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Polyamine Levels During Growth, Sporulation, and Spore Germination of *Bacillus megaterium*

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Spermidine was the major (>95%) polyamine of *Bacillus megaterium* in all stages of growth, although it could be replaced completely by spermine. Log-phase cells had 40 to 50% as much spermidine, based on ribonucleic acid (RNA) content, as did either stationary-phase cells or dormant spores; similar results were obtained in three other bacilli including an asporogenous mutant. Polyamine levels were essentially the same in *B. megaterium* grown in rich or poor media, or in media of high or low ionic strength. Polyamine levels were elevated three- to sixfold by exogenous spermidine without a major effect on growth, sporulation, or subsequent spore germination. During germination, the absolute amount of spermidine remained constant for almost 2 h until net RNA synthesis had lowered the polyamine/RNA ratio to a value close to that in log-phase cells. At this time, the spermidine level began to rise, and thereafter spermidine and RNA increased in parallel. This parallel relationship between the spermidine and RNA levels was abolished by actinomycin D, but not by chloramphenicol.

Polyamines such as putrescine, spermine, and spermidine are found in a wide variety of bacteria, although different species show different patterns and levels (8). Several roles have been proposed for these cations, including regulation of cellular osmotic pressure (12), ribonucleic acid (RNA) synthesis (4, 7), or protein breakdown (5); stabilization of RNA or ribosome structure (1, 6); and possibly ribosome function (2).

During sporulation and spore germination in *Bacillus* species, there are large variations in the content of small molecules and possibly also in cellular osmotic pressure (14); large variations in the rates of RNA and protein synthesis and protein breakdown (9, 19, 26); and possibly differences in the activity of ribosomes in cell-free, protein-synthesizing systems (10). It therefore was of interest to investigate polyamine levels during sporulation and germination in *Bacillus* species especially in view of the report that polyamine levels might be involved in controlling encystment in *Myxococcus xanthus* (31), a process with some similarity to sporulation in *Bacillus* species.

MATERIALS AND METHODS

Chemical reagents. Cadaverine, putrescine, spermine, spermidine, and chloramphenicol were purchased from Sigma Chemical Co. Actinomycin D was a gift from Merck, Sharpe and Dohme. Ninhydrin was purchased from the Pierce Chemical Co., and [^{14}C]-spermidine-trihydrochloride was purchased from New England Nuclear Corp.

Growth and isolation of cells and spores. The majority of the work described in this communication was carried out with *Bacillus megaterium* QM B1551, originally obtained from Hillel Levinson (U.S. Army Natick Laboratories, Natick, Mass.). *B. megaterium* JV 114, an asporogenous mutant derived from *B. megaterium* QM B1551 by nitrosoguanidine treatment, was kindly supplied by James C. Vary (University of Chicago Medical Center, Chicago, Ill.). This mutant gave <1% sporulation in supplemented nutrient broth and is probably blocked extremely early in sporulation (stage I or earlier, J. C. Vary, personal communication). *Escherichia coli* B was obtained from Parlane Reid (University of Connecticut Health Center, Farmington, Conn.).

Cells were grown at 30 C in three different media: supplemented nutrient broth (22), Spizizen medium supplemented with 0.1% Casamino Acids (25), and the sucrose-salts medium of Slepecky and Foster (24). Vegetative cells were harvested at 20 to 40% of maximal growth while still in log phase, and stationary-phase cells were harvested 3 to 6 h after the end of log phase. Harvesting was by centrifugation at room temperature (10 min, 8,000 × *g*), and pellets were immediately frozen in an ethanol-dry ice bath. Samples obtained at the different times in either log phase or stationary phase gave similar results.

Spores were obtained in the three growth media as described previously (13, 22), and in all media used (with or without exogenous polyamine) sporulation was >80%, as judged by counting in the phase-con-

trast microscope. Vegetative cells and cell debris were removed by extensive washing with water, and cleaned spore preparations were lyophilized and stored in a desiccator at room temperature.

Growth in the presence of exogenous polyamines (500 μM) was in Spizizen medium plus 0.1% Casamino Acids (25). Such cultures were harvested as described above but, in addition, cells were washed once and spores were washed three times with 50 volumes of 0.15 M NaCl at 25 C. All spore preparations used in this study were >95% refractile and were free from vegetative cells and cell debris as judged in the phase-contrast microscope.

