Cloning and disruption of the ornithine decarboxylase gene of *Ustilago maydis*: evidence for a role of polyamines in its dimorphic transition

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The gene encoding ornithine decarboxylase (ODC) from *Ustilago maydis* was cloned. A conserved PCR product amplified from *U. maydis* DNA was synthesized and used to screen a genomic library of the fungus. Alignment of its deduced protein sequence with those of other cloned ODCs showed a high degree of homology. Gene replacement was obtained by removal of a central part of the gene and insertion of the hygromycin resistance cassette. The null mutant thus obtained displayed no ODC activity and behaved as a polyamine auxotroph. This result is evidence that a single ODC gene exists in the fungus, and that *U. maydis* utilizes the ODC pathway as the only mechanism for polyamine biosynthesis. When grown in polyamine-containing media, the null mutant accumulated a polyamine pool which further sustained its normal rate of growth in polyamine-free media for approximately 12–16 h. When putrescine concentrations lower than 0.5 mM were employed, the mutant grew at a normal rate but was unable to engage in the dimorphic transition. Under conditions favourable for mycelial growth, the mutant grew with a yeast-like morphology in liquid media, and formed smooth colonies consisting of yeast cells on solid media. Reversion to normal dimorphic phenotype required high concentrations of putrescine or spermidine. These results are evidence that concentrations of polyamines higher than those necessary to sustain vegetative growth are required for the dimorphic transition in *U. maydis*.

Keywords: *Ustilago maydis* dimorphism, ornithine decarboxylase gene, ornithine decarboxylase mutants, polyamine synthesis

INTRODUCTION

Polyamines are basic cationic micromolecules essential for the growth of all organisms (Pegg & McCann, 1982; Tabor & Tabor, 1984, 1985). Because of their positive net charge, it has been considered that polyamines exert their multiple functions by binding to negatively charged molecules of the cell, mainly nucleic acids. Among the many roles played by polyamines, it has been described

that they protect DNA from enzymic degradation, X-ray irradiation, mechanical shearing and oxidative damage. They also stabilize RNA, prevent ribosome dissociation, and *in vitro* they stimulate DNA and RNA synthesis and improve the fidelity of translation (McCann *et al.*, 1987). A role for polyamines in cell differentiation has also been substantiated (Pegg & McCann, 1982; Tabor & Tabor, 1984, 1985; Ruiz-Herrera, 1994; Slocum *et al.*, 1984), although no clear-cut mechanisms for this role have been demonstrated. In general, it has been considered that this specific function is only an extension of their general effects on growth. In animals and fungi, the first enzyme specifically involved in polyamine biosynthesis is ornithine decarboxylase (ODC), responsible for the synthesis of putrescine

Abbreviations: DAB, 1,4-diamino-2-butanone; ODC, ornithine decarboxylase.

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(Davis *et al.*, 1992; Tabor & Tabor, 1984, 1985). Mutants affected in the catalytic polypeptide of ODC are polyamine auxotrophs (Davis *et al.*, 1987; Balasundaram *et al.*, 1994).

