Polyamine metabolism in the thermotolerant mesophilic fungus
*Aspergillus fumigatus*

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Received 16 May 1997; revised 16 June 1997; accepted 16 June 1997

**Abstract**

Biomass production by *Aspergillus fumigatus* was greatest at 40–45°C and was associated with an increase in concentration of the diamine putrescine and activity of its biosynthetic enzyme ornithine decarboxylase. Concentrations of the other amines, cadaverine, spermidine and spermine were considerably lower than putrescine concentration and did not change significantly over the temperature range 20–50°C. This is surprising in view of the greatly increased flux of label from ornithine through to spermidine at 45 and 50°C, indicating an increased formation of this triamine. It is suggested that there was increased formation of spermidine derivatives at these temperatures. Interestingly, there was greatly increased formation of the higher homologues of cadaverine, aminopropylcadaverine and N,N'-bis(3-aminopropyl)cadaverine, in *A. fumigatus* at 45 and 50°C.

**Keywords**: Polyamine; Cadaverine derivative; Polyamine biosynthesis; *Aspergillus fumigatus*

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1. **Introduction**

The diamine putrescine, the triamine spermidine and the tetraamine spermine, collectively known as polyamines, are important for the growth and development of all cells [17]. Spermidine and spermine, bearing three and four net positive charges respectively, are the most cationic small molecules of the cell. Thus, they bind to polyanionic macromolecules like DNA, RNA and phospholipids [18]. Since these amines have a distributed charge, unlike multivalent cations like Mg$^{2+}$, the charge spacing may allow them to interact more flexibly with the phosphate groups of nucleic acids [7]. In fungi, spermidine is usually the major polyamine and indeed, in *Neurospora crassa*, spermidine is considered to be an essential metabolite [13].

In most fungi, putrescine is formed by decarboxylation of ornithine in a reaction catalysed by the enzyme ornithine decarboxylase (ODC; EC 4.1.1.17), while spermidine and spermine are formed from putrescine by subsequent additions of aminopropyl groups [NHCH$_2$]$_3$ from decarboxylated S-adenosylmethionine (AdoMet). The formation of these aminopropyl groups from AdoMet is catalysed by AdoMet decarboxylase (AdoMetDC; EC 4.1.1.50), and the aminopropyl additions to putrescine catalysed successively by the aminopropyltransferase enzymes spermidine synthase and spermine synthase (EC 2.5.1.16) [18]. ODC can be inhibited by the suicide inhibitor α-difluoromethylornithine

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(DFMO), AdoMetDC by methylglyoxal bis(guanylhydrzone) (MGBG), and spermidine synthase by cyclohexylamine (CHA) [18].

The diamine cadaverine is formed by decarboxylation of lysine, in a reaction catalysed by lysine decarboxylase (LDC; EC 4.1.1.18) or by ODC [6]. Aminopropylation of cadaverine leads to the formation of the higher homologues aminopropylcadaverine (APC) and N,N′-bis(3-aminopropyl)cadaverine (3APC). These compounds have been reported previously in bacteria [5], human tumour cells [1], N. crassa [11] and in a number of mycorrhizal and plant pathogenic fungi [21]. Zarb and Walters [21] suggested that most fungi synthesise the higher homologues of cadaverine using AdoMetDC and the aminopropyltransferases, although some evidence for the operation of a route from L-aspartic-δ-semialdehyde was presented. Recently, Walters and Cowley [19] showed that the yeast Saccharomyces cerevisiae routinely formed APC and 3APC and that their formation was greatly increased at elevated temperatures. Formation of these cadaverine derivatives appeared to occur only partly via the action of AdoMetDC and the aminopropyltransferases [19].

