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Osmotic Effects on the Polyamine Pathway of *Neurospora crassa*

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DAVIS, R. H., AND RISTOW, J. L. 1995. Osmotic effects on the polyamine pathway of *Neurospora crassa*. *Experimental Mycology* 19, 314–319. In bacteria, mammals, and certain plants, the induction of the polyamine synthetic enzyme, ornithine decarboxylase (ODC), and the accumulation of its product, putrescine, follows osmotic manipulations of cells. In at least some of these cases, this response is indispensable for survival. We wished to determine whether the polyamine pathway of *Neurospora crassa* was regulated in response to hyper- or hypoosmotic conditions. Unlike ODC of most other classes of organisms, the *N. crassa* enzyme and the accumulation of putrescine appears to be relatively indifferent to these conditions, either during sudden transitions or in steady-state. We conclude that other mechanisms of osmotic adjustment or tolerance have evolved in *N. crassa* and perhaps other fungi that obviate the need for putrescine accumulation. © 1995 Academic Press, Inc.

The polyamines (putrescine, spermidine, and spermine) as a group are abundant organic cations in all cells (Pegg, 1986). In mammals and fungi, they are made via a pathway initiated by ornithine decarboxylase (ODC), which forms putrescine (1,4-diaminobutane) from ornithine. Putrescine is converted to spermidine in a subsequent reaction, spermidine synthase. The latter requires decarboxylated S-adenosylmethionine, made by a second, dedicated polyamine enzyme, S-adenosylmethionine decarboxylase. ODC, rate-determining in normal cells (Pitkin *et al.*, 1994), is highly regulated in response to growth stimuli, the polyamine status of the cell, and, in many organisms, the ambient osmotic potential (Davis *et al.*, 1992). In many bacteria and plants, an additional route of putrescine formation coexists with ODC, namely, the decarboxylation of arginine to form agmatine, followed by the agmatine ureohydrolase reaction, which yields putrescine and urea (Tabor and Tabor, 1985).

We wished to determine whether a sustained, low osmotic potential or hypoosmotic shock would lead to a substantial rise in ODC activity in *Neurospora crassa*. This response, leading to a large accumulation of putrescine, is character-

istic of mammalian (Poulin *et al.*, 1991), plant (Flores and Galston, 1982), and certain bacterial (Munro *et al.*, 1972; Munro and Bell, 1973) cells, and in the first two cases cited, it is required for survival of hypoosmotic shock or continued optimal growth. We extended our study to the effect of hyperosmotic stress and K⁺ starvation, because these conditions elicit increased putrescine synthesis in leaves of certain plant species (Flores *et al.*, 1984; Flores, 1991). In the plants studied, the increased synthesis of putrescine is correlated with elevated arginine decarboxylase activity.

In fungi, little is known about the effect of osmotic changes on the polyamine pathway. Fungi have a tough cell wall and may for this reason not require some of the biochemical adjustments to hypotonic or hypoosmotic conditions seen in mammals (Slayman, 1977). In addition, fungi have a well developed means of adjustment to hyperosmotic conditions, namely, the synthesis of polyols such as glycerol, erythritol, and mannitol (Beever and Laracy, 1986; Ellis *et al.*, 1991). We wished to know how these adaptations to hypo- or hyperosmotic conditions were correlated with the behavior of the polyamine pathway.

MATERIALS AND METHODS

Strains and media. Our standard (wild-type) strain in these studies, IC3, carries the *aga* (ar-

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ginaseless) mutation, which we take advantage of in studies of polyamine metabolism (Paulus and Davis, 1981). This has no metabolic significance here, because arginase is inactive in wild-type cells grown in the absence of arginine (Davis *et al.*, 1970). A strain, IC1894-53a, carrying the *aga* and *spe-1*(LV10) mutations, the latter eliminating ODC activity (Davis *et al.*, 1987), was used in one experiment. Two transformants of an ODCless strain were used, one (PL2) carrying a plasmid with the normal *spe-1* gene and the other (DP6) carrying a plasmid with a constitutively expressed *spe-1* gene (Pitkin *et al.*, 1994). Three mutants of the osmotic-sensitive type, *os-1*(M16), *os-2*(UCLA80), and *os-5*(UCLA84), were obtained from the Fungal Genetics Stock Center, University of Kansas Medical Center. Exponential cultures were grown overnight at 25°C in Vogel's medium N using heavy conidial inocula (Davis and De Serres, 1970). Osmotic and other variations in the medium were accomplished as indicated in the Results. Spheroplasts were prepared with Novozyme 234 (Orbach *et al.*, 1986).

