RESEARCH ARTICLE

Ustilago maydis spermidine synthase is encoded by a chimeric gene, required for morphogenesis, and indispensable for survival in the host

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Abstract

To analyze the role of spermidine in cell growth and differentiation of *Ustilago maydis*, the gene encoding spermidine synthase (Spe) was isolated using PCR. We found that the enzyme is encoded by a chimeric bifunctional gene (*Spe-Sdh*) that also encodes saccharopine dehydrogenase (Sdh), an enzyme involved in lysine biosynthesis. The gene contains a 5′ region encoding Spe, followed, without a termination signal or a second initiation codon, by a 3′ region encoding Sdh, and directs the synthesis of a single transcript that hybridizes with 3′ or 5′ regions' probes of the gene. The gene could not be disrupted in a wild-type strain, but only in a mutant defective in the gene encoding ornithine decarboxylase (*Odc*). Single *spe-sdh* mutants were isolated after sexual recombination *in planta* with a compatible wild-type strain. Mutants were auxotrophic for lysine and spermidine, but not for putrescine, and contained putrescine and spermidine, but not spermine. Putrescine in double mutants is probably synthesized from spermidine by the concerted action of polyamine acetyl transferase and polyamine oxidase. *spe-sdh* mutants were sensitive to stress, unable to carry out the yeast-to-mycelium dimorphic transition, and showed attenuated virulence to maize. These phenotypic alterations were reverted by complementation with the wild-type gene.

Introduction

Polyamines are cationic micromolecules present in all organisms, and essential for their growth and normal functions (reviews in Pegg & McCann, 1982; Tabor & Tabor, 1984, 1985). The most common polyamines found in all organisms are putrescine and spermidine, spermine being present mostly in higher eukaryotes. Synthesis of putrescine occurs by two pathways related to arginine metabolism: one that is almost universal and involves ornithine decarboxylation by ornithine decarboxylase (Odc), and another one, present in plants and bacteria, involving decarboxylation of arginine (Smith, 1985; Tabor & Tabor, 1985). Spermidine is synthesized through the transfer of an aminopropyl group from decarboxylated *S*-adenosyl methionine to putrescine, a reaction catalyzed by spermidine synthase, and spermine synthesis occurs by transfer of a second aminopropyl group, this time to spermidine, by the action of spermine synthase.

The role of polyamines is related to their cationic nature, and involves their interactions with cellular polyanions, mostly nucleic acids, and phospholipids. Polyamines protect DNA from enzymatic degradation and X-ray irradiation, stabilize RNA, and prevent ribosomal dissociation (Goldemberg & Algranati, 1977; Pingoud *et al.*, 1984; D'Agostino *et al.*, 2006). Deprivation of polyamines reduces the rate of synthesis of nucleic acids and proteins, and the fidelity of translation (Marton & Morris, 1987). Under prolonged polyamine starvation, chromosome aberrations occur, they are disintegrated, and alterations in nuclear morphology and loss of actin and microtubules take place (Pohjanpelto & Knuutila, 1982). Polyamines are also involved in development and cell differentiation, for example before any differentiation step in fungal development, there occur increases in polyamine pools and Odc activity (Calvo-Méndez *et al.*, 1987; Martínez-Pacheco *et al.*, 1989; reviewed by Ruiz-Herrera, 1994). Nevertheless, the mechanisms underlying

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their mode of action have not been defined (reviewed by Gerner & Meyskens, 2004).

Ustilago maydis (DC.) Corda, the causal agent of common smut in maize, has been considered an excellent model for the study of fungal pathogenicity and differentiation mechanisms (reviewed in Ruiz-Herrera & Martínez-Espinoza, 1998; Martínez-Espinoza *et al.*, 2001; Basse & Steinberg, 2004; Pérez-Martín *et al.*, 2006). Its life cycle involves two different stages: one occurring in the environment, where the fungus grows in the form of haploid yeast cells that reproduce by budding; and a second one, initiated by the fusion of sexually compatible haploid yeast cells to form a dikaryotic hypha that invades the plant host and grows in the form of mycelium (Kronstad & Leong, 1989, 1990; Schulz *et al.*, 1990; Bölker *et al.*, 1992), eventually forming diploid teliospores within the tumors characteristic of the disease (Banuett & Herskowitz, 1996). Teliospore germination outside the plant reinitiates the life cycle (reviewed in Banuett, 1995, 2002; Ruiz-Herrera & Martínez-Espinoza, 1998; Ruiz-Herrera *et al.*, 1999; Bölker, 2001).

Inhibition of putrescine biosynthesis in *U. maydis* by selective drugs that affect Odc activity inhibited germination of teliospores and the *in vitro* dimorphic transition (Guevara-Olvera *et al.*, 1997). Moreover, dimorphic transition of Δ*odc* mutants required putrescine concentrations higher than those necessary to support fungal growth (Guevara-Olvera *et al.*, 1997), suggesting a specific role of polyamines in dimorphism. Similar results were obtained with Δ*odc* mutants of *Candida albicans* (Herrero *et al.*, 1999) and *Yarrowia lipolytica* (Jiménez-Bremont *et al.*, 2001). Nevertheless, it was not possible to demonstrate as to loss of which of the several polyamines was responsible for the resulting phenotype. In the present study, we have proceeded to the isolation and disruption of the gene encoding spermidine synthase in order to obtain evidence on this subject.

Materials and methods

Strains, culture media, and growing conditions

Strains of *U. maydis* and *Escherichia coli* used in this study are described in Table 1. Strains of *U. maydis* 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 for *c.* 48 h at 28 °C under shaking conditions. These cultures were used as inocula, normally for minimal medium (MM; Holliday, 1974), and incubation proceeded as above. When necessary, media were supplemented with one or more of the following compounds: hygromycin (200 μg mL^{-1}), carboxin (20 mM), lysine (0.2 mM), 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 of decimal dilutions from cell suspensions containing 10^8 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 addition of ampicillin (100 μg mL^{-1}) or kanamycin (50 μg mL^{-1}) for plasmid selection.

Techniques for nucleic acid manipulation

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

Plasmid DNA isolation from *E. coli* was performed by standard procedures (Sambrook & Rusell, 2001). DNA enzymatic reactions such as digestion, ligation, and vector dephosphorylation were performed as recommended by the manufacturers of the reagents used (Invitrogen Co., Carlsbad, CA; New England Biolabs). 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 Co.), using 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 a 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) 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 Co.). The primers used are shown in Table 2.

