Regulation of *S*-adenosylmethionine decarboxylase during the germination of sporangiospores of *Mucor rouxii*

CARLOS CALVO-MENDEZ and JOSE RUIZ-HERRERA*

Departamento de Genética y Biología Molecular, Centro de Investigación y Estudios Avanzados, IPN, and Instituto de Investigación en Biología Experimental, Universidad de Guanajuato, Apartado Postal 187, Guanajuato, Gto. 36000, Mexico

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General properties of *S*-adenosylmethionine decarboxylase (SAMDC) from *Mucor rouxii* were studied. Dormant spores of the fungus did not contain detectable levels of the enzyme, but it started to be synthesized at early stages of spore germination. Kinetics of synthesis changed before emergence of the germ tube, with a corresponding increase in a second SAMDC activity which, in contrast to the one originally synthesized, was not activated by putrescine. Development of the second enzyme activity required *de novo* protein synthesis. Neither enzymic activity was stimulated by Mg^{2+} . Addition of the SAMDC inhibitor methylglyoxal bis-(guanyldrazone) (MGBG) stopped fungal development in growth phase Ia: cells became spherical and showed ultrastructural alterations. Although MGBG inhibited polyamine formation, it barely inhibited protein and RNA biosynthesis during the first hour of incubation. However, at later periods, biosynthesis of both macromolecules was strongly decreased. When MGBG was added to growth media 3 h after inoculation of spores, it did not affect spore germination and outgrowth. A hypothesis for two different roles of spermidine and putrescine in spore germination is discussed.

Introduction

In previous communications we have reported that high levels of polyamines are required during the germination of sporangiospores of *Mucor* (Calvo-Mendez *et al.*, 1987). In the presence of 1,4-diaminobutanone (DAB), an inhibitor of ornithine decarboxylase (ODC), spores are unable to proceed beyond phase Ib of spherical growth: they reach large diameters, and fail to produce germ tubes (Ruiz-Herrera & Calvo-Mendez, 1987). For a description of growth phases during germination of *Mucor* sporangiospores see Bartnicki-Garcia & Lippman (1977) and Cano & Ruiz-Herrera (1988). We have presented evidence that the requirement for high polyamine concentrations during development of the fungus is related to DNA hypomethylation, a process occurring during DNA replication, and required for the differential expression of genes involved in polarized growth of the fungus (Cano *et al.*, 1988).

In order to gain more information on the role of the different polyamines in *Mucor* development, we have investigated the regulation of *S*-adenosylmethionine

decarboxylase (SAMDC) during sporangiospore germination. SAMDC is responsible for the formation of decarboxylated *S*-adenosylmethionine, which serves as the aminopropyl donor to putrescine for the synthesis of spermidine (*Mucor rouxii* does not contain spermine: Nickerson *et al.*, 1977; Ruiz-Herrera & Calvo-Mendez, 1987). *S*-Adenosylmethionine decarboxylation constitutes one of the rate-limiting factors in spermidine formation, and is precisely regulated in several systems (Tabor & Tabor, 1984; Pegg, 1986). Three different classes of SAMDC have been described with respect to activation of the enzyme by Mg^{2+} and putrescine. The enzyme from *Escherichia coli* and plants is activated by Mg^{2+} but not by putrescine. In contrast, the mammalian enzyme is activated by putrescine but does not require Mg^{2+} , whereas slime mould and *Tetrahymena pyriformis* enzymes are activated by neither putrescine nor Mg^{2+} (Pegg, 1984). SAMDC from *Saccharomyces* and mycelial fungi behaves like the mammalian enzyme: it is activated by putrescine, but not by Mg^{2+} (Stevens & Winther, 1979).

In the present study we have investigated the general properties of SAMDC from *Mucor rouxii*, its regulation during the process of sporangiospore germination, and the effect of its *in vivo* inhibition on the development of the fungus.

Abbreviations: SAM, *S*-adenosylmethionine; SAMDC, *S*-adenosylmethionine decarboxylase; ODC, ornithine decarboxylase; MGBG, methylglyoxal bis-(guanyldrazone); DAB, 1,4-diamino-2-butanone.

Methods

Strains, culture media and growth conditions. *Mucor rouxii* IM-80 (ATCC 24905) was maintained on slants of YPG medium, and propagated in liquid YPG medium or synthetic medium (Bartnicki-Garcia & Nickerson, 1962), as described by Calvo-Mendez *et al.* (1987).

