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Polyamine Toxicity in *Neurospora crassa*: Protective Role of the Vacuole[1]

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We used mutant strains of *Neurospora crassa* to define the discretionary capacity of this species for excess putrescine. The *spe-3* mutant, which accumulates putrescine internally, and the *puu-1* mutant, which accumulates toxic levels of putrescine from the medium, both sequestered large excesses of putrescine in vacuoles. Concomitantly in *puu-1*, inorganic polyphosphate increased modestly and some of the monovalent cation of the vacuole was discharged. These two factors contribute to the increased capacity for polyamines in this fungus. Putrescine, however, can exceed the capacity of vacuoles such that they no longer protect the cytosol from toxic levels of the amine. The *puu-1* mutant illustrates the importance of the sequestration of intracellular polyamines, as well as the control of polyamine uptake through the cell membrane.

A mutation, *puu-1*, imparts to cells growing in normal medium the ability to concentrate polyamines, particularly putrescine, in the cell (1). The *puu-1* mutation renders the polyamine uptake system much less sensitive to external Ca^{2+} in the medium (1). The polyamine uptake system of the wild type, by contrast, is wholly inhibited in the growth medium, and polyamines enter the cell only by a nonsaturable, diffusional mechanism (1–3). Here, we use the *puu-1* strain and a strain, *spe-3* (4), which accumulates biosynthetic putrescine, to show that *Neurospora* has a sizable capacity for excess polyamines. When the medium contains a high concentration of putrescine (ca. 5 mM), the growth rate of *puu-1* strains diminishes markedly. We wished to identify the location of excess putrescine and some of the effects of toxic levels of this compound.

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MATERIALS AND METHODS

Strains. In addition to the wild type and *puu-1* strains [ORS-6a and IC2296-1a in the previous paper (1)], we also used two strains carrying the *spe-3* mutation, LV105. These were IC2572-41 (*spe-3 aga*) and IC2572-1A (*puu-1 spe-3 aga*). The *spe-3* mutation imposes a severe, but incomplete deficiency in the synthesis of spermidine, presumably at the spermidine synthase step (4). The *aga* mutation imposes an arginase deficiency, but is not relevant to this work.

General methods. Uptake and excretion of polyamines, and binding of polyamines to *n*-butanol-permeabilized cells, were measured as described previously (1–3). K^+ and other inorganic ions were determined by atomic absorption spectroscopy as described previously (1). Polyamine determinations on cell extracts and fractionated media were done by high performance liquid chromatography (1, 5).

Cell fractionation. Exponential cells were disrupted in osmotically stabilizing media, and the homogenates were fractionated by methods previously described (6). In some cases, the mixed organellar pellet collected at 15,000*g* was further resolved into vacuolar and mitochondrial fractions on discontinuous sucrose gradients (6).

Polyphosphate extraction and determination. In *N. crassa*, vacuoles contain almost all the polyphosphates (7). Their chain length distribution renders most of them intrinsically acid-insoluble. At neutral pH, however, most polyphosphates are soluble in solutions lacking divalent cation. Most of these polyphosphates become artifactually bound to a polygalactosamine-containing polymer of the cell wall, in neutralized extracts of cells (8). Only the longer chain polyphosphates that are in stoichiometric excess of cell wall polycations are retrieved in soluble, neutral solution. The remaining, bound polyphosphates can be determined after solubilizing them by partial hydrolysis, as described by Harold (9). The binding of polyphosphate to insoluble material is far less in preparations of organelles and pure vacuoles.

The specific extraction and estimation methods, based on those of Clark and Wood (10) as modified by Schuddemat *et al.* (11), and on that of Harold (9), were as follows: Acetone powders were made by grinding acetone-dried mycelial pads (12) in a mortar. Powders (25 mg) were extracted in 1.5-ml Eppendorf tubes in 0.3 ml 2% trichloroacetic acid and centrifuged. The pellet was successively washed in 1 ml 0.7% trichloroacetic acid–67% acetone and 1 ml 67% acetone. The three supernatants were combined and extracted with 2.3 ml of a 1:1 mixture of 90% phenol (equilibrated at pH 6.5 with 0.1 M NH_4^+ acetate) and chloroform and treated with 15 mg Norit A. The fraction contained the inorganic phosphate and acid-soluble polyphosphate and is denoted Fraction *a*.

