Life without putrescine: disruption of the gene-encoding polypeptide oxidase in Ustilago maydis odc mutants

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Abstract

In previous communications the essential role of spermidine in Ustilago maydis was demonstrated by means of the disruption of the genes encoding ornithine decarboxylase (ODC) and spermidine synthase (SPE). However, the assignation of specific roles to each polypeptide in different cellular functions was not possible because the spermidine added to satisfy the auxotrophic requirement of odc/spe double mutants was partly back converted into putrescine. In this study, we have approached this problem through the disruption of the gene-encoding polypeptide oxidase (PAO), required for the conversion of spermidine into putrescine, and the construction of odc/pao double mutants that were unable to synthesize putrescine by either ornithine decarboxylation or retroconversion from spermidine. Phenotypic analysis of the mutants provided evidence that putrescine is only an intermediary in spermidine biosynthesis, and has no direct role in cell growth, dimorphic transition, or any other vital function of U. maydis. Nevertheless, our results show that putrescine may play a role in the protection of U. maydis against salt and osmotic stress, and possibly virulence. Evidence was also obtained that the retroconversion of spermidine into putrescine is not essential for U. maydis growth but may be important for its survival under natural conditions.

Introduction

In the last few decades, the study of polypeptide metabolism has attracted the attention of many groups interested in the understanding of growth and differentiation phenomena in different organisms (Tabor & Tabor, 1985; Cohen, 1998; Morgan, 1999; Igarashi & Kashiwagi, 2000) because it is well known that these molecules play a role in cell and tissue proliferation, differentiation, and growth (Ruiz-Herrera, 1994; Auvinen et al., 1997; Farriol et al., 2001). Even though polypeptides are structurally simple, there is evidence of their important role in a variety of biological processes such as cell cycle regulation, gene expression, and signal transduction (Patel & Wang, 1997; Ray et al., 1999; Bachrach et al., 2001; Chattopadhyay et al., 2009; Koomoa et al., 2009).

The pathways of polypeptide metabolism in plants and animals have been extensively studied (e.g. Gerner & Meyers, 2004; Casero & Marton, 2007; Efrose et al., 2008; Kusano et al., 2008), but fungi may also constitute good models to study the physiological roles of these important micromolecules (Balasundaram et al., 1991; Walters, 1995). Our research group has obtained evidence of the direct relationship between polypeptide synthesis and differentiation in a large number of fungal species by means of the use of inhibitors of their synthesis (e.g. Calvo-Méndez et al., 1987; Martínez-Pacheco et al., 1989; reviewed by Ruiz-Herrera, 1994), and the isolation of mutants deficient in the gene-encoding ornithine decarboxylase (ODC) (Guevara-Olvera et al., 1997; Herrero et al., 1999; Jiménez-Bremont et al., 2001). An attractive model for these studies is the phytopathogenic basidiomycete Ustilago maydis, which contains only two (putrescine and spermidine) of the three widely distributed polypeptides (putrescine, spermidine, and spermine), simplifying their study (Valdés-Santiago et al., 2009).

One unsolved problem of polypeptide functions concerns the specific roles of each polypeptide in cell growth and differentiation. Previously, using single mutants in the
gene-encoding spermidine synthase (spe), and double odc/spe mutants, we obtained evidence that in *U. maydis*, spermidine can fulfill all the functions ascribed to polyamines (Valdés-Santiago et al., 2009). Nevertheless, the question of a specific role for putrescine remained unanswered, as those mutants still possessed the capacity to synthesize putrescine by the retroconversion of spermidine into putrescine. It is known that putrescine is not only synthesized by decarboxylation of ornithine by Odc (Sebela et al., 2001; Seiler, 2004), but also by the FAD-dependent polyamine oxidase (Pao), which catalyzes the oxidation of N1-acetylputrescine to produce N-acetyl-3-aminopropionałdehyde and putrescine (Pegg, 1988). In the present article, we describe the isolation and disruption of the gene-encoding Pao, and the phenotypic characteristics of single *pao* and double *pao/odc* mutants, as an approach to understand the specific roles of putrescine in *U. maydis* growth and development.