Preparation of cell-free extracts and toluene-permeabilized cells. These were prepared as described previously (Calvo-Mendez *et al.*, 1987).

S-Adenosylmethionine decarboxylase (SAMDC) assays. Erlenmeyer flasks (10 ml) containing a centre well were used for incubation. Enzyme preparations in phosphate buffer pH 7.5 containing 0.1 mM-EDTA and 1 mM-dithiothreitol (plus the additions indicated in each experiment) were dispensed into the outer chamber in a final volume of 395 μ l. A piece of folded filter paper containing 35 μ l 5 M-KOH was placed in the centre well to trap the released CO₂. The reaction was started by the addition of 0.2 mM (5 μ l) S-adenosyl[carboxyl-¹⁴C]methionine (sp. act. 59.5 Ci mol⁻¹; 2.2 TBq mol⁻¹). The flasks were closed with rubber stoppers and incubated at 37 °C for 60 min. When anaerobic conditions were used, two needles were inserted through the stoppers and N₂ was flushed through one of them for 5 min; they were then simultaneously removed. Anaerobiosis was checked by incubation in parallel of a methyl viologen solution reduced with sodium dithionite. The reaction was stopped by injecting 100 μ l of 2 M-HCl into the outer chamber. After 12 h, the filters were removed, dried at 60 °C and placed in vials with scintillation fluid containing 100 mg 1,4-di-2-(4-methyl-5-phenyloxazolyl)benzene and 2 g 2,5-diphenyloxazole per litre of toluene. Radioactivity was measured in a scintillation spectrometer. Activity was expressed in units (nmol CO₂ evolved min⁻¹). Specific activity was referred to 1 mg protein. All assays were performed on duplicate samples.

Diamine oxidase assay. Diamine oxidase was measured fluorimetrically with homovanillic acid as substrate, essentially as described by Guilbault *et al.* (1968). In a cuvette, 1 mM-putrescine, 10 units of horseradish peroxidase, 0.47 mM-homovanillic acid and enzyme extract were mixed in a final volume of 3 ml 0.1 M-phosphate buffer pH 7.5 containing 0.1 mM-EDTA. Fluorescence was measured during 30 min in a Perkin Elmer LS-5B spectrofluorimeter with excitation and emission wavelengths of 315 nm and 425 nm respectively. Activity was expressed in units, one unit being the amount of enzyme which oxidizes 1 μ mol putrescine in 1 h. Specific activity was related to 1 mg protein.

Determination of RNA and protein synthesis. For determination of RNA biosynthesis, spores (5 $\times$ 10⁶ ml⁻¹) were inoculated into 2 ml aliquots of liquid YPG medium. At 1 h intervals, 0.065 μ Ci of [5,6-³H]uracil (sp. act. 40 Ci mol⁻¹; 1.48 TBq mol⁻¹) were added, and after 1 h incubation, the reaction was stopped by the addition of 2 ml ice-cold 10% (w/v) TCA. Samples were incubated on ice for 1 h, filtered through glass-fibre filters (Schleicher and Schüll no. 8, 2.5 mm diameter) and washed with about 60 ml ice-cold 10% TCA, followed by 60 ml ethanol. Filters were dried at 60 °C and radioactivity was measured as described above. All assays were performed in duplicate.

To determine protein biosynthesis, spores (5 $\times$ 10⁶ ml⁻¹) were inoculated into 2 ml aliquots of liquid synthetic medium plus 0.002% Casamino acids. At 1 h intervals, 0.140 μ Ci of a [U-¹⁴C]protein hydrolysate (57 mCi matom⁻¹; 2.1 GBq matom⁻¹) were added, and after 1 h of incubation, the reaction was stopped by addition of 2 ml ice-cold 10% TCA and samples were kept on ice for 1 h. Samples were heated in a boiling water bath for 30 min, filtered and processed as described for RNA biosynthesis. All assays were performed on duplicate samples.

Determination of polyamines. Polyamines were measured as described by Calvo-Mendez *et al.* (1987).

Microscopic observations. Samples of cultures were mixed with an equal volume of 2% (v/v) glutaraldehyde in 0.05 M-sodium cacodylate buffer and observed under the microscope. Samples for electron microscopy were prepared as described by Ruiz-Herrera *et al.*, (1989).