Genetic transformation of *U. maydis*

We used the procedure described by Tsukuda *et al.* (1988), with slight modifications. Protoplasts (50 μL) were mixed with 1 μg of transforming DNA and 1 μL heparin (15 mg mL^{-1} in STC: 10 mM Tris-HCl pH 7.5, 100 mM $CaCl_2$, 1 M sorbitol), and incubated on ice for 10 min. At this time, 0.5 mL ice-cold 40% polyethylene glycol (PEG

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Table 1. Strains used in this work

Organism	Strain	Genotype	Source
U. maydis (wt)	FB2	*a2b2*	F. Banuett, California State University, Long Beach
U. maydis (wt)	FB1	*a1b1*	F. Banuett
U. maydis (wt)	031	*a1b2*	S. Gold, University of Georgia
U. maydis (wt)	032	*a2b1*	S. Gold
U. maydis	LG4	*a2b2*, Δ*odc*	Guevara-Olvera *et al*. (1997)
U. maydis	81	*a2b2*, Δ*odc/spe-sdh*	This work
U. maydis	LV7	*a1b2*, Δ*spe-sdh*	This work
U. maydis	LV54	*a2b1*, Δ*spe-sdh*	This work
U maydis	LV10	*a2b2*, Δ*spe-sdh/Spe-Sdh*	This work
U. maydis	LV8	*a2b2*, Δ*spe-lys/Spe-Sdh*	This work
U. maydis	LV4	*a2b2*, *Spe-Sdh*	This work
E. coli	Top 10	F′	Invitrogen Co.

Table 2. Primers used in this work

Primer	Orientation	T_m (°C)	Sequence
AC101	Forward	62	GCGGTACCCCCATAGATGATTTGCGTG
AC104	Reverse	62	GCGGCCGCCAACCAAACTTAGATGAAG
486	Forward	62	GATTGCGGTTGGTTCGTCA
489	Reverse	60	TCCAAAAGGCGGGTATCAC
488	Forward	60	TGGGTCTTTGCGATGCCT
SPDS3REV	Reverse	60	GCGGTCTCGATCTCAAACTTGTGCTG
pCBX	Reverse	62	GACCTGCCCCAAGAACCTCAACCCTG
AC102	Reverse	62	CCCAAGCTTCAAAACACCAAGTCCACCG
5′odc	Forward	63	CAACATGGACGAGCTCGAAAAGAT
3′odc	Reverse	63	GTAAGCGCCCATGTTTTCGTAGAC
Bw1-2F	Forward	66	CCGAACCAACAGGCCCTCAGCCG
Bw1-2R	Reverse	66	CCGAACCAACAGGCCCTCAGCCG
Bw2-2F	Forward	66	CCGAACCAACAGGCCCTCAGCCG
BW2-2R	Reverse	66	CGATGCTACCGCCACTGCCAAGAA
Pra1-F	Forward	66	TAGGCTCGTGCCCTTTATCGG
Pra1-R	Reverse	66	ACTAGGCTTCTCATTGACTTTTTG
Pra2.F	Forward	66	TTGCCTTATCCACCTGCAGCTAA
Pra2-R	Reverse	66	ACTAGGCTTCTCATTGACTTTTTG

MW 3350) was added, and incubation was continued for 15 min on ice. STC (0.5 mL) was added, and the material was mixed by inversion of the tube. Protoplasts were centrifuged at *c.* 600 ***g*** for 5 min, suspended in STC, and spread over DCMS double-strength selective medium agar plates containing 1 M sorbitol. After 4–5 days, colonies were transferred to fresh hygromycin or carboxin-containing plates to confirm their phenotype.

DNA sequencing and analysis

DNA sequencing was performed with double-stranded templates in an ABI PRISM 377 DNA automatic sequencer (Perkin Elmer). Homology searches were performed using the FASTA3 searches of EMBL, SWISS-PROT, and SWALL databases. Analysis of nucleotide sequences was performed using the DNASTAR, DNASTRIDER 1.1, and TRANSFAC programs. The sequence of the *Spe-Sdh* gene was submitted to EMBL with accession number: FN178523.

Plasmids

The genomic sequence of *Spe-Sdh* was obtained from the *U. maydis* genome sequence at http://mips.gsf.de/genre/proj/ustilago/ (Um05818). The complete gene was amplified with primers AC101 and AC104 (see Table 2), and the PCR product was cloned in TOPO 2.1 (Invitrogen Co.) to generate the pUmAC4 plasmid. For construction of pGEM-spe3, we amplified the *Spe-Sdh* gene from pUmAC4 with 2138 and 2139 primers, and the product was cloned at the EcoRI site in pGEM-T Easy. pUm-AC2 is a plasmid where most of the ORF was eliminated by digestion with HindIII, and substituted by a 3.1 kb hygromycin resistance marker [obtained by HindIII digestion of pJW42 (Mayorga & Gold, 1998)] flanked by the *HSP70* promoter and terminator. A second construction was made by substitution of the hygromycin resistance marker with a different hygromycin resistance cassette [2.3 kb, obtained from pHyg-tubulin (unpublished data) by HindIII digestion], where the gene was flanked by the tubulin promoter and terminator, to generate the pUm-AC3 plasmid. pCBX-AC4 (Δ*spe-sdh*::Cbx^R) was constructed by replacement of the hygromycin resistance cassette from pUmAC3 by a carboxin resistance cassette obtained by HindIII digestion of pCBX-AC2 [(obtained by EcoRI–HindIII digestion from pCBXND3 (Keon *et al*., 1991)].

Mating analysis

Experiments were carried out by the Fuz reaction (Banuett, 1992, 1995). Briefly, drops of suspensions of the mating strains to test were placed one over the other on charcoal-agar plates of MM with the added requirements,

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and incubated for 24 h at 25 °C. The appearance of aerial mycelium indicated that the strains were sexually compatible.

Determination of the virulence of *U. maydis* strains

We followed essentially the method described by Martínez-Espinoza *et al.* (1997). Compatible strains of *U. maydis* (either wild-type or *spe* mutants) were grown separately overnight at 28 °C in a complete liquid medium with gentle shaking. Cells were sedimented by low-speed centrifugation, washed by centrifugation with sterile-distilled water, suspended in sterile-distilled water, to a density of 10^8 cells mL^{-1}, and mixed in equal amounts. Aliquots (0.1 mL) were inoculated with a syringe and needle into 6-day-old maize plantlets cv. Cacahuazintle. Plants were incubated in a greenhouse. Control plantlets were injected with 0.1 mL of sterile-distilled water, while others were left uninoculated. At intervals, some inoculated plants were removed, and the presence of the fungus in plant tissues was investigated by microscopic observation of plant sections obtained with a scalpel, and stained with lactophenol blue. Disease symptoms (chlorosis, anthocyanin formation, and tumor formation) were recorded routinely after 5–15 days, unless otherwise specified.

Isolation of segregants from inoculated plants

The procedure described by Chavez-Ontiveros *et al.* (2000) was followed. Mature tumors were excised from infected plants 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 release teliospores, treated with 0.5% $CuSO_4$ for 1 h, and filtered through a cheesecloth. Teliospores were recovered by centrifugation, washed with sterile-distilled water, diluted to contain 10^4 cells mL^{-1}, and 0.1-mL aliquots 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 phenotypic analysis.

