNBGlc medium. Data are means of two different experiments.

by yeast cells, and the highest differences were those for spermidine and free putrescine.

We tested the effect of two ODC inhibitors on the activity of the enzyme. These were 1,4-diamino-2-butanone (DAB) and α -difluoromethylornithine (DFMO). They were added to two kinds of preparations; whole cells or cells permeabilized with DMSO. Other permeabilization methods which made use of toluene or digitonin, or preparation of cell-free extracts by shaking the cells with glass beads in a Braun homogenizer or by use of a sonicator lowered enzyme activity. The response of whole or permeabilized cells to the inhibitors was quite distinct. In whole cells, DAB or DFMO produced

Table 1. Polyamine content of *Yarrowia lipolytica*

Yeast cells grown in YNBGlc medium were inoculated into fresh medium to obtain either yeasts (YNBGlc) or mycelium (YNBGlcNAc, heat shocked). Samples of the inoculum and cells grown for 2 h under either set of conditions were recovered, and free, bound and conjugated polyamines were measured. Two sets of values are shown. Each is a mean value from two different experiments performed with triplicate samples, expressed as nmol polyamine (mg cell protein) $^{-1}$.

Sample	Putrescine				Spermidine				Spermine			
	Free	Conj.	Bound	Total	Free	Conj.	Bound	Total	Free	Conj.	Bound	Total
Inoculum	0.4	1.7	7.9	10.0	2.1	0.4	15.9	18.4	1.4	0.5	8.3	10.2
	0.6	2.2	8.5	11.3	3.7	0.7	17.6	22.0	1.2	0.6	10.7	12.5
Yeasts	5.0	4.5	3.6	12.6	12.2	9.3	5.2	26.7	5.2	6.8	8.1	20.1
	3.3	3.3	5.1	11.7	6.5	8.6	3.7	18.8	6.2	5.9	12.4	24.5
Mycelium	8.6	0.9	4.7	14.2	15.0	21.5	6.4	42.9	4.1	8.8	6.3	19.2
	8.1	0.5	5.2	13.8	12.5	19.7	4.3	36.5	5.5	8.9	7.8	22.2

Table 2. *Effect of DAB and DFMO on ornithine decarboxylase activity of intact and permeabilized cells of Yarrowia lipolytica*

Mycelial cells grown in YNBGlcNac medium for 8 h were harvested by centrifugation and divided into two aliquots. One of them remained as the control, whereas the other was treated with DMSO as described in Methods. Ornithine decarboxylase activity was measured and expressed as nmol CO₂ liberated per min per mg cell protein. Results are means $\pm$ SEM of at least 3 experiments.

Inhibitor	Intact cells		DMSO-treated cells	
	Activity	Inhibition (%)	Activity	Inhibition (%)
None	15.7 $\pm$ 2.7	—	24.4 $\pm$ 3.2	—
0.1 mM DAB	19.2 $\pm$ 1.1	0	6.3 $\pm$ 0.6	74.2 $\pm$ 1.1
1.0 mM-DAB	20.7 $\pm$ 2.1	0	2.2 $\pm$ 0.1	91.5 $\pm$ 1.5
20 mM-DAB	10.0 $\pm$ 1.6	29.3 $\pm$ 1.9	2.4 $\pm$ 0.2	88.3 $\pm$ 2.0
1.0 mM-DFMO	ND	ND	2.2 $\pm$ 0.1	91.5 $\pm$ 1.5
40 mM-DFMO	11.6 $\pm$ 0.8	31.1 $\pm$ 0.8	1.8 $\pm$ 0.1	94.0 $\pm$ 2.4

ND, not determined.

Table 3. *Effect of ornithine decarboxylase inhibitors on yeast-mycelial transition of Yarrowia lipolytica*

Cells were grown for 12 h in YNBGlc medium, centrifuged, washed, resuspended in water and kept at 4 °C. They were inoculated into YNBGlcNac medium in the presence or absence of the indicated inhibitors at a cell density of 3×10^6 cells per ml, and incubated at 30 °C for 12 h. Cell growth and cell morphology were measured. The data for DAB and DFMO are means $\pm$ SEM of four experiments. MO values are means of two different experiments.