We have described in several fungal models that the levels of ODC and polyamine pools increase previous to all differentiative steps: spore germination (Calvo-Mendez *et al.*, 1987), sporulation (Martinez-Pacheco *et al.*, 1989; Reyna-Lopez & Ruiz-Herrera, 1993) and dimorphic transition (Martinez-Pacheco *et al.*, 1989; Guevara-Olvera *et al.*, 1993). If this increase in the polyamine pools is blocked by addition of the ODC competitive inhibitor 1,4-diamino-2-butanone (DAB), differentiation is prevented, without serious alterations in cell growth (Ruiz-Herrera & Calvo-Mendez, 1987; Martinez-Pacheco *et al.*, 1989; Reyna-Lopez & Ruiz-Herrera, 1993; Guevara-Olvera *et al.*, 1993). Specificity of the effect of the inhibitor is supported by the observation that putrescine addition reverses the effect of DAB. More decisive evidence for the specific role of high levels of polyamines in cell differentiation would be provided by controlling their concentrations in auxotrophic mutants. In the present study, we have isolated an *odc* null mutant of *Ustilago maydis* by means of reverse genetics. *U. maydis* is a plant-pathogenic fungus responsible for corn smut. The fungus displays a complex life cycle with two alternating phases: a haploid stage, characterized by its saprophytic yeast-like growth; and a dikaryotic stage during which the fungus grows as a mycelium and invades the host plant (Banuett, 1992). Recently we have developed conditions which permit the dimorphic transition of haploid strains in liquid or solid media (Ruiz-Herrera *et al.*, 1995a). An especially attractive feature of *U. maydis* is that it has a well-developed genetic system, and all the molecular tools which permit its easy manipulation by recombinant DNA techniques (Fotheringham & Holloman, 1989, 1990; Wang *et al.*, 1988). We have thus proceeded to the cloning of the ODC gene of the fungus, its substitution, and the analysis of the phenotype of the null mutant obtained.

METHODS

Strains and culture media. FB1 (a_1b_1) and FB2 (a_2b_2) strains of *U. maydis* (F. Banuett, University of California, San Francisco, USA) were used in this study. They were maintained and propagated in the yeast or mycelial forms as described previously (Ruiz-Herrera *et al.*, 1995a). *U. maydis* grows with yeast-like morphology at pH 7, and in the mycelial form at pH 3 (Ruiz-Herrera *et al.*, 1995a). When necessary, hygromycin ($200 \mu\text{g ml}^{-1}$) and/or putrescine or spermidine were added to minimal medium (Holliday, 1974). *Escherichia coli* strains DH5 α mcr and KW258 were used for transformation, and for plasmid and phage propagation, respectively. They were grown in LB broth containing 10 mM MgSO_4 and 2% (w/v) maltose, or ETB media containing the necessary antibiotics for selection.

DNA techniques. Restriction enzymes and modifying enzymes were from BRL, except *SalI*, which was obtained from Boehringer. They were employed according to the instructions

of the suppliers. DNA-DNA (Southern) hybridizations utilizing [α - ^{32}P]dCTP-labelled probes were performed under high stringency according to Sambrook *et al.* (1989). Transformation of *U. maydis* was carried out by the method of Tsukuda *et al.* (1988).

DNA was sequenced by the dideoxynucleotide termination method (Sanger *et al.*, 1977) using the Sequenase kit (version 2.0; USB). Single- or double-stranded DNA was used as template. The terminal sequences of the cloned fragments were obtained using the forward and reverse universal primers. Additional primers were designed to extend the sequences.

Screening of the genomic library. The genomic library of *U. maydis* RK32 (a_2b_3) constructed in λ EMBL3 was obtained from Regine Kahmann (Universität München, Germany). The labelled *Umodc* PCR product (Torres-Guzman *et al.*, 1996) was used to screen the library. Positive phages were amplified in *E. coli* KW258. Inserts were recovered, digested with *SalI*, and probed by hybridization with the PCR product. The positive fragments of approximately 4–5 kbp were subcloned in Bluescript KS(+) (pBKS+) and propagated. Since similar restriction maps were displayed by three clones, one of them was selected for further studies.

RNA extraction and Northern hybridization. RNA was extracted using a slight modification of the lithium method (Sambrook *et al.*, 1989). RNA was separated by electrophoresis in formaldehyde/agarose gels, transferred to Hybond-N⁺ membranes (Amersham) and hybridized. A radio-labelled *PstI*–*XhoI* 0.9 kbp fragment of the gene was used as a probe under stringent conditions (Sambrook *et al.*, 1989). RNA concentrations were normalized by hybridization with the carboxin resistance gene (Keon *et al.*, 1991).