Stress assays

Different stress conditions were tested on solid media. Cells were grown in liquid MM containing lysine, but not spermidine, for two cycles in order to deplete the cell polyamine pools. They were then collected by centrifugation, and washed with sterile-distilled water by centrifugation. Cell suspensions were adjusted to contain 10^8 cells mL^{-1} (counted with a Neubauer chamber), 10-fold serial dilutions were prepared, and 10 μL was spotted on plates containing different additions. Plates were incubated at 28 °C for 48 h and photographed using a Cannon camera model Eorebeld.

Polyamine analysis

Polyamines were extracted from the cells with 6% perchloric acid for 3 h at room temperature. Derivatization of polyamines was made as described by Jin *et al.* (2002), and analyzed by HPLC using a Beckman C-18 Stering Ultrasphere column 4.6 × 150 mm. A mixture of methanol : water 60 : 40 (v/v) was used as a solvent, at 40 °C, 121 bar, and a flow of 1 mL min^{-1}. Detection was followed at 254 nm, and the concentration was measured from the absorption curve of the peaks, relative to curves obtained with known concentrations of benzoylated putrescine, spermidine, or spermine as standards.

Microscopic observations

Microscopic observations were made using a Leica DMRE microscope. Observations of colonial morphology were made using a Leica MZ8 stereoscope. Photographs were obtained using a Spot digital camera (Diagnostic Instruments).

Results

Isolation of the gene encoding spermidine synthase (*Spe*)

A search in the *U. maydis* genomic bank (http://mips.gsf.de/genre/proj/ustilago/) using known sequences of fungal spermidine synthases revealed the presence of a single ORF of 2310 bp in chromosome 20, contig 20, *locus* UM05818 with a high homology to genes encoding fungal Spe's. The relatively high *Mr* of the gene (compared with those from different sources) was similar to the one from *Cryptococcus neoformans* (Kingsbury *et al.*, 2004). Alignment of the *U. maydis* gene with other fungal homologues revealed that the first region of the gene (bp 1–768) showed a high homology to fungal genes encoding Spe, whereas a second region without any ATG starting codon (bp 963–2310) presented homology to the genes encoding saccharopine dehydrogenase (Sdh), an enzyme catalyzing a step of lysine biosynthesis. These data are schematized in Fig. 1a. These results are similar to *C. neoformans*, which was reported to contain a chimeric gene encoding a single ORF with two regions, one corresponding to Spe and the other to Sdh. The *U. maydis* gene is referred to here as *Spe-Sdh*. For its isolation, we designed primers AC101 and AC104 (see Table 2), which amplified by PCR a fragment of 4925 bp that contained the ORF plus 1635 bp corresponding to the 5′-noncoding region, and

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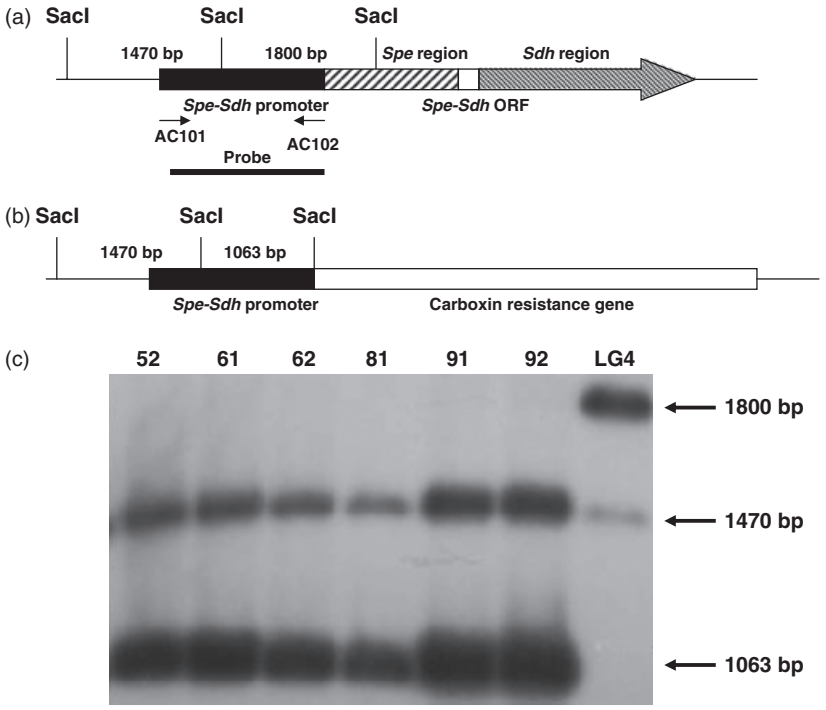

Fig. 1. Disruption of the *Spe-Sdh* gene. (a) Scheme of the chimeric *Spe-Sdh* gene showing the conserved domains, and the relative position of the SacI sites, and the probe used for Northern blot hybridization. (b) Schematic representation of the *spe-sch*::*Cbx* mutant gene. (c) Southern blot analysis of the parental LG4 strain and Δ*spe-sdh* mutants. DNA was digested with SacI and probed with a fragment from the *Spe-Sdh* promoter amplified with primers AC101 and AC102.

1000 bp to the 3′-noncoding region. The fragment was cloned in TOPO pCR 2.1, to obtain pUmAC4.

Disruption of the *Spe-Sdh* gene

Repeated attempts to disrupt the *U. maydis Spe-Sdh* gene by double recombination using two different cassettes containing the hygromycin resistance gene as the selective marker (see Materials and methods) were unsuccessful, independent of the several changes that we introduced into the protocol. This result was rather peculiar because it is known that *U. maydis* homologous recombination is rather high (20–65%). Accordingly, we considered the possibility that in *spe* mutants, putrescine would be accumulated, reaching toxic concentrations (Guarino & Cohen, 1979; Davis & Ristow, 1988; Tome *et al.*, 1997). Accordingly, we decided to use a previously isolated *odc* mutant (Guevara-Olvera *et al.*, 1997) as a recipient for the disruption cassette. Because this mutant is hygromycin resistant, we constructed a disruption cassette containing the gene providing carboxin resistance (see Materials and methods). The cassette was amplified by PCR using primers 2138 and 2139 (see Table 2) and used to transform protoplasts of the LG4 (*odc*) mutant (Guevara-Olvera *et al.*, 1997). Putative transformants were recovered in a medium containing hygromycin, carboxin, putrescine, spermidine, and lysine, and were probed by PCR using primers 486 and 489. A fragment of 3000 bp was expected for the *odc/spe-sdh* double mutants, and of 1500 for the *odc* parental strain. The results obtained revealed the appearance of several of the expected double mutants (not shown). These mutants were further verified using primers pCBX (whose sequence is within the carboxin-resistance gene) and AC101, which is outside the construction (Table 2). The amplification of a 2000-bp fragment confirmed the nature of the double mutants (not shown). Finally, a Southern blot of several mutants, using as a probe a sequence from the promoter amplified with primers AC101 and AC102, was performed. The hybridization pattern obtained was evidence that the mutants were deficient in the chimeric gene (Fig. 1). As expected, the selected *spe-sdh* mutants were resistant to carboxin and hygromycin, and auxotrophic to lysine and spermidine, but interestingly, not to putrescine. Data for a selected mutant are shown in Fig. 2.

