Isolation, characterization and expression analysis of the ornithine decarboxylase gene (ODC1) of the entomopathogenic fungus, *Metarhizium anisopliae*

Jaime Madrigal Pulido a, Israel Padilla Guerrero a, Isaura de J. Magaña Martínez a, Briseida Cacho Valadez a, Juan Carlos Torres Guzman a, Eduardo Salazar Solis b, J. Felix Gutierrez Corona a, Augusto Schrank c, Francisco Jiménez Bremont d, Angélica González Hernandez a, *

* a Departamento de Biología, Universidad de Guanajuato, Guanajuato, Gto., Mexico
  b Departamento de Agronomía, Universidad de Guanajuato, Irapuato, Gto., Mexico
  c Centro de Biotecnología, Universidad Federal do Rio Grande do Sul, Porto Alegre, Brazil
  d Division de Biología Molecular, Instituto Potosino de Ciencia y Tecnología, San Luis Potosí, SLP, Mexico

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**Abstract**

The gene ODC1, which codes for the ornithine decarboxylase enzyme, was isolated from the entomopathogenic fungus, *Metarhizium anisopliae*. The deduced amino acid sequence predicted a protein of 447 amino acids with a molecular weight of 49.3 kDa that contained the canonical motifs of ornithine decarboxylases. The ODC1 cDNA sequence was expressed in *Escherichia coli* cells; radiometric enzyme assays showed that the purified recombinant protein had ornithine decarboxylase activity. The optimum pH of the purified Odc1 protein was 8.0—8.5, and the optimum reaction temperature was 37°C. The apparent $K_m$ for ornithine at a pyridoxal phosphate concentration of 20 mM was 22 $\mu$M. The competitive inhibitor of ODC activity, 1,4-diamino-2-butanone (DAB), at 0.25 mM inhibited 95% of ODC activity. The ODC1 mRNA showed an increase at the beginning of appressorium formation in vitro. During the *M. anisopliae* invasion process into *Plutella xylostella* larvae, the ODC1 mRNA showed a discrete increase within the germinating spore and during appressorium.
Introduction

Polyamines are small polycation molecules essential for growth and differentiation in prokaryotic and eukaryotic cells (Heby 1981; Tabor and Tabor 1985; Ruiz-Herrera 1994; Herrero et al. 1999; Coffino 2000). These molecules have a key role in a variety of processes, such as nucleic acid packaging, DNA replication, transcription, translation, membrane stabilization, the functioning of certain ion channels and resistance to oxidative stress (Pegg and McCann 1982; Tabor and Tabor 1985; Williams 1997; Cohen 1998). Polyamines may have additional roles in the protection of DNA from enzymatic degradation, X-ray irradiation and mechanical shearing (Jimenez-Bremont and Ruiz-Herrera 2008; Shah and Swiatlo 2008). The first step in the biosynthesis of polyamines is the decarboxylation of ornithine by L-ornithine decarboxylase (ODC) to form putrescine, one of the most highly regulated enzymatic reactions in eukaryotic systems (Tabor and Tabor 1984; Pegg 2006). In plants and some microorganisms, a second mechanism for the synthesis of polyamines exists, which involves the action of arginine decarboxylase (ADC) to produce agmatine, which can also be converted into putrescine (Tabor and Tabor 1984).

In fungi, polyamine metabolism plays an essential role during differentiation. There are several-fold increases in activity of ODC and polyamine levels preceding each differentiation event (Martinez-Pacheco et al. 1989; Ruiz-Herrera 1994; Jimenez-Bremont et al. 2001). The importance of ODC activity during fungal dimorphic transition was determined by the addition of 1,4-diamino-2-butane (DAB), a competitive inhibitor of ODC activity, which blocks the yeast to hyphae transition in different fungi such as Mucor rouxii (Martinez-Pacheco et al. 1989), Paracoccidioides brasiliensis (Nino-Vega et al. 2004), Coccidioides immitis (Guevara-Olvera et al. 2000), Yarrowia lipolytica (Guevara-Olvera et al. 1993; Jimenez-Bremont et al. 2001), Ustilago maydis (Guevara-Olvera et al. 1997) and Candida albicans (Herrero et al. 1999).

Metarhizium anisopliae is an entomopathogenic fungus used for the biological control of a wide range of insect pests. The different stages of the pathogenic process include adhesion of conidia to the surface of the insect, germination, penetration, invasive growth and conidiation. These stages constitute the life cycle of the fungus in insecta (Clarkson and Charnley 1996; Pedrini et al. 2007). In each of these stages, the fungus undergoes differentiation events in which ODC activity may be involved. In previous work, we isolated a conserved fragment of a putative ODC1 gene from M. anisopliae; it showed high homology to genes encoding ornithine decarboxylase enzymes (Jimenez-Bremont et al. 2006). In this study we describe the isolation of the gene and its expression analysis during in vitro appressorium formation and during the infection process of its host, Plutella xylostella. The ODC1 gene was expressed in Escherichia coli, and biochemical characterization of the recombinant protein was performed. Moreover, the ability of the ODC1 gene to complement the null odc mutant of the dimorphic yeast, Y. lipolytica, was tested.