Other methods. Cell morphology of *U. maydis* grown in liquid medium was scored by microscopic observation of samples stained with cotton blue. At least 200 cells were scored in each experiment. Cells and colonies were observed and photographed with a Zeiss microscope (Jena). ODC was measured in whole cells as described previously (Guevara-Olvera *et al.*, 1993). Cell growth was measured by dry weight or protein determination as described by Ruiz-Herrera *et al.* (1995a). Protein was measured by the Folin method. *U. maydis*-infected corn ears were obtained by inoculation of young plants with a mixture of FB1 and FB2 *U. maydis* cell suspensions. Naturally infected corn ears were obtained from a local supplier.

RESULTS

Inhibition of teliospore germination and yeast-to-mycelium transition by DAB

Teliospores obtained from galls developed in artificially or naturally infected corn were incubated on solid plates of minimal medium with or without 20 mM DAB. Germination was followed for 48 h and scored. At the concentration used, DAB inhibited teliospore germination by more than 97% (Table 1). A similar degree of inhibition (88%) of the yeast-to-mycelium transition of the fungus was brought about by the same DAB concentration, whereas growth inhibition was only of the order of 30% (Table 1). These results suggested that, as described in other fungi (Ruiz-Herrera, 1994), poly-

U.m.	MSSITLSR-----LM-VTSPSITA-----TLTRS--VGVF-----STLTLYHG-----	35
N.c.	MVMPTVSD-RMGTFIDYTNHVFSSKQ-TDGLNTVNN--GSLKHDDYLHGLANGKLVAKQMGDALRQVDES	71
S.c.	MSSITQVGNALSSSTTLVDLSNSETVTQKQYKDGTELHNLLELKNQDLELLPHEQ--AHPKIFQALKARIOR	73
	* * * * *	
U.m.	-----CRANGENAFFVADLSEVTRQHLRWMRALPRIVFFYAVKCNPDYVLQLLGALGTGFCASWGEIEAVLKL	105
N.c.	IDSEFCFPGDEDTTFVADLGEVTRQHLRWKLNLPKVPFPIAVKCHPDERLLQLLAALGTGFCASKAEIEQVLRM	146
S.c.	INNRTCDPGEENSFFICDLGEVTRQHLRWKLNLPKVPFPIAVKCHPDERLLQLLAALGTGFCASKAEIEQVLRM	148
	* * * * *	
U.m.	GINPARIITYANPSKAASFVRHAAGHNGLTTFDNMDELEKMKRYHPSCKLVVRILTDDSKSACQLGLKFGAPIAS	180
N.c.	GVDPSRIITYAQCKTNSYLRYVAQGVQVMTFDNADELKRIARLYPDAELFLRLITDDSSSLCRFSMKFGASLDS	221
S.c.	NISPDRIIVANPCVASTIRYAASKNVVKSTFDNVEELHKIKKHPESQQLLRITDDSTAQCRLSTKYGCENEN	223
	* * * * *	
U.m.	VPRLLERARELELDVVGVSFHVSGCYDPSFRDAVTRACAF-ENGK-QAGF-SFDLLDVGGGFGHDN-FEMVAG	251
N.c.	TGGLLGLARQLGLNVVGVSFHVSGASDPTAFKAVQDAHVVFQQA-AYGY-SLKTLDVGGGFCSDSFEQMAN	294
S.c.	VDVLLKAIKELGLNLGVSFHVSGASDPTSLYKAVRDARTVFDKAANEYGLPPLKILDVGGGF-QFSEFKESTA	297
	* * * * *	
U.m.	VLGPAIDAYFPDEDFAPGKREVGVKNGLRITAEPRFTVHSAFALATNIIAVRRGEPSAANDHVEATPKAQ---	323
N.c.	VLRAALDEYFPAN-----TGVLNIAEPGRYASSAFTLACNIIARRTIQDGSAGVSVDSSSMDDGS	356
S.c.	VLRLALEFFFPVG-----CGVDIAEPGRYFVATAFTLASHVIAKRLSENEAMIYTDNGVYGNMNC	359
	* * * * *	
U.m.	VNY-----YQNDGLYGSFNCVIFDHVKFIQSSDAGPRVRVADDWVARGASGCGRLALQOEAMTLESCSVWG	391
N.c.	VNNGDARYMVYVNDGLYGNFSSIMFDHQ-----HPVAKI-LRAGGRTHYNSVA--AHSSAEDAIKY---SIWG	419
S.c.	ILFDHQE--PFRP-TLYHNL-EFHYDDF-----ESTTAV-L-----DSI---NKTRESEYPIYKV---SIWG	408
	* * * * *	
U.m.	PTCDSDICVRDHVELFKGLQVGDWLVYENMGAYTICAASFNGIRSEIRYTLGHQDADIVRQLMLQ	459
N.c.	PTCDGIDRITTSIRFREILDVGDWLYFEDMGAYTKSATTFNGFSNEH--DVIYVCSEPGAMALLGL	484
S.c.	PTCDGLDCIAKEYYMKHDVIVGDWYFYPALGAYTSSAATQFNGFEQA---DIVYIDSE-----LD-	466
	* * * * *	