Spore germination. All germination experiments utilized spores prepared in supplemented nutrient broth. Standard conditions for germination were as follows unless otherwise noted. Spores (20 mg [dry weight]/ml) were heat-shocked for 10 min at 60 C, cooled, and germinated at 30 C in Spizizen medium (25) without added Casamino Acids at a spore concentration of 500 μg (dry weight)/ml. Initiation of germination was >95% complete in 15 min, as judged in the phase-contrast microscope. I have called this initial 10 to 15 min the initiation of germination and have called the following 115 to 120 min germination. Germination is then followed by vegetative growth, and by 250 min of incubation 75% of the cells have undergone the first division. I have taken 130 min of incubation as the end of germination since in the germination medium used this is the time of initiation of rapid deoxyribonucleic acid synthesis (P. Setlow, unpublished results, 1973).

Extraction of polyamines. Polyamines were extracted from cells (1 to 4 g wet weight) with 10 ml of 5% trichloroacetic acid. After 4 h at 4 C, the solution was centrifuged and the pellet was re-extracted with an additional 5 ml of 5% trichloroacetic acid. The supernatant fractions were pooled and extracted five times with diethyl ether, with the addition of a few drops of concentrated HCl after each extraction. Polyamines were then extracted into alkaline butanol as described by Raina (15), the butanol phase was flash-evaporated, and the residue was dissolved in 0.5 ml of 0.1 M HCl. Dormant spores (100 to 400 mg dry weight) were extracted in an identical manner after rupture of the spores in a Wig-L-Bug with glass beads as the abrasive (20). The recovery of [^{14}C]spermidine added to the initial trichloroacetic acid extract of cells or spores was 87%, and all polyamine levels have been corrected using this value.

In experiments measuring polyamines during spore germination, spores (10 to 15 mg dry weight) were extracted with 2 ml of 5% trichloroacetic acid, the trichloroacetic acid was extracted with ether as described above, and the extract was lyophilized. Further purification by extraction into alkaline butanol was not required. Spermidine recovery in this procedure was 95%.

The levels of polyamines in cells were not affected significantly by the harvesting procedures used as shown by the following criteria. (i) Washing cell pellets with 0.15 M NaCl did not affect the levels of cellular polyamines (see Table 4). (ii) When vegetative cells (50 ml) growing in Spizizen medium plus 0.1% Casamino Acids were extracted with trichloroacetic acid (5.5 ml of 50%) without prior centrifugation, a value for cell polyamine level was obtained which was within 10% of the value obtained by extracting cells after centrifugation. Therefore, it appears that the routine harvesting procedures used permitted accurate measurements of polyamine pools.

Quantitation of polyamines. Polyamines were determined after separation by electrophoresis (75 min at 30 V/cm) on Whatman 3 MM paper in 0.1 M sodium citrate (pH 4.3) (18). Individual polyamines were quantitated by wetting the dried paper with a ninhydrin solution (ninhydrin, 1 g; cadmium acetate, 100 mg; ethanol, 85 ml; glacial acetic acid, 15 ml), drying for 10 min at 90 C to develop the color, and cutting out the colored spots and eluting them for 30 min in 3 ml of water-ethanol-acetic acid (1:4:5) containing 2 mg of cadmium acetate per ml. Polyamine concentrations were determined from the absorption at 505 nm by using a calibration curve constructed with known amounts (20, 50, and 100 nmol) of the polyamines in question which were also separated by electrophoresis on the same paper as the unknown. Background color was determined on each paper by eluting a spot identical in size to the polyamine spots. A 10-nmol amount of polyamines was easily detected with an error of ±1.5 nmol. All polyamine determinations reported in this paper are averages of at least duplicate determinations on extracts from at least two different cell or spore preparations.

Other workers have expressed polyamine levels relative to the amount of protein (12), RNA (16), or even dry weight (8). I chose to express polyamine content relative to the RNA level for several reasons. (i) Most polyamines in bacteria appear associated primarily with RNA, although this has not been definitively established (11). (ii) The work of Cohen and his associates has demonstrated a close parallel relationship between polyamine and RNA levels under a variety of conditions in *E. coli* (16, 17). (iii) A close relationship between polyamine and RNA levels has also been observed in a number of other systems (see reference 3 for discussion).