Materials and methods

Strains and growth conditions

The M. anisopliae Ma10 (CNRCB MaPL10), was obtained from the Centro Nacional de Referencia de Control Biológico, and was originally isolated from Geraeus senilis (Coleoptera: Curculionidae) in the state of Colima, Mexico. To obtain conidia or mycelia, the fungus was grown in minimal media (MM) or in Saboraud dextrose media (SDM), respectively, as previously described (Morales Hernandez et al. 2010). The harvested conidia were washed by centrifugation and suspended in sterile 0.1% Triton X-100 solution. The cultures were incubated in flasks at 28°C and were shaken at 160 rpm. The mycelia were then collected by filtration.

The following strains of Y. lipolytica were used: FJOD (Mat A, leu 2-270, ura3-302, Δodc) (Jimenez-Bremont et al. 2001); FJCO (Mat A, leu 2-270, Δodc/pFJ4 (YIODC+) ), which was derived from FJOD, complemented with the wild type YIODC.
gene, and up-regulated by the metallothionein (METI-II) promoter (Jimenez-Bremont et al. 2001); and YMaODC1, which was derived from FJOD, complemented with the cDNA of the MaODC1 gene, and up-regulated by the metallothionein promoter (METI-II). The strains were maintained on slants of YEPD medium (1% yeast extract, 2% peptone, 2% dextrose, and 2% agar) and propagated in liquid YEPD medium or in minimal medium (0.67% yeast nitrogen base without amino acids (DIFCO), 1% dextrose, supplemented with uracil and or leucine (50 μg mL⁻¹)). In some experiments, the minimal medium was supplemented with 1 mM or 5 mM of putrescine as required.

E. coli: The DH5α strain (Invitrogen, Carlsbad, CA, USA) was used for DNA manipulations and transformations. It was maintained in Luria–Bertani medium at 37°C (Sambrook and Russell 2001). Ampicillin (100 μg mL⁻¹) was added as required.

E. coli BL21(DE3)plysS (Invitrogen) was utilized for heterologous expression of the ODC1 cDNA.

Nucleic acid isolation and MaODC1 gene cloning

Fungal genomic DNA was isolated from mycelia grown for 24 h in SDM. DNA was extracted by friction following standard protocols (Sambrook and Russell 2001). Total RNA was extracted from cells of either M. anisopliae or Y. lipolytica grown in different culture media or from P. xylostella larvae previously inoculated with conidia of M. anisopliae, using TRIzol® Reagent (Invitrogen) according to the manufacturer’s instructions. All samples were DNase-treated using RNase-Free DNase (Promega Co., Madison, WI, USA) according to the manufacturer’s instructions. The concentration and purity of both total RNA and DNA were determined by the ratio of absorbencies at 260 and 280 nm. A pair of specific primers, invODCma1 (5′-CACCTGCTCAATCTCCCCTTT-3′) and invODCma2 (5′-GTTACTATGTCTCCACGGCGTTCA-3′), was designed according to the sequence of a fragment encoding a partial ornithine decarboxylase gene from M. anisopliae (Jimenez-Bremont et al. 2006). This was used in iPCR to amplify a fragment containing the complete open reading frame (ORF) from M. anisopliae according to a protocol previously described (Ochman et al. 1988). The reaction mixture contained total DNA, each primer and PCR SuperMix High Fidelity (Invitrogen). The amplification product was cloned into the pCR®2.1-TOPO® vector (Invitrogen) and sequenced. Both strands of the nucleotide sequence of the clone were generated using universal primers and specific primers based on the ODC1 genomic sequence deposited into the EMBL database (accession number FN689467). The DNA sequence was analyzed using the Lasergene 8 program (DNASTAR Inc., Madison, WI, USA), and its identity was confirmed by comparison to published ornithine decarboxylase sequences in the database (www.ebi.ac.uk).

Southern blot and northern blot analysis

For hybridization analysis, restricted chromosomal DNA or total RNA was prepared. Southern blot or northern blot hybridization was performed following standard procedures (Sambrook and Russell 2001). A radioactively labeled DNA probe from ODC1 gene was used as a homologous probe.