Isolation of *spe-sdh* single mutants

To isolate single *spe-sdh* mutants, we turned to the use of sexual recombination *in planta*. For these experiments we used an *a2b2 spe-sdh/odc* double mutant (81), and the FB1 wild-type strain (*a1b1*), following the procedure described in Materials and methods. Teliospores from the corresponding cross were inoculated on plates of MM containing spermidine, putrescine, and lysine, and the meiotic products were transferred to fresh plates to isolate smooth (haploid) colonies. These were further tested in media containing either carboxin or hygromycin. Only strains showing sensitivity to hygromycin and resistance to carboxin (Cbx^R,Hyg^S) were further selected, and their auxotrophy to lysine and spermidine was confirmed (Fig. 3a). The mating characteristics of the mutants were further analyzed using the Fuz

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reaction (see Materials and methods) using tester strains as partners (see Table 1). From these, two strains, LV7(*a2b1*) and LV54(*a1b2*), were selected for further experiments. The mating genotype of these mutants was confirmed by PCR of genes *bW* and *Pra* using the specific primers Bw1-2F and Bw1-2R (*bW1*); Bw2-2F and Bw2-2R (*bW2*); Pra1-F, Pra1-R (*Pra1*); and Pra2-F and Pra2-R (*Pra2*) (results not shown). The nature of their *Odc* genotype was further confirmed by PCR using primers 5′odc and 3′odc for the *Odc* gene (we expected an 857-bp fragment for the *wild-type* gene, and of 2700 bp for the mutant gene). The results (Fig. 3b) confirmed that single mutants contained the wild-type *Odc* gene.

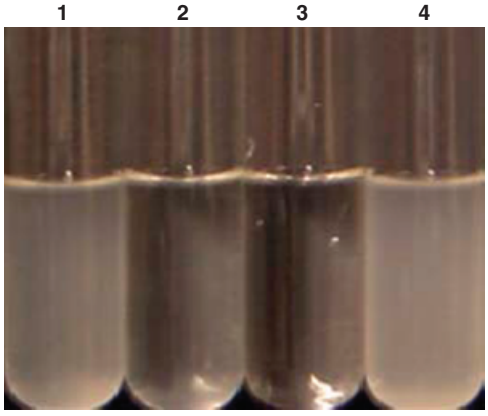

Fig. 2. Auxotrophic requirements of the double *odc/spe-sdh* mutant (81). The mutant was grown at 28 °C for 24 h in liquid MM pH 7 containing: 1, 0.5 mM spermidine (spd), 5 mM putrescine (put), and 0.2 mM lysine (lys); 2, put and lys; 3, spd and put; 4, spd and lys. All tubes contained 20 μM carboxin and 300 μg mL^{-1} hygromycin.

Expression of the chimeric *Spe-Sdh* gene

Wild-type and LV54 (*spe-sdh*) strains were grown for 19 h in MM containing 0.5 mM spermidine and 0.2 mM lysine. RNA was isolated, and Northern blots were performed using the following probes: (1) a fragment of 583 bp from the *Spe* region, obtained by digestion with XhoI/SacI of a 1102-bp PCR fragment amplified from pUmAC4 with primers 486 and 488 (Table 2) and (2) a 654-bp fragment from the *Sdh* region obtained by digestion with HindIII and EcoRI of a 2245-bp PCR fragment amplified from pUmAC4 with primers 486 and SPDS3REV (Table 2). The results obtained (Fig. 4) showed that, as expected, the LV54 mutant did not express any region of the chimeric gene. Data from the same figure also showed that the wild-type strain expressed a transcript of the same size recognized by either probe that roughly corresponded to the size of the whole ORF, evidencing that a single mRNA was expressed from the gene.

Phenotypic characterization of *spe-sdh* mutants

Growth rate

The growth of mutants LV7 and LV54 was compared with that of the FB2 wild-type strain by inoculation of decimal dilutions on plates containing spermidine and lysine, as described in Materials and methods. It was observed that the growth of both mutants was only slightly reduced in comparison with the wild-type strain (not shown).

Colonial and cellular morphology

We have previously shown that *U. maydis* displays a yeast-like growth at neutral pH, and mycelial growth at acid pH

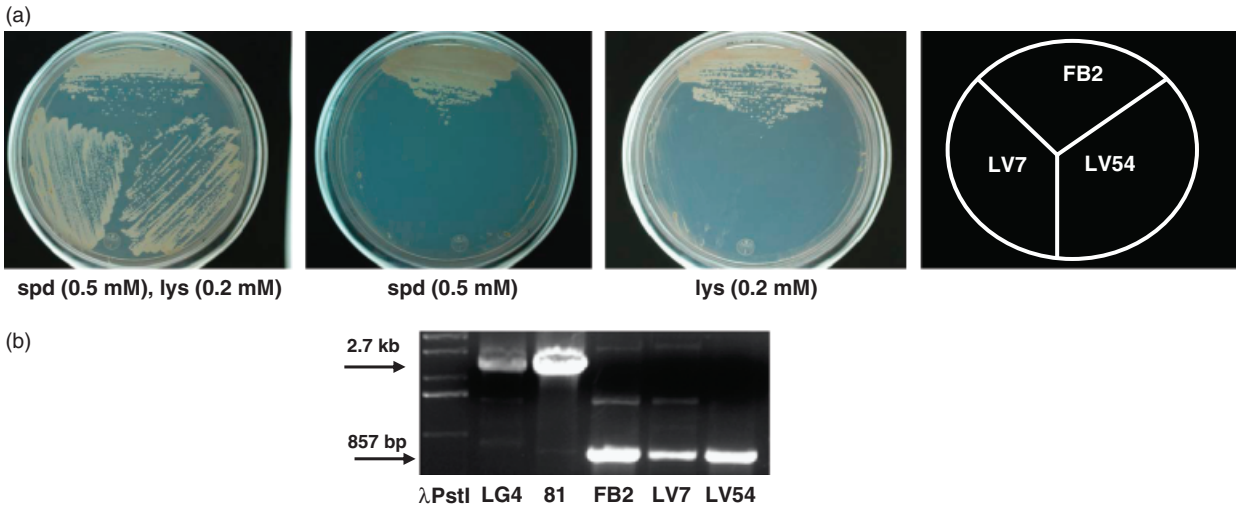

Fig. 3. Confirmation of single *spe-lys* mutants. (a) Wild-type and two *spe-lys* mutants (LV7 and LV54) were inoculated on plates of solid MM containing: 1, spd and lys; 2, spd; 3, lys, and incubated for 48 h at 28 °C. (b) PCR analysis to identify the presence of the wild-type *Odc* gene using primers 5′odc and 3′odc. A fragment of 857 bp corresponds to the wild-type gene and a fragment of 2.7 kb corresponds to the mutant gene.

