Phenotypic comparison of *samdc* and *spe* mutants reveals complex relationships of polyamine metabolism in *Ustilago maydis*

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Synthesis of spermidine involves the action of two enzymes, spermidine synthase (Spe) and *S*-adenosylmethionine decarboxylase (Samdc). Previously we cloned and disrupted the gene encoding Spe as a first approach to unravel the biological function of spermidine in *Ustilago maydis*. With this background, the present study was designed to provide a better understanding of the role played by Samdc in the regulation of the synthesis of this polyamine. With this aim we proceeded to isolate and delete the gene encoding Samdc from *U. maydis*, and made a comparative analysis of the phenotypes of *samdc* and *spe* mutants. Both *spe* and *samdc* mutants behaved as spermidine auxotrophs, and were more sensitive than the wild-type strain to different stress conditions. However, the two mutants displayed significant differences: in contrast to *spe* mutants, *samdc* mutants were more sensitive to LiCl stress, high spermidine concentrations counteracted their dimorphic deficiency, and they were completely avirulent. It is suggested that these differences are possibly related to differences in exogenous spermidine uptake or the differential location of the respective enzymes in the cell. Alternatively, since *samdc* mutants accumulate higher levels of *S*-adenosylmethionine (SAM), whereas *spe* mutants accumulate decarboxylated SAM, the known opposite roles of these metabolites in the processes of methylation and differentiation offer an additional attractive hypothesis to explain the phenotypic differences of the two mutants, and provide insights into the additional roles of polyamine metabolism in the physiology of the cell.

INTRODUCTION

Polyamines are organic polycations required by all living organisms (Pegg & McCann, 1982; Tabor & Tabor, 1984, 1985; Cohen, 1998). They have drawn interest because they are essential for cell growth and differentiation, one model of which is fungal dimorphism, which provides a useful system to study their role (Ruiz-Herrera & Calvo-Méndez, 1987; Ruiz-Herrera, 1993, 1994; Guevara-Olvera *et al.*, 1993; Herrero *et al.*, 1999; Jiménez-Bremont *et al.*, 2001; Blasco *et al.*, 2002). The most common polyamines in eukaryotes are putrescine, spermidine and spermine, but some fungi lack spermine, and contain only putrescine and spermidine (Nickerson *et al.*, 1977; Valdés-Santiago *et al.*,

2009). Putrescine, the smallest of the polyamines and precursor of the others, is the result of decarboxylation by ornithine decarboxylase (ODC). In *Ustilago maydis*, we observed that *odc* mutants were unable to carry out the pH-dependent dimorphic transition, even using concentrations of putrescine that were high enough to satisfy their growth requirements (Guevara-Olvera *et al.*, 1997). A similar behaviour was displayed by *Yarrowia lipolytica* (Jiménez-Bremont *et al.*, 2001) and *Candida albicans* (Herrero *et al.*, 1999) *odc* mutants. These mutants were able to carry out the yeast-to-mycelium dimorphic transition only in the presence of an exceedingly high concentration of putrescine. These results clearly demonstrated the role of polyamines in fungal differentiation, but they failed to identify the polyamine(s) involved in the process. This question was duly resolved in *U. maydis* by analysis of mutants that lack putrescine (Valdés-Santiago *et al.*, 2010), demonstrating that spermidine is the important

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polyamine required for *U. maydis* dimorphism. Synthesis of this polyamine requires the action of two enzymes, spermidine synthase (Spe) and *S*-adenosylmethionine decarboxylase (Samdc). The latter enzyme is responsible for the decarboxylation of *S*-adenosylmethionine (SAM) with formation of decarboxylated SAM (dcSAM), which serves as donor of a propylamine group to putrescine in a reaction catalysed by Spe (Pegg & McCann, 1982).

It is known that Samdc is synthesized as a proenzyme that subsequently undergoes an intramolecular cleavage at a serine residue to generate two non-identical subunits termed α and β, both of which are indispensable components of the mature enzyme (Pegg, 1986; Stanley, 1995). Genes encoding Samdc have been cloned from several organisms, including *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Leishmania donovani*, *Neurospora crassa* and mouse (Cohn *et al.*, 1978; Da'dara & Walter, 1998; Hoyt *et al.*, 2000; Roberts *et al.*, 2002; Nishimura *et al.*, 2002). Samdc is regulated by both putrescine, which increases the levels of enzyme activity, and spermidine, which reduces them. In fact, the intracellular levels of polyamines influence *SAMDC* expression at multiple steps, including transcription, translation, protein half-life and, as indicated, enzymic activity (Shantz *et al.*, 1992; Stanley & Pegg, 1991; Pegg *et al.*, 1998). Additionally, the study of *samdc* mutants in the systems mentioned above has demonstrated the essential role of spermidine. However, despite the characterization of some *samdc* mutants, little is known regarding the existence of differences in their phenotypic characteristics from mutants deficient in the *SPE* gene.

Besides this multiplicity, gene disruption studies have demonstrated that spermidine is essential for vegetative growth and differentiation, while putrescine is only the precursor of higher polyamines and appears to have a minor role in the stress response and/or virulence (Chattopadhyay *et al.*, 2008; Valdés-Santiago *et al.*, 2010). One specific function of spermidine is to serve as precursor of the translation initiation factor eIF-5A, although the precise mechanism of action at the molecular level is mostly unknown (Schnier *et al.*, 1991; Zanelli & Valentini, 2007).

U. maydis, a plant-pathogenic Basidiomycota fungus, is an excellent model for the study of different biological phenomena, such as fungal phytopathogenicity, DNA recombination and repair, long-distance transport in hyphal growth, mitosis, and dimorphism (Holliday, 1985; Sánchez-Martínez & Pérez-Martín, 2001; Bölker, 2001; Basse & Steinberg, 2004; Klosterman *et al.*, 2007; Steinberg & Pérez-Martín, 2008). It is also a model to understand polyamine functions, considering that it contains only two polyamines, putrescine and spermidine (Guevara-Olvera *et al.*, 1997, Valdés-Santiago *et al.*, 2009, 2010). *U. maydis* has a sexual cycle that is easy to reproduce in the laboratory or greenhouse, possesses an efficient transformation system, and there are accessible dominant selection markers that

provide the basis for gene replacement (Tsukuda *et al.*, 1988; Fotheringham & Holloman, 1990). In addition, its genome has been sequenced and annotated (Kämper *et al.*, 2006).