Expression analysis of the ODC1 gene by RT-PCR

The in vitro production of appressoria was performed according to the protocol previously described (St Leger et al. 1989) with some modifications. Conidia (1 × 10⁷ mL⁻¹) suspended in 10 mL 0.01% Triton X-100 were inoculated in 10 mL of YE media (0.125% yeast extract) in 8.5 cm polystyrene dishes (Falcon). The cultures were incubated at 28°C without shaking. At specific times (4, 8, 12, 18, 22, 24 and 28 h), the fungal material was collected by scraping, frozen immediately in liquid nitrogen and stored at −70°C until use. The material was used for RT-PCR analysis of the ODC1 gene, and AJ274118 (loading control) was used to compare transcript abundance. RT-PCR was performed using the SuperScript™ III One-step RT-PCR System with the Platinum® Taq DNA Polymerase kit (Invitrogen) according to the manufacturer’s recommendations. The reaction employed specific primers: oliODC1 (5′-AAAGGGAGAATGACGAGTTG-3′) and oliODC2 (5′-TGAACCGCTGGAGACATAAG-3′) for the ODC1 gene and conest100-sen (5′-GGGGGTGGTTGATTGATGTAC-3′) and conest100-anti (5′-TACCTCAGTCTGCCGTCG-3′) for the AJ274118 gene, the latter of which was used as a constitutive expression control (Freimoser et al. 2005; Morales Hernandez et al. 2010). Both oligomer pairs were used simultaneously in the same reaction tube. The reverse transcription reactions were carried out at 58°C for 30 min, and DNA amplifications were then carried out in a Gene AmpPCR System 9700 (Applied Biosystems). Aliquots of the RT-PCR products were analyzed on 3% agarose gels (UltraPure™).
were fed with a leaf disk of *Brassica oleracea* and incubated at 25°C. Every 48 h the leaf disk was changed. Different stages of infection were monitored over an 8-day period. The stages of infection were categorized as previously described (Morales Hernandez et al. 2010). Third-instar *P. xylostella* larvae were sprayed with a conidial suspension from a Potter spray tower (Potter-Precision Laboratory Spray Tower, Burkard Scientific, Uxbridge, Middlesex, UK). The larvae were fed with a leaf disk of *Brassica oleracea* and incubated at 25°C. Every 48 h the leaf disk was changed. Different stages of infection were monitored over an 8-day period. The stages of infection were categorized as previously described (Morales Hernandez et al. 2010): (1) 0 (conidia) to 24 h post-infection; (2) invasion of the infected insect (24—48 h); (3) death (48—72 h); (4) growth of mycelia on the surface of the cadaver (72—144 h); and (5) conidial growth on the surface of the cadaver (+144 h). At each time point in the observed infection, 10—40 larvae were collected and stored at −70°C until RNA extraction. Upon death, conidia from infected insect cadavers were collected with sterile 0.1% Triton X-100 solution. From a parallel control experiment, larvae treated only with 0.1% Triton X-100 solution were collected. RT-PCR analysis of the *ODC1* gene and AJ274118 (loading control) was performed to compare transcript abundance. RT-PCR was performed using the SuperScript® III One-step RT-PCR System with the Platinum® Taq DNA Polymerase kit (Invitrogen) according to the manufacturer’s recommendations and as previously described (Morales Hernandez et al. 2010).

Expression of the *ODC1* cDNA in *E. coli* cells

Expression of the *M. anisopliae ODC1* cDNA in *E. coli* BL21(DE3)pLysS (Invitrogen) was accomplished by sub-cloning the *ODC1* cDNA into a pRSETB expression vector (Invitrogen). The *ODC1* cDNA was obtained from *M. anisopliae* Ma10 amplified with two primers, ODCBamHIFd (5′-GGATCCGATGGTTATGGCAACGCTAT-3′) which contains a BamHI restriction site (underlined) and ODCEcORIrev (5′-GAATTCCTACATGTTCAAAAAGAAA-3′) containing an EcoRI restriction site (underlined), in a reaction mixture with PCR SuperMix High Fidelity (Invitrogen). The amplification product was digested with both BamHI and EcoRI restriction enzymes and ligated to the BamHI/EcoRI-digested pRSETB DNA plasmid, resulting in the recombinant plasmid pJCTG424. *E. coli* BL21(DE3)pLysS cells were transformed with plasmid pJCTG424, and colony selection occurred in LB medium supplemented with ampicillin and chloramphenicol. For Odc1p expression and purification, bacterial cells were grown to an OD600 of 0.4 at 37°C in LB liquid medium supplemented with 100 μg mL−1 ampicillin, 35 μg mL−1 chloramphenicol and 1 mM IPTG for 6 h at 37°C. Induced cells were collected by centrifugation (8000 × g for 5 min at 4°C) and suspended in binding buffer (20 mM phosphate, 500 mM NaCl, 20 mM imidazole with 0.2 mg mL−1 lysozyme, and 20 μg mL−1 DNAse). Collected cells were lysed in a Tyssue Lysser II (Qiagen, Mexico) following the manufacturer’s recommendations. Cell debris was separated by centrifugation (40,000 × g for 30 min at 4°C), and the supernatant was passed through a 1 mL HisTrap FF column (GE Healthcare, Piscataway, NJ, USA) equilibrated with 50 mM phosphate buffer at pH 7.5 and 20 mM imidazole according to the directions of the supplier. The proteins bound to the column were eluted with a gradient of 20—500 mM imidazole. Odc1p was detected at 300 mM imidazole. Fractions were saved at −70°C until their use in ornithine decarboxylase assays. ODC activity was determined by a radiometric assay of ODC based on the measurement of 14CO2 released from carboxyl-radiolabeled ornithine (Calvo-Mendez et al. 1987).