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with an optimum at pH 3 (Ruiz-Herrera *et al*., 1995), and that *odc* mutants required high concentrations of putrescine to carry out their dimorphic transition at acid pH (Guevara-Olvera *et al*., 1997). We observed that *spe-sdh* mutants, in contrast to the FB2 strain, grew yeast-like at either pH 7 or 3 in liquid or solid (smooth phenotype) media containing 0.5 mM spermidine and 0.2 mM lysine (Fig. 5). Different concentrations of spermidine from 5 μM to 2 mM were unable to support mycelial growth at pH 3 (not shown).

Determination of cellular polyamines

Polyamine contents were measured by HPLC in 81 and LV54 mutants and FB2 strain, as described in Materials and methods. It was observed (Fig. 6) that either strain contained only putrescine and spermidine; no spermine was detected. This last observation agreed with our *in silico* observation that the *U. maydis* genome does not contain a gene encoding spermine synthase. It was noticeable that both double (*spe-sdh/odc*) and single (*odc*) mutants were able to form putrescine when grown in MM to which spermidine was added, despite the lack of the ornithine decarboxylase gene. The LV54 mutant (*spe-sdh*) accumulated putrescine, but only about twofold higher than the wild-type strain, when grown in the presence of 1 mM spermidine (data not shown).

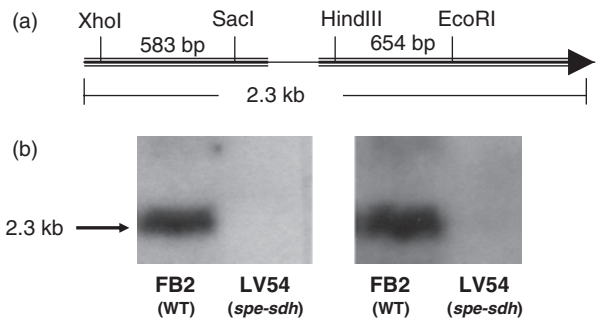

Fig. 4. Determination of *Spe-Sdh* transcription. (a) Schematic representation of the *Spe-Sdh* gene showing the relative size and location of the two fragments used as probes for hybridization. (b) Northern blot of the transcript. RNA was isolated from the wild-type strain and a single *spe-sdh* mutant (LV54), and probed with the two fragments of the *Spe-Sdh* gene schematically shown in (a), one (left) corresponding to the *Spe* region and the other one (right) to the *Sdh* region. WT, wild type.

Assays of susceptibility to stress

We tested four kinds of stress (osmotic with sorbitol, ionic with NaCl, and by SDS and Calcofluor white that affect the cell envelope) in order to further characterize the *odc/spe-sdh* and *spe U. maydis* null mutant. Growth of cells treated with 1 M KCl or 0.3 mM SDS was severely inhibited in simple and double mutants in comparison with the wild-type strain [Fig. 7(4) and (6), respectively]. The effect of a high osmotic concentration (1 M sorbitol) or 0.5% Calcofluor white on the growth of the mutants was less severe [Fig. 7(3) and (5), respectively].

Determination of virulence

Maize plantlets were inoculated with different mixtures of the mutant and wild-type strains: FB2(*a2b2*) × FB1(*a1b1*); 031(*a1b2*) × 032(*a2b1*); LV7(*a1b2*, *spe-sdh*) × LV54(*a2b1*, *spe-sdh*); LV7(*a1b2*, *spe-sdh*) × 032(*a2b1*); and LV54(*a2b1*, *spe-sdh*) × 031(*a1b2*). The results of gross symptoms are shown in Table 3 and Fig. 8. It was observed that in contrast to plants inoculated with wild-type cells, only a low number of those inoculated with sexually compatible isogenic mutants produced tumors, and these were scant and small. As expected, the *spe-sdh* mutation was recessive, because mixtures of a wild-type and an *spe-sdh* mutant were almost as virulent as the mixtures of wild-type cells. Microscopic observations of sections of the infected plants revealed that *spe-sdh* mutants were able to generate conjugation tubes (mating hyphae) *in planta*. Dikaryotic hyphae were initially able to grow on the surface of plant tissues. However, a significant difference in the proliferation between mutant and wild-type hyphae became evident during subsequent biotrophic growth. After 2–4 days postinoculation, we were able to observe extensive filament networks of wild-type

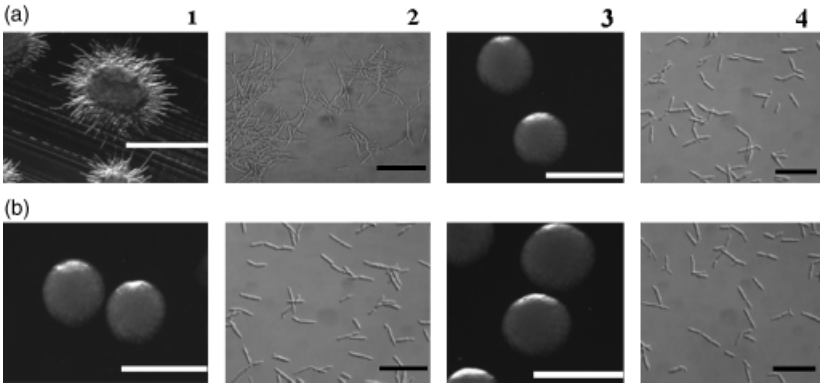

Fig. 5. Cellular and colonial morphology of wild-type and an *spe-sdh* mutant. Cells were grown in MM, pH 3 or 7, to which 0.5 mM spermidine and 0.2 mM lysine were added for the mutant. (a) FB2, wild-type strain. (b) LV54, *spe-sdh* mutant. a1 and b1, solid pH 3 medium. a2 and b2, liquid pH 3 medium. a3 and b3, solid pH 7 medium. a4 and b4, liquid pH 7 medium.

