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

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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

Abbreviations: dcSAM, decarboxylated S-adenosylmethionine; ODC, ornithine decarboxylase; SAM, S-adenosylmethionine.

The GenBank/EMBL/DDBJ accession number for the SAMDC gene sequence of U. maydis is HE582743.
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 catalyzed 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 \( \alpha \) and \( \beta \), 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-Martin, 2001; Bölk, 2001; Basse & Steinberg, 2004; Klosterman *et al.*, 2007; Steinberg & Pérez-Martin, 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 °C in liquid complete medium (CM; Holliday, 1961) supplemented with 50% glycerol, and were recovered on solid CM plates and incubated at 28 °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 °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 32P-labelled 1 kb EcoRI–HindIII *samdc* gene fragment (see below) was used as hybridization probe.

**Plasmid constructs.** To delete the gene encoding *U. maydis* Samdc (SAMDC), plasmid pAMsamdc 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 EcoRI–HindIII 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 (Table 2) using genomic DNA from *U. maydis* strain FB2 (Table 1) as template. The PCR product (3.1 kb) was cloned into plasmid pCR-2.1 (Invitrogen), generating plasmid pAMSAMDC. Next, the SAMDC gene was recovered as a BamHI–NotI fragment and cloned into the same sites of the episomal plasmid pHyg101 (Mayorga & Gold, 1998), generating plasmid pAMSAMDCHyg-20, which was used to transform protoplasts of *samdc::CbxR* 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><strong>U. maydis strains</strong></td>
<td></td>
<td></td>
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<tr>
<td>FB2</td>
<td>a2b2</td>
<td>Banuett &amp; Herskowitz (1989)</td>
</tr>
<tr>
<td>FB1</td>
<td>a1b1</td>
<td>Banuett &amp; Herskowitz (1989)</td>
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<tr>
<td>samdc6</td>
<td>a2b2 Δodc/samdc::HygR/CbxR</td>
<td>This study</td>
</tr>
<tr>
<td>samdc7</td>
<td>a2b2 Δodc/samdc::HygR/CbxR</td>
<td>This study</td>
</tr>
<tr>
<td>5-11</td>
<td>a1b1 Δsamdc::CbxR</td>
<td>This study</td>
</tr>
<tr>
<td>LV71</td>
<td>a2b2 Δsamdc::CbxR</td>
<td>This study</td>
</tr>
<tr>
<td>4samdcR</td>
<td>a2b2 Δsamdc::CbxR/pLV20</td>
<td>This study</td>
</tr>
<tr>
<td>7samdcR</td>
<td>a2b2 Δsamdc::CbxR/pLV20</td>
<td>This study</td>
</tr>
<tr>
<td>11samdcR</td>
<td>a2b2 Δsamdc::CbxR/pLV20</td>
<td>This study</td>
</tr>
<tr>
<td>LG4</td>
<td>a2b2 Δode::HygR</td>
<td>Guevara-Olvera et al. (1997)</td>
</tr>
<tr>
<td>LV54</td>
<td>a2b1 Δspe-sdh::CbxR</td>
<td>Valdés-Santiago et al. (2009)</td>
</tr>
<tr>
<td>LV7</td>
<td>a1b2 Δspe-sdh::CbxR</td>
<td>Valdés-Santiago et al. (2009)</td>
</tr>
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<td><strong>E. coli strain</strong></td>
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<td>Invitrogen</td>
</tr>
<tr>
<td>DH52</td>
<td>F'</td>
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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 % CuSO4 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’ode and 3’ode.

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 % perchorlic 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/TOPPAS suite, version 1.7.0 (1, 2). First, a pipeline was written for TOPPAS, executing the following tasks for all spectra: file conversion to mzXML, merging all spectra of a sample, NoiseFilter sglay 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 μg gave a correlation coefficient, R², 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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’−3’)</th>
<th>Orientation</th>
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<tbody>
<tr>
<td>Samdc5</td>
<td>GTACGTTTGACCTGTCGATGTG</td>
<td>Forward</td>
</tr>
<tr>
<td>Samdc3</td>
<td>ACATGCATAGCACAGGCCGAC</td>
<td>Reverse</td>
</tr>
<tr>
<td>pCBX</td>
<td>GACCTGGCCCCAGAACGTCTCAACCTGTC</td>
<td>Forward</td>
</tr>
<tr>
<td>AC110</td>
<td>CGCAGCGGGGAGTCAAGGCGCACAGAAAG</td>
<td>Reverse</td>
</tr>
<tr>
<td>5’odec</td>
<td>CAACATGGAGAGCTCGAAAAGAT</td>
<td>Forward</td>
</tr>
<tr>
<td>3’odec</td>
<td>GTAAGGGCCCATGTTTTCGTAGAC</td>
<td>Reverse</td>
</tr>
</tbody>
</table>
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 ± 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 U10792 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 Malazozia globosa, Schizophyllum commune and Coprinopsis cinerea was of the order of 40% at the amino acid level. The U. maydis protein has a higher Mr 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), AYLLESSMF, 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 pAsamdc (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 spermidine), 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 (Valde´s-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::CbxR/a1b1) and LV71 (samdc::CbxA2b2) 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 (4samdcR, 11samdcR, 7samdcR) resistant to carboxin and hygromycin. 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 H2O2, 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 CaCl2 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 4samdcR (revertant) strains (Fig. 3a). On the other hand, under some stress...
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 \( \text{samdc} \) strains (Fig. 3b). Interestingly, \( \text{Li}^+ \) addition resulted in growth inhibition only of the \( \text{samdc} \) and not of the \( \text{spe} \) mutant, FB2 or the complemented strain (Fig. 3c). \( \text{H}_2\text{O}_2 \) (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 \( \text{H}_2\text{O}_2 \) 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 \( \text{samdc} \) mutant being slightly more sensitive (Fig. 3f). Other concentrations tested were equally inhibitory for all strains (results not shown). Other tested compounds, sorbitol, \( \text{CaCl}_2 \) 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**

\( U. \text{maydis} \) 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 \( \text{odc} \) 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 \( \text{samdc} \) mutant 5-11 and \( \text{spe} \) mutant LV54, and the results are shown in Fig. 4. It was observed that low spermidine concentrations (5 \( \mu \text{M} \)) sustained vegetative growth of both mutants (\( \text{samdc} \) or \( \text{spe} \)), but addition of a higher concentration of spermidine (0.1 mM) was necessary to induce the dimorphic switch of the \( \text{samdc} \) mutant only, having no effect on the cell morphology of the \( \text{spe} \) 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 (\( a1b1 \Delta \text{samdc}::\text{Cbx}^R \) × LV71 (\( a2b2 \Delta \text{samdc}::\text{Cbx}^R \)) and LV54 (\( a2b1 \Delta \text{spe}::\text{Cbx}^R \) × LV7
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 4*samdcR* revertant with *samdc* mutant 5-11 reached the tumour-formation level of wild-type crosses: 90% of the 62 infected plants formed tumours.

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**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.

**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 H₂O₂, (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.
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. 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) 4samdcR 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.]

![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::CbxR a1b2) and LV7 (spe::CbxR a2b1), (3) cross between LV71 (samdc::CbxR a2b2) and 5-11 (samdc::CbxR 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 approximately 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: FEGPEKLE (1), PCGYSAN (2) and THVTPTE (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 fuzz 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-butanolone (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-difluoromethylnithine, 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 Fusó 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.

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