Effect of pH and temperature on ODC activity

The effect of pH on ODC activity of Odc1p was measured using the following buffers: 100 mM phosphate buffer at pH 6.5, 7.2 and 8.0; and 100 mM Tris—HCl at pH 8.5 and 9.0. The effect of temperature on ODC activity was measured at 24, 28, 37, 42 and 48°C using 100 mM Tris—HCl buffer at pH 8.5.

Effect of inhibitors on ODC activity

The effect of 1,4-diamino-2-butanone (DAB) and putrescine was assayed. First, 0.50 μg of purified recombinant Odc1p protein was incubated at 37°C with 100 mM Tris—HCl pH 8.5 in the presence of 5, 12.5, 25, or 75 μM DAB or putrescine. Next, ODC activity was assayed. To analyze the inhibitory effect of DAB during appressorium formation, suspensions of 5 × 105 conidia in 150 μL
of 0.125% yeast extract (YE) were prepared, and DAB was added 25, 50, 75 and 100 mM at either time 0 or 12 h. The cultures were placed in Petri dishes and incubated at 37 °C. The cells were observed under the microscope to follow appressorium formation at 20 and 22 h. The percentages of appressorium formation were quantified in at least 200 cells and repeated in three independent assays.

Fusion of ODC1 ORF with the metallothionein promoter, METI-II, of Y. lipolytica

A 1362-bp fragment of the MaODC1 cDNA was amplified by PCR using the primers oliODCBamfd (5' - CACTAGGGATTATGCAACGGCTAT-3'), which contains a BamHI restriction site (underlined), and ORFODC2 (5' - GGTCTAGACTACATGTTTC-3'). An 870-bp fragment, including the 3'-end proximal coding region (26 codons) and terminator region of the ODC1 gene, was amplified by PCR using two primers, oliODCfd2 (5' - TGCTCGGCCACCCAGTTCAAT-3') and oliODCrev3 (5' - CACGATCGTTTGGTGTGTCATTTTCA-3') which contains a BamHI restriction site (underlined). Both PCR products were fused by alignment and extension. This 2178-bp fusion product was used as a template; it was amplified by PCR using the two primers, oliODCBamfd and oliODCrev3 and cloned into pCR2.1TOPO (pJCTG407). Sequencing was used to corroborate the correct fusion. The 2178-bp BamHI fragment was recovered from pJCTG407 and ligated into the BamHI site of the pSG572 plasmid (Jimenez-Bremont et al. 2001). The correct orientation of ODC1 was tested by the restriction pattern of the pJCTG413 plasmid, which contains up-regulated ODC1 cDNA controlled by the METI-II promoter.

Transformation of a Y. lipolytica null mutant with the MaODC1 gene

The strain FJOD (Δodc) was transformed using the lithium acetate procedure (Chen et al. 1997) and the pJCTG413 plasmid; the transformants were recovered and maintained in minimal medium without uracil (2% dextrose, 0.67% Yeast Nitrogen Base without amino acids, 0.002% of leucine and 2% agar). The transformants obtained were grown in the presence of increasing concentrations of copper. The expression of the ODC1 gene was monitored by RT-PCR. We used the primers oliODC1 and oliODC2 to amplify the ODC1 gene, and we used the primers oliYlZNC1fd (5' - TGTGCTCAGCTTTTTTGGTTGGCATTTTTGA-3') and oliYlZNC1rev (5' - AGGGGTTGGAGCTGGATAGCCATAG-3') to amplify the YlZNC1 gene (as a constitutive control in the presence of copper) as described above. The RT-PCR reaction was developed using SuperScript™ III One-Step RT-PCR with Platinum® Taq (Invitrogen) as described above. Functionality of the ODC1 gene was determined by a radiometric assay of ODC. In order to test the prototrophy of the transformants to putrescine and their ability to show yeast—hyphae differentiation, it was necessary to decrease the levels of endogenous intracellular polyamines as follows: 100 μL of cells were transferred from complete medium (YEPD) to synthetic minimal medium without amino acids (SC) supplemented with either 0.002% leucine (for FJCO and YMaODC1 strains) or 0.002% leucine and 0.002% uracil (for FJOD strain). The cultures were incubated for 15 h at 28 °C, and the cells (OD600 1.7) were then transferred to fresh medium for three successive periods of growth. After each period of growth, cells dilutions of 1:10, 1:100 and 1:1000 were made and inoculated on the respective agar SC medium and incubated for 3 days at 28 °C. The morphology of the Y. lipolytica colonies was followed by stereoscopic observation, and the cells were followed by microscopic observation counting at least 200 cells.
Fig. 2. Nucleotide and deduced amino acid sequence of the *MaODC1* gene. The putative TATA and CAAT boxes are indicated by a square. The putative STREs, cccct, are indicated in boldface. The beginning and the end of the intron are indicated in boldface and underlined. The transcription termination motifs are indicated by a discontinuous square. The characteristic ODC motifs are shadowed, including the pyridoxal phosphate-binding site and the decarboxylases family 2 signature 2. Finally, the region corresponding to the conserved ornithine decarboxylase catalytic domain is also shadowed.