Previously we reported the isolation and phenotype of *U. maydis spe* mutants (Valdés-Santiago *et al.*, 2009), and in this communication we describe the isolation and mutation of the *SAMDC* gene, which permitted the determination of the similarities and differences that exist between the phenotypic behaviour of *samdc* and that of the previously obtained *spe* mutants.

METHODS

Strains and growth conditions. *U. maydis* haploid strains (Table 1) were maintained at $-80\ ^{\circ}\mathrm{C}$ in liquid complete medium (CM; Holliday, 1961) supplemented with 50 % glycerol, and were recovered on solid CM plates and incubated at 28 $^{\circ}\mathrm{C}$. *U. maydis* mutant strains were recovered on minimal medium (MM; Holliday, 1961) supplemented with 5 mM putrescine and/or 0.5 mM spermidine (Sigma-Aldrich), and 300 μg hygromycin B ml^{-1} (Calbiochem) and/or 20 mM carboxin. *spe* mutants were supplemented with 0.2 mM lysine (Valdés-Santiago *et al.*, 2009). Yeast or mycelial cultures were obtained as described by Ruiz-Herrera *et al.* (1995). Cell dry weight was measured after drying overnight at 65 $^{\circ}\mathrm{C}$. Protoplasts were prepared with lytic enzymes from *Trichoderma harzianum* (Sigma-Aldrich) as described by Tsukuda *et al.* (1988). *Escherichia coli* transformation was performed by standard procedures (Sambrook & Russell, 2001).

Nucleic acid manipulation. Isolation of genomic DNA was conducted as reported by Hoffman & Winston (1987). PCR was carried out using *Taq* DNA polymerase or, when required, PCR SuperMix High Fidelity (Invitrogen). Vector dephosphorylation, ligation and DNA digestion were done according to manufacturer's instructions (Invitrogen). DNA sequencing reactions were performed using an ABI PRISM 377 DNA automated sequencer (Perkin Elmer) with dsDNA as template, and primers M13F and M13R (Invitrogen) or other gene-specific primers (Table 2). Northern analyses were performed as described by Sambrook & Russell (2001). A ^{32}P-labelled 1 kb *Eco*RI–*Hin*dIII *samdc* gene fragment (see below) was used as hybridization probe.

Plasmid constructs. To delete the gene encoding *U. maydis* Samdc (*SAMDC*), plasmid pΔ*samdc* was constructed. Briefly, the full gene including its 5′ and 3′ flanking sequences was amplified by PCR with primers Samdc5 and Samdc3 (Table 2) using genomic DNA from *U. maydis* strain FB2 (Table 1) as template. The PCR product was cloned into plasmid pUC13, and the *Eco*RI–*Hin*dIII ORF fragment of the *SAMDC* gene was replaced with the carboxin-resistant cassette from plasmid pCBX-AC2 (Valdés-Santiago *et al.*, 2009).

To complement *U. maydis samdc* mutants, a plasmid was constructed as follows. The full *SAMDC* gene including its promoter and terminator was PCR-amplified using primers Samdc5 and Samdc3. The PCR product (3.1 kb) was cloned into plasmid pCR2.1 (Invitrogen), generating plasmid pSAMDC. Next, the *SAMDC* gene was recovered as a *Bam*HI–*Not*I fragment and cloned into the same sites of the episomal plasmid pHyg101 (Mayorga & Gold, 1998), generating plasmid pSAMDCHyg-20, which was used to transform protoplasts of *samdc*::Cbx^{R} mutant strains. The presence of the *SAMDC* gene sequence in transformants was confirmed by PCR analysis using a primer pair specific to the gene (data not shown). All constructs used were confirmed by DNA sequencing.

Table 1. Strains used in this study

Strain	Relevant genotype	Reference or source
U. maydis **strains**		
FB2	*a2b2*	Banuett & Herskowitz (1989)
FB1	*a1b1*	Banuett & Herskowitz (1989)
samdc6	*a2b2 Δodc/samdc*::Hyg^R/Cbx^R	This study
samdc7	*a2b2 Δodc/samdc*::Hyg^R/Cbx^R	This study
5-11	*a1b1 Δsamdc*::Cbx^R	This study
LV71	*a2b2 Δsamdc*::Cbx^R	This study
4*samdc*R	*a2b2 Δsamdc*::Cbx^R/pLV20	This study
7*samdc*R	*a2b2 Δsamdc*::Cbx^R/pLV20	This study
11*samdc*R	*a2b2 Δsamdc*::Cbx^R/pLV20	This study
LG4	*a2b2 Δodc*::Hyg^R	Guevara-Olvera *et al.* (1997)
LV54	*a2b1 Δspe-sdh*::Cbx^R	Valdés-Santiago *et al.* (2009)
LV7	*a1b2 Δspe-sdh*::Cbx^R	Valdés-Santiago *et al.* (2009)
E. coli **strain**		
DH5α	F′	Invitrogen

Mating analysis. Mating was analysed by the 'fuz' reaction (Banuett, 1992, 1995). Briefly, drops of suspensions of the mating strains to be tested were placed one over the other on charcoal-MM agar plates with the required additions, and incubated overnight at 25 °C. The plates were checked for the presence of aerial hyphae, which give the colony a white, fuzzy appearance indicative that the strains are sexually compatible.

Stress assays. To determine the sensitivity of *U. maydis* to different compounds, decimal dilutions of cell suspensions were inoculated on plates of solid media amended with the compound to be tested, and growth was assessed as described previously (Valdés-Santiago *et al.*, 2009).

Virulence assays. These were performed as previously described (Martínez-Espinoza *et al.*, 1997). Briefly, 10 day-old seedlings of maize cv cacahuazintle were inoculated using a syringe and needle with a mixture of sexually compatible strains. The plants were kept in a greenhouse, and symptoms were recorded for 15 days after inoculation.