Inhibitor	Growth (μ g protein ml ⁻¹)	Inhibition (%)	Yeast-mycelium	
			transition (%)	inhibition (%)
None	314 $\pm$ 60	—	90 $\pm$ 4	—
10 mM-DAB	297 $\pm$ 70	12 $\pm$ 5.5	30 $\pm$ 2.5	67 $\pm$ 3
15 mM-DAB	296 $\pm$ 60	9 $\pm$ 3.5	21 $\pm$ 4	77 $\pm$ 3
20 mM-DAB	289 $\pm$ 70	12 $\pm$ 5.5	14 $\pm$ 3	84 $\pm$ 2.5
None	534 $\pm$ 130	—	77 $\pm$ 3	—
40 mM-DFMO	483 $\pm$ 120	10 $\pm$ 1.0	14 $\pm$ 3.5	78 $\pm$ 7.5
None	720	—	81	—
40 mM-MO	780	0	57	30
70 mM-MO	560	22	43	47
100 mM-MO	519	28	13	84

at most 30% inhibition at the highest concentrations used (20 or 40 mM, respectively). ODC activity in permeabilized cells, on the other hand, was inhibited about 90% by such concentrations (Table 2). In addition, 1 mM-DFMO and 1 mM-DAB gave comparable levels of inhibition (Table 2). The low inhibition of ODC in whole cells was not due to the short time of contact between the drug and the cells. Preincubation of the cells with DAB or DFMO for 2 or 4 h did not increase the level of inhibition of ODC (not shown).

Based on these results, we tested the effect of ODC inhibitors on the morphogenetic transition of the fungus.

Fig. 4. Sensitivity of mycelial induction to DAB as a function of incubation time. Yeast cells maintained in the cold were transferred to YNBGlcNac medium. At the indicated periods of time, DAB was added to sets of flasks. After 12 h incubation, cell morphology was scored (○), and inhibition of mycelial transition was calculated (●). Data are means of two different experiments.

The inhibitors used were DAB, DFMO and α -methylornithine (MO). All were strong inhibitors of the dimorphic transition, at concentrations which caused almost no inhibition of cell growth (Table 3). Concentrations even higher than those used in the experiments described in Table 2 did not affect yeast growth (not shown).

The time of addition of the inhibitors during incubation in YNBGlcNac was decisive for its morphogenetic effect (Fig. 4). When the drug was added after 4 h of growth, at which time the dimorphic transition is not yet discernible, its inhibitory effect was reduced to about 30–40% of that when it was added to the medium together with the inoculum. When DAB was added after 6 h incubation, it had no effect on mycelial development and mycelial cells observed after 12 or 24 h were indistinguishable from those of the control without drug.

Table 4. Effect of DAB addition to the inoculum on the yeast-mycelial transition of *Yarrowia lipolytica*

Cells were grown for 12 h in YNBGlc medium in the presence or absence of DAB. Cells were then harvested by centrifugation, washed and resuspended in cold water. After 15 min, cells (3×10^6 per ml) were inoculated into YNBGlcNAc medium and grown at 30 °C. After 12 h, cell growth and morphology were measured. Data for 0.5 and 1.0 mM-DAB are means $\pm$ SEM of three different experiments. Data for 10 and 20 mM-DAB are means of two different experiments.

DAB concentration (mM)	Growth (μ g protein ml ⁻¹)	Inhibition (%)	Yeast-mycelia	
			Transition (%)	Inhibition (%)
None	453 $\pm$ 40	—	82 $\pm$ 1.5	—
0.5	540 $\pm$ 110	0	24 $\pm$ 2.5	71 $\pm$ 2.5
1.0	458 $\pm$ 55	0	17 $\pm$ 1.5	80 $\pm$ 2
10.0	410	9	0	100
20.0	400	12	0	100

Table 5. Partial reversal of the effect of DAB by putrescine

Yeast cells were grown for 12 h in YNBGlc medium containing 20 mM-DAB, harvested, washed and resuspended in cold water. After 15 min they were inoculated into YNBGlcNAc medium in the presence or absence of putrescine. After 12 h, cell growth and morphology were determined. Values are means $\pm$ SEM of three different experiments.