Fig. 2. Multiple amino acid alignment as derived by maximal homology of ODCs from *U. maydis* (U.m.), *N. crassa* (N.c.) and *S. cerevisiae* (S.c.). Asterisks indicate positions of complete identity among all sequences. The putative PEST regions are underlined.

Neurospora crassa (Williams *et al.*, 1992) (Fig. 2). The ODCs from *U. maydis* and *N. crassa* share 40.3% identical amino acids. This value is higher than the homology existing among *U. maydis* and *S. cerevisiae* (31.8%), but lower than the homology between *S. cerevisiae* and *N. crassa* (46%). Higher homology was observed in the central part, compared to the amino- and carboxy-termini of the proteins.

Two putative PEST regions, characteristic of proteins with a high rate of turnover (Rogers *et al.*, 1986), but with low PEST scores of -11.8 and -9.21, were identified, respectively, in residues 306-315 and 374-401 of the protein, using the algorithm described by Rechsteiner & Rogers (1996). The second one is located at a similar position to the second PEST region identified in the ODC from *N. crassa* (residues 405-427) (Williams *et al.*, 1992). This PEST region is absent in *S. cerevisiae*.

U. maydis DNA digested with *Bam*HI, *Eco*RI, *Hind*III, *Pst*I or *Xho*I was probed with a radiolabelled 900 bp *Pst*I-*Xho*I fragment of the gene. A single fragment was observed in all cases (Fig. 3). This result suggests that the fungus contains a single ODC gene, *Umodc*. Northern analysis of *U. maydis* RNA revealed a single transcript of *Umodc* with a size of approximately 2.5 kb. Transcript analysis during the yeast-to-mycelium transition revealed that no significant changes in mRNA levels occurred at this step (Fig. 4).

Null *odc* mutants of *U. maydis* obtained by gene substitution

We utilized a method of double homologous recombination (Fotheringham & Holloman, 1989). The *Bgl*II fragment containing the coding region of the gene was subcloned into plasmid pT7-7 in which the *Sma*I site had been previously deleted. A central *Stu*I-*Sma*I fragment of 584 bp in length was removed. A *Sal*I fragment

containing the hygromycin resistance cassette (Tsukuda *et al.*, 1988) was then ligated to the only *Xho*I site of the ODC sequence (see Fig. 5a). The plasmid was digested with *Bgl*II, and the linear DNA fragment obtained was utilized to transform the FB2 wild-type strain of *U. maydis*. Transformants were selected and purified in minimal solid medium containing hygromycin (200 µg ml⁻¹) and 1 mM putrescine. From eight original hygromycin-resistant transformants, three were selected for further analysis. Transformants were analysed by Southern hybridization, utilizing the whole *Bgl*II fragment or a *Bam*HI-*Cla*I fragment of the hygromycin resistance gene (1046 bp) as probes. In two of the mutants, the original 2.08 kbp fragment of the wild-type was substituted by a 4.0 kbp fragment containing the hygromycin resistance cassette, whereas in the third one an ectopic incorporation of the substitution cassette occurred (Fig. 5b). This last result agrees with the observation that recombination in *U. maydis* is not 100% homologous (Fotheringham & Holloman, 1989, 1990). One of the null mutants (LG4) was used for further experiments.