Isolation of segregants from inoculated plants. Teliospores produced in the tumours induced in maize plants inoculated with sexually compatible *U. maydis* strains were suspended in 1.5 % CuSO$_4$ for 2 h to kill vegetative cells, filtered through cheesecloth, washed twice with sterile distilled water, recovered by centrifugation and plated on solid CM. After 12–18 h, the sporidia formed by germination of teliospores were recovered by washing the plates with sterile distilled water, and inoculated on plates containing hygromycin B and/or carboxin to determine their phenotype (Chavez-

Ontiveros *et al.*, 2000). Mating type (fuz reaction, see above) and auxotrophy to spermidine were tested in segregants thus obtained. The presence of the wild-type or the disrupted *SAMDC* gene was analysed by PCR using primer pair pCBX and AC110, and the presence of the wild-type or mutant *ODC* gene was determined by PCR according to Valdés-Santiago *et al.* (2009) using primers 5′odc and 3′odc.

Determination of SAM and dcSAM levels. *U. maydis* cells were grown in liquid MM with addition of 0.1 mM spermidine or other requirements (see Methods and the legend to Fig. 6) for 48 h, harvested by centrifugation, washed twice with sterile distilled water, suspended in 6 % perchloric acid (1 ml) for 1 h at room temperature and recovered by centrifugation (Shobayashi *et al.*, 2006), and the supernatants were subjected to MS analysis. MS measurements were carried out on a Micromass ZQ 2000 Quadrupole instrument with MassLynx 4.0 as control software. Quantification was performed using electron spray ionization (ESI) in positive mode. The capillary voltage was set to 3 kV, the cone voltage to 60 V and the extractor voltage to 3 V. The RF lens was left at 0. A source temperature of 100 °C and a temperature of 350 °C were used at a desolvation gas flow of 250 l h^{-1} and a cone gas flow of 20 l h^{-1}. In the Analyzer section, LM and HM resolution of 0.15 and an ion energy of 0.5 were set. The multiplier was adjusted to a value of 650. The samples were directly injected with a flow rate of 10 μl min^{-1}. Continuous spectra were collected in the range of 15–2000 *m/z*, with a run duration of 1 min, a scan time of 10 s and an inter-scan time of 0.1 s. MassLynx raw spectra were converted to mzXML data format using MassWolf. The further analysis of mass spectra was performed using the OpenMS/TOPP suite, version 1.7.0 (1, 2). First, a pipeline was written [1] for TOPPAS, executing the following tasks for all spectra: file conversion to mzML, merging all spectra of a sample, NoiseFilter sgolay with a frame length of 21 and polynomial order of 4, followed by a PeakPicker with a signal to noise of 1 and a peak width of 0.15. Subsequently, the corresponding peak data were extracted manually from the processed spectra. Protonized SAM has a monoisotopic weight of 399.145 *m/z*. Peaks of this *m/z* were evaluated, with a mass tolerance of 0.3 *m/z*. The corresponding peak intensities were transferred to Microsoft Excel for further evaluation. A calibration curve for SAM in the range between 5 and 50 μM gave a correlation coefficient, R^2, of 0.9961. Based on the signal-to-noise ratios of calibrants and samples, the detection limit was estimated to be about 1 μM SAM in solution; based on the dry weight and the extraction volume from the respective experiment, the SAM content of the fungi

Table 2. Primers used in this study

Primer	Sequence (5′–3′)	Orientation
Samdc5	GTACGTTGACTCGTGACTGTG	Forward
Samdc3	ACATGCATAGCACAGGCGAAC	Reverse
pCBX	GACCTGCCCCAAGAACCTCAACCCTG	Forward
AC110	CGCACCGGGAGTCAAGCCAGAGAAAG	Reverse
5′odc	CAACATGGACGAGCTCGAAAAGAT	Forward
3′odc	GTAAGCGCCCATGTTTTCGTAGAC	Reverse

was calculated. For statistical analyses and graph drawing, SOFA Statistics 0.9.20 and R version 2.13.1 were employed. In the case of dcSAM, the corresponding peak data were extracted manually from the processed spectra. Protonized dcSAM has a monoisotopic mass of 355.155 m/z. Peaks of this m/z were evaluated, with a mass tolerance of 0.3 m/z. The corresponding peak intensities were transferred to Excel for further evaluation. The mean $\pm$ SEM of data corresponding to triplicates from six different SAM and dcSAM samples are reported.

RESULTS

Identification and cloning of the *U. maydis* SAMDC gene

The *U. maydis* gene encoding *S*-adenosylmethionine decarboxylase (Samdc) was identified in the *U. maydis* genome database at the Broad Institute (http://www.broadinstitute.org/annotation/genome/ustilago_maydis/Home.html), corroborated at the Munich Information Center for Protein Sequences (MIPS) (http://mips.helmholtz-muenchen.de/genre/proj/ustilago/), and, finally, the BLASTX algorithm (Altschul *et al.*, 1997) was used to search the databases with fungal Samdc proenzymes as queries: *C. albicans* (EEQ46120), *S. cerevisiae* (NP_014590) and *Y. lipolytica* (XP_504183). In this way the putative *U. maydis* SAMDC gene, corresponding to the annotation number Um10792 at MIPS, was identified. This gene showed no introns and encoded a predicted ORF of 556 aa in length, with the characteristic Samdc domain spanning almost the full sequence. Its degree of identity with enzymes from *Malazzesia globosa*, *Schizophyllum commune* and *Coprinopsis cinerea* was of the order of 40 % at the amino acid level. The *U. maydis* protein has a higher M_r than homologues from some Ascomycota species. Thus, Samdc proteins from *N. crassa* and *S. cerevisiae* contain 503 and 396 aa, respectively (Hoyt *et al.*, 2000). However, despite these differences, the amino acid residues predicted to be important for the proenzyme cleavage and formation of the pyruvoyl group (during the maturation process of Samdc, a specific serine residue is converted to a pyruvoyl residue), AYLLSESSMF, are conserved in all of them (Fig. 1, arrow). With this information, specific primers were designed, and the whole *U. maydis* gene with the estimated promoter and terminator elements was amplified by PCR, cloned into an episomal pHyg101 plasmid, and sequenced.