Nucleic acid extraction and assay. Cells or spores extracted with trichloroacetic acid to remove polyamines were treated further with 5 ml of 7% perchloric acid (PCA) for 20 min at 70 C to hydrolyze nucleic acids (21). The hydrolysate was centrifuged, the precipitate was treated with an additional 5 ml of PCA, and both supernatant fractions were combined. RNA was determined on the combined supernatants by using orcinol (21). All values for nucleic acids are averages of duplicate determinations on extracts from at least two different cells of spore preparations.

RESULTS

Polyamine levels in cells and spores. Spermidine comprised >95% of the polyamine found in all stages of growth of *B. megaterium* (Table 1). Less than 2% of the sperimidine was in the monoacetylated form, as determined by

TABLE 1. *Polyamines in B. megaterium*[a]

Polyamine	μmol of polyamine/extract		
	Vegetative[b]	Stationary	Spores
Spermidine	3.1	6.1	1.8
Spermine[c]	<0.15	<0.3	<0.08
Putrescine	<0.02	<0.02	<0.02
Cadaverine	<0.02	<0.02	<0.02

[a] Cultures were grown in supplemented nutrient broth and harvested, and polyamines were extracted and determined as described in Materials and Methods; ~4 g (wet weight) of cells and 240 mg (dry weight) of spores was extracted. All values are ±6%.

[b] Stage of growth.

[c] Lower limits of spermine are high due to interference by the large amount of spermidine present.

paper electrophoresis; acetylated putrescine and spermine were also absent, as shown by electrophoresis of polyamine samples after acid hydrolysis (24 h, 100 C, 6 M HCl). Indeed, the conditions for harvesting cells (room temperature centrifugation followed by quick freezing) were chosen to minimize the acetylation of polyamines reported to occur in *E. coli* (28).

The polyamine level (relative to RNA) in *B. megaterium* was almost identical in rich (supplemented nutrient broth) and poor (Slepecky and Foster [24]) growth media (Table 2). However, dormant spores and stationary-phase cells had 2 to 2.5 times more spermidine per unit of RNA than did log-phase cells (Table 2). In contrast to findings in *E. coli*, there was no decrease in the polyamine level in cells grown with a high NaCl concentration (Table 2; reference 12).

The similarity between the polyamine/RNA ratio of stationary-phase cells and spores was observed not only in *B. megaterium*, but also in *B. cereus* T and *B. subtilis* SB-133 (Table 3). The latter organisms also contained primarily spermidine as has been previously reported (8), and the polyamine/RNA ratio was 1.5 to 2-fold higher in stationary-phase cells than log-phase cells. *B. megaterium* JV 114, an asporogenous mutant derived from *B. megaterium* QM B1551, also had a higher polyamine/RNA ratio in stationary-phase cells, but the magnitude of this increase was slightly less than in the wild type. In addition, *E. coli B* showed an elevated polyamine/RNA ratio in stationary phase, and the percentage of the polyamine pool as putrescine also increased (Table 3). Values for polyamine levels in the *Bacillus* species and in *E. coli* are similar to those reported by other workers (8, 12, 16, 27), although exact comparison is difficult due to differences in bacterial strains, means of expressing polyamine content, etc.

Spermidine level during germination and subsequent vegetative growth. Since the spermidine/RNA ratio was significantly higher in spores than in vegetative cells, it was of interest to examine the changes in the polyamine level during spore germination. During the first 130 min of germination, the absolute amount of spermidine remained constant. However, the RNA level increased, thereby lowering the ratio of spermidine to RNA about twofold, close to the value for the log-phase cells (Fig. 1). At 130 min, the absolute level of spermidine began to

TABLE 2. *Spermidine levels in cells and spores of B. megaterium prepared in several media*[a]

Growth medium or reference for	μmol of spermidine/mg of RNA		
	Vegetative[b]	Stationary	Spores
Supplemented nutrient broth	0.058	0.144	0.145
Spizizen medium + 0.1% Casamino Acids (25)	0.054	0.121	0.127
Slepecky and Foster (24)	0.058	0.142	0.161
Supplemented nutrient broth + NaCl (0.5 M)	0.060	0.147	0.135

[a] Cultures were grown in the various media and harvested, and polyamines were extracted and determined as described in Materials and Methods. All values are ±10%.