Determination of the phenotype of the *odc* null mutant

The null mutant behaved as a polyamine auxotroph. When cells grown in a complex medium containing 1 mM putrescine were inoculated into polyamine-free synthetic medium, the mutant grew at a rate not different from the wild-type for about 12-16 h, before a reduction in growth rate occurred. When these cells were recovered, washed with water and reinoculated into fresh synthetic medium, no growth took place unless putrescine was added (Table 2). ODC activity from the wild-type and mutant strains was measured in cells grown for 6 h in polyamine-free minimal medium. ODC specific activity [as pmol CO₂ liberated min⁻¹ (mg cell

Fig. 3. Southern blot analysis of *U. maydis* genomic DNA. DNA digested with *Bam*HI (lane 1), *Eco*RI (lane 2), *Hind*III (lane 3), *Pst*I (lane 4) or *Xho*I (lane 5) was probed with a *Pst*I-*Xho*I radiolabelled fragment of 900 bp from *Umodc*. As controls, *Umodc* gene fragments were run in lanes 6–9. Lanes: 6, *Pst*I-*Xho*I fragment of 900 bp; 7, *Pst*I-*Pst*I fragment of 1.6 kbp; 8, *Xho*I-*Xho*I fragment of 2.6 kbp; 9, *Pst*I-*Xho*I fragment of 600 bp. H, *Hind*III; P, *Pst*I; S, *Sal*I; X, *Xho*I.

Fig. 4. Northern blot analysis of the *Umodc* transcript. Total RNA from yeast cells incubated for 4.5 h was isolated and blotted (lanes 1 and 2). Cells were transferred to fresh medium of pH 7.0 or 3.0 to obtain yeast or mycelial growth, respectively. After 3 h incubation, total RNA from yeast (lane 3) or mycelial cells (lane 4) was isolated and blotted. (a) RNA was hybridized with the *Pst*I-*Xho*I radiolabelled fragment of 900 bp from *Umodc*; (b) as a control, the membrane was hybridized with the carboxin resistance gene of *U. maydis*.

protein)⁻¹] in the wild-type was approximately 30. However, the specific activity of the mutant was approximately 0.5, a value probably representing background.

Fig. 5. Strategy for *Umodc* replacement. (a) Scheme of the strategy followed. An internal *Stu*I-*Sma*I fragment (584 bp) was deleted. After ligation, the hygromycin resistance cassette was inserted into the single *Xho*I site. The thin line is the pT7-7 vector. Bg, *Bgl*II; Sa, *Sal*I; Sm, *Sma*I; St, *Stu*I; Xh, *Xho*I; HFT, hygromycin resistance cassette. (b) Southern blot analysis of wild-type and transformants probed with the whole *Bg*/II fragment of the construction. Lanes: 1, wild-type; 2, LG3; 3, LG4; 4, LG7. The arrowhead indicates the ectopic integration.

Table 2. Requirement of putrescine for growth of the *U. maydis* null mutant

Cells were grown in minimal medium (pH 7.0). After 20 h, growth was measured, cells were washed and reinoculated into fresh minimal medium. After 20 h, more growth was measured again.

Incubation cycle	Putrescine (5 mM)	Growth ($\mu\text{g protein ml}^{-1}$)*	
		FB2 (parental wild-type)	LG4 (<i>odc</i> Δ)
1st	No	510	432
	Yes	600	582
2nd	No	395	19
	Yes	486	282

* Inoculum: 1st cycle, 17–22 $\mu\text{g protein ml}^{-1}$; 2nd cycle, 14–16 $\mu\text{g protein ml}^{-1}$.