Disruption of the SAMDC gene using an *odc* mutant as a recipient strain

Essentially we followed the method of Fotheringham & Holloman (1989). The disruption cassette from plasmid pΔ*samdc* (4.5 kb) was PCR-amplified with primers Samdc5 and Samdc3, and the fragment was used to transform protoplasts from the *U. maydis* LG4 *odc* mutant (Table 1), as indicated above. Putative transformants resistant to carboxin and hygromycin B were recovered on solid MM medium, pH 7, with added sorbitol and other requirements (5 mM putrescine and 0.5 mM sper-

midine), and confirmed by PCR-based screening using primers pCBX and pAC110 (Table 2), where the expected PCR product of ~1.5 kb was amplified (not shown). Auxotrophy to polyamines was confirmed (see below), and *odc/samdc* double mutants samdc6 and samdc7 were selected for further experiments.

Auxotrophic requirements of the *odc/samdc* double mutants

Taking into consideration that the SAMDC gene encodes an enzyme essential for spermidine synthesis, we expected that *odc/samdc* double mutants would require the enzyme to grow. Mutants were able to grow on two subcultures without polyamines, after which their polyamine pools were exhausted and they failed to grow in media without polyamines, although they grew in the presence of 0.1 or 0.5 mM spermidine at a rate comparable with that of the wild-type (results not shown). Although *odc/samdc* mutants were unable to produce putrescine through the ODC pathway, the spermidine acetylase-oxidase route (Valdés-Santiago *et al.*, 2009, 2010) provided enough of this polyamine to cover their requirements (Fig. 2).

Isolation of *samdc* single mutants by sexual recombination *in planta*

Using sexual recombination *in planta* between an *a2b2 odc/samdc* double mutant and the FB1 wild-type strain (*a1b1*), it was possible to isolate a set of single *samdc* mutants, selecting strains 5-11 (*samdc*::Cbx^R *a1b1*) and LV71 (*samdc*::Cbx^R *a2b2*) to conduct further studies. Mutants were confirmed by Northern analysis (results not shown).

Complementation of *samdc* mutants

Through transformation of a *samdc* mutant with a plasmid containing a functional copy of the SAMDC gene, it was possible to obtain SAMDC revertant strains (4*samdc*R, 11*samdc*R, 7*samdc*R) resistant to carboxin and hygromicin. The presence of the SAMDC gene in these strains restored the capacity to grow in the absence of spermidine.

Effect of different stress conditions on *samdc* mutants

The effect of 10 mM LiCl, 3 mM H_2O_2, different concentrations of menadione, 0.005 or 0.05 mM Rose Bengal (RB), 0.2 or 0.7 mM ascorbic acid, 1 M sorbitol, 0.5 M $CaCl_2$ or 1 M NaCl on cell growth was assayed as described in Methods. Polyamine pools of the *U. maydis* mutants were depleted by subculturing twice in polyamine-free medium, followed by inoculation on plates supplemented with 0.1 mM spermidine. In the absence of inhibitors only slightly reduced growth rates were observed for 5-11 (*samdc*) and LV54 (*spe*) mutants as compared with the FB2 wild-type (control) and 4*samdc*R (revertant) strains (Fig. 3a). On the other hand, under some stress
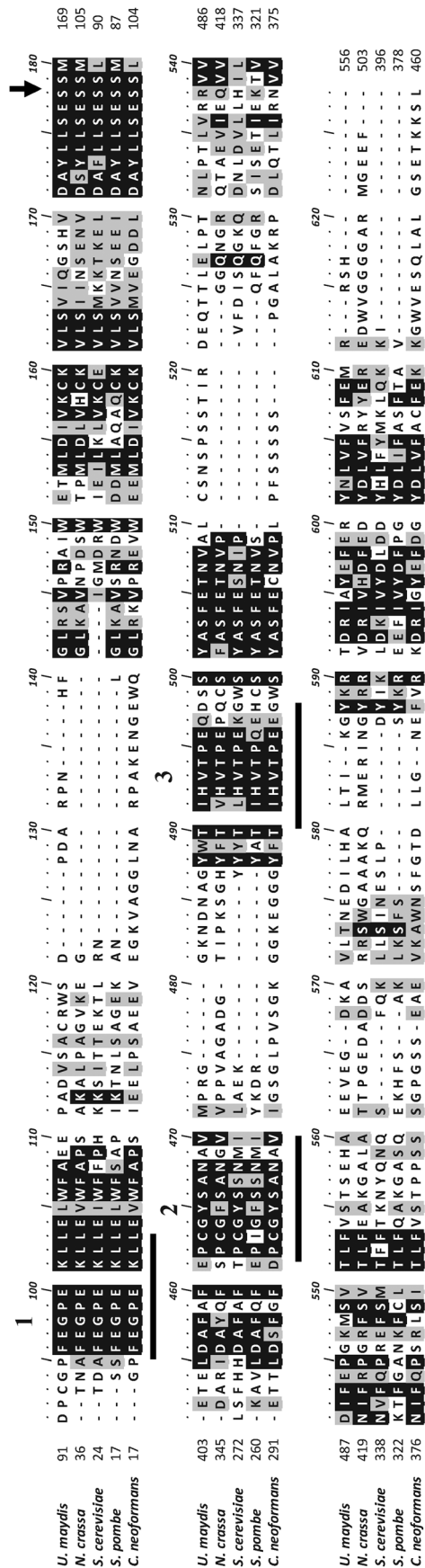
Fig. 1. Alignment of Samdc proenzyme sequences from *U. maydis* with those of several Basidiomycota and one Ascomycota fungi. The amino acid sequences were aligned by the CLUSTAL W method. Amino acid residue numbers refer to ***. Residues that are identical in *U. maydis* Samdc and in two other proenzymes are shaded in black, while less conserved positions are shaded in grey. Important amino acids involved in proenzyme cleavage and formation of the pyruvoyl group are indicated with an arrow. Other important motifs of the enzyme are indicated by black bars. For the significance of 1, 2 and 3, refer to the Discussion.