[b] Stage of growth.

TABLE 3. *Polyamine levels in different stages of growth of several bacteria*[a]

Organism	μmol of polyamine/mg of RNA[b]		
	Vegetative[c]	Stationary	Spores
B. megaterium	0.058	0.14	0.15
B. megaterium JV 114	0.062	0.11	
B. cereus T	0.074	0.14	0.13
B. subtilis SB 133	0.081	0.14	0.13
E. coli B	0.31[d]	0.76[e]	

[a] Cultures were grown in supplemented nutrient broth, harvested and polyamines extracted and determined as described in Materials and Methods. All values are ± 10%.

[b] >90% of the polyamine in all the *Bacilli* was spermidine.

[c] Stage of growth.

[d] ~80% of the polyamine was putrescine and 20% spermidine.

[e] ~95% of the polyamine was putrescine and 5% spermidine.

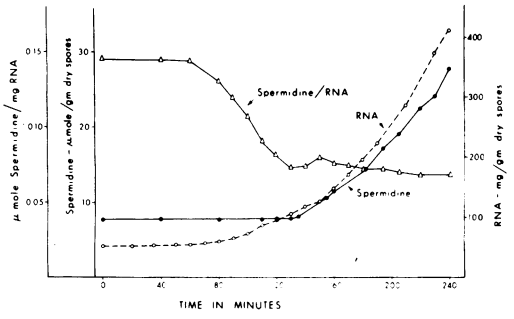

FIG. 1. *Spermidine and RNA levels during spore germination. Spores were germinated under standard conditions, and 30-ml samples were centrifuged (1.5 min, 10,000 × g) and frozen in dry ice-ethanol. The levels of spermidine and RNA were determined in the pellets as described in Materials and Methods.*

rise, and for at least the next 2 h spermidine and RNA increased almost in parallel.

Effect of inhibitors on spermidine and RNA levels late in germination and subsequent vegetative growth. Since spermidine and RNA increased almost parallel to one another during the vegetative growth following germination, it was of interest to determine whether this parallel relationship could be disrupted by addition of various inhibitors. As was observed in untreated cultures, spermidine and RNA levels also paralleled one another in cultures treated with chloramphenicol from 100 to 200 min of incubation (Fig. 2a,b). Despite significant or complete inhibition of spermidine accumulation (Fig. 2b), RNA synthesis was inhibited to the same extent, and therefore spermidine/RNA ratios were similar to those in untreated cultures. However, spermidine and RNA levels showed some independence of one another since inhibition of RNA synthesis at 190 min allowed further increase in sperimidine and a rise in the spermidine/RNA ratio (Fig. 2a,b).

Effect of exogenous polyamines in growth, sporulation, and germination. Because the polyamine/RNA ratio did not vary by more than 2.5-fold in different stages of growth, it was of interest to examine the effect of large variations in the type and amount of intracellular polyamine on growth and sporulation. Therefore, I grew cells in the presence of high levels of exogenous polyamine. Exogenous spermidine increased the polyamine/RNA ratio three- to fourfold in cells and sixfold in spores, and these increases were diminished only 20 to 25% by addition of high salt to the growth medium (Table 4). Exogenous spermine also elevated polyamine levels and completely (>95%) replaced spermidine (Table 4). However, neither putrescine nor cadaverine was taken up by cells or spores, and putrescine did not cause elevation of the spermidine content. Qualitatively,

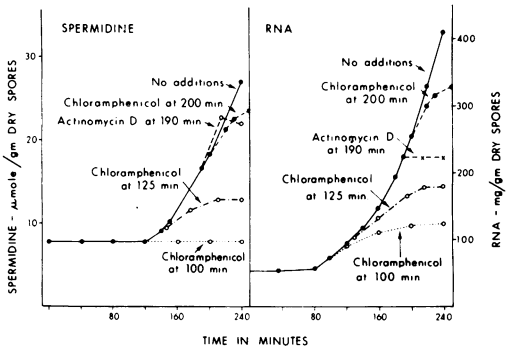

FIG. 2. *Effects of chloramphenicol and actinomycin D on spermidine and RNA levels in germinating spores. Spores were germinated under standard conditions, and at indicated times actinomycin D or chloramphenicol was added to part of the culture to a final concentration of 100 μg/ml. Samples (30 ml) were taken at various times and centrifuged (1.5 min at 10,000 × g), and spermidine and RNA were determined in the pellets as described in Materials and Methods.*