Assays. ODC activity assay and polyamine determinations were made as described (Davis *et al.*, 1985). ODC-specific activities are reported in nmol/h (mU)/mg protein. The very low, repressed ODC activity of the permeabilized cells used normally varies over a 2- to 2.5-fold range.

RESULTS

We first compared the ODC activities of mycelia grown in normal, hypoosmotic and hyperosmotic media (Table 1). ODC activity was similar in cultures grown continuously in normal medium (77 mU/mg), one-third strength medium (86 mU/mg), and Vogel's medium to which 1 M sorbitol was added (68 mU/mg). The differences in activity were not significant, given the standard deviations of about 15–20 mU/mg protein in the three determinations. Transfers of cells among these conditions had no significant effect upon ODC activity (Table 1).

We next applied extreme hypotonic shock by transfer of mycelia to distilled water or to water supplemented by very low levels of casamino acids and glucose. These conditions had no effect over 30 min, and in fact ODC declined at later times as internal nutrients were depleted (Fig. 1). Other experiments showed that ODC, an unstable enzyme, declines on a similar schedule after omission of individual sources of carbon, phosphorus, nitrogen, and sulfur (Jacobs and Davis, unpublished observations).

The putrescine content of cells transferred from Vogel's medium to Vogel's medium containing 4% NaCl or 1 M sorbitol or to distilled water did not change significantly (Table 2), consistent with the observation in the same ex-

TABLE 1
ODC Activity after Transfer between Various Media^a

Initial medium ^b	Transferred to	<i>T_d</i> (h) (initial)	<i>T_d</i> (h) (new)	ODC-specific activity (mU/mg)
Vogel's	Vogel's	3.0	3.0	77 ± 22
Vogel's	Vogel's + 1 M sorbitol	3.0	2.7	82 ± 30
Vogel's	1/3X Vogel's	3.0	2.2	78 ± 21
Vogel's + 1 M sorbitol	Vogel's + 1 M sorbitol	4.0	4.0	68 ± 15
Vogel's + 1 M sorbitol	Vogel's	4.0	3.0	54 ± 18
Vogel's + 1 M sorbitol	1/3X Vogel's	4.0	No growth	77 ± 14
1/3X Vogel's	1/3X Vogel's	2.6	2.6	86 ± 21

^a Mycelia were transferred at an early stage of growth (0.3 mg dry weight/ml); they were then sampled three times over the next 3–4 h (1–1.5 doublings).

^b Nominal osmotic strengths: 1/3X Vogel's = ca 100 mOsm; Vogel's = 300 mOsm; Vogel's + 1 M Sorbitol = 1300 mOsm.

FIG. 1. ODC activity before and after transfer to distilled water (closed circles) and to distilled water with added casein hydrolyzate (100 μ g/ml), tryptophan (12 μ g/ml), and D-glucose (0.1%) (solid circles). A culture growing continuously in normal Vogel's medium was maintained as a control (open squares).

periment that ODC activity changed little. Cells accumulated spermidine in the media in which growth did not continue (4% NaCl and water), and the accumulation was no more than 50% of normal. The accumulation may reflect the continued synthesis of spermidine after growth stopped.

