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

Lorenzo Guevara-Olvera, Beatriz Xoconostle-Cázares and José Ruiz-Herrera

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.

The GenBank accession number for the sequence reported in this paper is X88796.
We have described in several fungal models that the levels of ODC and polyamine pools increase previous to all differentiative steps: spor 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 et al., 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 (Fortheringham & 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 (a1b1) and FB2 (a2b2) 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 µg ml⁻¹) and/or putrescine or spermidine were added to minimal medium (Holliday, 1974). Escherichia coli strains DH5a mcr and KW258 were used for transformation, and for plasmid and phage propagation, respectively. They were grown in LB broth containing 10 mM MgSO4 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 [³²P]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 (a1b1) constructed in λEMBL3 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-
amines are required for both differentiative processes, and prompted us to analyse the phenomenon with an odc null mutant.

**Cloning of the Umodc gene**

The ODC gene fragment obtained by PCR utilizing U. maydis DNA (Torres-Guzman et al., 1996) was used as a probe to screen a genomic library of U. maydis (Schulz et al., 1990). Eight positive clones were obtained out of 12000 recombinant phages. SalI fragments of about 4-5 kbp were recovered and cloned into pBKS+. After restriction analysis and hybridization with the PCR fragment, plasmid F2 was selected for further studies. Nucleotide and amino acid sequences of a XhoI-PstI fragment comprising 3.18 kbp are shown in Fig. 1. A continuous ORF encoding a polypeptide of 460 amino acids with a molecular mass of 50600 Da was observed. No consensus splicing sequences were detected. A putative TATA box at -1107 bp, and two possible CAAT boxes were located at -479 and -461 bp. A consensus polyadenylation signal was also identified. The amino acid sequence of U. maydis ODC showed high similarities with other ODCs. The ones with the highest degree of similarity were those from Saccharomyces cerevisiae (Fonzi & Sypherd, 1987) and

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**Table 1. Inhibition of teliospore germination and yeast-to-mycelium transition by DAB**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DAB (mM)</th>
<th>Natural infection</th>
<th>Artificial infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Teliospore germination</td>
<td>0</td>
<td>67, 90</td>
<td>930, 950</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0, 2</td>
<td>0, 0</td>
</tr>
<tr>
<td>Dimorphic transition</td>
<td>0</td>
<td>NA</td>
<td>1-6</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>NA</td>
<td>1-7</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>NA</td>
<td>1-2</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>NA</td>
<td>1-1</td>
</tr>
</tbody>
</table>

NA, Not applicable.

* Mean number of teliospores germinated in three plates from two different experiments. Plates were inoculated with 500 or 1000 teliospores obtained from naturally or artificially infected ears, respectively.

† Dry weight (mg ml⁻¹). Means of three experiments.

‡ Means of three experiments.
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 residues 306-315 in U. maydis (Williams et al., 1992). This PEST region is absent in S. cerevisiae.

U. maydis DNA digested with BamHI, EcoRI, HindIII, PstI or XhoI was probed with a radiolabelled 900 bp PstI–XhoI 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, Umocdc. Northern analysis of U. maydis RNA revealed a single transcript of Umocdc 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 BglII fragment containing the coding region of the gene was subcloned into plasmid pT7-7 in which the Smal site had been previously deleted. A central Stul–SmaI fragment of 584 bp in length was removed. A Sall fragment containing the hygromycin resistance cassette (Tsukuda et al., 1988) was then ligated to the only XhoI site of the ODC sequence (see Fig. 5a). The plasmid was digested with BglII, 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 BglII fragment or a BamHI–ClaI 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.

**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.
The *Ustilago maydis* ornithine decarboxylase gene


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 *PstI-XhoI* radiolabelled fragment of 900 bp from *Umodc*; (b) as a control, the membrane was hybridized with the carboxin resistance gene of *U. maydis*.

Fig. 5. Strategy for *Umodc* replacement. (a) Scheme of the strategy followed. An internal *Stul-Smal* fragment (584 bp) was deleted. After ligation, the hygromycin resistance cassette was inserted into the single *XhoI* site. The thin line is the pT7-7 vector. Bg, *BgIII*; Sa, *Sall*; Sm, *Smal*; St, *Stul*; Xh, *XhoI*; HFT, hygromycin resistance cassette. (b) Southern blot analysis of wild-type and transformants probed with the whole *BgIII* 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

<table>
<thead>
<tr>
<th>Incubation cycle</th>
<th>Putrescine (5 mM)</th>
<th>Growth (μg protein ml⁻¹)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FB2 (parental wild-type)</td>
<td>LG4 (odcs)</td>
</tr>
<tr>
<td>1st</td>
<td>No</td>
<td>510</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>600</td>
</tr>
<tr>
<td>2nd</td>
<td>No</td>
<td>395</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>486</td>
</tr>
</tbody>
</table>

*Inoculum: 1st cycle, 17-22 μg protein ml⁻¹; 2nd cycle, 14-16 μg protein ml⁻¹.*

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.
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 *UmodcΔ* 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 *odu* *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 (*spe1Δ*) 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 *spe1Δ* 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
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.

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.
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