**Materials and methods**

**Strains, culture media, and growing conditions**

The strains of *U. maydis* and *Escherichia coli* used in this study are described in Table 1. *Ustilago maydis* strains were maintained in 50% glycerol at −70 °C. When necessary, they were recovered in complete medium (CM; Holliday, 1974) containing different additions (see Results), and incubated as above. Strain strains were tested for growth and development.

<table>
<thead>
<tr>
<th>Strains used in this study</th>
<th>References or sources</th>
</tr>
</thead>
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<tr>
<td><em>Ustilago maydis</em></td>
<td></td>
</tr>
<tr>
<td>FB2</td>
<td>a2b2</td>
</tr>
<tr>
<td>F81</td>
<td>a1b1</td>
</tr>
<tr>
<td>LV20</td>
<td>a2b2 Δpao::CbxΔ</td>
</tr>
<tr>
<td>LV39</td>
<td>a1b1 Δpao::CbxΔ</td>
</tr>
<tr>
<td>LV24(5)</td>
<td>a2b2 Δodc::pao/Hyg′/CbxΔ</td>
</tr>
<tr>
<td>LV24(7)</td>
<td>a2b2 Δodc::pao/Hyg′/CbxΔ</td>
</tr>
<tr>
<td>LV201</td>
<td>a2b2 Δpao::CbxΔ</td>
</tr>
<tr>
<td>LG4</td>
<td>a2b2 Δodc::HygΔ</td>
</tr>
<tr>
<td>LV54</td>
<td>a2b1 Δspe-sdh::CbxΔ</td>
</tr>
<tr>
<td>LV38C</td>
<td>a1b1 Δodc::pao/Hyg′/CbxΔ</td>
</tr>
</tbody>
</table>

**Escherichia coli**

| Top 10 F′                  | Invitrogen           |

When necessary (see Results), media were supplemented with one or more of the following compounds: hygromycin (200 μg mL⁻¹), carboxin (20 μM), and spermidine or putrescine in different concentrations. Yeast or mycelial growth in liquid or solid media was obtained as described (Ruiz-Herrera et al., 1995). Comparative growth of *U. maydis* strains was measured on plates of solid media inoculated with 10 μL drops of decimal dilutions from cell suspensions containing 10⁸ cells mL⁻¹. After 24 and 48 h of incubation at 28 °C, the relative growth was observed, and the plates were photographed.

*Escherichia coli* was grown at 37 °C in Luria–Bertani medium (1% tryptone, 0.5% yeast extract, and 0.5% sodium chloride) with ampicillin (100 μg mL⁻¹) or kanamycin (50 μg mL⁻¹ added for plasmid selection.

### Techniques for nucleic acid manipulation

Genomic DNA from *U. maydis* was isolated by a glass bead lysis method (Hoffman & Wriston, 1987). Total RNA was isolated according to Jones *et al.* (1985). Southern and Northern hybridizations of 10 and 30 μg of nucleic acid samples, respectively, were performed by standard techniques (Sambrook & Russel, 2001). DNA probes were labeled using the random primer labeling system and [³²P]dCTP (Amersham Biosciences, Little Chalfont, UK).

Plasmid DNA isolation from *E. coli* was performed by standard procedures (Sambrook & Russel, 2001). Plasmid isolation from *U. maydis* was performed according to Sobanski & Dickinson (1995).

DNA enzymatic reactions such as digestion, ligation, and vector dephosphorylation were performed as recommended by the manufacturers of the reagents used (Invitrogen, Carlsbad, CA, and New England Biolabs, Ipswich, MA). DNA for sequencing, ligation, and random primer labeling reactions was purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA).