Miscellaneous. Protein was measured with the Folin reagent. S-Adenosylmethionine (SAM), horseradish peroxidase, homovanillic acid, putrescine, hog kidney diamine oxidase and bovine liver catalase were obtained from Sigma. Components for culture media were from Difco. Radioactive compounds were obtained from Amersham. All other reagents were of the highest purity available. Experiments were usually repeated three times, and exceptionally twice. Representative data from reproducible experiments are presented.

Results

General properties of SAMDC from *M. rouxii*

Properties of SAMDC were analysed in the 27000 g supernatant obtained from cell-free extracts of the fungus. Activity was linearly dependent on time of incubation and protein concentration in the range of 1 h, and up to 0.8 mg protein in the reaction mixture, respectively. Optimum pH was 7.5, and optimum temperature, 37 °C. The addition of dithiothreitol to the reaction mixtures did not affect activity, but EDTA addition more than doubled it. Apparently the enzyme is very sensitive to contaminating divalent metals present in the enzyme preparations. The effect of putrescine on SAMDC activity was variable (see below). The inhibitor methylglyoxal bis-(guanyldiazide) (MGBG) was extremely active against SAMDC: 10 μ M-MGBG caused 50% inhibition of the enzyme activity and 100 μ M-MGBG about 95% inhibition. 1,4-Diamino-2-butanone (DAB) and difluoromethylornithine, which are inhibitors of ODC in general (Heby, 1981) including that of *M. rouxii* (Calvo-Mendez *et al.*, 1987) had, as expected, no inhibitory effect on SAMDC activity (but see below).

Effect of putrescine, DAB and Mg²⁺ on SAMDC activity

The effect of putrescine on SAMDC activity was variable. Normally, in extracts prepared from 8–9-h-old cultures, addition of putrescine had only a weak stimulatory effect. When younger cultures (less than 6 h incubation) were used as a source of the enzyme, both putrescine and its analogue DAB stimulated SAMDC activity in cell-free extracts or toluene-permeabilized cells. Higher concentrations of DAB were required to obtain the same degree of stimulation as putrescine, and activation declined at higher DAB concentrations (Fig. 1). DAB also activates SAMDC from *Aspergillus nidulans*, but to a lesser extent than putrescine (Stevens & Winther, 1979). The K_a value calculated for putrescine in cell-free extracts was 0.02 mM. These results suggested

Fig. 1. Effect of putrescine and DAB on SAMDC activity. Cell-free extracts (○, ●) or toluene-treated cells (△) were incubated in the presence of variable concentrations of putrescine (○, △) or DAB (●, ▲), and SAMDC activity was measured as described in Methods.

the presence of two forms of the enzyme, one stimulated, and another one unaffected by putrescine. Mg^{2+} did not stimulate SAMDC activity of toluene-permeabilized cells which had been grown for 3 or 6 h (not shown).

Suresh & Adiga (1977) have shown that in *Lathyrus sativus* seedlings activation of SAM-decarboxylase by putrescine was artifactually due to chemical decarboxylation of SAM by H_2O_2 generated through the action of diamine oxidase. Accordingly, we analysed whether putrescine activation of SAM-decarboxylase was due to a similar phenomenon in *M. rouxii*. Firstly we measured the levels of diamine oxidase in 3 and 7 h-old germlings of the fungus. It was observed that in agreement with previous data SAMDC from 3 h-old cells was activated

to a higher degree (about fourfold more) than the enzyme from 7 h-old cells. On the other hand diamine oxidase levels were higher in older cells (data not shown). As a second approach we determined whether a large excess of exogenous diamine oxidase or hydrogen peroxide increased the levels of SAM decarboxylation by cell-free extracts of *M. rouxii*. We also tested whether catalase decreased the putrescine-activatable liberation of CO_2 from SAM. Addition of diamine oxidase or H_2O_2 increased SAM decarboxylation, but only by about 40%, whereas catalase decreased it by 13% (Table 1). Finally we compared SAM decarboxylase activity of toluene-permeabilized cells under an atmosphere of nitrogen, with that under normal aerobic assay conditions. SAMDC activity was about the same under either condition (data not shown).