conditions, mutants showed growth impairment. Both mutants were completely inhibited by 1 M NaCl, but in contrast, no effect was observed on wild-type FB2 or complemented 4samdcR strains (Fig. 3b). Interestingly, Li$^+$ addition resulted in growth inhibition only of the *samdc* and not of the *spe* mutant, FB2 or the complemented strain (Fig. 3c). H$_2$O$_2$ (3 mM) or 0.05 mM menadione completely inhibited the growth of the mutants and that of the wild-type and complemented strains almost completely (Fig. 3d, e). Concentrations of H$_2$O$_2$ or menadione higher than 3 or 0.05 mM, respectively, completely inhibited the growth of all strains (results not shown). Rose Bengal (0.05 mM) had no effect on the wild-type and complemented strains, and was barely inhibitory for the mutants, the *samdc* mutant being slightly more sensitive (Fig. 3f). Other concentrations tested were equally inhibitory for all strains (results not shown). Other tested compounds, sorbitol, CaCl$_2$ and ascorbic acid, affected the growth of the wild-type, revertant and mutant strains to the same extent (results not shown).

Dimorphic transition induced by acid pH

U. maydis grows in the yeast form at neutral pH, and in the hyphal form at acid pH (Ruiz-Herrera *et al.*, 1995). Previously, it was demonstrated that *odc* mutants were unable to carry out the dimorphic transition, unless cultivated at high putrescine concentrations (Guevara-Olvera *et al.*, 1997). To determine whether spermidine-less mutants behaved in the same way, we carried out similar experiments with *samdc* mutant 5-11 and *spe* mutant LV54, and the results are shown in Fig. 4. It was observed that low spermidine concentrations (5 µM) sustained vegetative growth of both mutants (*samdc* or *spe*), but addition of a higher concentration of spermidine (0.1 mM) was necessary to induce the dimorphic switch of the *samdc* mutant only, having no effect on the cell morphology of the *spe* mutant. As expected, the wild-type strain and the revertant grew in the mycelial form in pH 3 medium in the absence of spermidine.

Mating analysis

We observed a concentration-dependent effect of spermidine on mating of homologous strains of both types of mutants: 5-11 (*a1b1 Δsamdc::*Cbx^R) × LV71 (*a2b2 Δsamdc::*Cbx^R) and LV54 (*a2b1 Δspe::*Cbx^R) × LV7

0.5 mM Putrescine   MM7

0.5 mM Spermidine

samdc7 (odc/samdc)
samdc6 (odc/samdc)
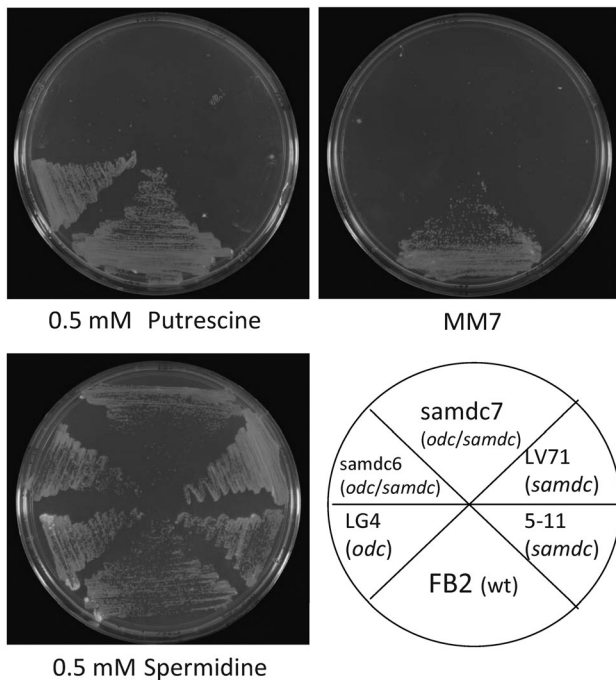LV71 (samdc)
LG4 (odc)
5-11 (samdc)
FB2 (wt)

Fig. 2. Auxotrophic requirements of *odc/samdc*, *samdc* and *odc* mutants. Strains were grown at 28 °C for 48 h on plates of solid pH 7 MM containing or not containing the indicated polyamines. Wt, Wild-type.

$(a1b2 \, \Delta spe::Cbx^R)$. In both cases, the intensity of dikaryon formation increased, as revealed by the appearance of white fuzzy filamentous colonies as we raised the concentration of spermidine (see Fig. 5). Nevertheless, visually, *samdc* mutant crosses did not attain the filamentous appearance of wild-type strains, even at the highest spermidine concentrations used, while *spe* mutant crosses behaved as the wild-type cells at 0.5 mM spermidine (Fig. 5).

Virulence studies

In contrast to *spe* mutants, which generate tumours in about 20 % of infected maize plants (Valdés-Santiago *et al.*, 2009), *samdc* mutants proved to be completely avirulent to maize plants: out of a total of 128 plants inoculated with a mixture of 5-11 and LV71 *samdc* sexually compatible mutants, not a single one developed tumours, whereas 76.9 % of maize plants (out of a total of 79 plants) inoculated with a mixture of FB1 and FB2 strains formed tumours. This avirulent phenotype agrees with the behaviour of *odc* mutants, which are also unable to induce tumours in maize plants (Guevara-Olvera *et al.*, 1997; Valdés-Santiago *et al.*, 2010). The mutation was recessive, since 82.5 % of 82 plants inoculated with a mixture of a wild-type and a *samdc* strain formed tumours. Also, crosses of the 4samdcR revertant with *samdc* mutant 5-11 reached the tumour-formation level of wild-type crosses: 90 % of the 62 infected plants formed tumours.
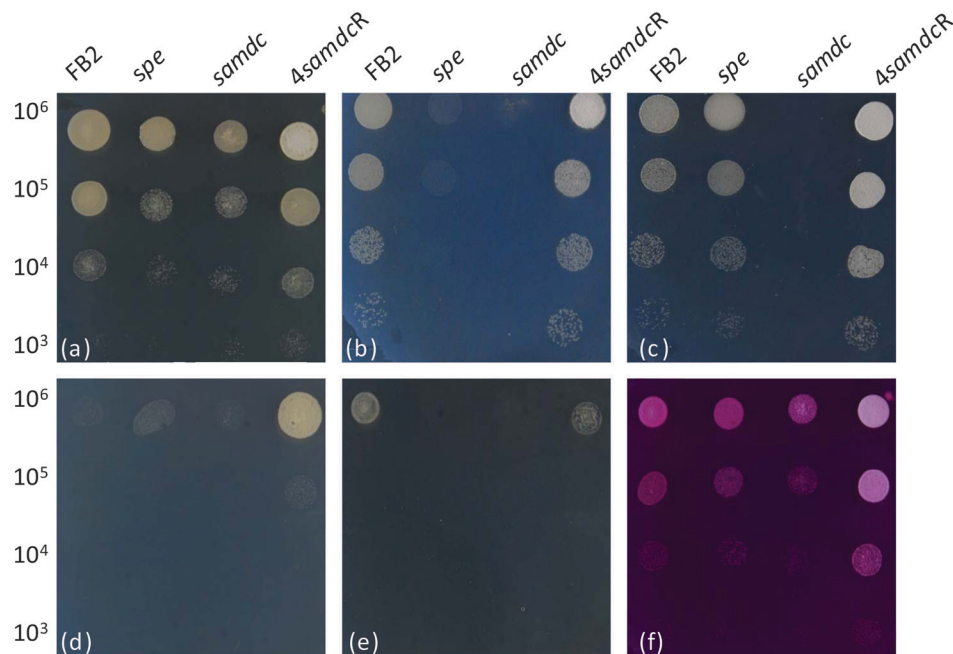