### PCR conditions

Routine PCR was conducted using Taq DNA polymerase (Invitrogen) and the following general program: an initial cycle of 94 °C for 5 min, amplification (30–35 cycles) at 94 °C for 30 s followed by annealing at primer-specific temperature for 60 s, and polymerization at 72 °C (1 min kb⁻¹ of DNA target length). When required, the expanded high fidelity PCR system (Boehringer, Mannheim, Germany) was used according to the manufacturer’s instructions. An extension period of 7 min at 72 °C was programmed for those PCR products that were cloned into pCR2.1 Topo (Invitrogen). The primers used are described in Table 2.
The sequence of gene \( \text{PAO} \) was obtained from the \( \text{U. maydis} \) genome sequence at http://mips.gsf.de/genre/proj/ustilago/(UM05850). The complete gene plus 1100 and 944 bp, corresponding to the 5'- and 3'-noncoding regions, respectively, was amplified with primers UEN16 and UEN15 (see Table 2), and the PCR product was cloned into pGEMT-easy to produce plasmid, pAC2108. This plasmid was digested with HindIII, which removed most of the ORF, substituted by the carboxin-resistance cassette obtained by HindIII digestion from pCBX-AC2 (Valdès-Santiago et al., 2009) to generate pLV24 (\( \text{pao}::\text{Ch}^R \)).

**Construction of a plasmid to transform \( \text{pao} \) mutants with the wild-type \( \text{PAO} \) allele**

\( \text{PAO} \) gene was recovered from pAC2108 by digestion with BamHI/NdeI and ligated into the same sites of the selectable autonomous replicating plasmid pHyg101 (Mayorga & Gold, 1998) to generate the pLV28 plasmid. This was used to complement \( \text{pao} \) mutants by electroportation.

**Mating and virulence assays**

Mating was analyzed by the Fuz reaction (Banuett, 1992, 1995). Virulence assays followed essentially the method described by Martinez-Espinoza et al. (1997). Disease symptoms (chlorosis, anthocyanin formation, and tumor formation) were recorded routinely after 5–15 days unless otherwise specified.

**Isolation of segregants from inoculated maize plants**

The procedure described by Chavez-Ontiveros et al. (2000) was followed. Mature tumors were excised from plants infected with sexually compatible strains, and surface sterilized with a commercial solution of 7% sodium hypochlorite for 5 min, followed by 70% ethanol for the same time, and washed twice with sterile distilled water. Tumors were crushed to liberate teliospores, which were treated with 0.5% CuSO\(_4\) for 1 h, filtered through cheesecloth, and recovered by centrifugation. Teliospores were washed with sterile distilled water, diluted to a density of \( 10^6 \) mL, and aliquots of 0.1 mL were spread onto plates of CM plus additions. After 24 h, teliospores had germinated to produce sporidia. These were recovered, appropriately diluted, and inoculated onto fresh plates. Smooth colonies were recovered for mating-type determination with tester strains and for phenotypic analysis.

**Stress assays**

Different stress conditions were tested on solid media. Cells were grown in liquid MM without polyamines for two cycles to deplete the cell polyamine pools (Guevara-Olvera et al., 1997). They were then collected by centrifugation and washed with sterile distilled water by centrifugation. Cell suspensions were adjusted to contain \( 10^6 \) cells mL\(^{-1}\) (counted with a Neubauer chamber), 10-fold serial dilutions were prepared, and 10 \( \mu \)L of each dilution was spotted on
plates containing different additions. In a different type of hydrogen peroxide (H₂O₂) sensitivity assay, U. maydis strains were plated on CM. Filter disks (eight per strain) of Whatman paper (5 mm in diameter) were soaked with 10 μL H₂O₂ (3% v/v) and placed over the plates. Plates were incubated at 28 °C for 48 h, photographed, and the diameter of the inhibition zones was measured.

**Determination of growth rate**

To monitor rate of cell growth, we incubated 10^6 cells mL⁻¹ in triplicate samples of 15 mL of MM at 28 °C under shaking conditions, and OD₆₀₀nm was measured at 2-h intervals for 24 h.