The remaining cell pellet was suspended in 0.5 ml 2 mM EDTA, followed by neutralization of the suspension with LiOH. Phenol–chloroform (0.2 ml) was added and the suspension was shaken vigorously. The su-

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pernatant of this step contained long-chain soluble polyphosphate, and was denoted Fraction *b*.

The residue was extracted successively with 1.0 ml ethanol and 1.0 ml ethanol–ether (3:1), the latter at 100°C for 1 min. The last residue was suspended in 1 ml 0.5 N $HClO_4$ and held at 70°C for 15 min. The supernatant was removed after centrifugation, and the step was repeated on the residue. The supernatants were combined and treated with Norit A. This fraction, largely hydrolyzed polyphosphate from cell wall and other polymers, was denoted Fraction *c*. This fraction varied very little, as though "insoluble" polyphosphate had saturated cell-wall binding sites of constant capacity. Controls showed that nucleic acids did not yield inorganic phosphate during the preparation of Fraction *c*, but that authentic polyphosphate (chain-length 200) did so. For that reason, Fraction *c* polyphosphate was taken as the sum of inorganic and acid-labile phosphate (determined after hydrolysis; see below) in standard determinations.

For Fractions *a* and *b*, phosphate determinations by the method of Onishi (13) were made before and after hydrolysis in 1 N HCl for 10 min at 100°C. Inorganic polyphosphate was calculated by difference and reported as nanomoles P.

RESULTS

Growth and Solute Pools of the puu-1 Mutant

The *puu-1* mutant grew almost as fast as the wild type in exponential culture (t_D ca. 2.7 h). After germination, however, there was often a pause in weight increase, not seen in the wild type, before vigorous exponential growth of *puu-1* began, and old, frozen conidial inocula often yielded cultures with a longer lag and slower growth. Addition of putrescine (1 or 5 mM) inhibited growth of the *puu-1* mutant, whereas the wild type grew normally (Table I). The *puu-1* mutant varied in its susceptibility to putrescine inhibition. This suggests that the mutant lives at the lower limit of good health.

Wild type cells grown in minimal medium normally have about 0.8 nmol putrescine and 18 nmol spermidine per milligram dry weight. [*N. crassa* makes little spermine (14).] Putrescine added to the medium simply equilibrates with cell water [2.5 ml per gram, dry weight (15)] in young cultures. Thus at 5 mM external putrescine, the initial internal concentration in the wild type is 10 nmol per milligram dry weight, or about 4 mM (Table I). Wild type concentrates putrescine somewhat at later times in this medium, a point pursued in the Discussion. The *puu-1* mutant, grown in unsupplemented medium, contained normal polyamine pools. Addition of 1 and 5 mM putrescine, however, caused *puu-1* to accumulate enormous internal pools of this amine (nominally over 80 mM in cell water, or 200 nmol/mg, dry weight) (Table I). The relative insensitivity of the spermidine pool of *puu-1* to putrescine indicates that putrescine saturates the enzymes of spermidine synthesis and may actually compete with spermidine for occupancy of the cell (compare 1 and 5 mM putrescine, Table I). The *puu-1* cells did not excrete detectable amounts of spermidine into the medium under these conditions (data not shown).

The normal spermidine pool of the wild type (ca. 18 nmol per milligram, dry weight) is largely bound to cellular constituents, about one-third of it to vacuolar polyphosphate (3, 16). In medium supplemented with 5 mM spermidine, the spermidine pool of the wild type was 30 nmol per milligram, and that of the *puu-1* mutant was 70 nmol (data not shown). With 18 nmol bound, the remaining 12 nmol in the wild type simply represents equilibration of free spermidine (5 mM = 12.5 nmol per milligram, dry weight) across the cell membrane as we have shown previously (3). The much higher concentration of free spermidine (ca. 52 nmol per milligram, nominally about 21 mM) in the mutant under these conditions shows that the mutant can concentrate spermidine as well as putrescine.