Results

Cloning of the MaODC1 gene and sequence analysis

In previous work, a fragment of the MaODC1 gene has been described (Jimenez-Bremont et al. 2006). In this work, we isolated the complete gene sequence by inverse PCR (Ochman et al. 1988). In order to select the restriction enzyme necessary to perform this particular method, we performed a Southern blot analysis. This technique shows that the ODC1 gene exists as a unique copy in the M. anisopliae genome (Fig. 1). We also selected the restriction enzyme, HindIII, for complete gene isolation. The genomic DNA was digested with HindIII and circularized. This was used as a template in the iPCR reaction with a pair of specific primers arranged in a back-to-back orientation. A DNA fragment of 2100-bp, containing both the 5′- and 3′-ends of the ODC1 gene, was amplified. The assembled sequence of the iPCR product, together with the 800-bp fragment previously reported, was corroborated by isolation and sequencing of the complete gene from genomic DNA and cDNA. The analysis of this sequence revealed the presence of a 2769-bp ODC1 gene with an open reading frame (ORF) of 1408-bp, interrupted by one intron of 65-bp (Fig. 2). The existence and location of the intron was corroborated by alignment of the genomic and cDNA sequences from the Ma10 strain. A putative TATA element in the 5′ untranslated region of the ODC1 gene was found 182 bp upstream from the proposed initiation codon. A possible CAAT box was located 162 bp upstream of the start codon. The 5′ UTR contains three putative STREs (stress responsive elements CCCCCT). Meanwhile, sequences matched to the transcription termination motifs, TAG, TAGT and TTT, were found 103, 116 and 137 bp downstream from the termination codon, respectively. Northern blot analysis revealed a hybridization signal approximately 1.8-kb long (Fig. 3). The sequence predicted a protein of 447 amino acids with a molecular weight of 49.3 kDa and an isoelectric point of 5.26. A PROSITE database search identified canonical motifs of ornithine decarboxylases, the sequence PFYAVKCPDFAPKLRL, was located between amino acid residues 90–110. This is thought to be the pyridoxal phosphate binding site (Fig. 2). The sequence, AAEGFTMRTLAVGGF, which is located between amino acids 248 and 265 and contains three glycine residues, is thought to be part of the proposed substrate-binding region (Fig. 2). A cysteine is located within the WGTCDGID consensus sequence at position 385 in the catalytic site (Poulin et al. 1992) (Fig. 2). BLAST analysis of MaOdc1p using the EMBL database (www.ebi.ac.uk) shows the highest homology of this gene with the ODC from the plant symbiont, Epichloe festucae (86%, accession number A883P2), and lowest homology with ornithine decarboxylases from the following filamentous fungi: Spe1p from Neurospora crassa (69%, accession number M68970), Odc1p from Tapeinia yallundae (65%, accession number Q96X19), Odc1p from Y. lipolytica (54%, accession number Q8WZM1), and Spe1p from Saccharomyces cerevisiae (51%, accession number P08432). Genes of low homology from other fungi also exist.

Expression of the MaODC1 cDNA in E. coli

To demonstrate that the ODC1 gene codes for an ornithine decarboxylase protein, ODC1 cDNA was expressed in E. coli BL21 cells using the pRSETB vector. E. coli BL21 cells were transformed by inserting the pJCTG424 plasmid with the ODC1 cDNA into the plasmid vector. After recombinant Odc1p purification on a Ni²⁺-agarose column, the recombinant protein (Fig. 4A and B) showed ornithine decarboxylase activity by radiometric enzyme assays. Meanwhile ODC specific activity in E. coli crude extract was 6.7±2.9 pmol CO₂ h⁻¹ μg protein⁻¹, the ODC specific activity in crude extracts from E. coli cells transformed with plasmid pJCTG424 was 351.2±21.6 pmol CO₂ h⁻¹ μg protein⁻¹; and in the purified recombinant MaOdc1p the ODC specific activity was 484.6±5.1 pmol CO₂ h⁻¹ μg protein⁻¹. This result confirms the predicted amino acid sequence analysis and indicates that ODC1 codes for an ornithine decarboxylase. The purified protein showed a molecular weight of approximately 50 kDa on SDS-PAGE, which is similar to the predicted MW of 49.3 kDa. The optimum pH was 8.0–8.5 (data not shown), and the optimum temperature of the enzyme reaction was...
Expression of the *MaODC1* gene during appressorium formation