Development of SAMDC activity during germination of sporangiospores of M. rouxii

Activity of SAMDC was measured at different times in germinating cultures of *M. rouxii*. Ungerminated spores had no detectable levels of the enzyme. Total SAMDC activity increased exponentially upon incubation of the spores in minimal medium. After 1 h incubation, specific activity increased to more than one-third of the maximal specific activity which was attained after 4 h incubation, when about 40% of the spores had germinated (Fig. 2). After this time specific but not total enzyme activity started to decline.

When SAMDC activity was measured in the presence or absence of putrescine during germination in minimal medium it was observed that at early periods of incubation the enzyme was strongly activated by the

Table 1. *Effect of different additions on SAM decarboxylation by cell-free extracts of M. rouxii*

Cell-free extracts from 7-h-old mycelium were incubated in standard assay mixtures for determination of SAMDC. Where indicated, 0.15 units of hog kidney diamine oxidase, 0.1 μ mol of H_2O_2 , 100 units of bovine liver catalase per assay, or 1 mM-putrescine were added to the incubation mixtures. After incubation for 60 min at 37 °C, radioactive $^{14}CO_2$ evolved was measured as described in Methods.

Additions to extract	Protein per assay (mg)	$^{14}CO_2$ liberated (c.p.m.)	Activation or inhibition (%)
None	1.29	1759	—
Diamine oxidase	1.29	2429	+38
H_2O_2	1.29	2501	+42
Putrescine	0.5	1189	—
Putrescine + catalase	0.5	1044	–13

Fig. 2. Synthesis of SAMDC during sporangiospore germination. Spores were inoculated into minimal medium. At timed intervals, samples were recovered for determination of spore germination, cell protein and SAMDC activity in toluene-treated samples in the presence of 1 mM-putrescine. ●, SAMDC specific activity; ○, SAMDC total activity (per 100 ml medium); △, spore germination.

polyamine, about 12-fold in 2- or 3-h-old cells, whereas in 6–7-h-old cells, putrescine produced less than twofold activation of the enzyme (Fig. 3a). Results expressed as differential rate of synthesis of the enzyme show that after a short delay, putrescine-activatable SAMDC is synthesized as an exponentially increasing fraction of total cell protein. At later times a decline in the rate of synthesis is observed, whereas an increase in the putrescine-insensitive enzyme was noticed (Fig. 3b).

To determine whether the putrescine-insensitive activity came from a permanent activation of the enzyme brought about by the putrescine accumulated at late periods of incubation (Ruiz-Herrera & Calvo-Mendez, 1987), we permeabilized 3-h-old cells with toluene and incubated them with or without putrescine. After 30 min at 25 °C, cells were washed, and SAMDC activity was measured in the presence or in the absence of putrescine. SAMDC was equally activated by putrescine, independently of whether or not the permeabilized cells had been incubated with the polyamine. We also explored the possibility that the putrescine-insensitive enzyme was the result of a post-translational modification of SAMDC. To test this hypothesis, spores were incubated in liquid YPG medium for 3 h, and then the culture was divided into two batches. One was treated with 100 µg cycloheximide ml⁻¹, the other remained as a control. At this time and after a further 2 and 4 h incubation, samples were removed to measure cell protein and SAMDC activity. SAMDC activity in cycloheximide-treated cells remained activatable by

Fig. 3. Development of putrescine-stimulated and putrescine-insensitive SAMDC activities during sporangiospore germination. Spores were inoculated into minimal medium. At timed intervals, samples were recovered for determination of spore germination (△), cell protein and SAMDC activity in toluene-treated samples both in the absence (○) or in the presence (●) of 1 mM-putrescine. (a) Development of SAMDC as a function of time of incubation. (b) Differential rate of synthesis of SAMDC.

putrescine in the same order of magnitude after 2 and 4 h incubation with the drug, although total activity declined somewhat. Cells growing in the absence of cycloheximide synthesized the putrescine-insensitive SAMDC as suggested by the fact that after 4 h incubation, activity of the enzyme measured in the absence of putrescine increased 20-fold, against a threefold increase in the activity measured in the presence of putrescine (Table 2).