TABLE I

Polyamine, Amino Acid and K^+ Pools of Wild Type ORS6A and IC2296-1A (*puu-1*)[a] Grown with Various Putrescine Supplements

Strain	[PUT]	t_D	Dry weight	PUT[b]	SPD	K^+	ORN	ARG
	(mM)	(h)	(mg/ml)			(nmol/mg dry wt[c])		
Wild type	0	2.5	0.39–0.94	1	22	436	26	28
	1	2.5	0.48–1.05	4	22	457	34	24
	5	2.7	0.46	10	28	442	50	28
			0.66	14	28	408	26	35
			0.97	22	38	436	30	41
puu-1	0	2.7	0.50–1.05	1	23	483	34	31
	1	3.1	0.4–0.77	132	29	334	13	33
	5	4.0	0.30	200	25	310	20	50
			0.39	218	25	297	13	47
			0.51	225	23	296	8	36

[a] Mycelia grew exponentially; three harvests were made and the values were averaged over the interval of increase indicated for the 0 and 1 mM putrescine cultures. Values for individual time points are shown for the 5 mM cultures to show significant changes that occurred during growth under that condition.

[b] Abbreviations used: ARG, arginine; ORN, ornithine; PUT, putrescine; SPD, spermidine; t_D, doubling time.

[c] Intracellular water is 2.54 μl per milligram (15); nominal solute concentrations (mM) are 0.4× the figures given.

TABLE II

Cations and Polyphosphate in Whole Cells and Organelles of *puu-1*

Culture[a] and fraction	ARG	PUT	SPD	Polyphosphate[b] a	b	c	Total	PUT/ARG[c]	PP/ARG[c]
Minimal									
Whole cells (a)	28	1	27	24	31	172	227	0.04	8.1
(b)	20	1	22	33	20	178	231	0.05	11.6
Organelles (a)	644	22	137	126	1462	2295	3883	0.03	6.0
Vacuoles (b)	776	16	229	177	1934	2105	4216	0.02	5.4
1 mM Putrescine									
Whole cells (a)	36	143	28	15	83	162	260	4.0	7.2
(b)	40	127	32	31	51	200	282	3.2	7.1
Organelles (a)	284	747	134	40	1710	2057	3807	2.6	13.4
Vacuoles (b)	330	805	196	173	1630	2210	4012	2.4	12.2

[a] Cultures were harvested at 1 mg per milliliter, dry weight. Values for whole cells are nmol per milligram, values for organelles (containing mainly mitochondria, vacuoles, and glyoxysomes) and for purified vacuoles are given as totals for the fraction studied. The data from two experiments, (a) and (b), are identified.

[b] Polyphosphate values are for fractions a (acid soluble), b (long chain soluble), c ("insoluble"), and the total (see Materials and Methods).

[c] Arginine is almost exclusively localized in vacuoles. Therefore other exclusively vacuolar materials will have about the same ratio to arginine in whole cells and in fractions containing vacuoles. Abbreviations as in Table I.

The Na^+, Ca^{2+}, and Mg^{2+} contents of *puu-1* grown in minimal medium or medium supplemented with 1 mM putrescine were normal (data not shown).

K^+ is the major cytosolic cation in *Neurospora* (15); little is found in vacuoles (16). External putrescine did not alter the K^+ pool of wild type cells. Mycelia of *puu-1* had normal K^+ pools in minimal medium, but putrescine added to 1 or 5 mM caused the K^+ pool to diminish 30 and 38%, respectively (Table I). Overall, the milliequivalents of putrescine + K^+ in *puu-1* in minimal medium (483 + 2 = 485) increased about 23% in medium with 1 mM putrescine (334 + 264 = 598), and 51% in 5 mM putrescine. Thus K^+ and putrescine compete somewhat for occupancy of the cytosol, and the growth rate of *puu-1* suffers from putrescine accumulation. However, the tolerance of *puu-1* for its huge putrescine pool suggests that it may not lie entirely in the cytosol.