Putrescine (mM)	Growth (μ g protein ml ⁻¹)	Mycelial transition (%)
None	184 $\pm$ 18	5 $\pm$ 1
0.25	456 $\pm$ 46	38 $\pm$ 3
0.50	495 $\pm$ 98	44 $\pm$ 4
0.75	422 $\pm$ 118	47 $\pm$ 4

These results indicate that the morphogenetic signal blocked by the ODC inhibitors operates at an early period. Also, it was not necessary for ODC inhibitors to be present during incubation in mycelial-induction medium for them to be effective. Concentrations even lower than those effective when added to YNB-GlcNAc inhibited mycelial growth if added to the medium where the inoculum was grown (Table 4).

This observation was used as the basis to determine whether the effect of ODC inhibitors was specific or not. Cells were grown in the presence of 20 mM-DAB in YNBGlc medium. The resulting yeasts were harvested and after holding at 4 °C, they were inoculated into YNBGlcNAc medium containing different concentrations of putrescine. When putrescine-free medium was used, only 5% of the inoculated cells turned into mycelium, whereas the inclusion of 0.25–0.75 mM-putrescine increased growth and the proportion of mycelial cells to 40–50% (Table 5). Higher concentrations of

Table 6. Partial reversal of the effect of DAB by 5-azacytidine

Yeast cells of *Y. lipolytica* were grown in YNBGlc medium for 8 h, then AC was added to some of the cultures, and incubation was continued for 4 h. Cells were harvested by centrifugation, washed, and maintained for 12 h in AC solution at 4 °C. They were then centrifuged, washed, and inoculated (3×10^6 per ml) into YNBGlcNAc medium in the presence or absence of 20 mM-DAB and incubated at 30 °C. After 12 h, cell growth and morphology were determined. Values are means $\pm$ SEM of three different experiments.

AC added to inoculum (μ M)	DAB added to growth medium	Growth (μ g protein ml ⁻¹)	Mycelial transition (%)
None	None	510 $\pm$ 46	83 $\pm$ 1
None	20 mM	420 $\pm$ 6	8 $\pm$ 0
2.0	20 mM	421 $\pm$ 21	27 $\pm$ 2
5.0	20 mM	270 $\pm$ 21	71 $\pm$ 2
10.0	20 mM	224 $\pm$ 20	29 $\pm$ 6

putrescine were inhibitory for cell growth and did not give rise to higher mycelial ratios. Mycelial cells formed in the medium containing putrescine were much longer than those observed in putrescine-free medium.

The effect of ODC inhibitors was reversed not only by putrescine, but also by the DNA-methylation inhibitor, 5-azacytidine (AC). The drug was extremely toxic, however, and its inhibitory effect was increased when added simultaneously with DFMO or DAB. Therefore, a special protocol had to be designed to measure the effect of AC on the inhibitory effect of DAB. After several trials, the following protocol was used: cells were grown in the yeast form for 8 h in the absence of any drug; AC was then added to the medium, and incubation continued for 4 h. Cells were harvested by centrifugation, washed and resuspended in a solution of AC. After 12 h at 4 °C, cells were centrifuged, AC was washed out, and the cells were inoculated into DAB-containing medium. The results are shown in Table 6. When cells were grown in AC-free medium and then resuspended into YNB-GlcNAc medium containing 20 mM-DAB, mycelial cells were decreased from 83% in the control, to less than 8% of stunted mycelial cells. But when 5 μ M-AC was included into YNBGlcNAc medium, the proportion of mycelial cells increased to 71%, and these mycelial cells were as long as those present in control cultures. Higher concentrations of AC were less effective in reversing the DAB effect, probably because of their toxicity.

Discussion

The similarities in the factors which affect the yeast-to-mycelium transition in *Yarrowia lipolytica* and *Candida albicans* are noticeable. Both organisms show mycelial growth when incubated in media containing GlcNAc

(Simonetti *et al.*, 1974; Rodriguez & Dominguez, 1984). In *C. albicans*, starvation predisposes the cells to grow as mycelium when they are further incubated at 37 °C (Soll, 1985). Similarly, we found that cells starved in plain water at 4 °C showed mycelial growth when transferred to a glucose-containing medium. The inducers appeared to be synergistic: when starved cells were inoculated into GlcNAc-containing medium, almost all the cells grew as mycelium. The small and constant proportion of yeast cells remaining under such conditions probably represent the non-viable cells originally present in the cultures. The effect of cooling and further heating of the cells appears to be related not to starvation or to synchronization in the cell cycle as suggested for *C. albicans* (Soll, 1985), but to a heat-shock phenomenon. Heat-shock induces the synthesis of specific heat-shock proteins (HsP) in almost all systems (Lindquist, 1986) including *C. albicans* (Dabrowa & Howard, 1984; Zeuthen & Howard, 1984). These proteins may well play a role in cell differentiation. However, the alternative that a heat-labile repressor of myceliation is destroyed during the heat-shock cannot yet be dismissed.