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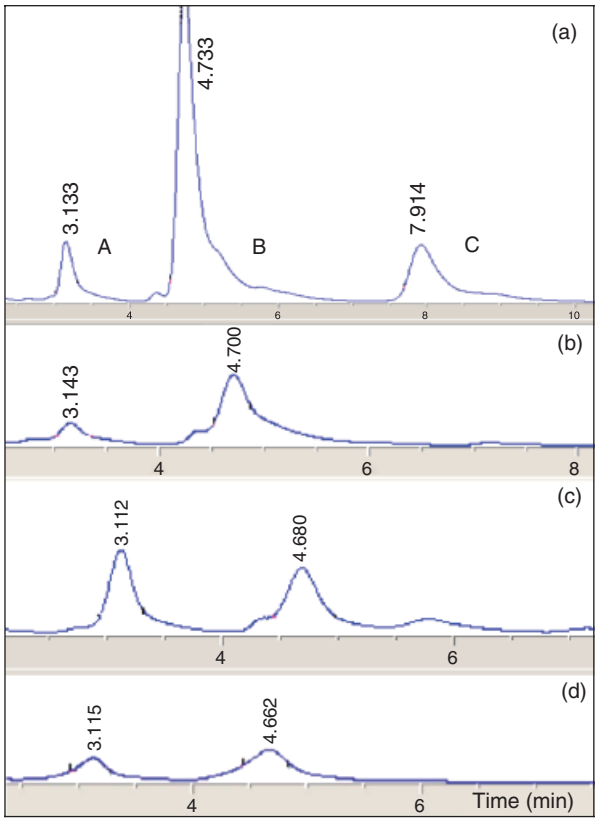

Fig. 6. Chromatographic separation of the benzoylated derivatives of polyamines. (a) Elution profile of mixture of polyamine standards. A, putrescine; B, spermidine; and C, spermine. (b) Analysis of polyamines in the wild-type strain FB2. (c) Polyamine determination in the *spe-sdh* single mutant LV54. (d) Analysis of polyamines in the 81 *odc/spe-sdh* double mutant. Elution times and profiles were taken directly from the recorder data.

hyphae in the plant tissues, followed by their proliferation and the initiation of tumor formation by the eighth day. By contrast, in plants inoculated with *spe-sdh* mutants, only a few predominantly unbranched hyphae among numerous yeast cells were detected in infected tissues only during the first 8 days postinoculation, mostly disappearing later on (data not shown). The exception were those few plants that developed small tumors where moderate accumulation of branched hyphae took place, and a single case where infection with a mixture of LV54 and LV7 strains gave rise to a large tumor (see Fig. 8b1) containing branched hyphae, but that took an unusually large time to mature.

Complementation of *spe-sdh* mutants with the wild-type gene

Strain LV54 was transformed with the whole *Spe-Sdh* gene obtained by PCR from pUmAC4 using primers AC101 and AC104 (Table 2). Transformants were recovered on solid MM without spermidine or lysine. Several prototrophic strains were recovered, and the presence of the wild-type gene was confirmed by PCR using primers 486 and 489 (Table 2). Thirteen transformants that had recovered the *Spe-Sdh* gene were identified. In five of them (LV4, LV9, LV11, and LV13), the event occurred by homologous integration at the *spe-sdh locus*, whereas in the rest (LV1, LV2, LV5, LV6, LV6a, LV8, LV10, LV12, and LV14) integration took place by ectopic recombination. These latter strains were merodiploids for the gene in question as demonstrated by PCR (not shown). Both types of transformants (*Spe-Sdh* or Δ*spe-sdh*/*Spe-Sdh)* recovered their wild-type phenotype with regard to prototrophy to spermidine and lysine, mycelial growth, and virulence (Fig. 9).

Discussion

Our results on the isolation of the gene encoding Spe in *U. maydis* revealed that, as reported for *C. neoformans* (Kingsbury *et al.*, 2004), it constitutes a chimeric gene that also encodes saccharopine dehydrogenase (Sdh). Apparently, this bifunctional gene is specific of basidiomycete fungi, because an *in silico* search for homologues gave similar results for all the basidiomycete species whose genomes have been sequenced, but not for any other eukaryotic organism belonging to different taxa. The functional advantages (if any) of having a single gene encoding both Spe and Sdh are obscure. Kingsbury *et al.* (2004) did not find evidence of any post-transcriptional regulation of the *C. neoformans* homologous bifunctional gene in response to exogenous addition or starvation of spermidine or lysine. It appears feasibly that once fusion of both genes occurred, probably after the separation of basidiomycetes from ascomycetes during evolution, the feature remained fixed in the descendants, possibly because their further separation led most likely to loss of both functions.

Disruption of the bifunctional chimeric gene (named here as *Spe-Sdh*) led to unexpected problems that were finally obviated on the hypothesis that a strain lacking *Spe* should accumulate high (and probably toxic) concentrations of putrescine, as reported for other systems. In *Neurospora crassa*, high putrescine levels were found to be toxic, an effect attributed to the interference on the accumulation of basic amino acids in the vacuole (Davis & Ristow, 1991). In other organisms, these same authors suggested that the polyamine was oxidized with the formation of a toxic aldehyde (Davis & Ristow, 1988), whereas in the cyanobacterium *Anacystis nidulans* toxicity was associated with its conjugation to ribosomes, affecting their protein-synthesizing activity (Guarino & Cohen, 1979).

That putrescine toxicity was probably the limiting factor for the isolation of *spe* mutants in *U. maydis* is suggested by the fact that mutants could be obtained in an *odc* genetic

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background. These mutants required both spermidine and lysine for growth, showing on the one hand the bifunctionality of the gene and on the other that it exists as a single copy in the genome. Our observation that the double mutants did not require putrescine for growth (irrespective of whether they were deficient in the *Odc* gene) led us to consider that putrescine was only required as a spermidine precursor.

Fungal mutants resistant to high concentrations of polyamines have been described in *N. crassa* (Davis *et al.*, 1991) and *Saccharomyces cerevisiae* (Serrano, 1991; Cunningham & Fink, 1994). These antecedents led us to consider the possibility to isolate single mutants deficient only in the *Spe-Sdh* gene, which had acquired a suppressor for the toxic effect of putrescine, a hypothesis that was confirmed when the expected single mutants were isolated by recombination of the double mutants with wild-type compatible strains during the sexual cycle carried out in maize.

According to our data, a single mRNA is synthesized from the *Spe-Sdh* gene, encoding either a single bifunctional polypeptide or two polypeptides emerging as products of post-transcriptional, translational, or post-translational modifications. The absence of both a terminal signal after the region encoding Spe and a starting codon before the region encoding Sdh favored the first hypothesis. Nevertheless, although Kingsbury *et al.* (2004) also identified a single transcript of the homologous *C. neoformans* gene, they were able to obtain *spe-lys9*, *spe3-LYS9*, and *SPE-lys9*, mutants auxotrophic to lysine and spermidine, spermidine, or lysine, respectively, suggesting that *SPE3-LYS9* encoded functional and separable spermidine synthase and saccharopine dehydrogenase gene products.