**Polyamine analyses**

Polyamines were extracted from the cells with 6% perchloric acid for 3 h at room temperature. Derivatization of polyamines and analysis were done as described previously (Valdés-Santiago et al., 2009), except that a gradient of methanol in distilled water of 40–65% was used to elute the polyamines from the HPLC column.

**Microscopic observations**

Microscopic observations were performed with a Leica DMRE microscope. Photographs were obtained with a Spot digital camera (Diagnostic Instruments, Livingston, UK).

**Results**

**Identification and isolation of U. maydis PAO gene**

A comparative analysis of the proteins involved in the catabolism of polyamines shows a high conservation between eukaryotes. We therefore used the human polyamine oxidase protein sequence to identify a polyamine oxidase in U. maydis, and its encoding gene. BLAST homology searching of the National Center for Biotechnology Information genome database revealed a single hypothetical homolog of the genome: UM05850. This was located in chromosome 20, contig 214: 152339–154072, denominated, LV24(5) and LV24(7) for further studies. These were further characterized by a Southern blot assay after digestion with SacI, using an 800-bp fragment belonging to the PAO ORF as a probe. The absence of bands in both mutants indicated the absence of the gene (Fig. 1). Double mutants of different mating genotypes were isolated by genetic recombination in planta with strain FBI (aib1), as described in Materials and methods, and an aib1 double mutant (LV38C) was selected for further studies. As expected, odc/pao mutants were resistant to carbboxin and hygromycin, and auxotrophic to putrescine or spermidine (Fig. 2). This last result is evidence that putrescine serves as an intermediate in spermidine biosynthesis, and is not required for vegetative growth of U. maydis.

**Determination of polyamines in odc/pao mutants**

To confirm that the double mutants LV24(5) and LV24(7) were unable to synthesize putrescine, we proceeded to measure their polyamine content by HPLC. As control we analyzed the parental LG4 strain. No putrescine was detected in odc/pao

To isolate the gene, a DNA fragment (3775 bp in length) containing the PAO gene ORF (1731 bp) plus 1100 bp upstream and 944 bp downstream of the ORF was amplified by PCR using specific primers UEN16 and UEN15 (see Table 1), and cloned into pGEMT, to obtain pAC2108 (see Materials and methods).

**Disruption of the gene-encoding U. maydis polyamine oxidase (PAO)**

To determine the specific role of putrescine in U. maydis, we proceeded to disrupt the gene-encoding polyamine oxidase using as background an odc mutant (LG4) that is unable to produce putrescine from ornithine (Guevara-Olvera et al., 1997). Primers UEN16 and UEN15 were used to amplify the cassette containing the carboxin-resistance gene flanked by the 5’- and 3’-untranslated regions corresponding to the promoter and terminator of PAO gene from pLV24 plasmid (see Materials and methods) and used to transform protoplasts of the LG4 (odc::HgyR) mutant. Transformants were recovered on plates of a medium containing hygromycin, carbboxin, putrescine, and spermidine, and were probed by PCR using primers PaoFow and UEN14, the first one located 840 bp upstream from the disruption cassette, and the second one located within the cassette. A band of 4619 bp indicated the presence of the mutant allele at the right locus, while a 3806 bp band corresponded to the wild-type gene. The putative transformants were verified using primers PaoFow and UEN9 (whose sequence is within the carboxin-resistance gene). Amplification of a 3761-bp fragment confirmed the nature of the double mutants. From the transformants obtained, we selected two odc/pao mutants, denominated, LV24(5) and LV24(7) for further studies. These were further characterized by a Southern blot assay after digestion with SacI, using an 800-bp fragment belonging to the PAO ORF as a probe. The absence of bands in both mutants indicated the absence of the gene (Fig. 1). Double mutants of different mating genotypes were isolated by genetic recombination in planta with strain FBI (aib1), as described in Materials and methods, and an aib1 double mutant (LV38C) was selected for further studies. As expected, odc/pao mutants were resistant to carbboxin and hygromycin, and auxotrophic to putrescine or spermidine (Fig. 2). This last result is evidence that putrescine serves as an intermediate in spermidine biosynthesis, and is not required for vegetative growth of U. maydis.
double mutants, contrary to what occurred in the parental strain (Fig. 3). It is notable that this is the first report of eukaryotic mutants that contain spermidine only, and constitutes evidence that putrescine is not essential for cell growth, at least in the case of U. maydis, and probably other fungi.  