Fig. 3. Stress response of spermidine auxotrophic mutants. Plates containing 0.1 mM spermidine and 0.2 mM lysine were amended with the following test substances: (a) no addition (control), (b) 1 M NaCl, (c) 10 mM LiCl, (d) 3 mM H_2O_2, (e) 0.05 mM menadione, (f) 0.05 mM Rose Bengal, and were spot-inoculated with decimal dilutions of suspensions of the indicated strains. The photograph was taken after 48 h of incubation at 28 °C.
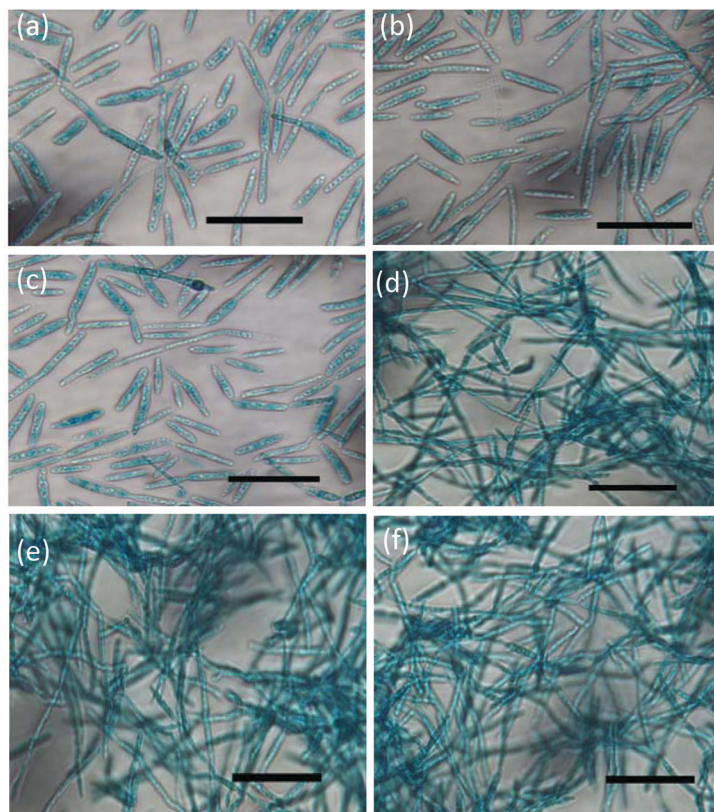
Fig. 4. Dimorphic transition of *spe* and *samdc* mutants compared with wild-type and revertant strains. Cells were grown in liquid pH 3 MM for 24 h. (a, c) *spe* LV54 mutant, (b, d) *samdc* 5-11 mutant, (e) 4*samdc*R revertant, (f) FB2 wild-type. (a, b) Medium containing 5 µM spermidine, (c, d) medium containing 0.1 mM spermidine. (a, c) Medium containing 0.2 mM lysine, (e, f) medium without additions. Bars, 15 µm.

Determination of SAM and dcSAM levels in wild-type and mutant strains

SAM was identified and quantified by MS in the mutants LV54 (*spe*; Valdés-Santiago *et al.*, 2009), 5-11 (*samdc*) and LG4 (*odc*; Guevara-Olvera *et al.*, 1997), and as a control, in the wild-type strain FB2. In strains FB2 and LG4, the SAM content was close to the detection limit of the method. The respective calculated means of 7.4 and 9.2 pmol (mg dry cell weight)$^{-1}$ were not significantly different, according to
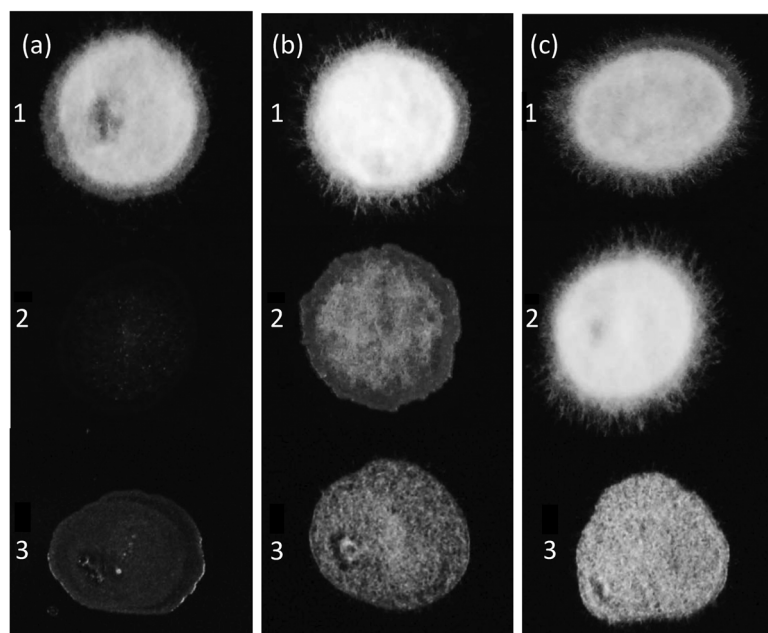