We reasoned that the tough cell wall of *N. crassa* might protect cells from swelling suffi-

ciently in hypotonic media to provoke a response by the polyamine pathway, as has been inferred for plant cells (Flores *et al.*, 1984). We therefore prepared and tested spheroplasts. Because spheroplast preparation uses 1 *M* sorbitol as an osmotic protectant, a parallel preparation of cells without cell-wall digestion was maintained in 1 *M* sorbitol until the osmotic changes were applied. Both sets of cells were diluted into Vogel's medium with 1.0, 0.75, 0.5, 0.25, and 0 *M* sorbitol and to $\frac{1}{3}$ Vogel's medium without sorbitol. The results (Table 3) show that the ODC activity of control cells (with cell walls) did not respond to any of the dilution media. Spheroplasts showed little difference in ODC activity in conditions in which they could be recovered.

We then determined the effect of different ODC activities upon recovery of growth rate of cells first transferred to water and then back to Vogel's medium. Two experiments were done. In the first, transformant strains were used, one (PL2) with normal ODC activity and the other (DP6) with 30-fold elevated ODC activity. In both cases, ODC activity declined 7-fold during

TABLE 2

Polyamine Content of Cells in Normal Medium and after Transfer to Water or to Media Containing 1 *M* Sorbitol or 4% NaCl

Condition	Time (h)	ODC act. (mU/mg protein)	Polyamine pools (nmol/mg, dry weight)		Doubling time (h) after transfer
			Putrescine	Spermidine	
Vogel's (con)	0.3	70	0.7	15	2.9
	0.9	50	0.7	15	
	1.4	50	0.5	16	
	1.8	47	0.6	16	
+ 4% NaCl	0.1	48	2.0	20	No growth
	0.7	30	1.3	16	
	1.2	20	1.5	20	
	1.6	20	1.2	19	
+ 1 <i>M</i> Sorbitol	0.1	49	1.1	15	3.2
	0.7	24	1.0	16	
	1.2	35	0.8	17	
	1.7	66	1.0	17	
To water	0.1	67	0.9	20	No growth
	0.8	44	0.7	22	
	1.2	36	0.6	24	
	1.7	3	1.6	25	

TABLE 3
ODC-Specific Activity after Dilution of Spheroplasts

Dilution medium	ODC activity (mU/mg protein) of	
	Spheroplasts	Intact cells
Vogel's + 1 M sorbitol	12	46
Vogel's + 0.75 M sorbitol	29	51
Vogel's + 0.5 M sorbitol	35	49
Vogel's + 0.25 M sorbitol	16	42
Vogel's only	No cells	38
1/3X Vogel's	No cells	31

a 90-min incubation in water. The recovery of ODC activity and growth was similar in the two strains, although at different absolute levels (data not shown). The growth rates during recovery, following 30 and 90 min in water, and restoration to normal medium could not be determined accurately, given the sliminess of the cultures. However, both continued growth; if anything, strain DP2 was slower to recover than the wild-type strain PL2 (data not shown).

In a second experiment, the wild-type strain, IC3, was compared to strain IC1894-53a, which lacked ODC activity. Here, both strains were first grown in medium containing spermidine (1 mM), a condition that rendered both equivalent in spermidine content. Moreover, the strains both lacked putrescine in these conditions, IC3 owing to further repression of ODC, IC1894-53a because it lacked ODC activity. The strains were washed and incubated 90 min in distilled water, after which Vogel's medium with 1 mM spermidine was restored. In both cases, growth resumed immediately at the prior growth rate (2.5–2.8 h per doubling; data not shown).

We tested the effect of hyper- and hypotonic shifts on three osmotic (*os*) mutants, *os-1*, *os-2*, and *os-5*. None of these strains could grow on normal medium to which 4% NaCl had been added. The three strains had normal ODC activity when grown in Vogel's minimal medium, and little change (less than two-fold) in ODC activity took place in either transition when cultures were subjected first to 4% NaCl for 1 h, and then returned to normal medium for 30 min more (Table 4).

Finally, because "potassium stress" (K^+ deprivation) causes an induction of polyamine synthesis in many plants (Flores, 1991), we tested the effect upon ODC and polyamine pools of growing cells in a growth-limiting K^+ concentration (0.1 mM vs the normal 37 mM) and then adding a saturating concentration (1 mM) of K^+ . (During K^+ starvation, Na^+ was substituted as a medium cation.) The results (Fig. 2) display a slow, modest increase in the cellular spermidine content, which is reversed to normal by reading K^+ . ODC activity, while affected, varies only over a 1.5-fold range.