In *M. anisopliae*, the early invasion of its host is characterized by discrete differentiation events, and conidia germination is followed by appressorium formation (adhesion structure). To determine if *ODC1* expression correlates with these differentiation processes, we evaluated *ODC1* mRNA levels by RT-PCR analysis during appressorium formation *in vitro*. Appressorium formation was developed according to a protocol previously described (St Leger et al. 1989). The conidia were inoculated on 0.125% YE on Petri dishes and incubated without agitation for 24 h. Samples were taken at different time points, and RT-PCR analysis was performed according to Morales Hernandez et al. (2010). *ODC1* mRNA increased at the beginning of germination and during appressorium formation (Fig. 5). To evaluate if this increase of *ODC1* expression is important in the differentiation process required to produce adhesion structures, we performed an assay of appressorium formation *in vitro*. First, we added the competitive inhibitor, DAB, at different concentrations (25, 50, 75 and 100 mM) at the start of the assay or 12 h after start. We observed that adding DAB initially inhibited appressorium formation up to 60% without affecting the germination process. The threshold for affect on germination is 150 mM. However, when DAB was added to the culture medium 12 h later, there was a much lower inhibition of only 10%. Further, addition of 5 mM putrescine restored appressorium formation ability, indicating that activity of the ODC is not only important for this differentiation process but also required during specific time points.

Expression of *ODC1* gene during invasion of *P. xylostella*

The above experiment was performed *in vitro*. To analyze *ODC1* expression in *insecta*, *P. xylostella* was infected with *M. anisopliae* conidia, and different stages of infection in the larvae were monitored. Observations were made over an 8-day period. The infected larvae were collected at different times post-inoculation, and the RNA was extracted. It was used to synthesize cDNA used as templates for RT-PCR assays, as previously described (Morales Hernandez et al. 2010). We simultaneously amplified *ODC1* and AJ274118 (loading control) in the same reaction tube (Fig. 6A). An *ODC1* amplification product of 520-bp and an AJ274118 amplification product of 100-bp were observed through the entire course of insect infec-

Fig. 5. Expression of the ODC1 gene during in vitro appressorium formation. For the production in vitro of appressoria, conidia of *M. anisopliae* Ma10 were inoculated in 10 mL of YE media (0.125% yeast extract) in 8.5 cm polystyrene dishes. The cultures were incubated at 28 °C without shaking; at specific times, the fungal material was collected and employed for RT-PCR analysis of the ODC1 gene to compare transcript abundances. (A) Gel electrophoresis. Lane M, molecular markers; lane 1, 4 h; lane 2, 8 h; lane 3, 12 h; lane 4, 18 h; lane 5, 22 h; lane 6, 24 h, lane 7, 28 h. (B) Relative RT-PCR analysis of ODC1 and AJ274118 (load control). (C) Appresorium formation at 24 h, arrows show the appressoria. The results are representative from three independent RNA extractions and RT-PCR analysis.

Fig. 6. Complementation of the odc null mutation of *Y. lipolytica* by the ODC1 gene

In fungi, ornithine decarboxylase genes are important during normal growth and differentiation processes. ODC1 gene expression analysis showed variation during appresorium formation and during mycelial growth inside *P. xylostella*. To prove that the ODC1 gene isolated from *M. anisopliae* encodes a functional ornithine decarboxylase, heterologous complementation of a strain of *Y. lipolytica* (FJOD) that lacked the ODC gene was carried out. The complementation of the mutant was carried out using the pJCTG413 plasmid. The plasmid up-regulates the ODC1 gene by the metallothionein promoter of *Y. lipolytica* (METI-II), which induces gene expression in response to increasing concentrations of copper sulfate in the medium. The independent transformant, YMaODC1, was selected because of its ability to grow in SC medium without uracil. PCR was used to confirm that the transformant contained the *MaODC1* gene (data not shown). Two *Y. lipolytica* strains, YMAODC1 and the null odc mutant (FJOD) were grown in the presence of increasing concentrations of copper sulfate. The levels of *MaODC1* gene expression were evaluated. We observed increased *MaODC1* mRNA in response to an increasing copper concentration, in YMAODC1 strain (Fig. 7).