Fig. 5. Mating capacity of spermidine auxotrophic mutants. Sexually compatible strains were inoculated on plates of charcoal-containing MM, pH 7, plus 0.2 mM lysine, incubated at 25 °C for 24 h, and photographed. (a) No additions, (b) 0.1 mM spermidine, (c) 0.5 mM spermidine. (1) Cross between FB1 *a1b1* and FB2 *a2b2*, (2) cross between LV54 (*spe*::Cbx^R *a1b2*) and LV7 (*spe*::Cbx^R *a2b1*), (3) cross between LV71 (*samdc*::Cbx^R *a2b2*) and 5-11 (*samdc*::Cbx^R *a1b1*).

Student's *t* test. On the other hand, mutants LV54 and 5-11 contained high SAM levels: 45.8 and 118.8 pmol (mg dry cell weight)$^{-1}$, respectively. These values are respectively 6.2- and 16.2-fold higher than that of the FB2 strain (Fig. 6a). According to Student's *t* test these differences are highly significant ($P < 0.001$). dcSAM content in the wild-type strain was close to the detection limit, agreeing with reports for mammals, where dcSAM content is very low (Pegg, 1988); similarly, in the LG4 mutant, the levels appeared to be below the detection limit. As expected, no dcSAM was present in mutant 5-11. In contrast, mutant LV54 showed an aproximately 46-fold higher signal intensity than the wild-type strain (Fig. 6b).

DISCUSSION

Our data indicate that *U. maydis* gene Um10792 encodes a functional Samdc proenzyme. The size of the polypeptide differs from those of Ascomycota, and even those of some Basidiomycota, but this is not surprising, as comparative analyses have revealed differences among bacterial, archaeal and eukaryotic Samdc polypeptides, which in different species range from 105 to 460 aa in length. In this sense, Kozbial & Mushegian (2005) concluded that the sizes of eukaryotic Samdc proteins and their characteristics may be directly related to the duplication of the ancestor of archaeal Samdc. Despite these differences, the Ser (Ser^{168}) that is the precursor of the pyruvoyl residue (Stanley *et al.*, 1989) is conserved in *U. maydis* Samdc. Additionally, the motif surrounding this residue, YVLSESS, is fully conserved in the *U. maydis* enzyme (Fig. 1). Other important conserved motifs, shown in Fig. 1, are also present in the *U. maydis* Samdc proenzyme: FEGPEKLLE (1), PCGYSAN (2)

and TIHVTPE (3). All these motifs are involved in the processing reaction to form the two subunits and the pyruvate prosthetic group (Xiong & Pegg, 1999). These data reveal that the *U. maydis* enzyme possesses a similar active site, and probably the same catalytic mechanism as that exhibited by eukaryotic Samdc proteins in general.

Previously, we obtained *spe* mutants in *U. maydis* only when we used the *odc* genetic background, possibly because of the toxic effect of accumulated putrescine in the single *spe* mutants (Valdés-Santiago *et al.*, 2009). Taking this precedent into consideration we used the same strategy to delete the *SAMDC* gene. The double (*odc/samdc*) mutants thus obtained were crossed *in planta* with sexually compatible wild-type partners to obtain *samdc* single mutants. The observation that these mutants behave as spermidine auxotrophs is evidence that the fungus contains a single Samdc-encoding gene. It also demonstrates that, as would be expected, the *SAMDC* gene is essential. This result is in agreement with data from *L. donovani*, *N. crassa* and *S. cerevisiae* (Pitkin & Davis, 1990; Balasundaram *et al.*, 1991; Hamasaki-Katagiri *et al.*, 1997; Roberts *et al.*, 2002).

Phenotypic analysis of *samdc* mutants revealed that they displayed the same basic characteristics as the *spe* mutants, but, interestingly, although both Spe and Samdc enzymes are required for spermidine biosynthesis, the mutants showed some interesting phenotypic differences. An important difference was that in contrast to *U. maydis* *spe* mutants, which are able to induce tumours in about 20 % of inoculated maize plants (Valdés-Santiago *et al.*, 2009), *samdc* mutants are totally avirulent. The most probable explanation for this discrepancy is the inability of *samdc* mutants to mate and form invasive dikaryons, as
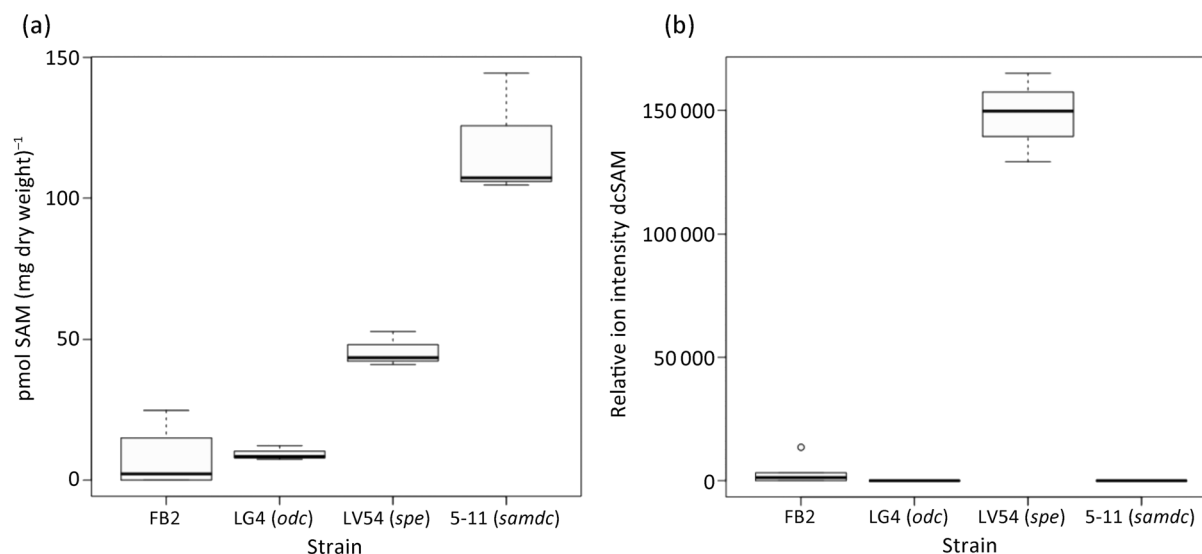