Effect of MGBG on cell growth and synthesis of macromolecules

Addition of MGBG to synthetic medium at concentrations in the range which inhibited SAMDC activity by more than 90% (see above) did not affect growth of the fungus, probably because MGBG has a low permeability. On the other hand, addition of 5 mM-MGBG inhibited growth almost completely. For SAMDC from *Acanthamoeba culbertsoni* MGBG had a K_i of 123 µM

Table 2. *Effect of cycloheximide on the formation of putrescine-insensitive SAMDC*

Spores were incubated for 3 h in YPG medium and then divided into two samples. One received 100 µg cycloheximide ml⁻¹ and the other remained as a control. Samples were removed to determine germination, cell protein and SAMDC activity, and the rest of the cultures were incubated further. After 2 and 4 h, samples were recovered to measure the same parameters. SAMDC activity is expressed as described in Methods.

Time of incubation (h)	Cycloheximide added	Spore germination (%)	Cell protein [mg (100 ml) ⁻¹]	Total SAMDC activity [units (100 ml) ⁻¹]	
				No putrescine	Plus putrescine
3	No	0	1.37	0.049	0.752
5	No	78	5.50	0.313	1.309
	Yes	0	1.29	0.032	0.389
7	No	95	15.62	0.921	2.59
	Yes	0	1.50	0.031	0.460

(Gupta *et al.*, 1987), but a concentration of 2.5 mM was necessary to inhibit growth of the amoeba (Gupta *et al.*, 1986).

Microscopic observations of inhibited cultures showed that spores became spherical with a mean diameter close to that of the large axis of the normal ellipsoidal sporangiospores (Fig. 4c), and did not proceed beyond this point in the germination process, in contrast to normal cells which after 3 h had enlarged significantly (Fig. 4a) and germinated after 4–5 h (Fig. 4b). Fig. 4 (d) shows the structure of a spore incubated for 12 h in the presence of MGBG. Inhibited cells did not increase in size, and appeared nearly spherical. The cell wall was relatively thick and with alternating electron-transparent and electron-opaque zones. The internal structure was relatively unaffected by the drug, except for the vacuole. Prolonged periods of incubation (more than 12 h) gave rise to severe alterations in the cell structure and eventually to cell lysis. Removal of MGBG at shorter periods of incubation (about 6–8 h) reversed the inhibition phenomenon, and spores germinated almost normally. Addition of polyamines to cultures grown in synthetic medium in the presence of MGBG partially reversed the inhibitory effect of the drug. Accordingly, 5 mM-putrescine or 5 mM-spermidine allowed 10 or 25% spore germination respectively. A mixture of spermidine plus putrescine was most effective, permitting about 60% germination. However, germination in all these cases was atypical, with production of distorted cells.

Interestingly, the period of sensitivity of *M. rouxii* cells to MGBG was limited to the first 2 h of the germination process in synthetic medium. When the drug was added after this time to germinating spores, they were completely unaffected and grew normally (Table 3).

Table 3. *Period of sensitivity of spore germination to MGBG*

Spores were inoculated into flasks containing liquid synthetic medium. At the indicated times, 5 mM-MGBG was added to five duplicate cultures; one duplicate remained as a control without MGBG. After 24 h incubation, germination and cell dry weight were measured. Data are mean values.

Time of MGBG addition (h)	Spore germination (%)	Cell growth [mg dry wt (100 ml) ⁻¹]
0	0	1.14
1	0	0.28
2	20	2.76
3	100	22.0
5	100	23.0
–	100	21.60

Determination of the effect of MGBG on the synthesis of RNA and protein demonstrated that the rate of RNA biosynthesis was only slightly affected by the drug during the first hour of incubation, it was reduced by more than 50% during the second hour and was almost completely abolished after this time (not shown). Similarly, the rate of protein biosynthesis was almost unaffected during the first hour of the germination process, decreased to about 50% during the second hour, and inhibition was almost complete after this time (not shown).

Effect of MGBG on polyamine biosynthesis

In normal cells, a rapid increase in the levels of spermidine occurs during the first hours of germination.

Fig. 4. Effect of MGBG on spore morphology. (a) Enlarged normal spores after 3 h of incubation. Bar, 10 μ m. (b) Normal germlings grown for 6 h. Bar, 10 μ m. (c) MGBG-treated cells incubated for 6 h. Notice their small size and their almost spherical shape. Bar, 10 μ m. (d) Electron micrograph of a thin section of a MGBG-treated cell incubated for 12 h in the presence of the drug. Notice the relatively thick cell wall with alternating electron-transparent and electron-dense zones. N, nucleus; M, mitochondrion; VA, vacuole; CW, cell wall. Bar, 1 μ m.