Very little is known about polyamine metabolism in fungi exposed to elevated temperatures, or in thermophilic filamentous fungi, i.e. those with growth minima at or above 20°C and maxima at or above 50°C [4]. The filamentous fungus Aspergillus fumigatus Fresenius, which has a temperature range of 12–52°C, might be called a thermotolerant mesophile [2]. We decided to use this fungus to examine the effects of growth at a range of temperatures on polyamine metabolism, and in particular, on formation of APC and 3APC.

2. Materials and methods

2.1. Growth of A. fumigatus

A. fumigatus was maintained on yeast phosphate soluble starch (YPSS) medium at 45°C. For all experiments, the fungus was grown in liquid YPSS medium. Conical flasks (250 ml) were inoculated with a 10 mm disc of mycelium and placed in a Gallenkamp orbital shaker (140 rpm) at the appropriate temperature. After 3 days, the fungus was washed with distilled water through a fine mesh sieve and centrifuged at 16 000 × g at 0°C for 10 min. The pellet obtained was weighed and used for analysis.

2.2. Polyamine analysis

The method used was adopted from Smith [14]. Briefly, fungal tissue (300 mg) was macerated in 3 ml 10% (v/v) perchloric acid and the homogenate centrifuged at 24 000 × g for 25 min at 0°C. To 100 μl of supernatant were added 200 μl saturated Na₂CO₃ and 400 μl dansyl chloride (10 mg/ml in acetone). This mixture was incubated in darkness at 60°C for 20 min, after which 100 μl proline (100 mg/ml) was added. After 10 min incubation in the dark at room temperature, dansylated polyamines were extracted in 500 μl toluene. Aliquots (25 μl) of the toluene phase were spotted onto activated LK6D silica gel plates (Whatman) and developed in chloroform/triethylamine (12:1, v/v). Polyamines were visualised under ultra-violet light and scraped off into 4 ml ethyl acetate. Fluorescence was measured at an excitation wavelength of 365 nm and an emission wavelength of 506 nm.

2.3. Enzyme assays

The activities of ODC and AdoMetDC were assayed as described by Foster and Walters [3]. For the LDC assay, fungal material (500 mg) was macerated in 1 ml of a buffer containing 50 mM Tris-HCl, 0.5 mM EDTA and 5 mM dithiothreitol, pH 8. Following centrifugation at 5000 × g for 15 min at 4°C, 50 μl of the supernatant was added to 200 μl of a reaction medium containing 10 mM Tris-HCl, 1 mM dithiothreitol, 0.1 mM EDTA, 0.1 mM pyridoxal phosphate, 5 mM lysine and 3.7 kBq [U-14C]lysine (11 GBq mmol⁻¹, Amersham International), at pH 8. Assays were done in 98 mm glass test tubes fitted with silicone rubber stoppers and 35 mm long, 22 gauge needles. A piece of filter paper (10 mm diameter) impregnated with 10 μl 2 M KOH was fitted to each needle to trap 14CO₂ released during the reaction. The test tubes were placed in a water bath at 45°C for 1 h after which 0.2 ml 10% perchloric acid was injected into each tube to stop the reaction. Tubes were then incubated for a further 30 min, after which the filter papers were removed.
and placed in scintillation vials containing 12 ml of Emulsifider Safe scintillant (Packard). The samples were counted for radioactivity using a Packard 1900 TR Liquid Scintillation analyser.

2.4. In vitro incorporation of \( {^{14}\text{C}}\text{l}ysine \) and \( {^{14}\text{C}}\text{ornithine} \) into polyamines

The in vitro incorporation of L-[\( ^{14}\text{C} \)]ornithine into putrescine, spermidine and spermine, and the in vitro incorporation of L-[\( ^{14}\text{C} \)]lysine into cadaverine, APC and 3APC were performed as described in detail previously [20,21].

2.5. Statistical analysis

Results were calculated as the means of 4 replicates. All experiments were repeated with similar results and statistical significance assessed using one-way analysis of variance. Differences among means were evaluated using the least significant difference (LSD) test [15].