Polyamines are important factors involved in cell proliferation and differentiation in a great number of systems (Heby, 1981; Tabor & Tabor, 1984, 1985), including fungi (Stevens & Winther, 1979). During differentiation the activity of the key regulatory enzyme for polyamine synthesis, ornithine decarboxylase (ODC), is increased several-fold. ODC activity increases during the germination of the spores of *Blastocladiella emersonii*, *Aspergillus nidulans*, *Neurospora crassa*, *Rhizopus stolonifer*, *Botryodiplodia* sp., *Dictyostelium discoideum* (Stevens & Winther, 1979, Tabor & Tabor, 1985), *Mucor racemosus* (Inderlied *et al.*, 1980), *Mucor rouxii* and *Mucor bacilliformis* (Calvo-Mendez *et al.*, 1987; Martinez-Pacheco *et al.*, 1989). Similarly, the transition of the yeast to mycelial cells in *Y. lipolytica* was accompanied by a transient increase in the levels of the enzyme. These results agree with the observation that ODC activity increased during the yeast-to-mycelium transition of *M. racemosus* (Inderlied *et al.*, 1980) and *M. rouxii* (Martinez-Pacheco *et al.*, 1989). Associated with the increase in ODC activity, reinitiation of active growth of the fungus was accompanied by an increase in the levels of polyamines. This increase was more noticeable for the mycelial than the yeast form.

Three inhibitors of ODC which have different modes of action all inhibited the yeast-to-mycelium transition of *Y. lipolytica* when added at concentrations which inhibited growth only slightly or not at all. DAB is a competitive inhibitor, and an analogue of putrescine, the ODC product. DAB inhibits ODC from different systems including *A. nidulans* (Stevens *et al.*, 1977) and *M. rouxii* and *M. bacilliformis* (Ruiz-Herrera & Calvo-Mendez,

1987). DFMO is an irreversible inhibitor which binds covalently to the enzyme (Metcalf *et al.*, 1978). Methyl-ornithine is an analogue of the substrate and a competitive inhibitor of ODC (Mamont *et al.*, 1976). These results agree with those obtained previously by our group. ODC inhibitors block the mycelial-to-yeast transition of *M. rouxii* (Martinez-Pacheco *et al.*, 1989) and *C. albicans* (Martinez *et al.*, 1990), and the germination of Mucoraceous fungi (Ruiz-Herrera & Calvo-Mendez, 1987). In agreement with the observations made in this latter system, the effect of DAB (and the other two ODC inhibitors) on the dimorphic transition of *Y. lipolytica* was restricted to a short period of time, immediately after the application of the morphogenetic stimulus and long before the appearance of any morphological indication of mycelial growth. When added after this critical period, which appeared to correspond to the transitory elevation of ODC activity, the drugs had no effect on mycelial growth. When cells were pre-grown in the presence of DAB, mycelial induction by the normal protocol followed in this study was prevented. It is probable that the drug is accumulated in the cells and prevents polyamine build-up during the induction period.

The data discussed above indicate that the effect of ODC inhibitors is due to interference with polyamine synthesis. Further evidence for this hypothesis is provided by the reversal of the DAB effect by the addition of putrescine. The sequential order of addition of both compounds, DAB during growth of the inoculum, and putrescine after transfer to GlcNAc medium, counters the possible criticism that putrescine is effective because it prevents the entrance of the inhibitor into the cell. In *C. albicans*, DFMO depleted polyamine pools after 20 h of exposure (Pfaller *et al.*, 1988). Polyamines are growth factors required for many different cells (reviews by Tabor & Tabor, 1976, 1984). This creates a paradox, since ODC inhibitors should inhibit growth and morphogenesis similarly. This, however is not the case. Using the appropriate drug concentrations, the processes of cellular growth and differentiation can be clearly separated, a phenomenon that we have already demonstrated in *M. rouxii* (Ruiz-Herrera & Calvo-Mendez, 1987; Martinez-Pacheco *et al.*, 1989). A possible solution to the paradox is provided by the differential effect of ODC inhibitors on intact compared with permeabilized cells. ODC appears to be located in at least two different cell compartments, only one of which is accessible to the inhibitors. A feasible hypothesis is that the accessible enzyme is somehow related to the morphogenetic phenomenon, whereas the other is essential for growth. We observed that only a fraction of the total cell polyamines are present in a free form and able to diffuse. This fraction, which is only 10–20% of the total in