To investigate if the ODC1 gene expressed in the yeast *Y. lipolytica* (strain YMAODC1) produces a functional ornithine decarboxylase capable of producing putrescine, we performed another assay. The polyamine pools were depleted in the cells, and we assessed the ability of these cells to grow and differentiate from yeast to mycelium. FJOD (null mutant) and FJCO (FJOD complemented by the ODC1 gene from *Y. lipolytica*) were used as controls. In Fig. 8, we show that the YMaODC1 strain, which contains the *MaODC1* gene, and the strain FJCO were capable of growing and differentiating in this medium without polyamines. In contrast, the FJOD strain, which is devoid of the ODC gene, was unable to grow and carry out the yeast-mycelium transition in a medium without polyamines. In the latter

Fig. 6. *In vivo* expression analysis of the ODC1 gene during *P. xylostella* infection. (A) The RT-PCR analysis of ODC1 and AJ274118 (load control) gene expression during progressive infection of third instar *P. xylostella* larvae with *M. anisopliae* Ma10. Lane M corresponds to DNA marker; lane 1 corresponds to RT-PCR from conidia; lanes 2–12 correspond to RT-PCR amplification products from total RNA of third instar *P. xylostella* larvae infected with *M. anisopliae* conidia at different time points post-infection (lane 2 (4 h), lane 3 (8 h), lane 4 (12 h), lane 5 (24 h), lane 6 (48 h), lane 7 (72 h), lane 8 (96 h), lane 9 (120 h), lane 10 (144 h), lane 11 (168 h), lane 12 (192 h)), lane 13 and 14 correspond to RT-PCR amplification products from total RNA of uninfected third instar *P. xylostella* larvae at 24 and 48 h. (B) Relative expression of ODC1 during invasion measured by densitometric analysis. (C) Morphology of the progression of the infection in third instar *P. xylostella* larvae at different post-infection time points: lane 1 (12 h), lane 2 (48 h), lane 3 (120 h), and lane 4 (192 h). Results are represented from two separate RNA extractions and RT-PCR analysis from independent infection assays.

In this work, we isolated a genomic copy and the cDNA of the ODC1 gene from *M. anisopliae* Ma10, using an internal fragment of the ODC1 gene of this fungus previously reported (Jimenez-Bremont et al. 2006). Based on sequence identity of the predicted protein, this gene encodes an ornithine decarboxylase (ODC) highly homologous (86%) to the ODC from the endophytic fungus, *E. festucae* (accession number A8B3P2). It is also homologous to the ODC from phytopathogenic fungi, such as the ODC from *Nectria haematococca* (*Fusarium solani*) (78%, accession number C7UY8), *Magnaporthe grisea* (71%, accession number A4R2T2), *Chaetomium globosum* (75%, accession number Q2HB10) and *Phaeosphaeria nodorum* (*Septoria nodorum*) (64%, accession number Q9UVG2). Notably, phylogenetic analysis of the ODC of the entomopathogenic fungus *M. anisopliae* was closely related to fungi that act as pathogens to other organisms in their environments.

The predicted protein sequence indicated that it contains typical domains of ornithine decarboxylase, including the binding site for pyridoxal phosphate, a cofactor of the enzyme; the con-
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Biochemical analysis confirmed that this protein is a PLP-dependent ornithine decarboxylase, it is inhibited in vitro by its end product (putrescine) or by the putrescine analogue, 1,4-diamino-2-butaneone (DAB), like most ODCs in fungi such as S. cerevisiae, N. crassa and A. nidulans (Tabor and Tabor 1985) or in the parasite protozoan Entamoeba histolytica (Arteaga-Nieto et al. 2002). This suggests that polyamine levels regulate the activity of this enzyme in vitro, as described in other fungi.

The complementation assays demonstrated that the protein encoded by the MaODC1 gene and expressed in the null mutant of the ODC gene of Y. lipolytica is functional. It fully complemented prototrophy and the fungus’s ability to differentiate itself from yeast to mycelium. These results suggest that ODC activity can provide sufficient amounts of putrescine to support these two events in the yeast, and, therefore, this protein was both adequately expressed and/or structurally competent. This result is in contrast with the ODC from S. nodorum, which only partially complemented the conidiation process of A. nidulans, possibly due to insufficient expression and/or structural incompetence of the protein (Bailey et al. 2000). In our case, the ODC1 gene was up-regulated by the promoter, YIMTLII, which responds to copper. Indeed, we observed that the promoter responded as expected, modulating ODC1 gene expression in relation to copper concentrations in the culture media. Interestingly, based on the complementation results, we can say that even basal expression of the ODC1 gene was sufficient to promote growth and differentiation in Y. lipolytica.