Fig. 5. Effect of MGBG on polyamine biosynthesis by germinating spores. Spores were incubated in YPG medium with ($\triangle$, $\blacktriangle$) or without ($\circ$, $\bullet$) 5 mM-MGBG. At intervals, samples were recovered for determination of cell protein and polyamines. $\bullet$, $\blacktriangle$, Putrescine; $\circ$, $\triangle$, spermidine. Concentrations are expressed as pmol per 100 ml aliquots.

The level then decreases. In contrast, accumulation of putrescine is delayed (Fig. 5). Apparently all putrescine synthesized during the initial periods of germination is rapidly utilized for spermidine biosynthesis. Addition of MGBG to spores inhibited the biosynthesis of spermidine and, indirectly, that of putrescine (Fig. 5). Since it inhibits protein biosynthesis it must block the increase in ODC formation which occurs at a late period of spore growth (Calvo-Mendez *et al.*, 1987).

Discussion

The results presented in this communication show that SAMDC is finely regulated during the germination of *M. rouxii* sporangiospores. In the initial periods of germination SAMDC activity increases. SAMDC is stimulated by putrescine, but not by Mg^{2+} . The possibility that activation of SAM decarboxylation by putrescine was artefactually due to H_2O_2 produced by oxidation of the diamine, as found in *Lathyrus sativus* (Suresh & Adiga, 1977) was ruled out. In fact Suresh & Adiga (1977) described putrescine-dependent SAMDCs from other sources which were not artefactual and concluded that the observation in *L. sativus* was not a general phenomenon.

The differential rate of synthesis of the putrescine-dependent enzyme changes before initiation of growth phase II. At the same time a second SAMDC activity which is insensitive to both Mg^{2+} and putrescine starts to increase. All organisms studied thus far contain a single SAMDC activity stimulated by either putrescine or

Mg^{2+} , or non-stimulatable by either effector (Pegg, 1984). Thus in this sense *M. rouxii* departs from the rule. The appearance of the second (putrescine insensitive) SAMDC activity is not the result of a permanent activation of the enzyme by the polyamine, but requires protein biosynthesis *de novo*, suggesting that it represents a different enzyme from the one which does respond to putrescine.

Causing polyamine shortage by the addition of the SAMDC inhibitor MGBG revealed the following. Firstly, the drug stopped the germination process at stage Ia, confirming our previous data that growth phases Ia and Ib represent different developmental stages during sporangiospore germination (Cano & Ruiz-Herrera, 1988). This effect was reversed by elimination of the drug or by polyamine addition. The drug did not significantly affect protein biosynthesis, and only slightly inhibited RNA biosynthesis during the time required to complete growth phase Ia, but it severely blocked the biosynthesis of both macromolecules after this time. MGBG inhibited polyamine biosynthesis in the fungus, and this is probably the basis for its inhibitory action in *Mucor* development. MGBG inhibits spermidine formation in mammary explants from mid-pregnant mice, and as a consequence blocks the stimulation of milk protein biosynthesis exerted by the combined action of insulin, hydrocortisone and prolactin (Oka & Perry, 1974). MGBG has also been shown to inhibit cell growth of *Entamoeba histolytica* (Kaul *et al.*, 1985) and *Acanthamoeba culbertsoni* (Gupta *et al.*, 1986), to block DNA biosynthesis in several animal systems, and to interfere in the cell cycle in different cell cultures (Pegg & McCann, 1982).

One important observation was that the effect of MGBG on spore germination was time-dependent. There was a 'window' of sensitivity to the drug restricted to the first 2 h of incubation. Addition of the drug after this time only delayed cell growth, so that after 24 h drug-treated cells were indistinguishable from untreated cells. We have reported similar results for the effect of DAB (an inhibitor of ODC) on the germination of *Mucor* spores (Ruiz-Herrera *et al.*, 1987). DAB addition decreases the levels of polyamines and stops development in growth phase Ib, preventing germ tube emergence, but addition of the drug after a critical time period has no further effect on cell growth. Based on these results it seems attractive to hypothesize that spermidine, but not putrescine, is required at an early stage of development to permit the expression of genes necessary for the establishment of growth phase Ib. In contrast, putrescine, but not spermidine, is required at a later step in development to inhibit DNA methylation and permit gene expression required for the establishment of the phase of polarized growth (Cano *et al.*, 1988).

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