N. crassa (Davis *et al.*, 1985) may be the only one involved in differentiative processes (Torrighiani *et al.*, 1987). Similar results which suggest the presence of ODC in more than one cell compartment have been obtained in *M. rouxii* (M. Martinez-Pacheco & J. Ruiz-Herrera, unpublished results).

The precise mode of action of polyamines in differentiation remains unknown. Recently, we have suggested that polyamines have a negative effect on DNA methylation (Cano *et al.*, 1989), the latter being associated with the regulation of gene expression in different systems (Dynan, 1989; Razin *et al.*, 1984) including fungi (Goyon & Faugeron, 1989; Selker *et al.*, 1987*a, b*). In agreement with higher eukaryotes, it has been suggested that DNA methylation plays an important role in fungal development (Juppe *et al.*, 1986; Magill & Magill, 1986; Russell *et al.*, 1987*a, b*). Solid evidence for this hypothesis was provided recently in the *M. rouxii* system where the effect of DNA hypomethylation and DAB inhibition were pinpointed to transcription of specific mRNAs (Cano-Canchola *et al.*, 1992). In agreement with our proposal on the mode of action of polyamines, it was observed that 5-azacytidine reverses the effect of DAB in *Yarrowia*. When cells were pre-grown in the presence of the optimal concentration of AC, further addition of DAB had only a slight inhibitory effect on mycelial formation. AC is a cytosine analogue which is incorporated into DNA and inhibits its methylation, apparently binding to methyltransferases (Tanaka *et al.*, 1980; Taylor & Jones, 1982). AC incorporation into DNA leads to gene expression in cells where it does not normally occur (Jones, 1985; Felsenfeld & McGhee, 1982). The fact that AC addition does not induce mycelial formation in *Yarrowia* indicates that DNA hypomethylation by itself is only a pre-requisite which permits the cell to become responsive to the morphogenetic stimulus. This result agrees with our previous observation that AC counteracts the inhibition in spore germination brought about by DAB, but is unable to accelerate germ tube formation (Cano *et al.*, 1988).