As observed in *spe* mutants from other fungi, *U. maydis* single (*spe-sdh*) mutants were auxotrophic to spermidine, but were able to grow in the absence of spermidine for three cycles, as described for *S. cerevisiae* (Balasundaram *et al.*,

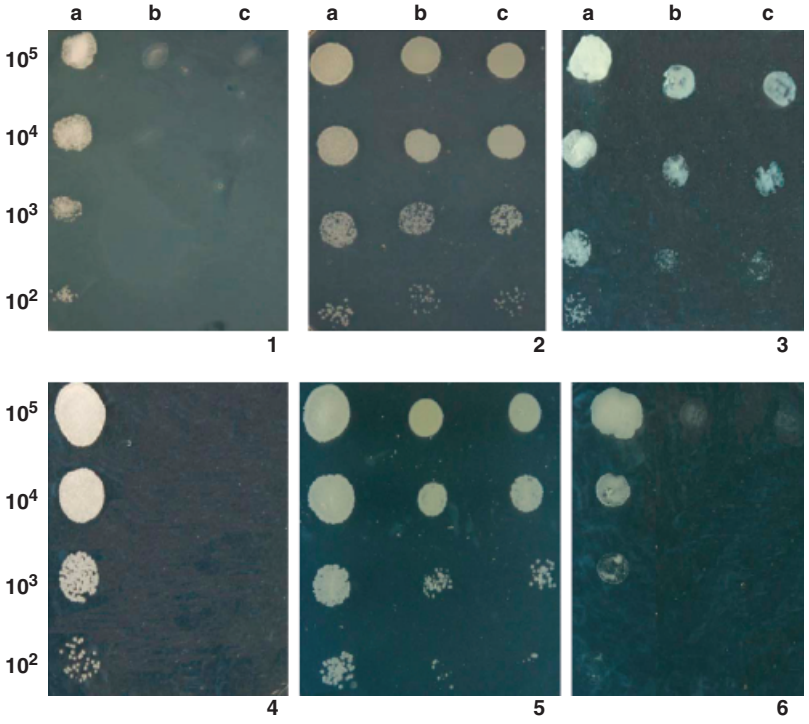

Fig. 7. Effect of different stress conditions on the growth of *Ustilago maydis*. Cells were grown for 24 h in MM containing, in the case of the mutants, 0.5 mM spermidine (spd) and 0.2 mM lysine (lys). Mutant cells were recovered by centrifugation and inoculated in MM containing 0.2 mM lysine only, for three cycles, to deplete their polyamine pools. Cell suspensions were decimally diluted and inoculated on solid MM containing 20 mM lysine in all cases, plus: 1, nothing; 2, 0.1 mM spd; 3, 0.1 mM spd and 1 M sorbitol; 4, 0.1 mM Spd plus 0.3 mM SDS; 5, 0.1 mM spd plus 0.5% Calcofluor white; and 6, 0.1 mM spd and 1 M KCl. (a) Wild-type strain FB2; (b) double *odc/spe-sdh* mutant 81; (c) single *spe-sdh* mutant LV54. Numbers of inoculated cells are indicated on the left. Plates were photographed after 48 h of incubation at 28 °C.

Table 3. Mating interactions and pathogenicity

Inoculum	Inoculated plants	Plants with tumors (%)	Plants with tumors < 1 mm	Plants with tumors > 1 mm	Plants with chlorosis (%)	Plants with anthocyanins (%)
FB1/FB2	59	49/59 (83%)	4/49	45/49	40/59 (68%)	32/59 (54%)
031/032	39	28/39 (71%)	2/28	26/28	24/39 (61%)	17/39 (44%)
LV7/032	72	60/72 (83%)	5/60	55/60	60/72 (83%)	38/72 (53%)
LV54/031	72	45/72 (65%)	4/45	41/45	38/72 (53%)	30/72 (42%)
LV54/LV7	73	15/73 (20%)	14/15	1/15	29/73 (40%)	11/73 (15%)

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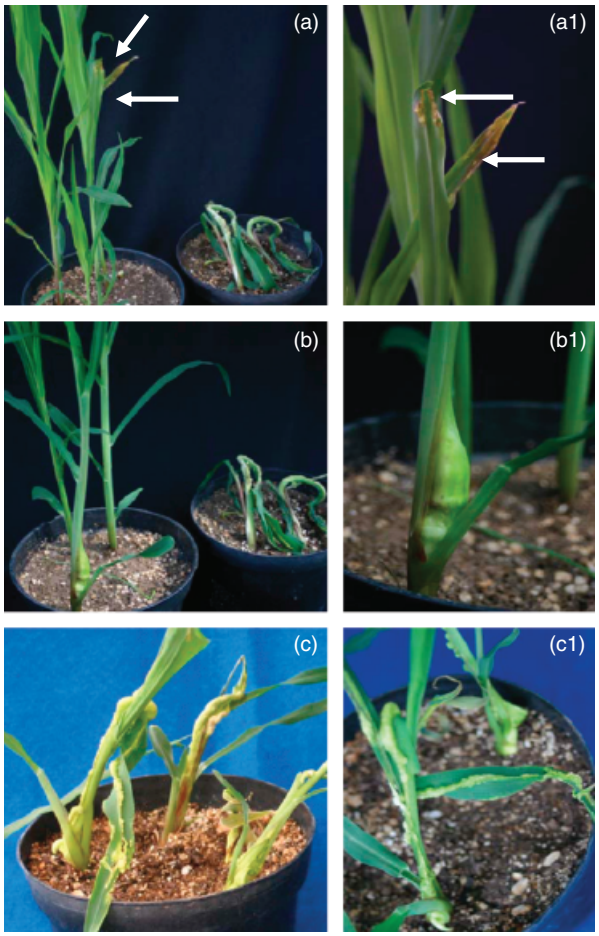

Fig. 8. Infection symptoms of maize plants inoculated with mixtures of different sexually compatible *Ustilago maydis* strains. (a and b) The pot to the left, plants inoculated with LV54(*a2b1*, *spe-sdh*) × LV7(*a1b2*, *spe-sdh*); the pot to the right, plants inoculated with FB1(*a1b1*)*wt* × FB2(*a2b2*)*wt*. (a1 and b1), closer view of tumors that developed in plants inoculated with mutant strains. (c) Plants inoculated with LV7(*a1b2*)*spe-sdh* × 032(*a2b1*)*wt*. (c1) Plants inoculated with 031 (*a1b2*)*wt* × 032 (*a2b1*)*wt*.

1991) and *C. albicans* (Herrero *et al.*, 1999), probably because they accumulate polyamine pools in the vacuole (Davis & Ristow, 1991), cytosol, and/or nuclei (Davis *et al.*, 1992).