TABLE 4. *Polyamine content of B. megaterium cells and spores grown with exogenous polyamine*[a]

Addition to medium	μmol of polyamine/mg of RNA[b]		
	Vegetative[c]	Stationary	Spores
None	0.06[d]	0.13[d]	0.13 (<0.01)
NaCl (0.5 M)		0.14	0.12
Spermidine	0.18	0.55	0.78 (<0.04)
Spermidine + NaCl (0.5 M)		0.42	0.65
Spermine	0.15[e]	0.48[f]	0.71[f] (<0.04)
Cadaverine	0.06[g]	0.13[g]	0.14[g]
Putrescine	0.07[h]	0.14[h]	0.13[h]

[a] Cultures were grown in Spizizen medium (25) plus 0.1% Casamino Acids. Polyamines were added to 500 μM, and NaCl was added to 0.5 M. Cells and spores were harvested and washed, and polyamines were extracted and determined as described in Materials and Methods. The values in parentheses were determined on extracts prepared by trichloroacetic acid extraction of dormant spores without prior rupture in the Wig-L-Bug. All values are ±15%.

[b] The polyamine present was spermidine unless otherwise noted.

[c] Stage of growth.

[d] These values were identical in cells which were washed with 0.15 M NaCl.

[e] ~90% spermine and 10% spermidine.

[f] >95% spermine.

[g] <5% cadaverine.

[h] <5% putrescine.

similar results have been reported previously in both gram-positive and gram-negative organisms (30). Although I have no explanation for the significantly higher uptake of exogenous spermine and spermidine into the dormant spores, the excess polyamine is inside the spore since it is not released by extraction with 5% trichloroacetic acid (Table 4), a procedure which does not extract small molecules from intact spores (23).

None of the exogenous polyamines had any observable effect on growth or sporulation in Spizizen medium other than inhibition of the rates of these processes. As observed in the phase-contrast microscope, sporulation appeared normal in the presence of exogenous polyamines as did the spores produced, which germinated as well as spores with normal polyamine levels. However, exogenous spermine and spermidine did result in an increased doubling time (from 30 to 43 min), and increased by 1.5- to 2-fold the time required for appearance of refractile spores. The inhibitory effect of polyamines on growth rates has been observed in a number of bacteria (30). Exogenous polyamines also increased by three- to fourfold, the time required for release of refractile spores from the sporangia. Inhibition by polyamines of lysis of bacteria has been reported (30).

As was observed with growing cells, germinating spores also took up exogenous spermidine, and the kinetics of this uptake paralleled the changes in spermidine levels in untreated cultures (Fig. 3a). The rapid spermidine uptake beginning at 130 min of germination accumulated this polyamine to levels five times higher than in control cultures (compare Fig. 1 and 3a); and since the harvested cells were washed with a high concentration of salt (~0.15 M), this accmulation almost certainly represents intracellular polyamine (29). Chloramphenicol gave either partial or complete inhibition of uptake depending on the time of its addition, and actinomycin D gave complete inhibition of spermidine uptake. As might have been predicted from previous results, spermine in 10-fold excess almost completely abolished spermidine uptake (Fig. 3b), whereas cadaverine and putrescine were not inhibitory. NaCl gave some inhibition, but this was probably not a direct effect on spermidine uptake, but rather a result of significant inhibition (20 to 30%) by NaCl of the rates of protein and RNA synthesis during germination (P. Setlow, unpublished results, 1973).