3. Results and discussion

The biomass of \( A. \text{fumigatus} \) increased with increasing temperature over the temperature range 15–50°C and was maximal at 45°C (Fig. 1). Fungal biomass decreased considerably at 50°C (Fig. 1).

Putrescine concentration in \( A. \text{fumigatus} \) grown at 20°C was 28.4 nmol [mg protein] \(^{-1} \) (Table 1) and increased significantly in fungus grown at 45 and 50°C (Table 1). The concentrations of cadaverine, spermidine and spermine were considerably lower than putrescine concentration in this fungus and were not significantly affected by growth at the higher temperatures (Table 1). This is unusual, since spermidine is generally considered to be the major polyamine in fungi, with putrescine and spermine usually present at 10% or less of the spermidine pool [16]. Increased growth is normally accompanied by increased polyamine levels [16,18], and indeed, putrescine concentration was greatest at 45°C, the temperature at which \( A. \text{fumigatus} \) produced maximum biomass. However, although fungal biomass was greatly reduced at 50°C, putrescine concentration remained high. These polyamine concentrations were associated with significant increases in activities of ODC, AdoMetDC and LDC in \( A. \text{fumigatus} \) grown at 45 and 50°C (Table 2). The increased ODC activity at the higher temperatures would have been responsible for the observed increases in putrescine concentration.

The flux of label from ornithine through to putrescine and spermidine was increased at 45 and 50°C, although most of the label appeared in spermidine.

<table>
<thead>
<tr>
<th>Temperature, °C</th>
<th>Putrescine (nmol [mg protein] (^{-1} ))</th>
<th>Cadaverine</th>
<th>Spermidine</th>
<th>Spermine</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>28.4</td>
<td>13.5</td>
<td>6.7</td>
<td>7.1</td>
</tr>
<tr>
<td>45</td>
<td>52.0</td>
<td>15.1</td>
<td>8.0</td>
<td>9.2</td>
</tr>
<tr>
<td>50</td>
<td>44.9</td>
<td>15.8</td>
<td>6.9</td>
<td>7.8</td>
</tr>
<tr>
<td>LSD (( P = 0.05 ))</td>
<td>2.89</td>
<td>1.96</td>
<td>2.80</td>
<td>1.66</td>
</tr>
</tbody>
</table>

Table 1

Polyamine concentrations in \( A. \text{fumigatus} \) grown at different temperatures

LSD, least significant difference.

Values are the means of 4 replicates.
Table 2
Enzyme activities in *A. fumigatus* grown at different temperatures

<table>
<thead>
<tr>
<th>Temperature, °C</th>
<th>Enzyme activity (pmol CO₂ [mg protein]⁻¹ h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ODC</td>
</tr>
<tr>
<td>20</td>
<td>212</td>
</tr>
<tr>
<td>45</td>
<td>382</td>
</tr>
<tr>
<td>50</td>
<td>982</td>
</tr>
<tr>
<td>LSD (P = 0.05)</td>
<td>58.1</td>
</tr>
</tbody>
</table>

LSD, least significant difference.
Values are the means of 4 replicates.

(Table 3). This is curious given that spermidine concentration was unaffected by growth temperature. It is possible that the formation of higher derivatives of spermidine is increased, since it is known that such compounds e.g. the ‘thermopolyamine’ norspermidine, are formed in thermophilic bacteria [7]. Future work in this laboratory will examine the formation of higher spermidine derivatives in *A. fumigatus*.