In steady-state cultures of the wild type, over 98% of the basic amino acids (arginine, ornithine, lysine, and histidine) is concentrated in the vacuoles, together with about one-third of the spermidine (14, 16, 17). These solutes constitute over 80% of the cationic equivalents of the vacuole (16). About half of the amino acids, and all of the spermidine, are bound to inorganic polyphosphate (16). The arginine and ornithine pools of the wild type were not affected by growth in the presence of 1 mM putrescine (Table I). This was expected, because little putrescine entered the cells. The addition of 1 mM putrescine reduced the ornithine pool of the *puu-1* strain to 38% of normal. The addition of 1 mM putrescine did not affect the pool of arginine, a stronger cation than ornithine, in the mutant. The addition of 5 mM putrescine to the mutant caused arginine initially to increase for unknown reasons (Table I). The effect of added putrescine upon the mutant's pool of ornithine (and, presumably, of vacuolar lysine and histidine pools) suggests that putrescine competes with the more weakly basic amino acids for occupancy of the vacuole.

Polyphosphate and the Localization of Putrescine in puu-1

Vacuoles contained most of the excess putrescine in *puu-1* cells grown in 1 mM putrescine (Table II). When normalized to arginine [which is wholly vacuolar (18)], putrescine rose to a similar extent in crude organelles (87-fold), purified vacuoles (120-fold), and whole cells (80- to 100-fold), compared to values for the culture grown in minimal medium.

Butanol-permeabilized *puu-1* cells, grown in the presence of 1 mM putrescine, retained their larger putrescine pools (Table III), as expected from previous data on the wild type (3). However, if polyamines were included in the permeabilization medium, *puu-1* cells that had accumulated polyamines during growth were able to bind much more polyamine (an additional 320 meq per milligram dry weight in the case of putrescine) than the wild type or *puu-1* cells grown in minimal medium (Table III). This indicated that cells conditioned by polyamine accumulation during growth contained a larger amount of accessible, fixed anion than cells with normal polyamine pools. Microscopic inspection of *puu-1* cells grown in 1 or 5 mM putrescine (particularly the latter) revealed greatly enlarged vacuoles, similar to those of cells with excess arginine (16). The polyamine-binding capacity and the engorged vacuoles suggested that there might be more vacuolar polyphosphate in putrescine-accumulating cells.

TABLE III

Retention of Polyamines by Butanol-Permeabilized Cells of Wild Type and *puu-1*[a]

Strain	Supplement to		Polyamine retained by cell pellet (nmol · mg^{-1} dry wt)	
	Growth medium	Permeabilization medium	Putrescine	Spermidine
Wild type	None	None	3.3	21.3
		1 mM PUT	34.6	18.9
		1 mM SPD	1.2	80.9
	1 mM PUT	None	5.1	28.0
		1 mM PUT	25.5	25.8
	1 mM SPD	None	0.6	36.2
		1 mM SPD	0.7	65.6
puu-1	None	None	0.4	28.3
		1 mM PUT	21.5	23.1
		1 mM SPD	1.9	76.9
	1 mM PUT	None	62.3	20.2
		1 mM PUT	185.9	20.0
	1 mM SPD	None	0.7	100.7
		1 mM SPD	0.7	146.6

[a] Exponential cultures, variously supplemented, were harvested, washed with 0.25 M NaCl to remove external polyamine, and placed in permeabilization medium. The latter was Na^+ Mops, pH 7.2 (20 mM cation), 0.2% glucose and 7.5% *n*-butanol, with [^{14}C]alanine as an internal marker. Cells were centrifuged, extracted, and the volume of supernatant retained by cell pellets determined by radioactivity. Polyamines in the extract were determined and corrected for ambient polyamine in the retained supernatant. Permeabilized cells bound only a small fraction of the 1 mM added polyamine. Methods from Refs. (2, 3).

Comparison of *puu-1* cells grown in minimal medium and with 1 mM putrescine revealed a small increase (about 40 meq phosphate) of total (vacuolar) polyphosphate, insufficient to neutralize the additional 270 meq putrescine (Table II). The observation conforms to the observed partial displacement of monovalent cations from the vacuole, and their replacement by polyamine. However, the displacement of resident cations is not enough to accommodate added putrescine, nor is the small increase of polyphosphate consistent with the much higher polyamine-binding capacity of permeabilized cells (Table III). We conclude that the polyphosphate of normal cells (such as the wild type and *puu-1* grown in minimal medium) was not nearly as accessible in permeabilized cells as the polyphosphate of polyamine-accumulating cells. The Discussion explores this matter further.