In contrast to its growth requirements, higher levels of putrescine than those accumulated in polyamine-containing medium were required by the mutant to grow in mycelial form. Cells incubated in synthetic medium of pH 3.0 (conditions at which the wild-type grows in the form of mycelium; Ruiz-Herrera *et al.*, 1995a) containing less than 0.5 mM putrescine grew with yeast-like morphology, even after 48 h incubation. However, cells incubated in medium containing 1 mM or more putrescine or 5 mM spermidine gave rise to a homogeneous mycelial population. In solid medium of pH 3, the null mutant failed to form mycelial ('fuzz') colonies as the wild-type does (Ruiz-Herrera *et al.*, 1995a); instead, smooth colonies were formed. Representative photographs illustrating this behaviour are shown in Fig. 6.

DISCUSSION

Inhibition of teliospore germination and yeast-mycelium transition in *U. maydis* by the ODC competitive inhibitor DAB agrees with results that we have obtained with other fungal systems (for a review see Ruiz-Herrera, 1994). According to these data, both differentiation processes require levels of diffusible polyamines higher than those necessary to sustain the growth of the organism. An interesting point is why differentiation is more sensitive than vegetative growth to the DAB effect. A plausible explanation lies in the observation that fungi may contain more than one pool of ODC, one of which is resistant to the drug *in vivo* but not *in vitro* (Martinez-Pacheco & Ruiz-Herrera, 1993; Guevara-Olvera *et al.*, 1993).

Cloning of the *Umodc* gene and construction of an *odc* null mutant of the fungus by reverse genetics allowed a more thorough examination of the role of polyamines in the dimorphic transition of the fungus. The deduced amino acid sequence of the *Umodc* gene product showed high homology with ODCs from other organisms. Highest homology was observed with the ODCs from *S. cerevisiae* and *N. crassa*, although this was lower than the homology existing between the enzymes from these two organisms. This would be expected since the latter are ascomycetes and *U. maydis* is a basidiomycete. Previously we have reported that ODC PCR fragments of fungi form a compact group when compared with the corresponding regions of ODCs from other sources, and that their homology agrees with their phylogenetic relationships (Torres-Guzman *et al.*, 1996). Most of the systems analysed (Fonzi & Sypherd, 1985, 1987; Heby & Persson, 1990; Williams *et al.*, 1992) contain only one ODC gene. Our results confirmed that in agreement with these data, *U. maydis* also contains a single gene encoding ODC. The observation that the *Umodc*Δ mutant is a putrescine auxotroph indicates that in *U. maydis*, as in other fungi and animals, the only functional pathway for polyamine biosynthesis involves ODC. Other organisms such as bacteria and plants possess an additional biosynthetic pathway which involves arginine decarboxylase (Tabor & Tabor, 1985; Slocum *et al.*, 1984).

Two putative PEST regions with a low score were identified in *U. maydis* ODC. These are sequences characteristic of proteins with a high rate of turnover (Rogers *et al.*, 1986; Rechsteiner & Rogers, 1996). In *S. cerevisiae* ODC, a single PEST region exists (Fonzi & Sypherd, 1987), whereas the *N. crassa* enzyme contains two of them (Williams *et al.*, 1992). Whether regions with the low values obtained are significant or not remains doubtful. If they were, regulation of the levels of ODC, at least in part, would occur through the control of its rate of degradation, as happens in other systems (Hayashi *et al.*, 1996).

U. maydis ODC transcript appears to display a long leader region. The only transcript identified was approximately 2.5 kbp, contrasting with the 1.38 kbp long coding region. This agrees with results generally obtained with ODCs (Heby & Persson, 1990; Williams *et al.*, 1992). These observations suggest that the putative TATA box located far upstream is functional (the one from human ODC, for example, is located at -763 bp; van Steeg *et al.*, 1989), but this remains undecided. Genes without TATA boxes have been described (e.g. Hudspeth & Grula, 1989; Moffat *et al.*, 1994). The same applies for the putative CAAT boxes identified in wrong order to the TATA box. The presence of a large leader region suggests a complex regulation of the transcript (Kameji *et al.*, 1993). Experiments from the group of Rowland Davis have revealed that the long leader of the *N. crassa* *SPE1* transcript is involved in the regulation of ODC levels by polyamines (Williams *et al.*, 1992; Pitkin *et al.*, 1994); whereas an upstream region (UAR) is responsible for transcription activation (Pitkin *et al.*, 1994). Interestingly, their data suggested that regulation of ODC activity was not essential for the growth of the fungus.