The ODC1 gene is expressed throughout the life cycle of the fungus during invasion and growth in its host P. xylostella. During the first 24 h (early invasion), the conidia germinate and differentiate to form the appressorium. This process is accompanied by a discrete increase in ODC1 gene expression. This increase is seen again during in vitro appressorium formation. In these in vitro assays, the inhibition of ODC activity by DAB caused a reversible inhibition of appressorium formation. This finding is congruent with those of Ruiz-Herrera (1994), who suggested that polyamines are essential in processes such as differentiation, germination, sporulation and dimorphism in fungi. In this particular case, the polyamines are necessary to form the appressorium. Interestingly, the ODC1 gene was more strongly induced during invasive fungal growth and death of the host than during early invasion. These data suggest that this fungus requires more reduced expression of the ODC gene for germination and appressorium formation than for invasive growth. This result contrasts with

Fig. 7. Induction of the ODC1 gene by copper. Cells from Y. lipolytica strains, YMaODC1 (Δodc/pJCTG413 (MaODC1)) and FJOD (Δodc), were grown for 16 h at 28 °C in liquid minimal medium with the required amino acids. Different CuSO4 concentrations were added, 3 h later the cells were recovered and total RNA was isolated and used for RT-PCR analysis of the MaODC1 and YlZNC1 genes to compare transcript abundances. M, DNA markers; lane 1 (0.0 mM CuSO4); lane 2 (0.2 mM CuSO4); lane 3 (0.4 mM CuSO4); lane 4 (0.8 mM CuSO4); lane 5 (1.2 mM CuSO4); and lane 6 (2 mM CuSO4). (A) Y. lipolytica strain, YMaODC1. (B) Y. lipolytica strain FJOD. (C) Relative RT-PCR analysis of MaODC1 and YlZNC1 (load control). Results are represented for two separate RNA extractions and RT-PCR analysis from two independent experiments.

served cysteine within the catalytic site (Poulin et al. 1992) and possible PEST sequences that are characteristic of high-turnover proteins in mammals (Rogers et al. 1986). These PEST regions have been described in several fungal ODC, such as ODC from N. crassa (Williams et al. 1992), S. cerevisiae (Fonzi and Sypherd 1987) and U. maydis (Guevara-Olvera et al. 1997). ODC from other fungi, such as P. brasiliensis (Nino-Vega et al. 2004) and C. immitis (Guevara-Olvera et al. 2000), do not have these PEST sequences. It is important to note that it is not clear whether fungal PEST regions play a similar role in the regulation of fungal ODC, because mammalian PEST sequences regulate mammalian ODC (Hayashi and Murakami 1995).

The expression of the cDNA of the ODC1 gene of M. anisopliae in E. coli facilitated the purification of this protein. Biochemical analysis confirmed that
findings reported for U. maydis (Guevara-Olvera et al. 1997) and Y. lipolytica (Jimenez-Bremont et al. 2001), in which higher concentrations of polyamines are required for dimorphic transition (a differentiation process), than for ensuring vegetative growth. However, it cannot be excluded that M. anisopliae could require higher concentrations of polyamines for other differentiation processes, including conidiation.

The induction of the ODC1 gene expression observed during early invasion, appressorium formation in vitro, and invasive growth suggests that the ODC1 gene is transcriptionally regulated. The molecular mechanism of this regulation is unknown. However, three STREs (stress responsive elements, CCCCT) have been observed within the ODC1 promoter, and they may be involved in this mechanism. STREs have been shown to mediate transcriptional activation in response to various stressors in S. cerevisiae (Siderius et al. 1997). Potentially, the STREs perform a similar function in M. anisopliae.

As shown in M. anisopliae, the inhibition of ODC inhibited appressorium formation without affecting germination, as reported in Uromyces viciae-fabae (Reitz et al. 1995). This structure has been associated with the ability of some fungi to infect their hosts, including Magnaporthe oryzae (Izawa et al. 2009) and M. grisea (Talbot et al. 1993).

Interestingly, in M. grisea, putrescine inhibited appressorium formation but not the ODC inhibitor, DFMO (Choi et al. 1998). The importance of ODC in the pathogenic process was showed in S. nodorum, in which an ODC mutant showed significantly reduced virulence in wheat, its host organism (Bailey et al. 2000). In the hemibiotrophic fungus, Colletotrichum higginsianum, disruption of the ODC gene also led to decreased pathogenicity (Huser et al. 2009). Obviously, it is interesting to study the role of the ODC1 gene in appressorium formation in relation to the virulence of M. anisopliae. In this fungi, it was reported that mutations that slowed the formation of the appressorium decreased virulence (Fang et al. 2009). It would be important to demonstrate the involvement of the ODC1 gene in each of the differentiation events the fungus must undergo during its life cycle in insects. Thus, null mutants and their complementation with the gene controlled by a regulated promoter may provide information about the role of the gene in each of these events. In order to generate the ODC1 null mutant we generated a disruption cassette containing the bar gene of Streptomyces hygroscopicus and transformed M. anisopliae. After isolation and PCR analysis of 120 transformants, we did not find a replacement for the ODC1 gene. There are multiple explanations for this finding, but the construction
of an ODC1 gene-null mutant will help to establish its role in the virulence of this fungus.

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