**Isolation of pao single mutants**

By use of genetic recombination in planta between the double mutant LV24(7) (odc/pao a2b2) and the wild-type strain FB1 (a1b1) (see Materials and methods), we were able to isolate the corresponding recombinants. Teliospores were germinated on plates of CM containing carboxin and spermidine, and the meiotic products were transferred to fresh plates to test their growth on media containing spermidine and either carboxin or hygromycin. Recombinants were further selected, and their mating type and auxotrophy to spermidine were determined. Several mutants were thus confirmed by PCR using primers PaoFow and UEN14. The presence of the wild-type ODC gene was further confirmed by PCR using primers 5'-odc and 3'-odc (we expected an 857-bp fragment for the wild-type gene, and a 2700-bp fragment for the mutant gene). Using this procedure we were able to isolate single pao mutants of different mating genotypes (Table 1 and Fig. 4).

**Phenotypic analysis of double and single pao mutants**

**Growth rate**

pao mutants showed only a slightly reduced growth rate at 28 °C in both MM and CM containing 0.1 mM spermidine as compared with the wild-type strain, whereas a higher
reduction in growth was observed in double odc/pao mutants than in pao single mutants in the same media (results not shown). It is important to remember that the byproduct of Pao catalysis (N-acetyl-3-aminopropanaldehyde) has been described as a precursor of β-alanine (a component of pantothenic acid) in Saccharomyces cerevisiae.
(White et al., 2001), but in most of the studied eukaryotes, including fungi, β-alanine is synthesized by β-alanine dehydrogenase as a degradation product of uracil (e.g. Gojkovic et al., 2001). Our search in the U. maydis genome (http://mips.helmholtz-muenchen.de/genre/proj/ustilago) also revealed the presence of a homolog gene-encoding β-alanine dehydrogenase in this fungus, suggesting that this, and not the pathway from spermidine, is the important source of β-alanine. This hypothesis is supported by the observation that pao mutants grow in a MM without any auxotrophic requirement.

**Dimorphic transition**

Wild-type haploid cells of *U. maydis* grow at pH 3 in the form of mycelium, and at pH 7 they display a yeast-like phenotype (Ruiz-Herrera et al., 1995). However, odc mutants require a high concentration of putrescine to undergo this morphological transition (Guevara-Olvera et al., 1997). When we induced the yeast-to-hypha transition by changing the pH in the LV20 (pao) mutant, we did not observe any difference with respect to the wild-type strain. In the case of LV24(7) (odc/pao) mutant, its behavior was the same as odc single mutants, and its mycelial growth occurred only in the presence of spermidine or high putrescine concentrations (Fig. 5).

**Response to stress**

When mutants LV24(7) (odc/pao) and LV20 (pao) were subjected to ionic or osmotic stress (1 M NaCl, 10 mM LiCl, or 1 M sorbitol), only the double mutant displayed a sensitive phenotype to these conditions, whereas the simple LV20 pao mutants showed no differences to the wild-type strain (Fig. 6). The response to oxidative stress was different. In the agar diffusion test, the halos of inhibition shown by LV20 and LG4 (pao and odc mutants, respectively) were wider in comparison with the wild-type strain (Fig. 7a and b), whereas, interestingly, the halo from the LV24(7) (odc/pao) mutant was narrower than the one from the parental strain. The same relative results were obtained when inhibition was measured on solid medium containing H2O2 and inoculated with decimal dilutions of the cell suspensions (Fig. 7c).