Once the *Umodc* gene was cloned, we proceeded to its substitution. To our knowledge, the *odc* *U. maydis* null mutants described here, and null mutants of the *S. cerevisiae* *SPE1* gene (Fonzi & Sypherd, 1987; Balasundaram *et al.*, 1994), are the only ones where ODC gene knockouts have been accomplished in fungi. As indicated above, the *odc* null mutant of *U. maydis* behaved as a putrescine auxotroph, but it was able to accumulate a polyamine pool large enough to permit cell growth for about 12–16 h. This behaviour agrees with that of *S. cerevisiae* *odc* null mutants (*spe1*Δ) which are able to grow for approximately 20 h in a polyamine-free medium (Balasundaram *et al.*, 1994). The observation that polyamines restore normal growth of the null mutant indicates that the only role of ODC in *U. maydis* is the synthesis of putrescine. This result agrees with data obtained with *spe1*Δ mutants of *S. cerevisiae* (Balasundaram *et al.*, 1994) and nonsense *odc* mutants of *N. crassa* (Davis *et al.*, 1987).

Since the null mutant of *U. maydis* was able to grow temporarily in the absence of exogenously added putrescine, we were able to determine its capacity to perform the dimorphic transition under those conditions. The observation that even in the presence of

Fig. 6. Cell and colonial morphology of wild-type and null *odc* mutant of *U. maydis*. Wild-type grown in liquid synthetic medium without polyamines of pH 3.0: (a) zero time; (b) 6 h; (c) 12 h. LG4 mutant grown as above: (d) zero time; (e) 6 h; (f) 12 h. Mutant grown with 0.4 mM (g), 1 mM (h) or 5 mM (i) putrescine. Colonial growth on charcoal-containing minimal medium of pH 3.0: (j) wild-type; (k) mutant. Bar, 10 µm.

putrescine concentrations lower than 0.5 mM, the fungus multiplied at an almost normal rate but was unable to carry out the yeast-to-mycelium transition is strong evidence for the role of polyamines in this process. Support for this conclusion is offered by the observation that larger amounts of putrescine or spermidine were necessary to restore the normal dimorphic phenotype of the fungus. These results eliminate the possibility that the phenotype was due to the accumulation of ornithine or products derived from it, or *S*-adenosylmethionine. It is generally accepted that the only role of putrescine in cell growth is to serve as a spermidine precursor (we have observed that *U. maydis* contains no spermine). Indeed, *S. cerevisiae* null mutants in the *SPE2* gene encoding *S*-adenosylmethionine decarboxylase are spermidine or spermine auxotrophs (Balasundaram *et al.*, 1991). Nevertheless, whether putrescine plays a specific

role or not in differentiation remains an open question, since it can be derived from spermidine by the action of polyamine oxidase (McCann *et al.*, 1987).

An important question to be answered in the future is the mode of action of polyamines in cell differentiation. It is known that due to their polycationic nature, polyamines bind to cellular anions. In *E. coli*, 90% of the total spermidine is bound to RNA, 5% to DNA and 0.8% to membrane lipids (Miyamoto *et al.*, 1993). Accordingly, a nonspecific role of polyamines has been suggested (Davis *et al.*, 1992). Nevertheless, we have described at least one specific role for them – the inhibition of cytosine-DNA methylases (Ruiz-Herrera *et al.*, 1995b). Accordingly, a tentative hypothesis may be that they contribute to the regulation of the transcription of genes involved in differentiative processes.

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