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References

- ADAMS, B. G. (1972). Method for decyptification of β -glucosidase in yeast with dimethyl sulfoxide. *Analytical Biochemistry* **45**, 137–146.
- BARTNICKI-GARCIA, S. (1963). Symposium on biochemical bases of morphogenesis in fungi. III. Mold-yeast dimorphism of *Mucor*. *Bacteriological Reviews* **27**, 293–304.
- CALVO-MENDEZ, C., MARTINEZ-PACHECO, M. & RUIZ-HERRERA, J. (1987). Regulation of ornithine decarboxylase activity in *Mucor bacilliformis* and *Mucor rouxii*. *Experimental Mycology* **11**, 270–277.
- CANO, C., HERRERA-ESTRELLA, L. & RUIZ-HERRERA, J. (1988). DNA methylation and polyamines in regulation of development of the fungus *Mucor rouxii*. *Journal of Bacteriology* **170**, 5946–5948.
- CANO-CANCHOLA, C., SOSA, L., FONZI, W., SYPHERD, P. & RUIZ-HERRERA, J. (1992). Developmental regulation of *CUP* gene expression through DNA methylation in *Mucor* spp. *Journal of Bacteriology* **174**, 362–366.
- CHENG, S. C. & OGRYDZIAK, D. M. (1987). Processing and secretion of the *Yarrowia lipolytica* RNase. *Journal of Bacteriology* **169**, 1433–1440.
- DABROWA, N. & HOWARD, D. H. (1984). Heat shock and heat stroke proteins observed during germination of the blastoconidia of *Candida albicans*. *Infection and Immunity* **44**, 537–539.
- DAVIS, R. H., KRASNER, G. N., DIGANGI, J. J. & RISTOW, J. L. (1985). Distinct roles of putrescine and spermidine in the regulation of ornithine decarboxylase in *Neurospora crassa*. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 4105–4109.
- DYNAN, W. S. (1989). Understanding the molecular mechanism by which methylation influences gene expression. *Trends in Genetics* **5**, 35–36.
- FELSENFELD, G. & MCGHEE, (1982). Methylation and gene control. *Nature, London* **296**, 602–603.
- GAILLARDIN, C. M., CHAROY, V. & HESLOT, H. (1973). A study of copulation, sporulation and meiotic segregation in *Candida lipolytica*. *Archives of Microbiology* **92**, 69–83.
- GAILLARDIN, C. M., FOURNIER, P., SYLVESTRE, G. & HESLOT, H. (1976). Mutants of *Saccharomycopsis lipolytica* defective in lysine catabolism. *Journal of Bacteriology* **125**, 48–57.
- GOYON, C. & FAUGERON, G. (1989). Targeted transformation of *Ascomobolus immersus* and *de novo* methylation of the resulting duplicated DNA sequences. *Molecular and Cellular Biology* **9**, 2819–2827.
- HERBY, O. (1981). Role of polyamines in the control of cell proliferation and differentiation. *Differentiation* **14**, 1–20.
- INDERLIED, C. B., CIHLAR, R. L. & SYPHERD, P. S. (1980). Regulation of ornithine decarboxylase during morphogenesis of *Mucor racemosus*. *Journal of Bacteriology* **141**, 699–706.
- JONES, P. A. (1985). Altering gene expression with 5-azacytidine. *Cell* **40**, 485–486.
- JUPPE, E. R., MAGILL, J. M. & MAGILL, C. W. (1986). Stage-specific DNA methylation in a fungal plant pathogen. *Journal of Bacteriology* **165**, 420–423.
- LINDQUIST, S. (1986). The heat-shock response. *Annual Review of Biochemistry* **55**, 1151–1191.
- MAGILL, J. M. & MAGILL, C. W. (1989). DNA methylation in fungi. *Developmental Genetics* **10**, 63–69.
- MAMONT, P. S., BÖHLEN, P., MCCANN, P. P., BEY, P., SHUBAR, F. & TARDIFF, C. (1976). α -Methylornithine, a potent competitive inhibitor of ornithine decarboxylase blocks proliferation of rat hepatoma cells in culture. *Proceedings of the National Academy of Sciences of the United States of America* **73**, 1626–1630.
- MARTINEZ, J. P., LOPEZ-RIBOT, J. L., GIL, M. L., SENTANDREU, R. & RUIZ-HERRERA, J. (1990). Inhibition of the dimorphic transition of *Candida albicans* by the ornithine decarboxylase inhibitor, 1,4-diaminobutanone: alterations in the glycoprotein composition of the cell wall. *Journal of General Microbiology* **136**, 1937–1943.
- MARTINEZ-PACHECO, M., RODRIGUEZ, G., REYNA, G., CALVO-MENDEZ, C. & RUIZ-HERRERA, J. (1989). Inhibition of the yeast-mycelial transition and the phorogenesis of *Mucorales* by diamino butanone. *Archives of Microbiology* **151**, 10–14.
- METCALF, B. W., BEY, P., DANZIN, C., JUNG, M. J., CASARA, P. & VEVERT, J. P. (1978). Catalytic irreversible inhibition of mammalian ornithine decarboxylase (EC 4.1.1.17) by substrate and product analogues. *Journal of the American Chemical Society* **100**, 2551–2553.
- ODDS, F. C. (1988). *Candida and Candidosis*. London: Bailliere Tindall.
- OGRYDZIAK, D., BASSEL, J., CONTOPOULOU, R. & MORTIMER, R. (1978). Development of genetic techniques and the genetic map of the yeast *Saccharomycopsis lipolytica*. *Molecular and General Genetics* **163**, 229–239.