**Determination of mating**

Fuz reaction of sexually compatible odc/pao double mutants occurred at all the concentrations of spermidine used, but required high levels of putrescine (5 mM). In contrast, mating of compatible pao mutants was not affected by the absence of spermidine (not shown).

**Virulence of mutants**

Maize seedlings inoculated with mixtures of sexually compatible odc/pao double mutants did not develop tumors, in contrast to plants inoculated with wild-type cells. On the other hand, inoculation of maize seedlings with compatible mixtures of pao mutants induced tumors, but these were noticeably...
Fig. 6. Effect of different stress conditions on the growth of *Ustilago maydis*. Cells were grown for 24 h in MM containing 0.1 mM spermidine followed by two growth cycles in the absence of spermidine to deplete polyamine pools. Cell suspensions were adjusted to a density of $10^5$ cells mL$^{-1}$, decimally diluted, and inoculated on solid MM containing 0.1 mM spermidine and (a) nothing (control), (b) 1 M NaCl, (c) 1 M sorbitol, or (d) 10 mM LiCl. 1, Wild-type strain FB2; 2, single odc mutant LG4; 3, double odc/pao mutant LV24(7); 4, single LV20 pao mutant. Plates were photographed after 48 h of incubation at 28 °C.

Fig. 7. Sensitivity of *Ustilago maydis* wild-type strain and mutants to oxidative stress. (a) Sensitivity of strains to H$_2$O$_2$ assessed in an agar diffusion test in which filters soaked with H$_2$O$_2$ (0.8 mM) were placed over plates of solid MM containing 0.1 mM spermidine, previously inoculated with the indicated strains. At the end of 48 h, the plates were photographed. (b) Diameter of the halos quantified for the strains given in (a). Error bars indicate SDs derived from three independent experiments consisting of eight replicas each. (c) Serial 10-fold dilutions of cell suspensions were spotted on solid MM plates supplemented with 0.1 mM spermidine only (left) or spermidine plus 0.8 mM H$_2$O$_2$ (right). (d) Similar spot test using a complemented strain of LV20 mutant with pLV28 (LV201). Left, control plate; right, plate containing 0.8 mM H$_2$O$_2$. 
smaller, and appeared in a smaller number of plants as compared with the wild-type strains. Moreover, infected plants appeared healthy at a time when those infected with wild-type strains looked dwarfed or dead (Fig. 8 and Table 3).

**Complementation of pao mutants**

Plasmid pLV28 carrying the PAO gene was introduced into the LV20 mutant (a2b2 pao::Cbx<sup>8</sup>), and transformants were recovered, and confirmed by their resistance to hygromycin, and by PCR using UEN13 and UEN15 and LVPPaoFow and UEN15 primers, which amplified a band of 800 bp corresponding to the terminator area, and the whole PAO gene, respectively (results not shown). We observed that transformants recovered resistance to H<sub>2</sub>O<sub>2</sub> to a higher level than the wild-type strain (see Fig. 7d), possibly because of their higher number of PAO copies in the autonomous replication plasmid. In the same way, when a transformant (LV201) was inoculated into maize plants together with a pao mutant LV39 (a1b1 pao::Cbx<sup>8</sup>), they were shown to be as virulent as the wild-type strains (Fig. 8e and Table 3).

**Discussion**

In different systems, evidence has been obtained suggesting that spermidine, contrary to putrescine, is absolutely necessary for eukaryotic cell growth (Balasundaram et al., 1991; Chattopadhyay et al., 2003; Valdés-Santiago et al., 2009). Nevertheless, whether putrescine serves only as a spermidine precursor or also has other functions in cellular metabolism, has been a matter of discussion, mainly because in the studied systems it is difficult to isolate its effects from those of the other polyamines present in the cell. Several authors have suggested that putrescine itself might have important physiological effects (Emanuelsson & Heby, 1978; Pegg, 1988), and in several plants putrescine catabolites have been found to serve as precursors of important alkaloids (Cohen, 1998; Hartmann, 1999). It is also known that, in vitro, putrescine stimulates both the cleavage reaction and the catalytic activity of the mature S-adenosylmethionine (SAM) decarboxylase (Stanley & Pegg, 1991). On the other hand, Yuan et al. (2000) demonstrated that putrescine by itself was not essential for growth and migration of intestinal epithelial crypt cells in vitro.