Internal Accumulation of Putrescine by the spe-3 Mutant

We compared Puu^+ and Puu^- strains that carried the *spe-3* mutation, LV105. The *spe-3* mutant has a substantial block in spermidine synthesis, but will grow normally for some time in minimal medium. In minimal medium, both *spe-3* and *spe-3 puu-1* accumulated internally synthesized putrescine equally (Table IV). Therefore the *puu-1* mutant resembled the wild type in its ability to accumulate putrescine produced within the cell. In particular, the results show that the Puu^+ strain had the capacity to accumulate a high level of putrescine. The expected difference between strains appeared, however, after putrescine was added to *spe-3* and *spe-3 puu-1* cultures (Table IV). The data show that the defect in *puu-1* lies in excessive transport through the cell membrane, not in its capacity to store excess polyamines. Further, it appeared that the rate of putrescine synthesis, though substantial, limited the amount of putrescine accumulated by the two strains in minimal medium.

The putrescine/arginine ratio of organelles of the *spe-3* and *spe-3 puu-1* strains grown in minimal medium was half that of whole cells (Table IV). This suggested that half of the higher putrescine pool in these strains lay in the organellar fraction, i.e., vacuoles. It is likely that in view of the severe spermidine deficiency caused by *spe-3*, the large amount of nonvacuolar putrescine inferred was bound to cytosolic sites normally occupied by spermidine. By contrast, the even greater excess of putrescine in the putrescine-supplemented culture of *spe-3 puu-1* appeared to lie in the vacuoles.

Of the putrescine made by cells grown to 1 mg, dry weight, per milliliter, similar proportions—8 and 6% for *spe-3* and *spe-3 puu-1*, respectively—had leaked into the medium. The difference between strains was not significant, owing to variation in polyamine determinations in cells and medium.

DISCUSSION

The *puu-1* mutant has an abnormal ability to concentrate putrescine and spermidine from the growth medium,

TABLE IV

Putrescine Pool and Its Vacuolar Location in *spe-3* Strains

Strain	Medium	Fraction	nmol per[a]	Pool			PUT/ARG
				ARG	PUT	SPD	
spe-3	Minimal	Cells	mg	37	58	1	1.5
		Organelles	Total	863	670	9	0.8
	1 mM PUT	Cells	mg	45	66	1	1.5
		Organelles	Total	1021	828	8	0.8
spe-3 puu-1	Minimal	Cells	mg	26	62	1	2.4
		Organelles	Total	1042	1435	8	1.4
	1 mM PUT	Cells	mg	21	231	1	10.8
		Organelles	Total	346	2983	1.3	8.6

[a] Values are given per milligram for whole cells, and per total fraction for organelles, owing to losses in isolation. The two are normalized to the vacuolar marker, arginine, in the last column.

while the *spe-3* mutant accumulates putrescine internally as the result of a genetic block. We demonstrated that the discretionary capacity of *N. crassa* for polyamines lay in vacuoles, and that this was a normal feature of Puu^+ cells. This interpretation confirmed our conclusion (1) that the *puu-1* mutant differs from the wild type in transport of putrescine and spermidine through the plasma membrane, not in its potential capacity for, or localization of, polyamines within the cell. Indeed, the mutation was isolated because it imparted an unusual ability to a Spe^- strain to use putrescine for growth; putrescine is used in the cytosol via the spermidine synthase reaction. Finally, if vacuolar uptake of putrescine were abnormally high in the *puu-1* mutant, the polyamine pools of cells grown in minimal medium should have been elevated. The primary defect of the *puu-1* mutation is discussed further in the accompanying paper (1).