- PFALLER, M. A., RILEY, J. & GERARDEN, T. (1988). Polyamine depletion and growth inhibition in *Candida albicans* and *Candida tropicalis* by α -difluoromethyl ornithine and cyclohexylamine. *Journal of Medical and Veterinary Mycology* **26**, 119–126.
- RAZIN, A., CEDAR, H. & RIGGS, A. D. (Eds.) (1984). *DNA Methylation: Biochemistry and Biological Significance*. New York: Springer-Verlag.
- RODRIGUEZ, C. & DOMINGUEZ, A. (1984). The growth and characteristics of *Saccharomyces lipolytica*: morphology and induction of mycelium formation. *Canadian Journal of Microbiology* **30**, 605–612.
- RUIZ-HERRERA, J. & CALVO-MENDEZ, C. (1987). Effect of ornithine decarboxylase inhibitors on the germination of sporangiospores of Mucorales. *Experimental Mycology* **11**, 287–296.
- RUSSELL, P. J., RODLAND, K. D., RACHLIN, E. M. & MCCLOSKEY, J. A. (1987a). Differential DNA methylation during the vegetative life cycle of *Neurospora crassa*. *Journal of Bacteriology* **169**, 2902–2905.
- RUSSELL, P. J., WELSCH, J. A., RACHLIN, E. M. & MCCLOSKEY, J. A. (1987b). Different levels of DNA methylation in yeast and mycelial forms of *Candida albicans*. *Journal of Bacteriology* **169**, 4393–4395.
- SELKER, E. U., CAMBARERI, E. B., JANSEN, B. C. & HAACK, K. R. (1987a). Rearrangement of duplicated DNA in specialized cells of *Neurospora*. *Cell* **51**, 741–752.
- SELKER, E. U., JENSEN, B. C. & RICHARDSON, G. A. (1987b). A portable signal causing faithful DNA methylation *de novo* in *Neurospora*. *Science* **238**, 48–53.
- SIMONETTI, N., STRIPPOLI, V. & CASSONE, A. (1984). Yeast–mycelial conversion induced by *N*-acetyl-D-glucosamine in *Candida albicans*. *Nature, London* **250**, 344–346.
- SISSONS, C. H. (1976). Improved technique for accurate and convenient assay of biological reactions liberating CO₂. *Analytical Biochemistry* **70**, 454–462.
- SMITH, T. A. (1985). Polyamines. *Annual Review of Plant Physiology* **36**, 117–143.
- SOLL, D. R. (1985). *Candida albicans*. In *Fungal Dimorphism: With Emphasis on Fungi Pathogenic to Humans*, pp. 167–195. Edited by P. J. Szanislo. New York: Plenum.
- STEVENS, L., MCKINNON, I. M. & WINTHER, M. D. (1977). The effects of 1,4-diaminobutanone on polyamine biosynthesis in *Aspergillus nidulans*. *FEBS Letters* **75**, 180–182.
- STEVENS, L. & WINTHER, M. D. (1979). Spermine, spermidine and putrescine in fungal dimorphism. *Advances in Microbial Physiology* **19**, 63–148.
- SZANISZLO, J. P. (Ed.) (1985). *Fungal Dimorphism: With Emphasis on Fungi Pathogenic to Humans*. New York: Plenum.
- TABOR, C. W. & TABOR, H. (1976). 1,4-Diaminobutane (putrescine), spermidine and spermine. *Annual Review of Biochemistry* **45**, 285–306.
- TABOR, C. W. & TABOR, H. (1984). Polyamines. *Annual Review of Biochemistry* **53**, 749–790.
- TABOR, C. W. & TABOR, H. (1985). Polyamines in microorganisms. *Microbiological Reviews* **49**, 81–89.
- TANAKA, M., HIBASAMI, H., NAGAI, J. & IKEDA, T. (1980). Effects of 5-azacytidine on DNA methylation in Ehrlich's ascites tumor cells. *Australian Journal of Experimental Biology and Medical Sciences* **58**, 391–396.
- TAYLOR, S. M. & JONES, P. A. (1982). Mechanism of action of eukaryotic DNA methyltransferase. Use of azacytidine-containing DNA. *Journal of Molecular Biology* **162**, 679–692.
- TORRIGIANI, P., ALTAMURA, M. M., CAPITANI, F., SERAFINI-FRACASSINI, D. & BAGNI, N. (1989). *De novo* root formation in thin cell layers of tobacco: changes in free and bound polyamines. *Physiologia Plantarum* **77**, 294–301.
- WICKERHAM, L. J., KURTZMAN, C. P. & HERMAN, A. I. (1970). Sexual reproduction in *Candida lipolytica*. *Science* **167**, 1141.
- ZEUTHEN, M. L. & HOWARD, D. H. (1984). Thermotolerance and the heat-shock response in *Candida albicans*. *Journal of General Microbiology* **135**, 2509–2518.