Fig. 6. Content of SAM (a) and dcSAM (b) in strains FB2 (wild-type), LV54 (*spe*), LG4 (*odc*) and 5-11 (*samdc*). Cells were grown for 48 h in pH 7 liquid MM, but the medium for 5-11 was amended with 0.1 mM spermidine, and the medium for LV54 was amended with 0.1 mM spermidine plus 0.2 mM lysine.

revealed by their negative fuz reaction in comparison with *spe* mutants. Since only dikaryotic or diploid *U. maydis* strains are virulent, a mixture of sexually compatible strains unable to mate, as occurs with *samdc* mutants, would be unable to infect their host.

Several authors have shown that polyamines are essential for the stress response (Gill & Tuteja, 2010), and, according to our data, *U. maydis* polyamine-deficient mutants show a higher sensitivity than the wild-type strain to a range of stress conditions (see Results and Valdés-Santiago *et al.*, 2009, 2010). In the present study we observed that *samdc* cells are more sensitive than *spe* mutants to ionic stress induced by LiCl. It is possible that *samdc* mutants are affected to a higher degree than *spe* mutants in their capacity to control the mechanisms of ion transport across the plasma membrane. A possible hypothesis to explain this phenotypic difference between the two types of mutants is a dissimilarity in their capacity to transport spermidine from the culture medium. Accordingly, it is probable that *samdc* mutants have a reduced capacity to take up the polyamine from the medium, reducing their ability to mate and maintain their ionic equilibrium.

Our previous data revealed that *spe* mutants were unable to carry out the dimorphic yeast-to-mycelium transition (Valdés-Santiago *et al.*, 2009), and the same phenotype was displayed by *samdc* mutants, although unlike *spe* mutants they recovered the wild-type phenotype by addition of a higher spermidine concentration (0.1 mM). This effect was not due to growth impairment in the presence of the lower levels of spermidine, since the same growth rate was obtained in both mutants using 0.1 mM spermidine. Several hypotheses can be invoked to explain this difference in behaviour of *spe* and *samdc* mutants, but the most simple one would be the different cellular locations of the two enzymes and the existence of different pools of the polyamine. This phenomenon has been demonstrated *in Mucor rouxii*, where it explains the different sensitivities of growth and dimorphism to the ODC inhibitor 1,4-diamino-2-butanone (Martínez-Pacheco & Ruiz-Herrera, 1993).

One interesting, although expected, characteristic of *samdc* mutants is that, unlike the wild-type, they accumulate high levels of SAM, the substrate of Samdc, but in contrast it was surprising that *spe* mutants accumulated SAM to levels as high as half those of *samdc* mutants. In contrast, *odc* mutants not only did not accumulate SAM, but their SAM content was almost the same as that of the wild-type, a result that agrees with the report that F9 teratocarcinoma stem cells treated with α-difluoromethylornithine, an inhibitor of ODC, exhibit a lower SAM content, probably due to a compensatory increase in Samdc activity, which consumes the substrate (SAM) (Stjernborg *et al.*, 1993; Frostesjö *et al.*, 1997). It has been established that Samdc is not only critical for polyamine biosynthesis but also plays a key role in determining the disposition of the cellular SAM pools (Pegg *et al.*, 1998). A possible explanation of this

accumulation in *spe* mutants is that the accumulated dcSAM (the product of Samdc) is unable fully to regulate Samdc activity (Kashiwagi *et al.*, 1990; Li *et al.*, 2001).

Another collateral metabolite in the pathway of polyamine biosynthesis, dcSAM, was found to be accumulated in the *spe* mutant (46-fold increase in comparison with the FB2 wild-type strain), and was absent in *odc* and (as expected) in *samdc* mutants. The relative contents of both SAM and dcSAM in the *spe* and *samdc* mutants are important in relation to DNA methylation, since DNA methylases and Samdc share SAM as a common substrate (Fraga *et al.*, 2002; Ruiz-Herrera, 1994). For this reason, SAM accumulation is related to the methylation of low-molecular-mass compounds, nucleic acids and proteins (for reviews on this topic see Chiang, *et al.*, 1996; Fontecave *et al.*, 2004; Lieber & Packer, 2002; Lu, 2000; Mato *et al.*, 1997; Loenen, 2006). Additionally, a negative relationship between the levels of dcSAM and the state of DNA methylation, and a positive relationship with cell differentiation, have been established (Frostesjö *et al.*, 1997). Accordingly, a plausible hypothesis to explain the differences observed in the phenotypes of *samdc* and *spe* mutants might be related to their different levels of SAM and dcSAM, which have important effects on different cellular functions. Examples of the effects of dcSAM are the study of Duranton *et al.* (1998), who reported that treatment of a Caco-2 cell line with an inhibitor of Samdc gave rise to an increase in global DNA methylation and the expression of a differentiation marker, and the observation that depletion of polyamine biosynthesis in F9 teratocarcinoma stem cells gave rise to an increase in the level of dcSAM, leading to an induction of differentiation that was counteracted by specific inhibition of Samdc (Frostesjö *et al.*, 1997). Regarding SAM, it has been reported that inhibition of its synthesis by 3-deazaadenosine promotes hypomethylation and differentiation of muscle (Scarpa *et al.*, 1996), and Fuso *et al.* (2001) have suggested the possibility of silencing genes regulated by DNA methylation through the administration of exogenous SAM. Whether the observed phenotypic differences between *spe* and *samdc* mutants might be related to the differential effects of SAM and dcSAM on the methylation of micro- and macromolecules is an interesting possibility that deserves to be analysed.

ACKNOWLEDGEMENTS

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