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**Table 3. Virulence of mutants compared with wild-type strain**

<table>
<thead>
<tr>
<th>Strains</th>
<th>No. of plants</th>
<th>Total no. of plants</th>
<th>Plants with tumors &gt; 5 mm</th>
<th>Plants with tumors &lt; 5 mm</th>
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<tr>
<td>FB1 × FB2</td>
<td>79</td>
<td>60/79 (75.9%)</td>
<td>9/79 (11.4%)</td>
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<tr>
<td>LV24(7) × LV38C</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LV39 × LV20</td>
<td>143</td>
<td>81/143 (56.6%)</td>
<td>28/143 (19.6%)</td>
<td>53/143 (37%)</td>
</tr>
<tr>
<td>LV39 × LV201</td>
<td>40</td>
<td>35/40 (87.5%)</td>
<td>5/40 (12.5%)</td>
<td>30/40 (75%)</td>
</tr>
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</table>
An additional source of difficulties in determining specific roles for putrescine is that it is synthesized by two mechanisms: (1) directly by decarboxylation of ornithine by Odc and (2) by the catabolism of spermine and spermidine. Polyamine catabolism is catalyzed by two enzymes: spermidine/spermine N\textsuperscript{1}-acyetyltransferase and the FAD-dependent polyamine oxidase (Pao). Pao catalyzes the oxidative splitting of acetyl derivatives of spermidine or spermine to form putrescine or spermidine, respectively (Seiler, 1995; Wang et al., 2001). There exists evidence suggesting that this retroconversion is involved in the regulation of intracellular polyamine levels, and also in different cellular processes such as apoptosis. In plants it contributes to the hypersensitivity response leading to induced cell death (Cohen, 1998; Yoda et al., 2003). Accordingly, to obtain a decisive answer to the question as to whether putrescine has some specific roles other than merely to serve as a spermidine precursor, it was necessary to obtain a system completely free of intracellular putrescine, by inhibition of the two pathways leading to its biosynthesis.

In this study, we took advantage of the fact that _U. maydis_ does not contain spermine, and proceeded to isolate mutants devoid of Odc and Pao (odc/pao double mutants), which did not contain putrescine as demonstrated by HPLC analysis, and analyzed their phenotype. These double mutants behaved as putrescine or spermidine auxotrophs, the same as the single _odc_ mutants, showing that putrescine is not necessary for vegetative growth, and that spermidine is the most important polyamine in this respect. This result of course does not invalidate the role of putrescine in the activation of SAM decarboxylase, a function that was not possible to analyze in our _odc/pao_ double mutants, which absolutely require the addition of putrescine or spermidine to grow.

Another phenomenon where putrescine appears not to be directly involved is the dimorphic transition of _U. maydis_, as _odc/pao_ double mutants behave like the single _odc_ mutant (LG4; Guevara-Olvera et al., 1997) in their polyamine requirements for dimorphic transition under acid conditions.