The flux of label from lysine through to the cadaverine derivative APC was increased significantly at 45°C, but reduced considerably at 50°C, while flux through to 3APC was increased at both 45 and 50°C (Table 4). Although *A. fumigatus* is a thermostolerant mesophile, it is interesting to note that APC and 3APC formation was increased at elevated temperatures in the mesophile *S. cerevisiae* [19]. Since one of the pathways for the formation of these compounds is via LDC, AdoMetDC and spermidine synthase [21], the increased activities of LDC and AdoMetDC at these temperatures could have been responsible for the increased formation of APC and 3APC. Interestingly, no evidence could be found for the formation of the higher cadaverine derivatives from aspartate in this fungus (Walters and McPherson, unpublished results), suggesting that AdoMetDC and the aminopropyltransferases represent the only route for APC and 3APC formation in *A. fumigatus*. When the fungus was grown at 45 and 50°C in the presence of 5 mM of the ODC inhibitor DFMO, flux of label from lysine through to APC was increased at both 20 and 45°C, but was reduced at 50°C (Table 4). Flux of label through to 3APC in the presence of 5 mM DFMO was increased at 20 and 50°C, but reduced at 45°C (Table 4). Since DFMO reduced ODC but not LDC activity in *A. fumigatus* (data not shown), these data suggest first that inhibition of ODC activity led to a compensatory increase in LDC activity, and second, that LDC and not ODC is responsible for the formation of cadaverine in this fungus. This is different to the situation in animal cells and *N. crassa*, where cadaverine formation is catalysed by ODC, albeit inefficiently [11,12].

The function of the higher derivatives of cadaverine in cells is unknown. Nevertheless, uncommon polyamines have been postulated to play protective roles in bacteria adapted to extreme environments [7]. Uncommon, long-chain polyamines are essential in thermophiles for in vitro protein synthesis at elevated temperatures [9]. These amines are required for activation of the ribosomal ternary complex during protein synthesis, and they influence the rate of chain elongation [8]. The precise complement of uncommon polyamines produced by thermophiles is dependent on growth temperature, with higher molecular mass compounds produced with increasing.

Table 3
Incorporation of radiolabelled ornithine into polyamines in *A. fumigatus* grown at different temperatures

<table>
<thead>
<tr>
<th>Temperature, °C</th>
<th>Radioactivity in polyamine (dpm [mg protein]⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Putrescine</td>
</tr>
<tr>
<td>20</td>
<td>25.1</td>
</tr>
<tr>
<td>45</td>
<td>36.0</td>
</tr>
<tr>
<td>50</td>
<td>34.4</td>
</tr>
<tr>
<td>LSD (P = 0.05)</td>
<td>2.4</td>
</tr>
</tbody>
</table>

LSD, least significant difference.
Values are the means of 4 replicates.

Table 4
Incorporation of radiolabelled lysine into APC and 3APC in *A. fumigatus* grown at different temperatures ± 5 mM DFMO

<table>
<thead>
<tr>
<th>Temperature, °C</th>
<th>Radioactivity in polyamine (dpm [mg protein]⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>APC</td>
</tr>
<tr>
<td>20</td>
<td>250</td>
</tr>
<tr>
<td>20+5 mM DFMO</td>
<td>513</td>
</tr>
<tr>
<td>45</td>
<td>491</td>
</tr>
<tr>
<td>45+5 mM DFMO</td>
<td>948</td>
</tr>
<tr>
<td>50</td>
<td>81</td>
</tr>
<tr>
<td>50+5 mM DFMO</td>
<td>11</td>
</tr>
<tr>
<td>LSD (P = 0.05)</td>
<td>48.1</td>
</tr>
</tbody>
</table>

LSD, least significant difference.
Values are the means of 4 replicates.
temperature [7]. It would be useful to determine the complement of uncommon polyamines produced by *A. fumigatus* at elevated temperatures.

In conclusion, these data show that *A. fumigatus* produces maximum biomass at 45°C and that putrescine, not spermidine, is the major common polyamine, although it is suggested that this fungus might form higher derivatives of spermidine. Cadaverine biosynthesis is catalysed by LDC in *A. fumigatus* and there is also considerable formation of the higher derivatives of cadaverine, APC and 3APC, using AdoMetDC and the aminopropyltransferases.

4. Unlinked reference

[10]

Acknowledgments

SAC receives financial assistance from the Scottish Office Agriculture, Environment and Fisheries Department.

References