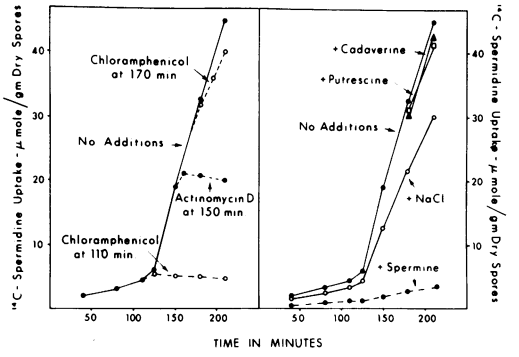

FIG. 3. *Effect of several compounds on uptake of exogenous spermidine during spore germination. Spores were germinated under standard conditions with 0.1 mM [^{14}C]spermidine (10^8 counts per min per nmol) present from zero time. At indicated times, 200-μliter samples were passed through a membrane filter (Millipore Corp.), which was then washed with 2 ml of fresh germination medium (salt concentration ~0.15 M) at 30 C. The filter was then dried under an infrared lamp and counted in a scintillation counter. Chloramphenicol and actinomycin D were added to a portion of the culture to a concentration of 100 μg/ml. Separate cultures contained spermine (1 mM), cadaverine (1 mM), putrescine (1 mM), or NaCl (0.5 M).*

DISCUSSION

Lack of involvement of polyamines in controlling sporulation. The 1.5 to 2.5-fold increase in the polyamine/RNA ratio in stationary-phase cells compared to log-phase cells was seen in all spore-forming *Bacillus* species tested. I have no explanation for this phenomenon, although it might be related to the rapid metabolism of organic acids taking place at this time. However, since the increase was observed not only in *Bacillus* species but also in *E. coli*, it is probably not related specifically to sporulation, but rather to some other property of the stationary phase of growth. This contention is strengthened further by the observation that an asporogenous mutant of *B. megaterium* also had an increased polyamine/RNA ratio in stationary phase as compared to log phase, although the magnitude of this increase was not quite as great as in the wild type. Increasing the polyamine/RNA ratio threefold by growth in exogenous spermidine did not cause sporulation in the mutant (P. Setlow, unpublished results, 1973).

Polyamines have been shown to be required for growth in a number of bacteria (30), and almost certainly there is some minimal amount necessary for sporulation. However, above this minimal amount the exact magnitude of the polyamine level does not seem crucial for sporulation since the process appeared normal (albeit slower) in cells containing fivefold-elevated spermidine levels. Furthermore, there was no absolute specificity in the polyamine necessary

for sporulation, since spermine could completely replace spermidine. However, neither putrescine nor cadaverine could replace spermidine.

Possible roles for spermidine in B. megaterium. It has been suggested that putrescine, but not spermidine, is involved in the regulation of osmotic pressure in *E. coli* (12). As was found in *E. coli*, growth of *B. megaterium* in a medium of high osmotic pressure also caused no lowering of the normal spermidine level (Table 2), suggesting that this polyamine does not regulate osmotic pressure in *B. megaterium*. This is suggested further by the inability of high salt to inhibit more than 25% of the extensive uptake of exogenous polyamine in either growing or sporulating cells or germinating spores (Table 4, Fig. 3).

A second role which has been suggested for polyamines is complex formation with RNA, causing gross regulation of the rate of RNA synthesis and/or stabilization of the RNA. The proposed functions would predict a close (possibly parallel) relationship between levels of polyamines and RNA, and in *E. coli* changes in RNA levels often produce similar changes in polyamine levels (16, 17). In *B. megaterium* there is clearly not a constant relationship between polyamine and RNA levels. This is demonstrated by the different polyamine/RNA ratios in different stages of growth (Table 2), the marked elevation of the polyamine/RNA ratio by exogenous polyamine (Table 4), and the continuation of spermidine accumulation in cultures of germinated spores treated with actinomycin D (Fig. 2). However, that there may indeed be some interrelationship between polyamine and RNA levels is seen in early vegetative growth where uptake of exogenous spermidine was abolished by actinomycin D, but not by chloramphenicol (Fig. 3a), and where spermidine accumulation did not begin until the value of the polyamine/RNA ratio reached that of the vegetative cell (Fig. 1). However, it is possible that the latter finding is only a coincidence and that the onset of spermidine accumulation at 130 min of germination is correlated with some other process. Indeed, in this germination medium, deoxyribonucleic acid synthesis begins at about this time (P. Setlow, unpublished results, 1973). It seems likely that the expectation of a direct one-to-one correlation between spermidine and RNA levels is an oversimplification of the role of spermidine, especially in view of the other cellular components which might affect polyamine-RNA binding, such as Mn^{2+}, Mg^{2+}, and possibly the intracellular pH. Possibly, knowledge of these latter variables might aid in outlining the precise relationship between spermidine and RNA levels.

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