DISCUSSION

Our most surprising observation is the stability of ODC-specific activity in the face of severe environmental shifts. ODC activity is regulated over a 50-fold range by the polyamines themselves (Davis *et al.*, 1985), and in normal cultures, the protein turns over with a half-life of ca. 50 min (Barnett *et al.*, 1988). It was expected that the balance of synthesis and turn-over would be disturbed by some of the manipulations used, even if the changes were not adaptive. However, except for extreme starvation conditions and K^+ starvation, little change in ODC activity was seen. Our observations conflict with one unpublished study (mentioned in Yang *et al.*, 1993), which reported that *N. crassa* displays a "large" increase in ODC activity after hypoosmotic shock. The strains and conditions used in that study were not noted, and therefore we may have failed for some reason to uncover a potential response of the polyamine pathway.

TABLE 4
ODC Activity of Osmotic Mutants

Strain	ODC specific activity (mU/mg protein) in		
	Vogel's	Vogel's + 4% NaCl (1 h)	Return to Vogel's (0.5 h)
(<i>os-1</i>)	79	75	142
(<i>os-2</i>)	93	64	45
(<i>os-5</i>)	86	62	42
IC3 (control)	100	65	126

FIG. 2. Growth, polyamine pools, and ODC-specific activity during growth on limiting K⁺ (open symbols) and their response to restoration of unlimiting K⁺ (at vertical arrow; closed symbols). The normal, wild-type levels of spermidine and putrescine are indicated by horizontal arrows in the center panel, labeled S and P, respectively.

With that caveat, we conclude that *N. crassa* ODC activity is not normally subject to significant regulation by the osmotic strength of the medium and that polyamines are not involved in osmotic adjustment. This conclusion contrasts markedly with observations on mammalian cells, in which the response of ODC and the attendant accumulation of putrescine is required for continued viability after hypoosmotic shock (Poulin *et al.*, 1991). It also contrasts with findings in some bacterial strains, in which putrescine accumulates after hypoosmotic shock (Munro *et al.*, 1972). In the latter case, a substantial loss of K⁺ follows the shock, and several authors have conjectured that putrescine²⁺

restores the charge balance with a lesser osmotic potential in bacteria (Munro *et al.*, 1972; however, see Csonka, 1989). Similarly, K⁺ deficiency is followed by an accumulation of putrescine in plants, and the same reasoning about restoration of charge balance has been offered there (Flores *et al.*, 1984). Conversely, addition of excess K⁺ has been shown to be the most effective external ion in depressing the putrescine content of wheat seedlings (Reggiani *et al.*, 1993). Finally, the findings are unlike those with cells of many species, cereals among them, in which hyperosmotic or salt stress also evokes putrescine accumulation (Flores and Galston, 1982; Das *et al.*, 1995).

The osmotic potential of *N. crassa* and certain other fungal cells is maintained during growth in hyperosmotic media mainly by synthesis of glycerol, erythritol, and mannitol (Ellis *et al.*, 1991; Beever and Lacey, 1986; Latterich and Watson, 1993). A loss of these polyols, and perhaps other solutes, may follow hypoosmotic shock. The cell wall is clearly necessary to maintain cell integrity in media of low osmotic potential (see Table 3), and microscopic observations indicate a swelling of cells with an increase in turgor following the decrease in the ambient osmotic potential. Whatever other adjustments are made, *N. crassa* cells appear to need no special contribution from the polyamine pathway in these conditions.

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Note added in proof. K. Y. Chen, an author of the paper by Yang *et al.* (1993), reports (personal communication) that the *N. crassa* strain used in unpublished studies cited in the paper was the multiply mutant, cell-wall-deficient *slime* variant (FGSC 4761). ODC activity rose fivefold in the 2 h following dilution of the medium with 50% water. The *slime* variant, therefore, appears to have revealed or acquired a potential that we failed to observe in our studies with the normal strain. The *slime* variant is described in Perkins *et al.*, *Microbiol. Rev.* **46**: 426–570 (1982).

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