Of particular importance was the observation that double mutants did not accumulate high concentrations of putrescine, suggesting the existence of a mechanism that controlled its cellular levels under conditions that avoided its transformation into spermidine. The unexpected observation that double *odc/spe-sdh* mutants contained putrescine suggests that this is synthesized by the conversion of part of the spermidine added for the growth of the mutants into putrescine as described in other eukaryotic cells (Pegg, 1986). This suggestion is supported by *in silico* data that reveal the existence of several genes with homology to genes encoding polyamine acetyl transferase, and one with homology to a gene encoding a polyamine oxidase (Pao). It is known that these enzymes participate in the transformation of spermidine into putrescine, a reaction suggested to be involved in the homeostatic regulation of cell polyamines (Seiler, 1995). This result leaves still unanswered the possibility that putrescine may play a specific role in cell physiology, other than merely being an intermediate in the synthesis of spermidine. Isolation of *odc/pao* double mutants might be a useful approach to answer this question.

The sensitivity of *spe-sdh* or *odc/spe-sdh* mutants to different stress demonstrates a role of spermidine in the protection of the fungus to different deleterious environmental factors. A role of polyamines in plant and bacteria protection to stress has been known for a long time (e.g. Flores & Galston, 1982; Schiller *et al.*, 2000; Jung & Kim, 2003). Although the mechanism for this effect is not yet clear, possibly it may be related to the activation of the transduction pathways responsive to stress conditions or the synthesis of the responsive elements.

There are reported evidences about the role of polyamines in pathogenesis, in some cases suggesting that they might be adequate targets to control different infections. Examples of this are the observation that *Agrobacterium tumefaciens* polyamines play a role in the induction of the *vir* gene and T-DNA transfer (Vinod-Kumar & Rajam, 2005), the demonstration that a membrane lipoprotein, which is a polyamine-binding component and a polyamine transporter in *Treponema pallidum*, constitutes a virulence factor (Machius *et al.*, 2007), and the demonstration that *Trichomonas vaginalis* cytotoxicity was decreased by treatment with the Odc inhibitor diaminobutanone (Alvarez-Sánchez *et al.*, 2008). In the specific case of phytopathogenic fungi, contrasting results have been published. *Uromyces viciae-fabae* appressoria formation was inhibited by the Odc inhibitor difluoro methyl ornithine, while conidial germination remained unaffected (Reitz *et al.*, 1995); *Septoria nodorum odc* mutants were almost avirulent to wheat (Bailey *et al.*, 2000), and we observed that *U. maydis odc* mutants were completely avirulent to maize (unpublished data). In contrast, *odc* mutants of *Tapesia yallundae* showed a reduction in infection plate formation *in vitro*, but were not reduced in their virulence to wheat (Mueller *et al.*, 2001). It is probable that the different behavior of these fungal mutants (*spe-sdh* mutants included) reveals the different capacities of the pathogens to obtain polyamines from their hosts.

The observation that *spe-sdh* mutants were unable to grow in the mycelial form at pH 3 confirms the polyamine requirements for dimorphism described previously for *odc* mutants (Guevara-Olvera *et al.*, 1997), but in contrast to those mutants, addition of high polyamine concentrations did not revert the phenotype. This behavior is difficult to

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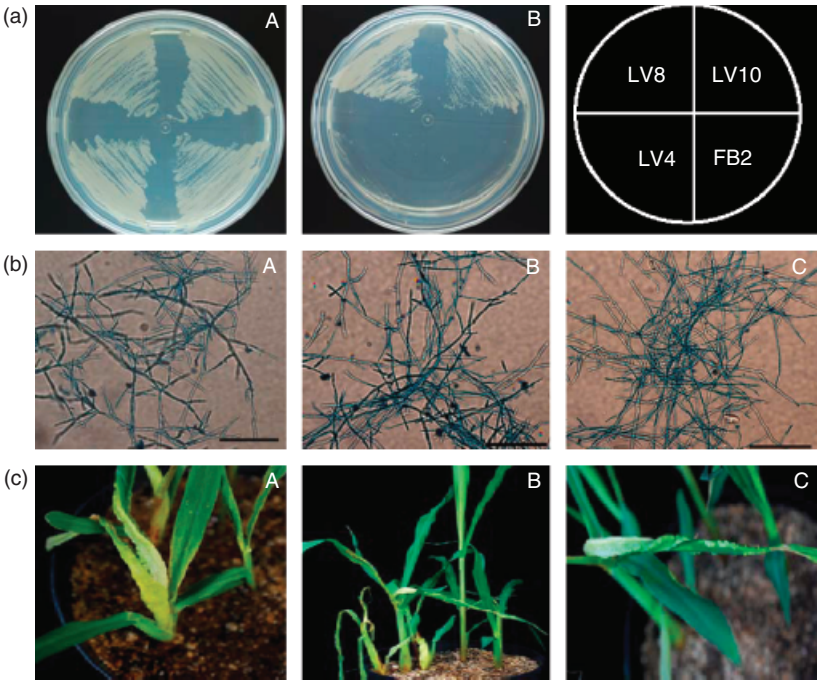

Fig. 9. Complementation of *spe-sdh* mutants with the *wt Spe-Sdh* gene. (a) Growth characteristics. A, MM without additions; B, MM with 20 mM carboxin. LV10 and LV8, Δ*spe-sdh*/*Spe-Sdh* transformants; LV4, *Spe-Sdh* transformant. FB2, wild-type strain. (b) Mycelial growth (lactophenol blue staining) of strains cultivated in pH 3 MM (magnification bar, 50 μm). A, FB2; B, LV4; C, LV8. (c) Virulence in maize plantlets. A, plants infected with FB1(*a1b1*) × FB2(*a2b2*); B, plants infected with LV4(*a2b1*, *Spe-Sdh*) × LV7(*a1b2*, Δ*spe-sdh*); C, LV8(*a2b1*, *Spe-Sdh*) × LV7(*a1b2*, Δ*spe-sdh*).

explain, but the observation that complementation of the mutation by the native gene reverted the phenotype demonstrates that the alteration observed is solely due to mutation of the specific gene. Because auxotrophy of the mutants was satisfied by the addition of exogenous spermidine, it is tempting to suggest that endogenous formation of the polyamine leads to a cellular distribution of spermidine different from that obtained by its exogenous addition. In *Mucor rouxii*, the existence of three different pools of Odc and polyamines that displayed different behavior towards the addition of an exogenous substrate or Odc inhibitors was described (Martínez-Pacheco & Ruiz-Herrera, 1993).

In summary, our data reveal that *U. maydis* Spe is encoded by a chimeric gene specific of basidiomycetes, which also encodes Sdh, and is expressed in the form of a single transcript that gives rise to one or separate functional proteins. We also demonstrated a specific role for spermidine in the growth of *U. maydis*, and its requirement in dimorphism and virulence. Whether putrescine also plays a specific role, or is only an intermediate in spermidine synthesis, remains an open question on whose answer we are now engaged.

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