Vacuoles took up excess putrescine, even in Puu^+ cells. Where putrescine was accumulated to growth-inhibiting levels by the *puu-1* mutant, the ornithine pool became lower as cellular putrescine rose, while the major cytosolic solute, K^+, was less affected. This indicates that vacuoles can sequester excess polyamines and minimize derangement of cytosolic ion balance up to a point. This capacity is limited, and beyond it, polyamines flood the cytosol, displacing K^+. The inhibition of growth of the *puu-1* mutant by putrescine demonstrated the toxicity of a high concentration of cytosolic putrescine. None of these effects were seen in the wild type, which did not concentrate polyamines very much from the growth medium. We do not know the basis of putrescine toxicity in *Neurospora*. Toxicity has been correlated with aldehyde formation in polyamine oxidase reactions in other organisms, but *Neurospora* does not catabolize polyamines to any great extent (2). Putrescine toxicity in the cyanobacterium, *Anabena*, however, has been correlated with conjugation of putrescine to ribosomes, leaving them incompetent to synthesize protein *in vitro* (19).

The modest increase in polyphosphate contributed only slightly to neutralizing excess putrescine in the vacuole. Displacement of basic amino acids, the major cationic occupant of the vacuole (16), was more significant in retaining electroneutrality within the organelle. A change of state of the polyphosphates in permeabilized cells that had accumulated putrescine may be inferred from our data. In normal cells, a substantial part of the polyphosphate forms a condensed granule in the vacuoles. The composition and state of the granule is not known. Its cationic counterions may include spermidine, Ca^{2+}, Mg^{2+}, and possible other nonpolyamine multivalent cations (16), and these might be retained by permeabilized cells. With the hydration of vacuoles full of excess polyamine, this granular form may have changed to a more open, soluble state with the displacement of more of the original counterion. In fact, *in vitro*, spermidine is known to cause granule formation when added to polyphosphate; putrescine does not do so (20). Conceivably, putrescine may neutralize more equivalents of polyphosphate that are accessible to the normal counterions in the condensed granule. This may account for the ability of permeabilized, putrescine-accumulating cells to bind more polyamine despite the fact that there was not much more polyphosphate. This is not a wholly satisfactory hypothesis, and only more study will resolve the matter.

Finally, the swollen vacuoles demonstrated an increased osmotic content of the organelle (16). This in turn suggested that putrescine entry (in meq) greatly exceeded basic amino acid efflux and the available polyphosphate, in which case small anions must also have been drawn into the vacuole as counterions.

We reported previously that increased basic amino acids in wild type cells did not lead to increased polyphosphate levels (7). Our more sophisticated method of estimating polyphosphate also revealed an increase in polyphosphate (about 80 nmol P per milligram dry weight) in mycelia grown in arginine as a nitrogen source (R. H. Davis and

J. L. Ristow, unpublished experiments). It was not nearly enough to neutralize the 220 cationic equivalents by which the basic amino acid pool increased. Thus the same principles governing putrescine/polyphosphate ratios (seen in *puu-1*) also govern arginine/polyphosphate ratios in wild type.

The *puu-1* mutant accumulates vast amounts of putrescine owing to a peculiar insensitivity of the transport system to membrane-bound Ca^{2+}. This provokes vacuolar uptake of putrescine. The behavior of the *spe-3 puu-*$^{+}$ mutant demonstrates that this is a natural response of *Neurospora* cells to excess putrescine. Indeed, the vacuolar pool accounts for a large part of the excess polyamine. We must ask why the wild type, in the presence of 5 mM putrescine, does not also accumulate putrescine to high levels, even though the entry of polyamine through the cell membrane is diffusional and nonconcentrative, at least in young cultures. The data of Table I show that wild type, grown with 5 mM putrescine, actually do concentrate the amine 1.6-fold (to 21 nmol per mg, or 8 mM) by the time they reach 1 mg per milliliter dry weight. The difference between this culture and those of the *puu-1* and *spe-3* mutants suggests that the vacuolar uptake system—whatever its mechanism—has extremely low affinity for putrescine, and significant rates of uptake are seen only when the cytosolic concentration is high. The leakage of putrescine into the medium by *spe-3* strains also indicates that the cytosolic concentration is relatively high under conditions of vacuolar putrescine accumulation.

In conclusion, we have shown that control of polyamine uptake through the cell membrane (1), and the ability to sequester polyamines in vacuoles, mitigates toxicity of amines in *Neurospora*. These mechanisms are all the more important because, unlike many other microorganisms, *Neurospora* lacks significant capacity to degrade or convert polyamines to nontoxic or further metabolizable compounds (2).

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