Regarding virulence, we have reported that _U. maydis_ _spe_ mutants showed only attenuated virulence to maize, whereas _odc_ mutants were completely avirulent (Valdés-Santiago et al., 2009), similar to the _odc/pao_ double mutants. This difference suggests that mutants are able to obtain a low supply of spermidine but not putrescine from the plants (maize plants contain higher amounts of spermidine and spermine than putrescine; Rodríguez-Kessler et al., 2008). The most plausible explanation for this difference in virulence is that putrescine is somehow required, at least partially, for _U. maydis_ virulence, possibly due to its role in protection to stress (see Results). Considering the background of the _odc/pao_ double mutants, their avirulence was anticipated (Valdés-Santiago et al., 2009), but the reduced virulence of _pao_ mutants was unexpected. It is possible that the defect in virulence of _pao_ mutants may be related to their increased sensitivity to oxidative stress. It is known that the oxidative killing of pathogenic fungal cells by the host defense mechanisms represents an important line of elimination of pathogenic microorganisms (Montesano et al., 2003; Nürnberg et al., 2004). _Ustilago maydis_ mutants affected in Yap, a transcription factor involved in oxidative response showed increased sensitivity to _H\textsubscript{2}O\textsubscript{2}, and their virulence was attenuated (Molina & Kahmann, 2007). An alternative possibility is that these mutants have an altered polyamine homeostasis, and become more sensitive to the stress imposed by the host.

One peculiar observation was the increased resistance of _odc/pao_ mutants to _H\textsubscript{2}O\textsubscript{2} as compared with single _odc_ or _pao_ mutants, an odd result difficult to explain. A possible explanation would be that, _in vitro_, spermidine was more efficient than putrescine in providing protection to oxidative stress, indicating a competition between the polyamines. As only the double mutant lacks putrescine, in a medium with an excess of spermidine it would be more resistant to _H\textsubscript{2}O\textsubscript{2} than would either single mutant.

It is important to recall that no general agreement exists on the role of specific polyamines in the protection against oxidative stress; for example, putrescine protected _E. coli_ single _samdc_ and double _odc/samdc_ mutants against oxidative stress (Chattopadhyay et al., 2003), but on the other hand, spermine caused a reversion of an apoptotic phenotype induced by oxidative stress in _spe/pao_ double mutants of _S. cerevisiae_ (Chattopadhyay et al., 2006). Similarly, in plants, putrescine, Odc, and arginine decarboxylase levels increased in response to oxidative stress (Szügeti et al., 1996; Ye et al., 1997), but _Trypanosoma cruzi_ lipoperoxidation induced by _H\textsubscript{2}O\textsubscript{2} was avoided by spermine, and putrescine had no effect at all (Hernández et al., 2006).

The observation that _U. maydis_ _odc/pao_ double mutants showed increased sensitivity to osmotic and ionic stress as compared with _odc_ mutants, even when growing in the presence of spermidine, suggests that putrescine is involved in the resistance of the fungus to these types of stress. This hypothesis agrees with the observation that under salt stress, different cereals showed increased levels of putrescine and decreased levels of spermidine (Simon-Sarkadi et al., 2002). Likewise, putrescine content in barley, corn, wheat, and oat was increased up to 60-fold when plants were exposed to osmotic stress (Flores & Galston, 1982).

Systems where polyamine catabolism has been affected show only slight changes in polyamine homeostasis (e.g. Niiranen et al., 2006), suggesting the existence of other regulatory mechanisms for polyamine accumulation, possibly at the levels of biosynthesis or excretion (Casero & Pegg, 1993; Seiler, 2004). These data disagree with the phenotype of _U. maydis_ _pao_ mutants, which showed a modest but significant reduction in growth rate with increased...
sensitivity to oxidative stress, and reduced virulence. These results indicate that if indeed polyamine oxidase is not of vital importance to the cell, it is still important for the ecological success of U. maydis in its natural environment.

From the results obtained in this study, we may conclude that putrescine is not necessary for the growth of U. maydis, its main role being to serve as an intermediary in the biosynthesis of spermidine, which is absolutely required by the fungus. In addition, putrescine is not required for the dimorphic transition, but appears to be involved in the protection against some forms of stress and, interestingly, in virulence. Evidence was also provided that the retroconversion of spermidine into putrescine may not be very important under laboratory conditions, but taking into consideration some aspects of the phenotype of pao mutants, it appears to be important for the success of the fungus in nature.

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